The oxidases and other oxygen - catalysts concerned in biological oxidations / by J. H. Kastle.

TREASURY DEPARTMENT
Public Health and Marine-Hospital Service of the United States
Walter Wyman, Surgeon-General

HYGIENIC LABORATORY.—BULLETIN No. 59

DECEMBER, 1909

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United States Public Health and Marine-Hospital Service.

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TABLE OF CONTENTS.

THE OXIDASES AND OTHER OXYGEN-CATALYSTS CONCERNED IN BIOLOGICAL OXIDATIONS.[a]

By Joseph H. Kastle,

Chief, Division of Chemistry, Hygienic Laboratory, U. S. Public Health and Marine-Hospital Service.

INTRODUCTION.

During the past eight years I have devoted a considerable part of my time to a study of the oxidases and of certain phases of biological oxidation, with the result that I have come to appreciate as never before the need of a somewhat more comprehensive and complete treatment of this subject than is ordinarily to be found in most treatises on fermentation. Furthermore, a study of the earlier literature of the subject has revealed certain rather curious mistakes, which probably found their way into the literature originally as the result of typographical errors, but which pervade the whole subject with an annoying persistency. During the past four years, as the result of easy access to the departmental libraries in this city, I have had unusual facilities for familiarizing myself with the literature of this subject, and it was partly with the view of giving to other workers in this field the benefits of my opportunities along this line, that this monograph has been written. No special claims are made for originality either in the mode of treatment of the subject, or in some cases even with regard to phraseology, my only object being to present the subject as accurately and comprehensively as possible within the time at my disposal. To this end I have made free use of such treatises on fermentation as "The soluble ferments and fermentation," by J. Reynolds Green (Cambridge Natural Science Manuals, London, 1899); "Die Fermente und ihre Wirkungen," by Carl Oppenheimer, (Leipzig, 1900); "Traite de Microbiologie," by E. Duclaux (Paris, 1898); "Biochemie der Pflanzen," by Fred. Czapek (Jena, 1905); "Theorie der Fermentwirkungen," by Moritz Traube (Berlin, 1858); "Les Enzymes et leurs Applications," by Effront (translation by Prescott, New York, 1902); "Recent advances in physiology and biochemistry," by Leonard Hill and others (London, 1906); "Das Sauerstoff-Bedürfniss des Organismus," by Paul Ehrlich (Berlin, 1885); "Les Oxydations de l'Organisme," by E. Enriquez and J.-A. Sicard (Paris, 1902); "Die Bedeutung der Katalyse für die Medicin," by H. Schade (Kiel, 1907); and "Kritische Studien über die Vorgänge

[a] Manuscript submitted for publication October 12, 1909.

der Autoxydation," by C. Engler and J. Weissberg (Bräunschweig, 1904). I am especially indebted for much valuable information to a dissertation by Pierre Sée, entitled "Contribution a l'Etude des Applications Therapeutiques des Oxydases et des Metaux Ferments" (Thèse, No. 239, Faculté de Médicine de Paris, 1905), and to recent articles by Engler and Herzog, "Zur chemischen Erkenntnis biologischer Oxydationsreaktionen" (Hoppe-Seyler's Zeitschrift für physiologische Chemie, 1909, vol. 29, pp. 327–375), and to Moore and Whitley, "The properties and classification of the oxidizing enzymes and analogies between enzymic activity and the effects of immune bodies and complements" (Bio-Chemical Journal, 1909, vol. 4, pp. 136–167). In many instances in dealing with original contributions to the subject I have adhered as closely as possible to the words of the author in order that the exact sense and meaning of the original article might be preserved to at least as great an extent as possible within the limits of the present communication. Furthermore, no claim is made for the completeness of this production. It has been impossible, through lack of time and space, to refer to all original communications on the subject, even in the bibliography. Even the current numbers of the journals contain many contributions bearing on the subjects treated of in the following pages, and out of this vast and constantly growing literature I have been compelled to select what seemed to me the most essential to the proper presentation of the subject.

In the writer's opinion the subjects considered in the following pages are strictly germane in their general scope to the work of the Hygienic Laboratory. The oxidases and related oxygen-catalysts belong to the same group of bodies as the serums, viruses, toxins, and antitoxins. Any facts throwing light on the one class of substances will doubtless assist ultimately to a better understanding of this whole group of biologically active substances. Portier is of the opinion that the oxidases play an important rôle in the defense of the organism against pathogenic micro-organisms, and according to Sieber they are able to destroy toxins, such as the toxins of diphtheria and tetanus. Furthermore, they are of importance as enabling us to form correct conclusions regarding the condition and character of certain foodstuffs—as to whether they are cooked or raw—and in enabling us to determine, within certain limits of temperature, whether a given sample of milk has been pasteurized or not. For the biological chemist, the physician, and the sanitarian, all of these matters are of considerable importance.

I am greatly indebted for much valuable material to various authors, whose works I have freely consulted and made use of in the preparation of this monograph, and also to Mr. F. A. McDermott, one of the assistants in the division of chemistry of the Hygienic Laboratory, for much valuable assistance.

Chapter I.

THEORIES OF OXIDATION.

In the period which elapsed between the classic researches of Lavoisier on Combustion (1774–1785) and the discovery of ozone by Schoenbein in 1840, a number of interesting and important observations had accumulated in the literature concerning the influence exerted by one substance on the oxidation of another by air or oxygen. Thus in 1806 Désormes and Clement [137] pointed out that nitric acid is not the principal agent concerned in the complete oxidation of the sulfur in the leaden-chamber process for the manufacture of sulfuric acid, but its "base," nitric oxide (*le gaz nitreux*), which takes oxygen from atmospheric air in order to offer it to the sulfurous acid in a condition capable of accomplishing its oxidation. A few years later it was observed by Vogel [443] that hydrogen and oxygen combine at low temperatures under the influence of charcoal. Then followed the numerous and interesting observations by Sir Humphrey Davy [134], Edmund Davy [132–133], Erman [167], Pleischl [328], and others on the remarkable power of platinum and other metals of the platinum group, such as iridium, osmium, and palladium, to effect the slow combustion, or under certain conditions even the actual ignition, of combustible gases such as hydrogen, carbon monoxide, etc., in an atmosphere containing oxygen, and the rapid conversion of alcohol into acetic acid, observations which culminated in Davy's lamp without flame (*see* Erman [167]), Doebereiner's lamp [141], and the rapid method for the production of acetic acid.

The effect of various substances on the oxidation of combustible gases and vapors was also exhaustively investigated by Dulong and Thenard [151–152], with the result that various metals, such as iridium, palladium, rhodium, gold, silver, mercury, nickel, cobalt, and iron, in spongy or pulverulent form, were found to accomplish the same changes as platinum, and that to a degree at least such changes can also be brought about by certain nonmetallic substances, such as carbon, pumice stone, porcelain, glass, and quartz crystals, at temperatures under 350° C., while salt, fluorspar, and marble did not appear to act to a sensible extent within these limits. These changes, they concluded, could not be ascribed entirely to electrical phenomena, as was at first supposed, and they explained them on the assumption of the condensation of large volumes of gas on the surface of the sub-

stance capable of causing the oxidation—as, for example, the condensation of oxygen upon platinum, whereby heat was liberated and the body (platinum) attained the temperature necessary to accomplish the oxidation.

The discovery of ozone by Schoenbein ([367]) in 1840 and the finding of this remarkable substance among the oxidation products of readily oxidizable substances gave a new impulse to the study of oxidation phenomena and led ultimately to a more careful study of the whole subject of induced oxidations and of the phenomenon of oxygen carrying. This distinguished observer seems to have been the first to clearly recognize that in the final products of chemical combination, particularly those in which oxygen is concerned, we see, as he put it, only the closing scene of a chemical drama which is in reality composed of several intermediate acts, and that for a correct understanding of such processes it is quite as essential to know these intermediate acts as it is to know the beginning and the end.

To the investigation of ozone and to the task of learning more of the initial and intermediate acts of the oxygen drama, Schoenbein set himself with remarkable assiduity and success. It is beyond the scope of this communication to attempt anything like a chronological account of the numerous investigations on the subject of ozone, which in themselves form one of the most interesting chapters in the history of chemical science. (*See* Engler and Weissberg[164].) It is sufficient to say in this connection that from the time of its discovery until its composition and nature were finally settled through the labors of Odling, Soret, and Andrews and Tait, Schoebein himself held different notions at different times respecting its composition. As the net result of his many researches on the subject, Schoenbein came to look upon ozone and the ozonides as containing an atom of negatively polarized oxygen, which in its conduct toward oxidizable substances was far more active than oxygen in its ordinary form, and as we would interpret these phenomena at the present day, he accounted for its production from ordinary oxygen through the action of electricity upon the supposition that as a result of the electrical discharge the ultimate particle (molecule) of oxygen is resolved into two atoms, one of which is negatively polarized and the other positively polarized, thus:

$$(O) = \ominus + \Theta \text{ or, } O_2 = \ominus + \Theta$$

The negatively polarized atom them combines with a particle of ordinary oxygen to form ozone, $O_2\Theta$, whereas the positively polarized atom (antozone) combines with water to form hydrogen peroxide, $H_2O.\Theta$. These two substances, ozone and hydrogen peroxide, were regarded as the prototypes of a whole series of compounds to which Schoenbein gave the name ozonides and antozonides, respectively.

According to Hagenbach [203], Schoenbein did not hold that the oxygen molecule consisted of two atoms. Therefore, according to Schoenbein, the chemical polarization of neutral oxygen is no true decomposition, but only a calling into being of two oppositely active states of the element—

$$(O) = \oplus + \ominus$$

The $\oplus$ combines with water to form H_2O_2, and the $\ominus$ goes partly to form ozone, but in greatest part to oxidize the metal or phosphorus.

Like ozone, the ozonides were believed to contain a part of their oxygen in a negatively polarized condition, whereas the antozonides were supposed to contain a part of their oxygen in a positively polarized condition. Like ozone itself, the ozonides were supposed to contain a part of their oxygen in an intensely active condition. On the other hand, in antozone and the antozonides the antozonic oxygen was supposed to be less active chemically than the active oxygen in ozone and the ozonides, at least in most of its chemical relations, and to exhibit chemical activities of a different order. The following is a list of some of the common ozonides and antozonides included in this classification:

$O_2.\ominus$, ozone.	$\ominus$, antozone.
$PbO.\ominus$, lead peroxide.	$H_2O.\ominus$, hydrogen peroxide.
$MnO.\ominus$, manganese peroxide.	$BaO.\ominus$, barium peroxide.
$HCl.\ominus$, hypochlorous acid.	etc.
etc.	

This view regarding the nature of ozone and antozone was apparently in harmony with a great many facts, among which may be mentioned:

1. Modes of formation from ordinary oxygen by the action of electricity and as a result of the autoxidation of readily oxidizable substances such as phosphorus, its production in the electrolysis of water and as the result of heating various oxides and peroxides and in the decomposition of highly oxygenated compounds by acids.

2. The greater oxidizing power of ozone and the ozonides as compared with the oxidizing power of antozone and the antozonides. Thus it was proven by Schoenbein that ozone and the ozonides can accomplish the oxidation of a number of metals, guaiacum, etc., which are not acted upon by hydrogen peroxide or other antozonides.

3. The mutual decomposition of ozone and the ozonides by antozonides resulting in the formation of ordinary oxygen. Such decomsitions are those met with in the decomposition of hydrogen peroxide by lead and manganese dioxides and by potassium permanganate, and also the remarkable decomposition of ozone itself by hydrogen peroxide, whereby water and ordinary oxygen are formed.

4. The apparent occurrence of antozone in nature in certain varieties of fluorspar, and its apparent formation (or the actual formation of hydrogen peroxide, an antozonide) by the action of an acid on an antozonide like barium peroxide.

While the differences existing between the so-called ozonides and antozonides and also their mutual decomposition, can be explained upon grounds[a] other than those involving the idea of oppositely electrified oxygen atoms, Schoenbein's theories respecting the nature of this interesting group of substances were more or less in harmony with the dualistic theory of Berzelius and with the fact that the molecule of oxygen consists of two atoms. Their principal interest in this connection is that erroneous as they were in some particulars, they afforded a satisfactory explanation of oxygen activation. A few examples will serve to make this clear: When a solution of indigo blue is exposed to oxygen or atmospheric air it suffers no change. On the other hand, as is well known, phosphorus is readily oxidized under these conditions. And now, what is more remarkable, if indigo be brought in contact with slowly oxidizing phosphorus, not only does the phosphorus continue to be oxidized but under these new conditions the indigo is oxidized as well. In other words, oxidizing phosphorus has the power to excite or induce the oxidation of a substance which alone is incapable of undergoing oxidation by simple contact with oxygen or atmospheric air. As shown by Schoenbein, however, when phosphorus is slowly oxidized in the air ozone is produced and it is the latter substance which oxidizes the indigo. When a solution of potassium iodide and starch or a suspension of guaiacum resin in water is shaken with air, no change of color occurs. If, however, as shown by Schoenbein ([380]), a drop or two of oil of bitter almonds be added to these solutions and the solutions afterwards shaken with air, each of them becomes blue in color, due in the first case to the oxidation of the potassium iodide with the setting free of iodine and the formation of the blue iodide of starch, and in the second case to the oxidation of the guaiacum with the production of guaiacum blue. According to Schoenbein, the oil of bitter almonds ozonized the air and the resulting ozone oxidized these substances which are not oxidizable by oxygen in its ordinary form.

He also made the interesting observation that certain of the higher fungi, as well as other plants, contain substances which apparently have the power of ozonizing the air to a remarkable degree, thereby accomplishing the oxidation of substances not oxidizable by ordinary

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[a] Mendelejeff ([299]) divides the peroxides into two classes, 1, superoxides, in which the oxygen atoms are directly united to one another as well as to the oxidized element, and 2, polyoxides, in which the oxygen atoms are not directly united with one another. The superoxides yield hydrogen peroxide when dissolved in acids; the polyoxides do not. Similar views are held by Traube and by Richarz ([349]).

oxygen, and in certain instances resulting in the development of characteristic vegetable colors (378). He proved, further, that just as a large number of substances have the power of activating the oxygen of the air, so also an even larger number have the power of activating the oxygen of hydrogen peroxide and other antozonides. Thus while hydrogen peroxide and old oil of turpentine are without action on guaiacum or a solution of potassium iodide and starch, the oxidation and bluing of these reagents are readily accomplished by these oxidizing agents through the action of platinum black, extract of malt, the red coloring matter of the blood, and the juices and extracts of many plants, for the reason that, according to Schoenbein (383), they are converted into ozonides, in the same way that an ozonide, like lead peroxide, results from the action of lead acetate on hydrogen peroxide.

He also proved that ozone is formed during many processes of combustion, an observation which led to the conclusion that every combustion is accompanied by the formation of ozone, and finally to the view that no oxidation can proceed of itself without the previous conversion of the common inactive oxygen of the air into active oxygen or ozone. In this manner he readily accounted for the phenomenon of oxygen carrying, and arrived at the further conclusion that through the agency of living things and the organic matter on the surface of the earth, the inactive oxygen of the air is constantly being transformed into ozone. This gradual formation of ozone through these agencies was deemed sufficient to account for the slow oxidations continually taking place on the surface of the earth, and in speaking of the oxygen-carrying power of certain substances contained in the blood of animals, which like phosphorus and oil of bitter almonds can activate the inactive respired oxygen, he says that "as a matter of fact, without the presence of such substances as convert ordinary oxygen into ozone, animals would be suffocated in the midst of an ocean of the purest but inactive oxygen as quickly as in a vacuous space." (Schoenbein (380).)

Indeed, upon every hand, apparently, facts and observations multiplied, indicating the importance of ozone in the economy of nature. Hare (205) observed its production as the result of rubbing a piece of flint. For example, the odor of ozone is very evident when flint is struck with a piece of steel. Brame (93) detected ozone in rain water. Scoutetten (394, 395, 396, 397) observed its production in the evaporation of impure waters. As a matter of fact ozone became the chemical fad of the day, with the result that its importance in nature was greatly overestimated. Thus during the years 1866–67 daily observations were made on the amount of ozone in the atmosphere of Paris and other localities, and everywhere men were busily engaged in studying its relation to health and disease. Its absence from the

atmosphere of certain localities, or its presence therein in diminished amounts was associated with several violent outbreaks of Asiatic cholera that occurred during this period, and according to Doctor Moffatt ([303]), an English physician, the approach of an ozone period, during which the quantity of ozone in the atmosphere suffers a considerable increase, is followed by a corresponding increase in the luminosity of the glowworm and of certain phosphorescent protozoa, and even of phosphorus itself, when exposed to the air; and in man by a decided increase in the output of phosphates in the urine. Further, according to Doctor Moffatt, the advent of an ozone period was found to be marked by the approach of thunderstorms and unsettled weather conditions generally, and by a marked increase in the number of cases of toothache, neuralgia, apoplexy, etc. Finally, as the result of a large number of observations extending over a period of ten years, the most exact agreement was established between the warnings of the admiralty cautionary telegrams, as the British weather forecasts were then called, and the readings of the Doctor's ozonometer. The Doctor himself had evidently fallen a victim to ozone.

Despite, however, the great number of faulty and imperfectly controlled observations on this remarkable substance and the erroneous theories arising therefrom, the fact remains that this remarkably active form of oxygen is produced under a great variety of conditions, and that it is responsible for the oxidation of many substances not ordinarily oxidized by atmospheric oxygen (*see* Wurster ([464])); and the fact that it is formed as a by-product in certain processes of autoxidation affords a simple explanation of oxygen carrying on the part of such substances as give rise to ozone during autoxidation.

From the ozone theory of Schoenbein as a point of departure, our theoretical conceptions regarding processes of autoxidation and the phenomenon of oxygen-carrying have been developed mainly along three different lines. Briefly stated these conceptions are: (1) The ionization theory of Van 't Hoff; (2) the theory of Hoppe-Seyler; and (3) the peroxide theory of Traube, Engler, Bach, and Manchot.

Van 't Hoff 's theory of oxygen-activation rests upon the assumption that in slow oxidation, such as that met with in the gradual oxidation of phosphorus, the oxidizable substance enters into combination, not with molecular oxygen, but with the very small amounts of atomic (ionic) oxygen which are constantly being produced from molecular oxygen in the sense of the equilibrium $O_2 \rightleftharpoons 2O$, and that in a gaseous system such as that furnished by acetic aldehyde and gaseous oxygen, the velocity of the reaction should be exactly proportional to the square root of the oxygen pressure.

The idea that molecular oxygen dissociates as the result of electrification and under the influence of heat and chemical action, is by

no means a new one, nor did it originate with Van't Hoff. As a matter of fact, it had been promulgated by Clausius ([119, 120, 121]) upon theoretical grounds and as affording a simple explanation of the differences between active and common oxygen, as early as 1857 to 1863, and has since been employed by other physicists (among them Schuster, Von Helmholtz, Richarz, Thomson, and others), as affording a simple explanation of the phenomena observed in the electrification of gases, including oxygen, and in the condensation of aqueous vapor in the steam jet as the result of the oxidation of various substances in close proximity thereto. Thus Clausius ([119]) in a communication entitled "Ueber die Art der Bewegung, welche wir Wärme nennen," had put forward the suggestion that in common oxygen the atoms are not detached, but combined in twos to form molecules, a conclusion which also derived support from the views of Gerhardt on the constitution of gaseous molecules. He reached the conclusion therefore that the active oxygen which common oxygen sometimes contains and which at this time was not distinguished from ozone, consists not of atoms combined in pairs, but of single atoms distributed among the molecules of the element in its ordinary form, and in a later communication on the difference between active and common oxygen ([121]) this author reiterates the view that common oxygen consists of paired atoms, and active oxygen of unpaired atoms, and further that the two atoms which go to form ordinary oxygen are in oppositely electrified states. Hence, according to Clausius, the molecule of ordinary oxygen is diatomic, containing one electro-positive and one electro-negative atom. On the other hand, active oxygen consists of unpaired atoms which may exist either free or loosely bound together (*lose gebunden*). If electro-negative, these atoms form ozone; if electro-positive, antozone.

Long afterwards it was pointed out by Schuster ([392]) in connection with his investigations on the electrification of gases that the passage of electricity from one molecule to another in a gas is always accompanied by an interchange of atoms composing the molecule. According to this author, physicists of the Faraday-Maxwell school had long considered it probable that the conduction of electricity through gases is due to something similar to the electrolytic conduction in solutions, or, in other words, to the migration of ions under the influence of the current.

Similarly, according to Von Helmholtz and Richarz ([208]) the interesting phenomena observed during the electrification of a steam jet and the influence of certain chemical reactions on condensation occurring in the jet, can only be explained on the supposition that molecular oxygen or the gaseous substances in the immediate vicinity of the jet are dissociated to a greater or less extent into free atoms or ions, or into molecular groups containing free valences, and that these are

responsible for the phenomena observed. It has been observed by these and other investigators that when a jet of steam emerges from a small orifice into dust-free air, there is very little if any condensation to be observed within half an inch or so of the orifice. On the other hand, if an electrode from which electricity is escaping be placed near the origin of the jet, dense condensation occurs right up to the orifice, and the jet itself appears colored, the color being due to the scattering of light by a great number of very small particles of water, the diameter of which is very small compared with the wave length of light.

According to J. J. Thomson ([422]), the electricity which escapes into the gas is carried by the charged atoms of the gas, and since in the region immediately around these atoms there will be a very intense electric field there will be a tendency for the steam to deposit in these regions. Thus around these charged atoms there will be very small drops of water, which will scatter the blue light more than the red, so that the jet when seen by transmitted light will appear reddish.

Von Helmholtz and Richarz ([208]) found further that condensation in the steam jet is also brought about by chemical action going on in its neighborhood. The reactions investigated were of the most varied character, involving such processes as the combination of nitric oxide and oxygen, ammonia, and hydrochloric acid, the oxidation of phosphorus, sulphur, and other readily combustible substances. All of these were found not only to effect the condensation of live steam, but also to cause the condensation of other vapors, such as those of alcohol and acetic acid. As a matter of fact so universal is this influence that Von Helmholtz and Richarz proposed to employ the steam jet as a means of detecting ionic dissociation in gases. Thus Von Helmholtz ([207]) found that condensation is effected in the steam jet by various glowing metals, such as platinum foil which had been heated to redness and which had not been artificially electrified. Similarly other metals such as silver, iron, copper, etc., after heating in the flame, color the steam jet for a long time. This is also shown in a high degree by all glowing organic bodies, such as wood, paper, tobacco, and especially by glowing sulphur. It was also proved that the production of smoke has nothing to do with the phenomenon observed. Tobacco smoke, especially, was observed to be without influence. Von Helmholtz and Richarz ([208]) found that condensation in the steam jet could be brought about by a piece of glowing phosphorus at a distance of several decimeters away, and that at this distance it acted remarkably powerfully, but less actively, on the vapors of alcohol, anilin, formic, and acetic acids. They also proved that the thick white clouds which are produced when moist phosphorus is exposed to the air or oxygen do not exhibit this phenomenon when the glowing phosphorus is quenched. So, also, the oxidation products of phosphorus already prepared had no influence on the condensation.

On the other hand, the active agent produced during the slow oxidation of phosphorus diffuses with considerable velocity, even against a current of air. Potassium and sodium burning in contact with water also exert an influence on the steam jet through great distances. As has been observed in the case of phosphorus, the resulting cloud, which consists of finely divided potassium or sodium oxide or hydroxide, is of itself inactive. It is also known that potassium and sodium glow on their freshly cut surfaces as the result of autoxidation. Hence all these phenomena are analogous to those observed in the burning of phosphorus.

According to these observers, it can readily be understood that in all oxidations occurring in atmospheric air there are produced so many substances that it is often difficult or impossible to obtain experimental proof by the exclusion of all other causes, that the action on the steam jet is only brought about by ions, especially those of active oxygen. Of the three substances, however, which are always produced during combustions in air, namely ozone, nitrous acid, and hydrogen peroxide, none were found to have any action on the steam jet.

In this connection it is of interest to note that Meissner ([207]) as early as 1863 had observed that under the excitation of oxygen by electrical induction there is produced, besides ozone, still another substance, which exerts a highly remarkable action on water vapor. Further investigations led him to believe that this substance was Schoenbein's antozone.

According to Von Helmholtz and Richarz ([208]), in all such oxidation processes as effect a condensation of aqueous vapor in the steam jet, the oxygen is dissociated into single atoms (ions), O^{+}_{+} and O^{-}_{-}, or into groups of atoms such as $-O-O-$. In terms of the electro-chemical theory both of these kinds of particles have free valences and are carriers of an excess of positive or negative electricity, and hence serve to conduct the current through the gas during electrification, or, as explained by Thomson (see p. 16), they cause a condensation of aqueous vapor when they come in contact with the steam jet. According to Von Helmholtz and Richarz ([208]), chemists have ascribed to active oxygen only a momentary existence; on the other hand, Clausius ([121]), on purely physical grounds, postulated the existence of dissociated atoms of oxygen at least as a transitional form enduring certainly for a short time, and if we judge from their conduct toward steam these particles of active oxygen exist for a sensible interval. By means of tetramethyl-p-phenylene-diamin, which is colored blue by contact with monatomic oxygen (antozone), Wurster ([464]) was able to obtain evidence of the existence of the dissociation products of oxygen in the vicinity of gas flames. Thus Wurster's reagent is instantly colored intensely blue in the Bunsen burner flame; more slowly, but still visi-

bly, in the luminous zone of flames and combustible gases, and also in the flame of burning alcohol. Similarly it was observed by Von Helmholtz (207) [a] that Wurster's paper is colored blue in the neighborhood of a glowing platinum spiral, pointing to the presence of active oxygen in the surrounding atmosphere.

It has also been shown by Elster and Geitel (158) that the electrification of gases is brought about by glowing bodies. They (159, 160, 161, 162) likewise observed that air in which moist phosphorus is oxidizing conducts the electric current, while ozone does not conduct it. Hence they conclude that this conduction is accomplished by ions, or split oxygen molecules, and that the production of ozone must result from the previous splitting of the oxygen molecule. That ozone differs essentially in its conduct from atomic oxygen is also indicated by the fact that ozone itself has no effect on the steam jet, whereas decomposing ozone, like decomposing oxygen, affects it most actively. (*See also* Von Helmholtz and Richarz (208), pp. 194–195, and also Meissner (296).

It is evident, therefore, from these observations and others of similar import, that the dissociation of oxygen, whereby active or ionic oxygen is produced under a great variety of conditions, had been recognized for some time before it was employed by Van't Hoff in explanation of oxygen activation. In reality the idea that in oxygen gas we have an equilibrium represented by the equation, $O_2 \rightleftarrows 2O$, dates from the time of Clausius. On the other hand, we are indebted to Van't Hoff and his coworkers (214, 236), especially to Ewan (169), for experimental proof that in the oxidation of acetic aldehyde the velocity of the reaction is as indicated by the above equation, proportional to the square root of the oxygen pressure, and to Van't Hoff himself for experimental proof than an atom of oxygen is rendered active for every two atoms of phosphorus oxidized, irrespective of the nature of the acid of phosphorus produced.

The fact that oxygen can distribute itself equally between two oxidizable substances, one of which is autoxidizable, while the other is not, is supported by a vast amount of experimental evidence, all of which, to a degree at least, supports Van't Hoff's hypothesis. Thus, to take an actual case studied by Jorissen (234, 235, 236). 131.5 milligrams of tri-ethyl phosphine were found to require 17.8 milligrams of oxygen to completely oxidize it in the sense of this equation,

$$(C_2H_5)_3P + O = (C_2H_5)_3PO,$$

whereas when this same amount of this compound was oxidized in the presence of 2 grams of sodium indigo-sulfonate, 35.3 milligrams of oxygen were consumed, or approximately twice as much. Similar results were obtained by this observer with propionic and benzaldehydes. (*See also* Van't Hoff (215).

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[a] See also Gorup-Besanez (196).

So also Meyer and Recklinghausen ([301]) and Hirtz and Meyer ([212]), in their study of the oxidation of hydrogen and carbon monoxide by means of potassium permanganate, have made the interesting observation that approximately as much oxygen is liberated in gaseous form as is absorbed by the reducing substance during the oxidation. These authors are therefore of the opinion that these phenomena probably stand in close relation to the phenomena described by Van't Hoff and his coworkers. The fact that ordinary oxygen dissociates at least to a slight extent into positive and negative ions, even at ordinary temperatures, enables us, according to Van't Hoff, to understand why the oxidation of one substance promotes the oxidation of another. All ions of one kind enter into combination with the autoxidizable substance during the process of autoxidation, thereby leaving the ions of the opposite kind free to combine with a second substance, the acceptor, which ordinarily in the absence of a carrier, will not combine with oxygen at all. Thus in the oxidation of indigo by oxidizing phosphorus, we would have, according to Van't Hoff:

$$O_2 = \oplus + \ominus,$$

and

$$P + \oplus = PO,$$

and

$$\text{Indigo} + \ominus = \text{Isatin};$$

or in general,

$$O_2 = \oplus + \ominus,$$

and

$$A + \oplus = AO,$$

and

$$B + \ominus = BO.$$

According to this view neither A nor B can combine with molecular oxygen, but since this dissociates to a slight extent, even under ordinary conditions, and since $\oplus$ can combine with A, it leaves the other ion, $\ominus$, free to combine with B.

The assumption, however, that the oxygen molecule dissociates into two oppositely polarized atoms is by no means rendered necessary by these facts alone, since the equal distribution of oxygen between two oxidizable substances, one of which functions as the autoxidator and the second only in the capacity of acceptor, can, as we shall see, be equally well if not better explained on the assumption that the autoxidizable substance combines with a molecule of oxygen to form an unstable peroxide, which in turn gives up half of its oxygen to the acceptor, thus:

$$A + O_2 = AO_2$$

and

$$AO_2 + B = AO + BO,$$

and certainly for most, if not all, of the actual cases thus far investigated, such an assumption is, as we shall see, more in harmony with the facts, since the formation of such peroxides as AO_2 rests on indisputable experimental evidence.

From his studies on the electrification of gases, Schuster [392] assumes that under the influence of the electric current the molecules of gaseous substances are broken up at the negative pole. A number of chemists in attempting to account for the activation of oxygen have thought it necessary to assume the decomposition or disruption of the oxygen molecule through the action of oxygen carriers. Thus in 1870, Loew [276] advanced the view that in slow and rapid oxidation the splitting of the oxygen molecule into its atoms occurs. Fudakowsky [177] concurs in this view.

From his study of fermentation, more especially of those changes occurring in putrefaction and anaerobic fermentation, Hoppe-Seyler [217] in 1878, arrived at the notion that oxygen is rendered active through the agency of active (nascent) hydrogen, which in all processes of this kind appropriates to itself an atom of oxygen to form water, thereby leaving the other atoms of oxygen free or in the active condition. Thus,

$$H + H + O_2 = H_2O + O$$

and

$$R + O = RO,$$

where R represents a substance which can not ordinarily combine with molecular oxygen. In many processes of putrefaction and anaerobic fermentation, he observed that hydrogen is a constant product. Furthermore, he found it to be *active*, in the sense that it has the power of accomplishing the reduction of many substances not ordinarily reducible by molecular hydrogen. In other words, Hoppe-Seyler [217] assumes that the nascent hydrogen splits the oxygen molecule, forming water and liberating atomic oxygen, which can then combine with easily oxidizable substances, or if no such substances be at hand, with water to form hydrogen peroxide, or with oxygen to form ozone, or with carbon monoxide to form carbon dioxide, as was shown by Baumann [39].

In the field of pure chemistry many facts of similar import were already known, so that in seeking for an explanation of his own results, Hoppe-Seyler found many analogies to draw upon. For example, as early as 1853, Osann [314,315,316] had made the observation that carbon and platinum which had been charged with hydrogen at the cathode in the electrolysis of dilute sulfuric acid, could effect the reduction of silver compounds in solution, etc., and somewhat later Beketoff [42] had shown that copper could be reduced from solutions of copper sulfate and silver from solutions of silver nitrate by passing hydrogen through such solutions containing a piece of

platinum foil. On the other hand, such solutions, in the absence of platinum, suffered no reductions in the current of hydrogen alone. Then followed the beautiful discovery of Graham [197-198] in 1866–68, that platinum and particularly palladium, and other metals to a slight extent, have the power of absorbing considerable amounts of hydrogen under the influence of moderate amounts of heat, or as the result of electrolysis. Furthermore, the hydrogen in these metallic combinations was recognized as active, in the sense that it could reduce iodine to hydriodic acid, ferric salts to ferrous salts, etc., changes which can not be effected by ordinary molecular hydrogen in the cold. According to Graham, the hydrogen under these conditions becomes polarized, its affinity for oxygen thereby being greatly increased. Such hydrogen, for example, was found to combine with oxygen even in the cold, with the production of water. Now in the course of his own investigations Hoppe-Seyler found that freshly prepared palladium hydride has the power of reducing copper sulfate to metallic copper, that it decolorizes a solution of indigo, and reduces potassium permanganate in neutral solution to manganese dioxide, or in acid solution to a manganous salt; that it converts quinone into quinhydrone and changes oxyhemoglobin to methemoglobin. He found further that these powerful reductions are brought about only when the palladium hydrogen compound is fresh; if allowed to stand it loses these properties, while it is still found to contain hydrogen.

These reducing actions correspond entirely to those exhibited on dissolving zinc or tin in dilute acids; in other words, they are effected by nascent or active hydrogen. According to Hoppe-Seyler the most interesting reduction which can be effected by active hydrogen is that of molecular oxygen to water, whereby $-OH$ or $-O-O-H$, and finally water and active oxygen, are formed successively. He found further that if one shakes some palladium foil saturated with hydrogen with a dilute starch paste containing potassium iodide and air, there is produced in a few moments a dark-blue solution of the iodide of starch. In other words, through the influence of the hydride of palladium, potassium iodide is oxidized by the oxygen of the air, a change which ordinarily takes place only with extreme slowness. If now the foil be boiled with water and heated to redness to expel the hydrogen, it no longer has the power to form the iodide of starch when shaken with a solution of potassium iodide and starch in contact with air.

Similarly, indigo is oxidized to isatin and ammonia to ammonium nitrite, benzene to phenol and toluene to benzoic acid, when these several substances in aqueous solution are shaken with air and palladium hydride. All of these changes go to show that we have here to deal with very vigorous oxidations, and it can not be doubted

that they are brought about indirectly by active hydrogen. According to Hoppe-Seyler there is no probability of another explanation than that the active hydrogen renders the oxygen active, and since it, the hydrogen, combines with oxygen to form water, the process can scarcely be conceived otherwise than that when active hydrogen appropriates unto itself one atom of oxygen of the oxygen molecule to form water, it sets free the other atom of oxygen, thereby rendering it active, and that just as the free atom of hydrogen can not remain free, so this atom of oxygen can not remain free, and hence when no other oxidizable substance is present, it unites with the water to form hydrogen peroxide, or with molecular oxygen, O_2, to form ozone. The action of hydrogen upon indifferent oxygen corresponds to the action thereupon of many other substances having a strong affinity for oxygen, such, for example, as magnesium, phosphorus, etc. Hence it is that when magnesium burns in the air nitrous acid is produced (Kämmerer [237]), and in the slow oxidation of phosphorus one atom of oxygen is rendered active for every two atoms of phosphorus oxidized. This latter change Hoppe-Seyler represented in the following manner:

$$2P + 3H_2O + 2O_2 = 2(PO_3H_3) + O,$$

so that for every two atoms of phosphorus oxidized at least one atom of oxygen is rendered active. According to Hoppe-Seyler, the observation by Schoenbein that hydrogen peroxide is formed by shaking zinc dust or iron powder with air and water can scarcely be interpreted otherwise than as resulting from the reduction of indifferent oxygen by the metal. Thus in the oxidation of iron in the presence of water we would have, according to Hoppe-Seyler, the following reaction:

$$Fe + O]:[O + H_2O = FeO + H_2O_2.$$

According to this author, however, none of these processes exhibit these changes as clearly and as simply as palladium hydride. Hoppe-Seyler saw, therefore, in this remarkable compound the chemical prototype of those complex unstable hydrogen compounds resulting from the anaerobic changes occurring in living matter, the decomposition of which gives rise to active hydrogen, and upon the conduct of palladium hydride this author based his conclusions respecting animal oxidation. During putrefaction, hydrogen is produced, and while in the absence of air powerful reductions occur, in the presence of air, powerful oxidations also occur in the putrefying liquid, particularly in the upper or exposed portions thereof. He was also of the opinion that similar changes occur in all living cells. The weak point in this theory of oxygen-activation is that it accounted

for the formation of hydrogen peroxide in certain oxidations on the supposition that this compound is formed by the oxidation of water by an atom of active oxygen. Thus he explained the formation of hydrogen peroxide during the oxidation of metals in the following manner:

$$M + O_2 = MO + O,$$

and

$$H_2O + O = H_2O_2.$$

The principal objections which have been urged against this theory are, first, that it fails to take into account the formation of peroxides other than hydrogen peroxide as the result of processes of autoxidation, and, second, that it accounts for the formation of hydrogen peroxide in processes of autoxidation on the assumption that this compound results from the oxidation of water. It is now known that many peroxides other than hydrogen peroxide are produced during processes of autoxidation, and it was pointed out by Weltzien [446, 447] as early as 1860 that hydrogen peroxide can not be regarded as oxidized water. (*See also* Bach [18].) While it was afterwards claimed by Richardson [345, 347, 348] that water is oxidized during the oxidation of ether by oxygen in sunlight, apparently the precise conditions for accomplishing this oxidation have never been described, and Dunstan and Dymond [153] have shown that hydrogen peroxide is never produced by the action of oxygen on water under the influence of light and heat, even in the presence of dilute sulfuric acid. We know now that hydrogen peroxide is formed during the autoxidation of various substances, sometimes as the primary product of the oxidation of labile hydrogen atoms or hydrogen ions; more frequently as a secondary product resulting from the hydrolysis of another peroxide previously formed during the autoxidation, but never by the oxidation of water.

In a series of remarkably interesting and suggestive communications extending over a period of eleven years, from 1882 to 1893, and published for the most part in the Berichte der Deutschen Chemischen Gesellschaft, Mauritz Traube [431] laid the foundations of the peroxide theory of oxidation and enriched the nomenclature of the subject with many valuable terms, such as "autoxidation," "autoxidizable," "holoxide," etc. The clue to an understanding of Traube's peroxide theory of oxidation is to be found in the fact that water is necessary for most if not all oxidations proceeding spontaneously at ordinary temperatures and that hydrogen peroxide is produced as one of the products in all or at least in nearly all of those oxidations that are effected by molecular oxygen. He [433] made the interesting observation that pure metallic sodium retains its bright luster for forty hours in an atmosphere of dry oxygen, whereas it is instantly tarnished the moment that a trace of moisture is admitted.

He also discovered that in perfectly pure air-free water, metals like zinc and iron retain their luster undiminished, whereas if air be admitted they soon rust. Evidently, therefore, two factors are concerned in the rusting of metals, viz, water and oxygen.

It was also recognized about this time that water is essential to the oxidation of combustible gases. Dixon (140) had observed that a mixture of perfectly dry carbon monoxide and oxygen does not explode under the influence of the electric spark, nor do they combine when passed over red-hot platinum gauze, and Traube (434) himself made the observation that a flame of burning carbon monoxide is extinguished on being brought into perfectly dry air. He therefore reached the conclusion that "no substance can act upon dry oxygen at ordinary temperature." (Traube (433), p. 1881.) Other chemists, among them Nef (311) and Armstrong (13), have arrived at essentially similar conclusions respecting the necessity for water in many such processes of oxidation, and recently Dunstan, Jowett, and Goulding (154) have found that both water and oxygen, the former in the liquid condition, are necessary for the rusting of iron. Cushman (128) also, in a recent communication on the "Corrosion of Iron," states that while not agreed as to the mechanism of the process, all investigators are agreed that both oxygen and water are essential to the rusting of iron.

That hydrogen peroxide is formed during the oxidation of oxidizable substances by molecular oxygen was known even to Schoenbein (*see* Traube (431), supra), and since his time instances of its formation during oxidation had greatly accumulated in chemical literature. When, for example, finely divided zinc is shaken with air and water, the metal is gradually oxidized and hydrogen peroxide is found in considerable quantities in the solution. These observations on the production of hydrogen peroxide as the result of oxidation by molecular oxygen acting in the presence of water were greatly extended by Traube. Thus he (432) observed its production in large amounts at the cathode during electrolysis when oxygen or air is passed through the solution surrounding the negative pole, whereas it is never produced by the action of molecular hydrogen on the oxygen liberated at the anode. Schuller (391) had shown that hydrogen peroxide results from the burning of hydrogen, and on repeating these experiments Traube (435) succeeded in obtaining as much as 0.0108 gram of hydrogen peroxide by the burning of 1 liter of hydrogen. Traube (434) also made the interesting observation that hydrogen peroxide is produced by allowing a flame of burning carbon monoxide to impinge on the surface of water. These and many facts of similar import led him to believe that in all oxidations it is the molecule of oxygen and not the atom which first enters into combination with the autoxidizable substance.

To Traube, therefore, we owe the introduction into the science of a number of distinctly new ideas respecting oxidation phenomena. These are as follows:

First. That in all processes of autoxidation the oxygen molecule as a whole combines with the oxidizable substance, or with the hydrogen of water under the influence of the oxidizable substance, to form a peroxide (holoxide). Thus when hydrogen burns in air or oxygen, hydrogen peroxide is the primary product of the oxidation, the formation of water resulting from the reduction of the peroxide by the action of the hydrogen; thus:

$$H_2 + O_2 = H_2O_2,$$

and

$$H_2O_2 + H_2 = 2H_2O.$$

Second. That water actively participates in all or at least in the greater number of autoxidations, and that hydrogen peroxide is formed as a primary product of such autoxidations. When, for example, zinc oxidizes at ordinary temperatures in the presence of water the following changes occur:

$$Zn + \begin{matrix} HO \\ HO \end{matrix}\Big] . \Big[\begin{matrix} H \\ H \end{matrix} + O_2 = Zn(OH)_2 + H_2O_2.$$

Similarly, according to Traube, traces of moisture are necessary for the burning of carbon monoxide for the following reasons:

$$CO + \begin{matrix} HO \\ HO \end{matrix}\Big] . \Big[\begin{matrix} H \\ H \end{matrix} + O_2 = H_2CO_3 + H_2O_2,$$

and

$$CO + H_2O_2 = H_2CO_3,$$

and

$$2H_2CO_3 = 2CO_2 + 2H_2O.$$

Third. That the phenomenon of oxygen-carrying is due primarily to the oxidation of the second oxidizable substance, the acceptor, by the hydrogen peroxide resulting from the oxidation of the autoxidizable substance (the carrier) in the presence of water. When, for example, palladium hydride is shaken with air and water large amounts of hydrogen peroxide are formed. If, however, a second oxidizable substance is present, such as carbon monoxide or potassium iodide, it, as well as the hydrogen of the palladium hydride, is oxidized for the reason that under the influence of the palladium these are oxidized by hydrogen peroxide; thus:

$$\begin{matrix} Pd_2H \\ Pd_2H \end{matrix} + \begin{matrix} HO.H \\ HO.H \end{matrix} + O_2 = 4Pd + 2H_2O + H_2O_2,$$

and

$$CO + H_2O_2 = H_2CO_3,$$

or

$$2KI + H_2O_2 = 2KOH + I_2.$$

Fourth. Contrary to the views of Hoppe-Seyler it is not the oxygen molecule which is decomposed in autoxidations, but the molecule of water, whereby atomic hydrogen is formed, which then combines directly with the molecular oxygen to form hydrogen peroxide. Digramatically the essential points of difference between these two theories of oxidation may be represented as follows:

$$M+O]:[O+H_2O=MO+H_2O_2 \text{ (Hoppe-Seyler)},$$

and

$$M+\begin{matrix}HO\\HO\end{matrix}\Big].\Big[\begin{matrix}H\\H\end{matrix}+O_2=M(OH)_2+H_2O_2 \text{ (Traube)}.$$

The weak point in Hoppe-Seyler's theory is that it explains the formation of hydrogen peroxide by the oxidation of water. On the contrary, everything points the other way, viz, that water results from hydrogen peroxide by loss of oxygen, either as the result of decomposition or through the action of oxidizable substances.

The objection to Traube's theory is that it has not been possible to prove the presence of hydrogen peroxide among the products of all autoxidations. Thus Cushman ([128]) obtained no evidence of it in his recent study of the corrosion of iron. Traube, however, explains its absence in certain autoxidations on the ground that it is decomposed as fast as formed by the other substances produced during the oxidation. Thus in the case of the slow oxidation of iron it is probable that the hydrogen peroxide resulting from the first phase of the oxidation is partly consumed in the further oxidation of the ferrous hydroxide to iron rust. It should also be borne in mind that hydrogen peroxide is also decomposed into water and oxygen by most, if not all, of those substances whose oxidation it can accomplish, so that, all things considered, it is not surprising that it should occasionally be found to be absent from solutions in which autoxidation processes are taking place.

During recent years the peroxide theory of oxidation has been considerably extended through the labors of Bach, Engler, and Manchot, and their coworkers in this field. In the main the work of these several observers has consisted in the extension and elaboration of Traube's peroxide theory of oxidation. Thus in 1897 Bach ([18]) investigated the slow oxidation of a large number of substances and arrived at the conclusion that readily oxidizable substances combine with partially dissociated molecular oxygen, $-O-O-$,[a] to form peroxides, and that these latter substances when once formed promote and accelerate the oxidation of any other less readily oxidizable substances that may happen to be present. He also came to the conclusion that the oxidizing ferments of the blood are in reality readily oxidizable substances, having a special aptitude for forming peroxides. Similar conclusions have been reached by other chemists respecting the nature of the plant oxidases.

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[a] *See* von Helmholtz and Richarz ([208]).

According to Ostwald ([317]) Bach's theory of slow oxidation derives support from the energy relations existing between the combining substances and the intermediate and final products of the combustion. He (Ostwald) is also of the opinion that the production of unstable intermediate products is not be looked upon as an uncommon occurrence, but rather as the rule, and that as a general thing in chemical processes it is not the most stable products which are first produced, but ofttimes the most unstable.

More recently Manchot and Herzog ([282, 283]) have quantitatively studied the oxidation of indigo-white, hydrazobenzene, and a number of other complex organic compounds in alkaline solution, by air or oxygen. All of their results have gone to show that as much oxygen may be obtained from the metallic peroxides resulting from the oxidation as enters into the oxidation of the oxidizable compound itself. In other words, they found that one-half of the total oxygen consumed in such processes went to oxidize the autoxidizable substance, while the other half went to form hydrogen peroxide. Thus in the case of hydrazobenzene we would have

$$\begin{matrix} C_6H_5NH \\ | \\ C_6H_5NH \end{matrix} + O_2 = \begin{matrix} C_6H_5N \\ \| \\ C_6H_5N \end{matrix} + H_2O_2.$$

As the oxygen molecule is composed of two atoms, he also reached the conclusion that a molecule of oxygen either united with the oxidizable substance to form a primary oxide, which is subsequently decomposed by water into a simpler oxide and hydrogen peroxide, or, as in the case of indigo-white, the molecule of oxygen unites with the hydrogen of the leuco-compound, forming indigo and hydrogen peroxide. If the oxidation take place in a solution of barium hydroxide, the hydrogen peroxide is removed as fast as formed in the form of barium peroxide, equivalent amounts of indigo and barium peroxide being formed, thus:

$$In H_2 + O_2 + Ba(OH)_2 = In + BaO_2. + 2H_2O.$$

In case the hydrogen peroxide is not removed in some insoluble combination, it reacts with a second molecule of indigo-white, in which case we would have ultimately two molecules of indigo formed, thus: [a]

$$In H_2 + O_2 = In + H_2O_2,$$

and

$$In H_2 + H_2O_2 = In + 2H_2O.$$

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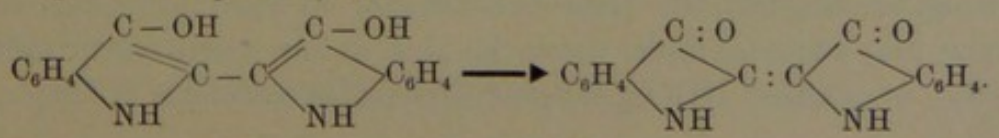
[a] Falk ([170]) in discussing the oxidation of indigo-white has offered the interesting and plausible suggestion that indigo-white may be looked upon as a phenol, which on oxidation passes to a quinone, thus:



These experiments afford quantitative proof of the peroxide theory of oxidation.

A similar case has been studied by Bamberger ([34]), who showed that when an aqueous solution of phenylhydroxylamin is exposed to the air it is oxidized to nitrosobenzene with the formation of hydrogen peroxide, thus:

$$C_6H_5NHOH + O_2 = C_6H_5NO + H_2O_2.$$

Thus three grams of phenylhydroxylamin in 40 grams of water yield, after treatment with a current of air for seventy hours, 2.5 grams of azoxybenzene.

Finally, during the last ten or twelve years Engler and his coworkers ([164, 165]) have done a great deal to extend the peroxide theory of oxidation. According to these authors every oxidation consists primarily in the union of molecular oxygen with the substance undergoing the oxidation, and that, contrary to Traube, the primary product of the autoxidation is not necessarily hydrogen peroxide but a peroxide of the substance undergoing oxidation. Thus when rubidium burns in air it is converted quantitatively into rubidium peroxide—

$$Rb + O_2 = RbO_2,$$

and, as shown by Baeyer and Villiger ([33]), when benzaldehyde is exposed to oxygen or air it is first converted into benzoyl-hydrogen peroxide, thus:

$$C_6H_5CHO + O_2 = C_6H_5CO.O.OH.$$

Engler and his coworkers have also pointed out that hydrogen peroxide, so frequently encountered in processes of autoxidation, may be produced in several entirely different ways; first, as the primary product of the autoxidation—as, for example, the burning of hydrogen or by the action of oxygen on the hydrogen liberated at the cathode during electrolysis, or in the oxidation of substances like indigo-white and hydrazobenzene, which contain labile hydrogen atoms, thus:

$$\begin{matrix} H- & -O \\ H- & -O \end{matrix} = H_2O_2,$$

and

$$In H_2 + O_2 = In + H_2O_2;$$

or, as Traube pointed out, it may result from any autoxidation in which water is essential to the oxidation; thus:

$$Zn + \begin{matrix} HO \\ HO \end{matrix}\Big] . \Big[\begin{matrix} H \\ H \end{matrix} + O_2 = Zn(OH)_2 + H_2O_2.$$

According to Engler, the true autoxidator in such processes is the hydrogen ion, while he looks upon the zinc as the pseudo-autoxidator. Secondly, as pointed out by Engler, hydrogen peroxide is frequently

a secondary product resulting from the decomposition or hydrolysis of the primary peroxide by the action of water. Thus, Clover and Houghton ([122]) have observed that succinic peroxide acid is readily hydrolyzed by water, yielding succinic acid and hydrogen peroxide, in the sense of the equation—

$$\begin{array}{l}CH_2COOH\\|\\CH_2CO\\CH_2CO\\|\\CH_2COOH\end{array}\!\!\rangle O_2 + 2H_2O = 2\begin{array}{l}CH_2COOH\\|\\CH_2COOH\end{array} + H_2O_2.$$

Finally, Engler and Weissberg ([164]), have laid considerable stress upon the idea that dissociation or the liberation of free valences, both in the oxygen molecule and in the substance undergoing autoxidation, are necessary for autoxidation processes, and in this way they explain the effect of heat and light in accelerating oxidations and also account for the ease with which unsaturated organic compounds undergo oxidation. They assume, with von Helmholtz and Richarz ([208]) and Bach ([18]), a partial dissociation of the oxygen molecule, resulting in the complex $-O-O-$, thereby enabling them to account for the direct addition of the whole molecule of oxygen to the oxidizing substance.

In the light of these considerations the phenomenon of oxygen-activation is easily explained. According to Engler and his followers it is due primarily to the oxidation of the second substance—the acceptor—by the peroxide (moloxide) resulting from the autoxidation of the carrier. Thus, when an autoxidizable substance, A, finds itself in contact with oxygen and a second oxidizable substance, B, the following changes would occur:

$$A + O_2 = AO_2,$$

and

$$AO_2 + B = AO + BO.$$

In this way a substance incapable of combining directly with oxygen may be oxidized through the intervention of another substance, and a given quantity of oxygen equally distributed between them. To take the case of the oxidation of benzaldehyde and the changes which may be accomplished through the oxidation of this compound at ordinary temperatures in the air. When exposed to the air this compound is converted into benzoyl-hydrogen peroxide (*see* Baeyer and Villiger ([33]))—

$$C_6H_5CHO + O_2 = C_6H_5CO-O-O-H.$$

If allowed to remain in contact with water, benzoyl-hydrogen peroxide is hydrolyzed with the production of benzoic acid and hydrogen peroxide—

$$C_6H_5CO.O.OH + HOH = C_6H_5COOH + H_2O_2.$$

On the other hand, if allowed to remain in contact with a second molecule of benzaldehyde, it oxidizes the second molecule of the aldehyde with the production of two molecules of benzoic acid,

$$C_6H_5CO.O.OH + C_6H_5CHO = 2C_6H_5COOH;$$

or finally, if an oxidizable substance, such as indigo, be present, it oxidizes the indigo with the production of benzoic acid and isatin, thus—

$$C_{16}H_{10}N_2O_2 + C_6H_5CO.O.OH = 2C_8H_5NO_2 + 2C_6H_5COOH.$$

Hence when a solution of indigo is shaken with benzaldehyde both are oxidized, whereas the indigo alone is unchanged by molecular oxygen.

Chapter II.

THE OXIDIZING FERMENTS.

In its present shape, therefore, the peroxide theory of oxidation accounts for the phenomena of autoxidation and oxygen carrying upon the supposition that spontaneously oxidizable substances have the power of combining with partially dissociated molecules of oxygen to form peroxides. These peroxides may then react either with additional amounts of the autoxidizable substance itself or with some other substance to form simpler oxides. Thus, it may happen that as the result of its oxidation one substance, *A*, may effect the oxidation of a second substance, *B*, which latter is not directly oxidizable under ordinary conditions. In this way the modern theory of oxidation affords a simple explanation of the formation of peroxides and of the phenomenon of oxygen carrying.

The numerous applications of this theory are by no means confined to the inorganic world. In the life cycle of the plant and animal we meet with many changes which, in so far as oxygen is involved, find their simplest explanation in terms of this hypothesis. The affinity for oxygen which is displayed not only by the warm-blooded animals, but even by plants, is often remarkable. That such is the case may be gathered from the fact that Phipson ([320]) in 1896 employed one of the higher fungi, *Agaricus atramentarius*, as an agent wherewith to effect the analysis of atmospheric air. It has so happened, therefore, that in the history of our science both a mouse and a mushroom have been employed as oxygen absorbents wherewith to determine the quantity of oxygen in atmospheric air. During the absorption of oxygen by plants various substances are produced which are capable of effecting oxidations which ordinarily can not be brought about by molecular oxygen alone, such, for example, as the bluing of guaiacum, the conversion of hydroquinone into quinone, the liberation of iodine from potassium iodide, etc. Like many of the chemical agents of the living cell, these powerful oxidizing substances are characterized by great instability. They are destroyed by heat and mineral acids and by various poisons, such as hydrogen cyanide, sulphur dioxide, phenylhydrazin, etc. It is claimed by some observers that they act catalytically, and hence are believed to partake of the nature of ferments. These substances are the *oxidases*,[a] or oxidizing ferments,

[a] The following general references to the literature of this subject will be found of interest in this connection: Remarques sur les matières oxydantes, que l'on peut rencontrer chez les êtres vivants, by Bourquelot ([85]); Ueber Oxydationsfermente, by

and closely associated with this remarkable group of substances are the *peroxidases* and *catalases*, which have the power of decomposing hydrogen peroxide or rendering active the oxygen which it contains.

While in many instances, as pointed out by Wender [448], the precise rôle of these catalysts in the life of the cell has not as yet been determined, it is known that they are very widely distributed in the plant and animal kingdoms and that they are concerned in many important bio-chemical processes. Thus, according to Tolomei [423] and Martinand [286] the oxidation of the coloring matter of the grape and the production of those aromatic substances which confer upon old wine its delightful aroma and taste is due to the action of oxidases which are present in the grape and in the yeast. Aso [15] has shown that oxidases are concerned in the curing of tea. They probably also play a part in the curing of tobacco, and in the change of color of any vegetable tissue from green to brown, which results finally in the production of humus substances. According to Grüss [201], the oxidases play an important rôle in the formation of starch grains. Hahn [204] found an oxidase in *Arum maculatum* which had the power of oxidizing grape sugar and which apparently assists in intramolecular respiration, and Raciborski [337] is of the opinion that the vegetable oxidases play the same rôle in the vasculiferous plants as that played by hemoglobin in the higher animals and hemocyanin in the lower animals, viz, that of the oxygen-laden vehicle of respiration. Woods [462] has shown that oxidases are responsible for the etiolation of green leaves.

Palladin [317a] and also Miss Wheldale [449a] are of the opinion that anthocyanin, the red pigment of certain flowers, results from the action of an oxidase on some colorless, chromogenic substance contained in the flower.

The oxidases are by no means confined to the plant, but are found in many animal tissues, where they are assumed to play an equally important rôle in oxygen metabolism. Thus, Dubois [147] attributes the phosphorescence of phosphorescent animals and plants to the action of an oxidase to which he has given the poetic name of "Luciferase," and this same observer [148] has traced the formation of a purple dye by the mollusc *Murex brandaris*, to the action of an oxidase which he calls "Purpurase." Carnot [111] found that the saliva and other secretions contain certain oxidases. Portier [330] has pointed out that oxidases are concentrated in the epidermal and exposed structures of

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Steudel [411]; Zur chemischen Erkenntnis biologischer Oxydationsreaktionen, by Engler and Herzog [163]; Contribution a l'Etude des Applications Therapeutiques des Oxydases et des Metaux Ferments, by Pierre Sée [398]; Die Bedeutung der Katalyse für die Medicin, by H. Schade [360]; Les Oxidations de l'Organisme, by Enriquez and Sicard [166]; Ueber die Oxydationsfermente der Leber, by Jacoby [224]; Die Oxydasen, by Neumann Wender [448]; The Properties and Classification of the Oxidizing Enzymes, and Analogies between Enzymic Activity and the Effects of Immune Bodies and Complements, by Moore and Whitley [306]; Les Ferments Oxydants, by Chodat [116].

the organism. He is of the opinion, therefore, that their physiological rôle both in animals and plants is one of defense against invasion by micro-organisms. In this connection Sieber (402) has shown that the oxidases have the power of destroying toxins. According to her observations, the fibrin of the blood of a normal horse gives no oxidase reactions, whereas the fibrin obtained from the blood of a horse immunized against diphtheria gives a blue coloration with guaiacum, indicating the formation of oxidases in the blood as the result of immunization. As we shall see, one of the oxidases, tyrosinase, is the active agent in the production of melanins, and hence of profound significance in its relation to certain pathological conditions of melanogenesis in man. In this connection Hougonenq and Paviot (220) claim to have found that certain malignant tumors give the guaiacum reaction. Still other facts of interest, pertaining to the occurrence of the oxidases or to their mode of action, are set forth in the following pages.

THE GUAIACUM REACTION.

Our first knowledge of the oxidizing ferments is closely associated with what is known as the guaiacum reaction, viz, the production of a blue coloring matter when gum guaiacum or its tincture is treated with certain oxidizing agents. According to Binz (68) our knowledge of guaiacum dates back to the sixteenth century when in 1508 it was first imported into Spain from Santo Domingo as a remedy for syphilis by Consolvus Ferrand, himself a syphilitic, soon after which it became known all over Europe as an antisyphilitic. (*See* Monograph of Ulrich von Hutten, De Guajaci Medicina et Morto Gallico; Liebermann, Mainz, 1519, p. 74).

That guaiacum resin becomes bluish-green in color on standing in the air and light and the liquid around the stoppers of bottles containing tincture of guaiacum generally acquires a bluish-green color are matters of everyday experience to those who have ever had occasion to use these substances. In 1804 Wollaston (461) was the first to show that this color change on the part of guaiacum is brought about by the air under the influence of light. According to this author, rays of light which cause the emission of oxygen by silver chloride cause its absorption by gum guaiacum. A few years later William Brande (94) showed that guaiacum becomes blue in pure oxygen gas more rapidly than in air.

Shortly afterwards a number of interesting observations on the bluing of guaiacum were made by the French pharmacists. Thus it was pointed out by Boullay (73) that a mixture of simple sirup, distilled water, gum arabic, and tincture of guaiacum takes on the color of a suspension of verdigris, and further that certain dentifrices containing guaiacum, when taken into the mouth, become intensely blue or green. This change of color had been attributed to impuri-

ties in the mouth cavity; he proved, however, that gelatin and albumin produced similar changes of color with alcoholic guaiacum. He therefore reached the conclusion that this change of color is without doubt due to the albumin of the saliva, since no change of color is observed if the mouth be deprived of saliva by previous washing with water. Similarly, according to Marc (285), it was pointed out by Goettling that a mixture of guaiacum resin, gum arabic, sugar, and water of peppermint becomes sensibly blue; that certain acids impart a bluish tint to guaiacum resin, and that this same change is brought about by sweet spirits of niter.

In 1810 Planche (326) in a note on the sophistication of Jalap Resin and a means of recognizing the same, calls attention to the fact that guaiacum resin takes on an intense blue color when exposed for a few minutes to the vapors of nitric acid, and further that the fresh root of the horse-radish also has the power of turning the tincture of guaiacum resin blue. In order to show this, he says, it is only necessary to plunge a little piece of the fresh root into a glass containing the tincture of guaiacum, when little by little the liquid acquires the color of indigo in sulfuric acid. In 1819 Taddey (sometimes spelled Taddei) (416), having had occasion to knead together several species of gum resins and resins proper with different sorts of flour, observed that the mixture of wheat flour and guaiacum becomes blue, especially after water has been added to the mixture exposed to the air. At his suggestion Rudolphi (416) followed up the investigation of this subject. He found that a mixture of guaiacum resin with pure starch does not develop a blue color when moistened and exposed to the air, nor is this blue color developed by other vegetable materials which do not contain *zimôme*, the name proposed by Taddei for the constituent of gluten insoluble in alcohol. He also observed that guaiacum is not colored, or at least only slowly, by flours poor in gluten and that it is not colored by those flours in which the gluten has suffered any great alteration. He observed further that when gluten or pure *zimôme* is mixed with guaiacum it develops a superb blue color instantly, but that such a mixture only develops this color in atmospheric air. Rudolphi therefore proposed to make use of guaiacum as a reagent for judging of the purity and quality of different kinds of wheat flour, and conversely, he recommended wheat flour as a reagent for testing the purity of guaiacum resin.

In 1820 Planche (327) undertook to determine the nature of the substance which produces the blue coloration with guaiacum. He conceived the idea that air and light had nothing to do with the bluing of guaiacum for the reason that if this change of color is due solely to the action of air and light, why is it, he asks, that when guaiacum resin is mixed with certain substances it is colored blue or green, whereas when mixed with other substances its natural color is

not altered? In attempting to answer this question he was led to test the conduct of a large number of substances toward guaiacum, among them a large number of roots, fresh and dried, several gums, milk, soap, etc. Among the fresh roots tested the following were found by this observer to blue guaiacum: Comfrey (*Symphytum consolida*, L.), dandelion (*Leontodon taraxacum*), common iris (*Iris germanica*), chicory (*Cichorium intybus*), thistle (*Eryngium campestre*), white water lily (*Nymphyaea alba*), potato (*Solanum tuberosum*), bryony (*Bryonia dioica*), elecampane (*Inula helenium*), marshmallow (*Althea officinalis*), carrot (*Daucus carota*), licorice (*Glycyrrhiza glabra*), turnip (*Napis sativa*), burdock (*Arctium lappa*), colchicum (*Colchicum autumnale*), soapwort (*Saponaria officinalis*), scurvy grass (*Cochlearia officinalis*), fumitory (*Fumaria officinalis*), figwort (*Scrophularia officinalis*), sorrel (*Rumex acetosa*), viper's grass (*Scorzonera hispanica*), asparagus (*Asparagus officinalis*), borage (*Borago officinalis*), angelica (*Angelica archangelica*), onion (*Allium caepa*), wild radish (*Cochlearia armoracia*, L.), little radish (*Raphanus sativus*). The fresh root of the chicory, especially in the fall of the year, was found to give with guaiacum a magnificent blue color. So also the fresh root of the water lily produced a very intense coloration at first, which faded rapidly on standing. For the most part the dried roots gave no color with guaiacum. Neither did the fresh roots of the following plants: Patience (*Rumex acutus*, L.), male fern (*Polypodium filix mas*), strawberry (*Fragaria vesca*). Furthermore he found that light was without influence on the development of the blue color of guaiacum by roots capable of effecting this change. Thus the parsnip caused the bluing of guaiacum in the dark. Similarly when guaiacum and white soap were mixed together there resulted a greenish mass which finally took on the color of verdigris, and the interior of the mass acquired a deep blue color. Obviously the light could not have been responsible for this increase of the color in the interior portions of the mixture. He also observed that milk has the power of bluing guaiacum, and that this change of color takes place in a vacuum. He concludes, therefore, that in the bluing of guaiacum by milk, air is not responsible for the change of color. On boiling, however, milk lost its power to blue guaiacum, and in general, those substances, gums and plant roots, which have the power of bluing guaiacum, lose this power by heat. Thus the potato, parsnip, and beechnut (*Fagus castanea*), cooked in closed vessels in their own juices, do not react with either the powdered resin or the tincture. He therefore concluded that the substance or substances causing the bluing of guaiacum are volatile and possibly condensable. He was therefore led to test the conduct of the distillate obtained from milk toward guaiacum, with the result, however, that no change of color was produced. He therefore had to renounce the idea that the

principle responsible for the bluing of guaiacum is volatile. He was led to believe, therefore, that this particular species of cyanogen (*cyanogene*), which is responsible for the bluing of guaiacum, whatever its nature, is absorbed by certain substances which in its ordinary state permit of this action, but that when exposed to certain temperatures it enters into new combinations and obeys other laws which do not permit of its coloring guaiacum.

To anyone at all familiar with the oxidizing ferments it is evident how close Planche came to the discovery of this remarkable group of substances. As a matter of fact he seems to have been the first to encounter them, and when he proved that heat destroys the power of milk and certain plant juices to blue guaiacum, he discovered one of their most remarkable characteristics, and had the state of knowledge regarding oxidation and fermentation been at this time what it was in the time of Schoenbein and Traube, there is scarcely room for doubt that he, and perhaps Taddei also, would have recognized the true nature of these substances and the part which they play in oxidation processes.

From the time of Planche to that of Schoenbein and Traube there appears to have been but little advance in our knowledge of the guaiacum reaction, except the discovery of various substances, organic and inorganic, which effect this change of color to a greater or less extent. Thus Regimbau, ([340, 341]) in letters to Planche, described the bluing of pills containing guaiacum resin, mercuric chloride, and white soap, and advanced the view that this bluing was due to the liberation of chlorine from the mercuric chloride, a view which Planche pointed out as erroneous; and Lodibert ([274]) reported results with dentifrices similar to those observed by Boullay ([73]). Pelletier and Deville ([318]) also published a paper on guaiacum in 1844, in which, however, there is nothing of any particular interest.

The further elucidation of the guaiacum reaction and the final discovery of the oxidizing ferments awaited the discovery of ozone and the renewed interest in the general subject of fermentation awakened by the writings of Pasteur and Liebig. In this connection it will be recalled that ozone was first recognized as a definite substance by Schoenbein ([368, 369]). In 1845 Schoenbein ([370]) showed that guaiacum is colored blue by ozone. The interest attaching to Schoenbein's earlier observations on the bluing of guaiacum by ozone is such in this connection that I shall present the subject in practically the author's own words. In his "Note on guaiacum resin" he goes on to say that—

> It has long been known that guaiacum resin under certain conditions becomes blue and that chlorine has the power of producing this change of color. I have found that bromine and iodine produce the same change. In consequence of the close similarity which chlorine, bromine, and iodine exhibit to ozone, one would naturally

expect that the latter substance would blue guaiacum resin, and my experiments have shown that such is really the case. If one brings a strip of filter paper which has been saturated with guaiacum tincture into strongly ozonized air, prepared by means of phosphorus, the paper at once takes on a blue color. As a matter of fact, guaiacum resin is as sensitive a reagent for ozone as the potassium iodide and starch mixture. If one allows the strips to remain for a somewhat longer time in the ozonized atmosphere, the color changes from blue to yellowish brown, as is the case with chlorine. Ozone therefore conducts itself towards guaiacum resin in precisely the same way as chlorine. It is scarcely necessary to point out that ozone produced by the other two methods, namely, by the electrolysis of water and by the action of the electric discharge on air or moist oxygen, acts in the same way toward guaiacum. Since the bluing of guaiacum undoubtedly depends on the action of oxygen, and since free oxygen does not act on the resinous mass, this element must first be gotten into a state of chemical activity in order to oxidize the guaiacum. This condition seems to be called forth even by sunlight. It is not known, however, whether dry oxygen can act on the water-free resin in the sunlight. It may well be that in the absence of water the guaiacum resin can, as is the case with other organic substances, take up oxygen at ordinary temperatures to a slight extent. Be this as it may, it is nevertheless a fact that the oxygen which is in association with certain other substances has such chemical activity that it can act upon guaiacum, or upon a substance with which it is in combination, at ordinary temperatures. If one believes, as indicated by the older theories, that chlorine, bromine, and iodine are the peroxides of murium, bromium, and iodium, one must believe that there exists in these compounds an equivalent of oxygen which is in this chemically active state, and that it is this oxygen which calls forth the above-described color change in guaiacum resin. If, as we hold, ozone is to be looked upon as a compound of oxygen with water, it is the chemically excited oxygen of this compound which blues the guaiacum. As I have elsewhere pointed out, compounds of certain of the metals act upon guaiacum in the same way as do chlorine and ozone. For example, if one mixes pure lead or manganese peroxide in water with a solution of guaiacum, the latter is instantly colored blue just as if brought into chlorine water. It is remarkable, and so far as I know a new fact, that guaiacum which has been blued in this way loses its color again if it is introduced into an atmosphere containing hydrogen sulfide or sulfur dioxide, or when added to a solution of stannous chloride; under these conditions the oxygen in combination with the guaiacum appears to be again removed. Finally, I have observed that all those substances which color a potassium iodide solution yellow to brownish red, or blue the potassium iodide and starch mixture—that is, all substances which have the power to cause the separation of iodine; for example, chlorine, bromine, ozone, nitrous acid, and the peroxides of manganese, lead, and gold—have also the power to blue the tincture of guaiacum. Conversely, those substances which have the power to remove the yellow color from potassium iodide solutions and the blue color from the potassium iodide and starch mixture—for example, hydrogen sulfide, sulfur dioxide, and stannous chloride—have also the power to destroy the blue color of guaiacum resin.

In a further note on guaiacum resin Schoenbein ([371]) shows that guaiacum is also blued by the products of the slow combustion of ether, for the reason that an ozonid is produced. Schoenbein ([372]) next makes use of the bluing of guaiacum as a reaction for indicating the presence of an electric current, he ([373]) having observed that strips of paper saturated with tincture of guaiacum are colored blue when exposed to the oxygen liberated from water by electrolysis and to the

ozone produced by the electric discharge in air or oxygen. In 1847 Schoenbein [374] seems to have published a résumé of his work on the bluing of guaiacum up to that time, mentioning the reduction of the blue compound by hydrogen sulfide and other reducing agents, and the general similarity between guaiacum-blue and the blue iodide of starch. In other still later communications on guaiacum resin [375.376], he calls attention to the considerable number of substances which have the power to blue guaiacum, among which may be mentioned chlorine, bromine, iodine, ozone, certain peroxides such as those of manganese and lead, silver oxide, and acetate, cupric chloride, ferric chloride, mercuric chloride, potassium ferricyanide, the bichromates and permanganates, and even finely divided platinum. He also points out that guaiacum which had been blued by any one of these substances gradually loses its blue color on standing, but that this may be restored by adding fresh quantities of the reagent. This may be repeated a certain number of times, but finally a colorless or brownish product of the resin is obtained which is no longer capable of being blued by ozone or similar substances.

He calls attention to the fact that the blue color of guaiacum is destroyed by phosphorus, the metals, hydrogen sulfide and selenide, sulfurous acid and hyposulfites, ferrous and stannous salts, and by acids and alkalis. He also points out that the majority of those substances capable of bluing guaiacum contain their oxygen in the peculiarly active condition in which this element is met with in ozone. In other words, these substances contain *oxylisirten* or *erregten* oxygen, or can give rise to the same. He points out a number of analogies between guaiacum blue and the iodide of starch, as to color, general methods of formation by the action of oxidizing agents, conduct towards reducing agents, etc., and he reached the conclusion that the blue material resulting from the action of ozone on guaiacum is a compound of ordinary guaiacum with a hydrogen peroxide (ozone), of much the same nature as the loose chemical combination met with in the iodide of starch. He goes on to say further that—

> I need scarcely remark that for those who with De la Rive and Berzelius look upon ozone as nothing but modified oxygen, it is only necessary to assume that guaiacum blue consists of a loose combination of ordinary guaiacum and this uncommon oxygen. In other words, that it possesses the nature of an organic peroxide which contains at least a part of its oxygen in the chemically excited or active condition in which it is met with in hydrogen peroxide, ozone, and manganese dioxide.

Finally, he explained the spontaneous decolorization of guaiacum blue and its ultimate conversion into a substance no longer capable of yielding the blue compound by treatment with ozone or other oxidizing agents, upon the supposition that the chemically active oxygen in loose combination in the blue resin can exist for only a short time as such in this compound and that this form of oxygen at ordinary

temperatures, or even at 0°C., acts slowly on the oxidizable constituents of the resin, probably extracting hydrogen and possibly carbon, and thereby altering the original composition of the guaiacum.

Schoenbein (377) next turned his attention to the bluing of guaiacum by those substances contained in the fresh tissues of plants, and in 1848 he gave to the chemical world the results of his first researches in this highly fruitful field of investigation in a communication entitled "On certain chemical reactions of the potato." In this paper he refers to the observations of Planche[a] and Taddei on the bluing of guaiacum by many roots and tubers, and to the fact that the latter appeared to consider air as necessary for the reaction. His own experiments indicate that there is unevenly distributed throughout the potato some substance having the power of bluing guaiacum, the most rapid bluing being produced by the under side of the potato peel and at the points where the "eyes" or sprouts occur. According to Schoenbein, the starch and expressed juice of the potato, however, do not possess this power to the slightest degree, and, upon boiling, all parts of the potato entirely lose their power to blue guaiacum. On the other hand, the freshly cut peel of the potato blues guaiacum as quickly as does manganese dioxide or lead peroxide, and the blue solution shows all of the properties of that obtained by the latter method, fading slowly in the air at ordinary temperature, and rapidly upon boiling being decolorized by hydrogen sulfide and other reducing agents and precipitating a blue resin when added to water. He observed also that the potato, like the other substances having the power to blue the guaiacum resin and tincture has the power of decomposing potassium iodide with the liberation of iodine, the latter reacting with the starch of the potato to form the blue iodide of starch. For these reactions Schoenbein offers two alternate explanations; either that the potato contains a substance analogous to the peroxides, ozone, etc., or that it contains a substance having the power of activating the oxygen of the air, and thus rendering it capable of producing these phenomena.

On the other hand, Nasse and Framm (310) (*see also* Nasse (309)) claim that fresh extracts that have been completely freed of oxygen by the prolonged action of hydrogen or carbon dioxide still give a blue

[a] In all of Schoenbein's original communications on this subject, this author (Planche) is referred to under the name of "Blanche." Thus a good deal of confusion has been introduced into this literature, and this is met with repeatedly, even in recent writings on the subject. Thus this error is repeated by Nasse and Framm in an article entitled "Bemerkungen zur Glykolyse" (Pflüger's Archiv., 1896, vol. 63, p. 207), in which the mechanism of the guaiacum reaction is discussed; and very recently again by Engler and Herzog, in "Zur Erkenntnis der biologischer Oxydationsreaktionen" (Hoppe-Seyler's Zeitschrift für physiologische Chemie, 1909, vol. 59, p. 357). For the benefit of those who may be interested in the historical development of this subject, the writer is glad to be able to correct this error.

color with guaiacum. Hence, according to these authors, the bluing of guaiacum by plant extracts and ferments, such as diastase and emulsin, is due not to an oxidation of the guaiacum by the oxygen of the air, but to a hydrolysis (*hydroxylirung*) of the guaiacum by the plant extract or ferment. Bertrand (51), however, is of the opinion that the bluing of the guaiacum is due to the combined action of oxygen and laccase, and Kastle and Loevenhart (244) were unable to obtain any evidence of the bluing of guaiacum by the freshly abraded surface of the raw potato in an atmosphere of hydrogen, carbon dioxide, or nitrogen. On the other hand, in air or oxygen, the freshly abraded surface of the potato was always found by these observers to develop a blue color instantly on the application of tincture of guaiacum.

OXYGEN-EXCITERS AND OXYGEN-CARRIERS ("SAUERSTOFFERREGERN" AND "SAUERSTOFFTRÄGERN," *Schoenbein*).

In 1855 Schoenbein (378) made a study of the spontaneous bluing of certain fungi. The results of these interesting observations were given to the world in a communication entitled "Ueber die selbst Bläuung einiger Pilze und das Vorkommen von Sauerstofferregern und Sauerstoffträgern in der Pflanzenwelt," and also in a letter to Faraday on "Ozone and ozonic actions in mushrooms." The titles of these two communications give a good idea of their contents. In brief his results were as follows: It had long been recognized that certain varieties of the higher fungi, notably the *Boletus luridus*, have the remarkable property of rapidly turning blue when the head or stem is broken or bruised in any way. Schoenbein now conceived the idea that in such color changes we have to do with phenomena similar to the bluing of guaiacum by the fresh tissue of the potato and other fresh roots. As a matter of fact he found this species of boletus to contain a colorless principle easily soluble in alcohol, and exhibiting the closest analogy to guaiacum, in that all oxidizing agents which blue a tincture of the latter also blue an alcoholic solution of the chromogenic substance of the mushroom, and, further, that all deoxidizing agents which discharge the color of guaiacum blue also discharge the color of the blued fungus extract. From these observations he was led to conclude that the chromogenic principle of the fungus, like guaiacum, is capable of combining with ozonized oxygen, Ô, whereas it is not affected by ordinary oxygen (O).

The fact that the alcoholic solution of this coloring principle of the Boletus is not spontaneously colored by atmospheric air, but is colored by air in the parenchyma of the fungus, led him to suspect that there exists in the fungus another substance endowed with the property of exalting the chemical power of ordinary oxygen, thereby causing this element in its active form, Ô, to associate itself with the

coloring matter of the fungus. As a matter of fact, he found in the expressed sap of this and other fungi, notably in *Agaricus sanguineus*, an organic matter which has this remarkable power of transforming ordinary oxygen (O), into ozonized oxygen, Ô, and of forming with the latter a compound analogous to lead peroxide (PbO + Ô), which readily gives up this active oxygen, Ô, to a number of easily oxidizable substances, both organic and inorganic, among them guaiacum and the chromogenic substance of *Boletus luridus*. He observed, further, that after having been deprived of its active oxygen, this peculiar compound may be charged with it again by simply passing a current of air through its solution. He then goes on to say that this peculiar substance may well be compared with nitric oxide, which enjoys to a remarkable extent the power of instantaneously transforming inactive oxygen, (O), into active oxygen, Ô, thereby forming a peroxide containing Ô, and from which this peculiar form of oxygen may easily be transferred to a multitude of oxidizable substances. In other words, in addition to the chromogenic substance which it contains and through whose oxidation it ultimately becomes blue, the *Boletus luridus* and other fungi, notably the *Agaricus sanguineus*, contain a substance capable of ozonizing the oxygen of atmospheric air; in other words, they contain a *Sauerstoff-erreger*, or oxygen-exciter. This substance enters into a loose combination with the ozone thus produced, forming therewith a compound analogous to a peroxide containing active oxygen, which in turn is capable of giving up its active oxygen to guaiacum or to the chromogenic substance contained in the fungus itself, thus turning it blue. In other words, the substance responsible for the bluing of the chromogenic substance contained in the boletus is not only a *Sauerstofferreger* but also a *Sauerstoffträger*, or a true carrier of oxygen.

Schoenbein was also impressed with the instability of these remarkable *Sauerstofferregern* and *Sauerstoffträgern* of the plant world. Thus he points out that on heating to boiling an aqueous solution of the fungus which has the power of deeply bluing a tincture of guaiacum, the solution not only loses this power, but was also found to lose the power of ever again becoming an oxygen-exciter or carrier, no matter how long it was kept in contact with atmospheric air. Finally he goes on to say that these substances which have the power of bringing into activity the inactive oxygen of the air must play an important rôle in the oxidation processes of vegetable materials, and that it is not an unwarrantable supposition that in the animal world as well there exist substances capable of ozonizing atmospheric oxygen at ordinary temperatures, thereby promoting the oxidation of other animal substances, and in a later article ([380], [386]) on the conduct of oil of bitter almonds to oxygen, in which he proved that this compound belongs to the class of ozonizing substances, he says that new

observations have made it in a high degree probable that there exist in the blood of animals substances having the power to change the respired inactive oxygen in a similar way to that accomplished by phosphorus and oil of bitter almonds—that is, substances which render the oxygen active, whereby the oxidation phenomena observed in the life cycle are brought about. He adds, further, that without the presence of such substances as convert (O) into Ȯ the animal would be suffocated in the midst of an ocean of the purest, but inactive, oxygen as quickly as it would be in a vacuum.

Schoenbein's remarkable observations in this field, however, did not end here. From his extended investigations on ozone and hydrogen peroxide he had reached the conclusions that oxygen can exist in three forms, viz, common oxygen, which he represented by O or (O), ozone, Θ or (Θ), and antozone, ⊖ or (⊖); and that deriving from these three modifications of oxygen, respectively, we have three classes of oxides, viz, (1) ordinary oxides, such as water; (2) ozonides, which included most if not all of the peroxides of the heavy metals, such as manganese dioxide and lead peroxide, and (3) antozonides, like hydrogen peroxide and barium dioxide

O	Θ	⊖
HO (old formula),	$MnO + \Theta$,	$HO + \ominus$,
CaO,	$PbO + \Theta$,	$BaO + \ominus$,

Of these several forms of oxygen, the ozonic modification was the most active. He had observed, further, that just as the common oxygen is transformed into ozone through the influence of ozonizers, such as phosphorus, platinum, etc., so the oxygen of the antozonides ⊖ may be transformed into the ozonic modification, Θ, by the action of certain substances such as platinum or lead salts, etc., and that finally as a result of the neutralization of their electrically opposite potentials, an ozonide reacts with an antozonide with the production of common oxygen. Thus ozone and hydrogen peroxide give rise to common oxygen and water:

$$\Theta + HO + \ominus = HO + 2O.$$

He found, for example, that guaiacum blue results from the action of finely divided platinum on tincture of guaiacum and air or hydrogen peroxide, whereas neither of these substances can effect this alteration in the color of the guaiacum, or can do so only with extreme slowness, when used alone. It had been previously observed by Thenard that hydrogen peroxide is decomposed by finely divided platinum into water and common oxygen. All of these facts found a simple explanation in terms of Schoenbein's theory. According to this theory, neither common oxygen nor antozone nor an antozonide can blue guaiacum, for the reason that the resin only combines with the ozonic

form of oxygen, Θ. On the other hand, ozone or any ozonide can accomplish this change for the reason that any of these substances can supply Θ, and platinum can accomplish this oxydation by common oxygen or hydrogen peroxide for the reason that it can transform common oxygen or the ⊖ of an antozonide into ozone, Θ. This theory also enabled one to understand the catalytic decomposition of hydrogen peroxide by platinum into water and common oxygen, since the ozone resulting from the action of the platinum on the antozonide would react with another portion of the antozonide, giving rise to water and common oxygen ([382]):

$$Pt + HO + \ominus = Pt + HO + \Theta,$$

and

$$HO + \Theta + HO + \ominus = 2HO + 2O.^{a}$$

In 1857 Schoenbein published a paper on "Chemical contact actions" ([379]), in which he discusses the action of various oxygen-carriers and oxidizing agents on guaiacum, and states that blood corpuscles separated from fibrin and serum and dissolved in water give the guaiacum reaction strongly in the presence of hydrogen peroxide, as does also the gluten of wheat, but that both are inactive in the absence of the peroxide. He regards this oxygen-carrying power of blood corpuscles as important to respiration. In this article he also gives the results of experiments with platinum and guaiacum. Three years later he ([381]) called attention to the fact that iron salts act toward a mixture of guaiacum and hydrogen peroxide in much the same way as do blood corpuscles and that the activity of the blood corpuscles toward guaiacum is proportional to their content of hemoglobin, and that it therefore seemed not improbable that the guaiacum reaction of blood is dependent on the iron content of the pigments. This, he says, is a matter of importance to physiological chemists.

In view of his previous work on the presence of oxygen exciters and oxygen carriers in certain of the higher fungi and in the potato, it was only natural that it should have occurred to Schoenbein, especially in the light of the earlier observations of Planche, that substances capable of bluing a mixture of hydrogen peroxide and guaiacum and of decomposing hydrogen peroxide into water and common oxygen should be widely distributed in the plant and animal kingdom. The results of his investigations on the decomposition of hydrogen peroxide by plant and animal extracts and ferments and on the bluing of guaiacum by hydrogen peroxide through the agency of organic

[a] A good discussion of Schoenbein's ozone and antozone theory is to be found in his paper on the "Conduct of blood toward oxygen" ([384]), a translation of which is given in the Medical Press and Circular, June 20 and July 4, 1866, and in Day's paper ([135]) on "Polarized or allotropic oxygen," in the Australian Medical Journal, vol. 12, 1867, pp. 141–149.

materials were first presented in a paper entitled "Ueber die katalytische Wirksamkeit organische Materien und deren Verbreitung in der Pflanzen- und Thierwelt" (383) in 1863. He calls attention to the fact that in 1863 the group of phenomena called by Berzelius "catalytic actions" were as yet but very imperfectly understood, especially such as are concerned with changes in the composition of organic compounds brought about by the action of ferments, such as the alcoholic fermentation and the conversion of starch into sugar through the action of diastase. He was therefore inclined to the opinion that the decomposition of hydrogen peroxide into water and common oxygen by the action of platinum and similar metals may be regarded as the prototype of all catalytic phenomena. Indeed, as he points out in a later part of this article, Berzelius had already compared the decomposition of hydrogen peroxide by the noble metals to the fermentation of grape sugar by yeast. It was therefore with the view of throwing still further light on the causes of catalysis in general that these studies on the decomposition of hydrogen peroxide by substances of animal and vegetable origin were first undertaken. He observed that platinum and the noble metals generally rapidly blue a tincture of guaiacum containing hydrogen peroxide, and that a certain amount of the hydrogen peroxide present is decomposed into water and common oxygen. It follows from this that the power of these substances to catalyze hydrogen peroxide is so intimately associated with their power to blue the tincture of guaiacum containing hydrogen peroxide that both properties are referable to the same cause, and it was for this reason that a tincture of guaiacum containing small amounts of hydrogen peroxide proved to be a valuable reagent in the investigation of these phenomena. By means of it he proved that these catalytic agents have the widest possible distribution in vegetable and animal tissues. Among the substances tested with the guaiacum hydrogen peroxide reagent were a number of the common enzymes, such as diastase, emulsin, myrosin, rennin, etc., yeast, gluten, and over 100 species of plants, including certain fungi and molds, and also a number of seeds and substances of animal origin, such as fibrin, red blood corpuscles, and saliva. Without exception, all of these substances were found by him to actively decompose hydrogen peroxide, and with only two exceptions, yeast and rennin, all of them likewise gave rise to the production of guaiacum blue. Schoenbein (387) therefore concluded that the power to decompose hydrogen peroxide and to blue guaiacum thereby is intimately associated with the specific activity of the unorganized ferments and with such vital phenomena as the sprouting of seeds, etc., inasmuch as all of these biologic properties are lost by heating to 100° C., by exposure to hydrogen sulphide, and also, as shown in a later paper (385), by exposure to hydrocyanic acid.

In still another communication in the year 1863, on "The conduct of blood toward oxygen" ([384]), he points out that ozone and hydrogen peroxide are formed simultaneously in a large number of slow oxidations, such as the oxidation of phosphorus in a moist atmosphere. In a large number of cases, however, only hydrogen peroxide is met with as the by-product of the oxidation, as, for example, in the oxidation of metallic substances, pyrogallic acid, etc.; and in still another large group of cases neither ozone nor hydrogen peroxide are produced, for the reason, probably, that the latter oxidations are accomplished by common oxygen without activation in any way. He then goes on to say that the view has long been prevalent that in all oxidations resulting in the formation of ozone or hydrogen peroxide the conversion of common inactive oxygen, (O), into $\oplus$ or Θ takes place. The former, $\oplus$, then unites with water to form hydrogen peroxide, $HO + \oplus$, whereas the latter, Θ, is either consumed at once in the oxidation or appears as ozone. Indeed, according to Schoenbein, Θ is ozone. He therefore arrived at the conclusion that the neutral oxygen which is taken into the body during respiration must obviously suffer such a change of state before it can accomplish the oxidations ordinarily taking place in the animal organism, and since both ozone and hydrogen peroxide result from the oxidation of many substances, he expected to find these substances in the blood of animals. As a matter of fact, however, although he employed the most delicate reagents in testing for these substances in the blood, he was unable to find a trace of either ozone or hydrogen peroxide therein. In this connection he points out, however, that Hiss had shown that ozone readily oxidizes the albumin and fibrin of blood and also the blood corpuscles, and that long ago Thenard had shown that fibrin readily decomposes hydrogen peroxide into water and common oxygen, and he himself had shown that blood corpuscles obtained from defibrinated blood also rapidly decompose hydrogen peroxide, and that a solution of defibrinated blood of such dilution as to no longer exhibit the characteristic color of blood rapidly blues tincture of guaiacum containing hydrogen peroxide. He concludes, therefore, that while the common oxygen consumed in respiration is resolved into $\oplus$ and Θ, the latter, Θ, is at once consumed in oxidations, and that the former, $\oplus$, unites with water to form hydrogen peroxide ($HO + \oplus$); which, however, is rapidly decomposed by the red blood corpuscles into water and common oxygen. This again enters into the ordinary cycle of changes met with in the absorption and utilization of the respired oxygen, viz, into the formation of $\oplus$ and Θ, so that ultimately all of the oxygen would be gotten into a form available for oxidations, viz, Θ.

It should likewise be borne in mind that the blood corpuscles not only have the power of decomposing hydrogen peroxide into water and common oxygen, but also of ozonizing the oxygen, $\oplus$, which it

contains, after the manner of a lead salt, as indicated by the fact that a solution containing the peroxide and red blood corpuscles rapidly blues a tincture of guaiacum. In the light of these facts, therefore, one can readily understand, he says, why the red blood corpuscles have the power of rapidly decomposing hydrogen peroxide, nor should one be inclined to look upon this as a useless property, since the blood corpuscles also have the power of ozonizing a part of the oxygen of the peroxide after the manner of platinum or a lead salt. According to Schoenbein, therefore, the red blood corpuscles, by virtue of their activating and catalyzing properties, play an important rôle in all processes of oxidation occurring in the organism of the higher animals, inasmuch as it is scarcely conceivable that their oxidizing power should be confined entirely to guaiacum resin or pyrogallic acid.

So far as the historical development of the subject is concerned, the work on the oxidases is approximately divisible into three periods. The first period begins with the earlier observations by Planche, Taddei, and Schoenbein, on the bluing of guaiacum. The second period has to deal with the work of Moritz Traube. The third period begins with the work of Yoshida and Bertrand on laccase, and includes all recent investigations on the subject. Obviously, as is the case with all things historically considered, the work of each of these periods overlaps somewhat the work of the other periods.

To review briefly the salient facts brought to light during the first period, we note that Wollaston was the first to call specific attention to the bluing of guaiacum and to the part played by air and light in effecting this change. William Brande also ascribed this change of color to oxygen, and succeeded in showing that pure oxygen gas effects the change somewhat more rapidly than atmospheric air. Then followed the earlier observations of Planche, that guaiacum is rapidly blued by the vapors of nitric acid and also by the fresh root of the horse-radish. This last-mentioned observation, made in 1809, marks the first recognition of a change brought about by an oxidizing ferment, although the ferment-like nature of the substance causing the change was not definitely recognized until many years afterwards. Other French observers noted a bluing of guaiacum by gums, such as gum arabic, and also by saliva. Taddei showed that under the influence of air and moisture the gluten of wheat flour blues guaiacum, and Planche showed that this reaction is characteristic of the roots of a large number of plants, and also of fresh milk, and, further, that the power to blue guaiacum on the part of these substances was lost on boiling. Thus, without fully appreciating its significance, this observer discovered one of the most characteristic properties of the oxidases as a class, viz, the loss of oxidizing power on boiling. Planche however failed to recognize the part played by atmospheric

oxygen in these changes, and attributed the bluing of guaiacum by plant tissues to a peculiar unstable principle which he called *cyanogene*.

After the work of Priestley and Lavoisier, the greatest impetus to a better understanding of the processes of oxidation was given by the discovery of ozone by Schoenbein in 1840. We have seen that this observer recognized this active variety of oxygen among the products of slow oxidation, and that he explained the phenomenon of oxygen carrying as due to the production of ozone or of an ozonide. He greatly extended our knowledge of the bluing of guaiacum, and also the earlier observations of Planche on the presence of oxygen exciters in plants and animals, and he accounted for the remarkable oxidizing activity of such substances on the supposition that they, in common with platinum, nitric oxide, and other oxygen carriers, ozonize the air, combining with the ozone thus produced to form an active ozonide, which in turn can give up its oxygen to other less readily oxidizable substances. These were his *Sauerstofferregern* and *Sauerstoffträgern*. They are what we to-day term the oxidases, or oxidizing ferments. Through the action of these substances on certain chromogens he was able to explain the changes of color observed in the growth and decay of certain species of the higher fungi, and similar changes occurring in certain fruits, such as take place in the apple when peeled or crushed. He also recognized the wide distribution of substances capable of decomposing or catalyzing hydrogen peroxide and of greatly activating the latent oxidizing powers of this substance; and all of these facts he was able to explain more or less satisfactorily in terms of his theory respecting the relation existing between common oxygen, ozone, and antozone, according to which ozone and antozone were oppositely electrified; that under the influence of various substances, like platinum, etc., common oxygen and antozone or an antozonide could be transformed into ozone or an ozonide, and finally that ozone could react with an antozonide, such as hydrogen peroxide, to form common oxygen. In this connection it may be observed that no one can read the writings of Schoenbein without being impressed with the great wealth of his observations, and his theory regarding the nature of ozone will always serve to remind the thoughtful student of chemistry how clearly and apparently how accurately a great variety of phenomena can ofttimes be explained by an hypothesis altogether erroneous in its premises.

It is evident, therefore, that during the first period, covering approximately the first six decades of the nineteenth century, the presence of oxygen activators and carriers in the plant and animal world had been recognized, and the more important characteristics of the oxidases and peroxidases had been discovered, so far as we have an accurate knowledge of these properties to-day, and yet the terms "oxidase" and "oxidizing ferment" had not up to this time been introduced into

the science, the nearest approach to them being the use of the terms *Sauerstofferreger* and *Sauerstoffträger* employed in the later writings of Schoenbein, and the recognition of the fact by this author that the power to activate oxygen and hydrogen peroxide is more or less intimately associated with the zymotic activity of the unorganized ferments and with vital phenomena generally. He accounted for all of these phenomena on the supposition that by means of various substances and under various influences the oxygen of the air becomes ozonized.

In the meantime other chemists and physicists (see Soret [405], Andrews and Tait [12], Brodie [102], Odling [313]) had gradually come to distinguish more clearly between the three forms of oxygen, viz, ordinary oxygen O_2, ozone O_3, and active (atomic) oxygen, O (antozone). The result has been that Schoenbein's original views regarding the manner in which the *Sauerstofferregern* and *Sauerstoffträgern* effect the activation of ordinary atmospheric oxygen, and the transfer of oxygen to other less readily oxidizable substances thereby, have been considerably modified and our knowledge of what we know as the oxidizing ferments considerably extended.

OXIDIZING FERMENTS ("VERWESUNGSFERMENTE" AND "OXYDATIONSFERMENTE," *Traube*).

During the second period of the development of this subject our knowledge of processes of slow oxidation and oxygen-activation, as well as of fermentation in general, was considerably extended through the labors of Moritz Traube. It remained for Traube to introduce into the science the term "oxidizing ferment" (*Oxydationsferment*) as a generic term for the unstable *Sauerstofferreger* and *Sauerstoffträger* of Schoenbein, which were found by Planche and Schoenbein to be so widely distributed among plants and animals.

In 1858 Traube [425] gave to the world his "Theory of Fermentation" (Theorie der Fermentwirkungen, von Moritz Traube, Berlin, Ferd. Dümmlers Verlagsbuchhandlung, 1858). A brief outline of this theory is also given in a paper published in Poggendorf's Annalen [426], and later he developed his ideas in a series of articles in the Berichte der Deutschen Chemischen Gesellschaft. In paragraph 175, page 61, of Traube's "Theorie" it is stated that an essential distinction between putrefactive and vital ferments—that is, those present in the living organism—does not appear to exist.

In this treatise and in his later papers he developed the idea that the ferments are not bodies in a state of decomposition which impart decomposition to other substances, as had been supposed by Liebig, but are, in all probability, chemical compounds originating from proteids which, while not as yet isolated in pure condition, have, without

doubt, as have other compounds, a definite chemical composition, and which, through the exercise of definite chemical affinities, produce alterations in other compounds. He held also that Schwann's hypothesis, as subsequently developed by Pasteur, namely, that fermentation is the result of the action of the life phases of lower organisms is insufficient, and that rather the converse of this hypothesis is really correct, namely, that the ferments are the causes of the important bio-chemical processes and are not confined to the lower organisms, but are present also in the higher forms of life. (*See* Traube's "Theorie," supra, paragraph 175, p. 61.) In this connection he pointed out that the ferments are produced at ordinary or slightly elevated temperatures from the decomposition of protein substances by the action of water, probably under the influence of oxygen. Thus fresh meal prepared from dry grain contains no diastase. If, however, the grain acts upon water in a limited supply of air, chemical changes occur and diastase is generated. (Par. 172, p. 60, Traube's "Theorie.") He also pointed out that in many instances at least, the ferments are ideal catalysts, acting as chemical go-betweens between free or combined oxygen and the fermentable substances. Thus the blood is an ideal carrier of free oxygen, in that in the lungs it becomes saturated with oxygen, which is used for the purpose of direct oxidation in the capillaries, from which it is returned to the lungs to recombine with oxygen.

He was led to believe further, as a logical corollary of his theory, that various organic and inorganic substances other than those elaborated in the living cell may under certain conditions function as ferments. That such is the case is shown by the fact that platinum is a carrier of oxygen; that is, it may exert the same sort of action as the *Verwesungsfermente* (oxidizing ferments), and that in indigo-sulphuric acid we have a substance capable of exhibiting changes altogether analogous to certain kinds of fermentation. Later he [428] showed in this connection that platinum black at 150° to 160° C. decomposes solutions of sugar with the production of carbon dioxide and of a light oil, which gives the iodoform reaction.

Briefly put, Traube's theory of fermentation is based upon two distinct chemical propositions; first, that the ferments are definite chemical compounds elaborated from proteid as a result of the combined action of heat, water, and oxygen, and present not only in the lower organisms but also in the tissues of the higher forms, where they are responsible for bio-chemical processes; second, that the ferments are powerful reducing agents and oxygen carriers, capable in their capacity of chemical go-betweens (*Vermittler*) of effecting the transfer not only of free oxygen to easily oxidizable substances but also the transfer of combined oxygen from one compound to another.

He was led to divide ferments into three classes:

(*a*) *Verwesungsfermente*, those which combine loosely with fre oxygen, forming unstable compounds which give up their oxygen t other less readily oxidizable substances.

(*b*) *Reductionsfermente*, those which combine with the oxygen c water, the hydrogen going to effect the reduction of the passive body

(*c*) *Hochstes Faulnissfermente*, those which cause putrefactions i which hydrogen is set free.

He was led to believe further that all of these ferments have th power of carrying to other substances the oxygen with which they hav combined, thereby becoming reduced and gotten into the conditio whereby they can again combine with fresh quantities of oxygen an again carry it to oxidizable substances. In this way they are able t carry free and combined oxygen in practically unlimited quantitie to other substances, and in that way bring about gradual deca (*Verwesung*) and fermentation proper (*Gährung*). (Traube, [425, 426] In the one case (*Verwesung*) the oxygen required for the oxidatio comes from the air; in the other case (*Gährung*) it comes from th water. Hence in all true fermentations water acts not merely a a solvent, but actively participates in the process. In this discus sion, however, we are primarily concerned only with his first clas of ferments, viz, the *Verwesungsfermente*. These he divided int two groups: *Vitale*, those met with in the higher life forms, an *faulige*, putrefactive, those concerned in the change and decay of dea material. In the first class he placed the guaiacum bluing fermen of the potato and the red coloring matter of blood. In the secon class he placed the ferments concerned in the transformation o alcohol into acetic acid and the nitrogenous compounds of dung int nitrates. As nearly as I have been able to determine, the term "*Verwesung*," as employed by Traube in this connection, seems t have been borrowed from the writings of Liebig, and in terms of th present nomenclature employed for processes in which the oxidizing ferments are concerned, it is practically untranslatable. As ordin arily employed, it signifies putrefaction or decay. In reality, a employed by Liebig, it referred primarily to the spontaneous decay o vegetable matter, whereby this sort of material is ultimately con verted into brown mold or humus-like substances. Other Englis writers have encountered this difficulty in dealing with this word an its derivatives. Thus in translating Liebig's Agricultural Chemistr Gregory was forced to coin the word *eremacausis* (from the Greek meaning "slow-burning") as the equivalent of *Verwesung*, the intro duction of this term being made with Liebig's consent. In his earlie writings on the subject Traube made constant use of the term *Verwes ungsfermente* as signifying the oxidizing ferments, to the exclusion o practically all other names for these substances. In Paragraph 132

page 49, of his "Theorie," it will be seen, however, that he employs the term "*Sauerstoffübertragenden Fermente*" (oxygen-carrying ferments) for the vegetable oxygen carriers described by Planche and Schoenbein, and later, in 1874, in an article on the conduct of yeast in oxygen-free media (429), referring to his earlier work, he says:

> I have given several examples to show that there are substances (in animal and vegetable tissues) which, like platinum, nitric oxide, and indigo-sulphuric acid, carry free oxygen to other substances and thereby accomplish their oxidation. (*Sauerstoffübertrager, oxydationsfermente.*)

It was in his later writings (430), however, especially in his controversy with Hoppe-Seyler that he insists upon the use of the term *Oxydationsfermente* (oxidizing ferments) as preferable to the term *Verwesungsfermente,* which latter term he had previously employed as signifying those ferments which possess the power of taking up free oxygen and carrying it to other passive substances, thereby accomplishing the oxidation of the latter.

As the result of their action, the oxidizing ferments suffer no chemical alteration, playing merely the part of go-betweens in oxidation, in that they alternately combine with oxygen and give it up to other substances. Obviously, according to Traube, free oxygen is essential to their action. In this connection his earlier observations on the oxidizing ferments of the potato are of interest. He showed that if thinly cut potato peel is rubbed up in a mortar with a small amount of distilled water, a solution is obtained which blues guaiacum intensely. If the blue solution thus obtained be allowed to stand in a reagent glass it bleaches, except on the upper surface where it is in contact with the air. On shaking with air, however, the whole solution again acquires a deep blue color, which on standing again bleaches. This alternate bluing of the solution by shaking with air and subsequent decolorization of the lower layer of the solution upon standing was observed upon one and the same solution for a period of fourteen days, indicating that the potato peel contained a true oxygen carrier through the instrumentality of which the oxygen of the air was transferred first to the guaiacum and subsequently to other substances. He also showed that the potato loses its power to blue guaiacum at 80° C., and that the unstable oxygen carrier which it contains is soluble in dilute alcohol. He points out that a body of such remarkable chemical conduct and of such wide distribution in the plant kingdom must play a significant rôle in the growth of vegetation. Similarly, he also recognized the red coloring matter of the blood as a powerful oxygen carrier, and was led to regard muscular activity as a respiratory act in which the muscle fiber functions as an oxidizing ferment in that it unites with the oxygen supplied by the blood to form an unstable compound, which in turn gives up its oxygen to other less readily oxidizable compounds contained in the

muscle fluids both in rest and active work, resulting finally in the pro
duction of animal heat and work. In other words, muscular activit
and the production of animal heat were accomplished, like all *Ve*
wesungsprocessen, through the action of *Verwesungsfermente* (oxidi
ing ferments) on the free oxygen of the air. (Traube [427, 430].)

In plants, under the influence of oxidizing ferments as go-between
oxygen accomplishes the conversion of soluble carbohydrates int
cellulose, and in animals it converts soluble proteids into ferment
He goes on to say further that the lessons to be learned concernin
oxygen are only limited by those to be learned of organic life itself.

OXIDASES (Laccase, *Bertrand*).

A new impulse to the study of the oxidases was given by the beauti
ful researches of Yoshida, and later by those of Bertrand, on th
changes occurring in the sap of the lac tree, *Rhus vernicifera*, an
allied species. It had long been known to the Japanese that th
milk-like sap of the lac tree dries and hardens on exposure to the ai
and turns brown and finally black, thereby yielding a lustrous
translucent varnish, which is highly prized on account of its luste
and stability. In 1883 Yoshida [467] for the first time undertook a
accurate investigation of these interesting changes. He found tha
the lac tree yields a strongly corrosive sap, which he called "urushi;"
this he found to consist essentially of four substances, viz., water
urushic acid, $C_{14}H_{18}O_2$, gum, and a peculiar diastatic matter whic
possesses the power of transforming the urushic acid into oxy
urushic acid, $C_{14}H_{18}O_3$, through the action of the oxygen of the air, i
the presence of moisture. He found that this nitrogenous compoun
loses this property when heated to 63° C. He was therefore led t
conclude that in this substance we have an enzyme which has th
power of carrying oxygen to the urushic acid, thereby transforming
it into oxy-urushic acid which is the basis of lac varnish. He also
showed that urushic acid can be transformed into oxy-urushic acid
by chromic acid.

Eleven years later a more exhaustive investigation of the changes
occurring in the sap of the lac tree was undertaken by the French
chemist Bertrand [48, 49], with the result that Yoshida's previous
results were confirmed. According to Bertrand, the changes ob-
served when the fresh juice of the lac tree is allowed to stand in the
air are brought about by the oxygen of the air under the influence
of an oxidizing ferment (*diastase oxydante*) to which he gave the name
of laccase. After boiling laccase loses the power of bringing about
these changes. Hence he regards laccase as the provocative agent of
the oxidation, and found it not only in the fresh juice of the lac tree
but also in gum arabic and gum senegal. According to Bertrand, the
oxidizable substance present in the juice of the lac tree and which

Yoshida had called urushic acid, shows many resemblances to the polyatomic phenols. He therefore called this substance "laccol," and in view of this analogy he was led to study the conduct of laccase toward a number of the polyatomic phenols and related compounds with the result that he found many of these to be oxidized under these conditions. Thus, hydroquinon is converted by laccase into quinon and quinhydron, with the absorption of oxygen (50). Thus, a 1 per cent solution of hydroquinon alone or with boiled laccase does not absorb oxygen from the air nor is it altered in any way. In the presence of a small amount of laccase, however, such a solution absorbed 25.4 c. c. of oxygen in three hours. With tannin and pyrogallol the oxidation was always attended with the disengagement of considerable amounts of carbon dioxide. Thus, in two experiments oxygen was absorbed and carbon dioxide disengaged in the following amounts:

	Oxygen Absorbed.	Carbon dioxide disengaged.
(1)	23.3 c. c.	13.7 c. c.
(2)	29.8 c. c.	16.4 c. c.

He also found guaiacum to be a useful reagent for laccase, and by means of it he was able to detect the enzyme in many plants, such as potatoes, apples, peas, quinces, lucerne, clover, asparagus, turnips, chestnuts, and various rhizomes (57).

Bertrand and Bourquelot(63), by the use of guaiacum, found laccase in a large number of species of mushrooms. Out of 18 species examined at this time only two, *Polyporus sulfureus* Bull. and *Squamosus* Huds., were found not to give the guaiacum reaction. Hence laccase is not confined to the chlorophyllous plants, but occurs in the nonchlorophyllous plants also.

The oxidations occurring in the juice of the apple, observed in cider-making, were also studied about this time by Lindet (271). This author also claims to have reached the conclusion, as early as 1893, that a soluble ferment governs this oxidation, and in a later article he (272) concludes that the changes of color occurring in fresh cider are due to the oxidation of tannin by a ferment, of the type of laccase, contained in the tissues of the apple.

In the course of his investigations Bertrand found that laccase always contains small amounts of manganese and that its oxidizing power is proportional to the amount of manganese present; and further that oxidations accomplished by laccase are greatly accelerated by small amounts of manganese salts and that no other metal is capable of accelerating the oxidation brought about by laccase.

In the course of these investigations the old observations by Schoenbein (378) that various fungi become colored in the air was also confirmed by Bertrand and Bourquelot (64). They arrived at the

notion that the change of color is brought about by an oxidizing fer ment which they held to be identical with laccase. The chromoge of the blue substance was later isolated by Bertrand and calle boletol ([60]). On the other hand it was known that certain fung become red and then black (not blue) on exposure to the air. I such fungi Bertrand and Bourquelot ([64]) found tyrosin. Now, sinc this compound is not oxidized by laccase but is oxidized by the fres extract of those fungi in which it occurs, they ascribed its oxidation t a second oxidizing ferment which they called "tyrosinase." Bour quelot found tyrosinase in many plants, including the potato an sugar beet.

From these observations Bertrand was led to regard laccase as on of a group or class of oxidizing ferments to which he gave the genera name of "oxydases." Thus in an article on the relationships exist ing between the constitution of certain organic compounds and thei oxidizability under the influence of laccase he ([52]) says in footnote 2 on page 793:

> I propose to apply the generic term *oxydases* to soluble oxidizing ferments in ord to distinguish them from the true diastases (ferments) which produce double decom position with the fixation of water.

To Bertrand, therefore, we owe the introduction into the scienc of the term *oxydase*.

Boutroux ([92]) has objected to the use of the term *oxydase* as pro posed by Bertrand for such oxygen carriers as laccase and that con tained in bran, on the ground that the termination *-ase* has been em ployed exclusively to indicate a hydrolytic ferment, and for th further reason that the formation of such a word as *oxydase* is con trary to the general rule governing the nomenclature of enzyme according to which the stem of the word thus employed designate the substances upon which the enzyme exerts its action; thus *maltas* signifies the ferment which hydrolyzes maltose. On the other hand i should be borne in mind that this nomenclature for the unorganize ferments has never been rigorously applied, as indicated by the use o the terms *invertase* and *diastase*. Furthermore, the term *oxydase* wa well chosen, since these ferments all act upon oxygen or a peroxid and such terms as *laccase* and *tyrosinase*, etc., serve to differentiat the oxydases from one another and likewise indicate the substance upon which the oxygen acts.

As is the case with other soluble ferments, the oxidases have prob ably never been obtained in a condition of purity. Indeed, we hav no reliable criteria whereby to judge of their purity, and hence bu little is known regarding their composition. From the fact that lac case always contains manganese, and that a salt of this metal greatl increases the activity of the oxidase, Bertrand reached the conclusio that laccase consists of manganese in combination with a protei radicle. The former functions in the capacity of a co-ferment

whereas to the latter the laccase owes its zymotic characteristics, such as destructibility by heat, poisons, etc.

According to Portier (330), the following properties are characteristic of an oxidase:

(1) The power to oxidize certain substances in the presence of free oxygen, gaseous or dissolved.
(2) The power to cause the absorption of oxygen during oxidation.
(3) Destruction by heat.
(4) Nondialyzability.

According to Bourquelot (84), the oxidases belong to the class of enzymes and possess the following characteristics common to enzymes in general:

(1) Catalytic power, viz., power to effect the transformation of an indefinite amount of material by means of an infinitesimal quantity of the ferment.
(2) Like other ferments, their activity is subject to regular and constant influence by heat, increasing in oxidizing power with rise in temperature to 42°–45° C. (optimum temperature), then falling off in activity with further rise to 60°–70° C., and finally completely destroyed at 100° C.
(3) Insolubility in alcohol.
(4) Solubility in water, even after precipitation by alcohol and desiccation.
(5) Adsorption by precipitates (colloids).
(6) Absence of power to dialyze.

In addition to these general characteristics, they possess, according to this author, the following properties properly belonging to the oxidizing ferments:

(1) Power of accomplishing oxidation by means of gaseous or dissolved oxygen.
(2) The accompaniment of the oxidations accomplished by them with a notable absorption of oxygen.

Duclaux (150, vol. 2, p. 565), has defined oxidases as substances which at ordinary temperatures and under physiologic conditions, carry oxygen rapidly to substances upon which, without the intervention of oxidases, ordinary oxygen would act very slowly.

The oxidases are very readily soluble in water; they are also readily soluble in glycerin, but in other organic solvents thus far investigated they seem to be insoluble. Thus I (238) have found the oxidase of *Lepiota americana* to be very soluble in water, glycerin, 40 per cent formaldehyde, and mixtures containing water and alcohol, but insoluble in ethyl, amyl, and allyl alcohols. This fungus also gave up its oxidase to a preservative solution containing equal quantities of water and alcohol to which a small amount of formaldehyde had been added. Some observations seemed to show that it was soluble in toluene to some extent. It has also been my own experience that aqueous solutions of the oxidases are very unstable even in the presence of mild antiseptics. Thus aqueous extracts of the potato soon lose their power to blue guaiacum, even when preserved under antiseptic conditions. Bourquelot observed that an aqueous extract of *Russula delica* when preserved with chloroform first loses its power to

oxidize tyrosin, then guaiacol, and finally, after eight weeks, its power to oxidize guaiacum. I have found aqueous extracts of *Lepiota americana* to oxidize guaiacum at the end of eighteen weeks. Bertrand found tyrosinase to be very unstable. On the other hand, according to Bourquelot (75), aqueous solutions of tyrosinase to which chloroform had been added were found to be active at the end of two or three months.

In glycerin the oxidizing ferments are much more stable. Thus Gessard (181) has found that a solution of tyrosinase in glycerin, obtained from mushrooms by Bourquelot's method, is stable, and in his work he employed such an extract which had retained its activity for ten months. Bourquelot (81) found that glycerin extracts of *Lactarius velutinus* retained their oxidizing properties for a year or longer. In the course of my own work on this subject I have found that glycerin extracts of *Lactarius piperatus* and *Lactarius volemus* prepared in the summer of 1905, both of which, in the fresh state, were found to be quite active toward guaiacum and tyrosin, were still active toward these substances in the summer of 1909.

As a general thing, the oxidizing power of an oxidase is not confined to one particular oxidizable substance. Thus laccase has been found to oxidize not only laccol, the oxidizable principle of the juice of the lac tree, but also to oxidize guaiacum, guaiacol, hydroquinone, phenolphthalin, and a large number of phenols and aromatic amino compounds. (See pp. 59–61.) So also tyrosinase oxidizes not only tyrosin but also a large number of related compounds. (See pp. 81–84.) In the same way an oxidase which will oxidize benzaldehyde to benzoic acid will also oxidize salicylic aldehyde, benzyl alcohol, and related substances. On the other hand laccase will not oxidize tyrosin, nor will tyrosinase oxidize guaiacum or phenolphthalin. So in the same way many animal tissues which have been found to oxidize salicylic aldehyde are without effect on guaiacum, hydroquinone, and tyrosin. While thus not absolutely confined in their action to any one particular oxidizable substance, the oxidases exhibit a certain degree of specificity in the sense that they are more or less limited in their action to certain groups of substances which are more or less closely related chemically.[a]

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[a] From his studies on the spontaneous oxidation of the sugars, Mathews (289) concludes that two distinct groups of substances have been confused under the name of *oxidases*; one group, the oxidases proper, activates oxygen more or less generally toward all oxidizable substances, while the other group of ferments, which are more specific in their action, activates certain oxidation processes, such as the oxidation of sugars, by causing a dissociation of the sugar molecule. For the latter he proposes the name *metabolase*, since they hasten metabolism. According to this author the failure on the part of the organism to burn glucose under certain conditions is probably not due to the absence of oxidases, but to the loss of its power to dissociate glucose into easily oxidizable substances.

CLASSIFICATION OF THE OXIDASES.

Various attempts have been made from time to time by different observers to effect a classification of the oxidases. None of these are altogether satisfactory, but are probably as good as can be made in the present state of our knowledge of these substances.

According to Grüss (200) there are three classes of oxidases in the higher plants, namely α-oxidases, β-oxidases, and γ-oxidases. The α-oxidases (1) act directly upon guaiacum and tetramethyl-para-phenylene diamin with the fixation of oxygen; (2) they are soluble in glycerine and are partially precipitated from their solutions by acetate of lead, and are easily destroyed by alcohol; (3) they are found in the parenchymatous tissue of the potato and in most dicotyledenous plants. The β-oxidases (1) activate the oxygen of peroxides, and hence they only blue guaiacum or act upon tetramethyl-para-phenylene diamin in the presence of hydrogen peroxide or a similar substance; (2) they are soluble in glycerine and are precipitated from their solutions by alcohol and ether without being destroyed; (3) they have been found in the resting, mature tubers of the potato. The γ-oxidases (1) also activate the oxygen of peroxides, and hence only blue guaiacum or act upon tetramethyl-para-phenylene diamin in the presence of hydrogen peroxide; (2) they are not destroyed even by boiling alcohol, and in order to demonstrate their presence the tissue must be boiled in alcohol for a short time before adding the hydrogen peroxide and oxidase reagent; (3) they have been found in old wounds in plants such as the potato, in the diastase of the barley grain, in association with cytase, in the leptome of growing roots, in *Astragallus glycophylloides*, etc.

Grüss has also classified certain of the vegetable oxidases according to differences in conduct toward certain oxidase reagents. Thus he observed that certain oxidases can act upon tetramethyl-para-phenylene diamin, but not upon guaiacum, whereas other vegetable tissues are capable of oxidizing both. He therefore divided the oxidases into two groups: (1) the guaiacum-oxidases, and (2) the amino-oxidases. An example of the latter is furnished by the oxidase of yeast. In this connection Rey-Pailhade (342) was able to find only two kinds of oxidases in plant tissues, (1) those which oxidize guaiacum and (2) those which only oxidize Röhmann-Spitzer's reagent (α-naphthol and para-phenylene diamin). The first is identical with Bertrand's laccase; the second with the indophenol-forming oxidases which occur so generally in animal tissues. In this connection Pohl (329) found certain plant extracts to give the indophenol reaction, but to be incapable of oxidizing formaldehyde.

Rosell ([352]) distinguishes between extra-and intra-cellular ferments, and divides the oxidases into the following groups:

(1) Aldehydases (Jacoby), such as salicylase, etc.
(2) Indophenol-oxidase (Spitzer), found in aseptic pus (Achalme).
(3) Guaiacum oxidase (Schoenbein), found in plant tissues.
(4) Hydrogen peroxide ferment (Rosell), peroxidase (Linossier), indirect oxidase (Bourquelot), and possibly includes catalase under this head.
(5) The glycolytic ferment (Lepine).
(6) The purin oxidase (Spitzer).

The following classification of the oxidases and other oxygen-carriers concerned in biological oxidations would seem to be more in line with present requirements:

(1) Laccase; ferments oxidizing guaiacum, guaiacol, hydroquinone, phenolphthalin, tannin, etc., directly by means of atmospheric or dissolved oxygen and without the intervention of hydrogen peroxide. According to Bach and Chodat, laccase consists of a peroxidase, together with an oxygenase (a peroxide). In this connection, see also a recent communication by Moore and Whitley ([306]).
(2) Tyrosinase; ferments acting on tyrosin and related substances.
(3) Aldehydase; ferments oxidizing aromatic aldehydes and related compounds.
(4) Indophenol oxidase (Spitzer), ferments oxidizing a mixture of α-naphthol and para-phenylene diamine to indophenol and other substances.
(5) The purin oxidases.
(6) Glycolytic ferments, causing the disappearance of sugar from animal tissues.

In addition to the oxidases proper, we have among related ferments and carriers the following:

(1) Peroxidases; oxidizing oxidase reagents only in the presence of a peroxide, such as hydrogen peroxide. These are the indirect oxidases (Bourquelot) and the β-oxidase of Grüss. According to Moore and Whitley ([306]) the peroxidase is the only type of enzyme concerned in oxidizing processes occurring in living cells and tissues.
(2) Catalases (Loew). These ferments actively decompose hydrogen peroxide, but are incapable of effecting the oxidation of oxidase reagents by means of the peroxide.
(3) Oxygen carriers (not true ferments). This class includes such substances as the iron-containing pigments of the blood, hemocyanin, and the γ-oxidases of Grüss. They activate the oxygen of a peroxide, but differ from the true peroxidases in having greater stability. (*See* Kastle ([240]).)

Whether the laccase, tyrosinase, aldehydase, etc., obtained from different vegetable and animal sources are really in all cases the same substances can not be determined with certainty in the present state of our knowledge respecting these substances. As already indicated, a very large number of plant tissues contain an oxidase capable of bluing guaiacum and oxidizing hydroquinone; whether this is the same chemical substance which is responsible for these oxidations in all cases can not be definitely decided. For certain reasons it would seem probable that the substances responsible for such oxidations, in case there were several, are at least closely related chemically. On the other hand, certain facts are known which would seem to indicate that they might have the greatest diversity of composition and yet

all be capable of activating oxygen toward guaiacum, hydroquinone, etc. From what is known regarding the effect of manganese on laccase, it would seem that almost any colloidal solution of manganese might exhibit essentially the same reactions as laccase. (See pp. 122–131.)

The conduct of the oxidases and peroxidases toward a large number of substances has been investigated by Bertrand, and also by Bourquelot and others. In this connection Bertrand [52] has studied the influence of the chemical constitution of certain organic compounds on their oxidizability by laccase.

Thus, he has shown that the oxidizability of the polyatomic phenols by laccase depends upon the relative ease with which they are converted into quinones. In this respect they conform in general to the findings of the Brothers Lumière [279] respecting the action of such substances as photographic developers. Thus, para-amido-phenol is an excellent developer and is also readily oxidizable by laccase, whereas meta-amido-phenol is not a photographic developer and is not acted upon by laccase. Similar results have been obtained with para-phenylene diamin and meta-phenylene diamin. On the other hand, the aromatic monophenols and monamins are not as a rule easily oxidized by laccase; the only substances easily oxidized by laccase are those of the benzene series containing hydroxyl or amino groups in the ortho or para positions. This rule defines the oxidizing power of laccase and serves to distinguish this soluble oxidizing ferment from the oxidases which attack compounds of different chemical constitution.

Bourquelot [74] has also investigated the action of the oxidizing ferments found in *Russula delica*, namely, laccase and tyrosinase, on a large number of compounds. He found the oxidizing power of this mixture of oxidases to be dependent upon the reaction of the medium. Thus, a solution of anilin was scarcely oxidized at all, owing to its alkalinity, while in the presence of small amounts of acetic acid the oxidation proceeded rapidly up to a certain limit of acidity, beyond which it was again checked, owing to the presence of an excess of acid. He found that the mixture of oxidizing ferments from *Russula delica* is capable of oxidizing a large number of phenols [77, 79] and aromatic amino compounds [82], among which may be mentioned phenol, the cresols, xylenols, thymol and carvacrol, alpha- and beta-naphthol, also the ethers of various phenols [79], anisol, phenetol, guaiacol, acetyl-guaiacol, veretrol, eugenol, acetyl-eugenol, vanillin, and vanillic acid; and by means of aqueous extracts of *Russula delica* and *Lactarius velutinus* he was able to obtain powerful oxidations with anilin, sulfate of anilin, methyl-, ethyl-, and diethy-anilin, meta- and para-toluidin, the xylidins, naphthylamin, and veratrylamin.

According to Bertrand and Bourquelot [64, 78] tyrosin is the best reagent for tyrosinase.

OXIDASE AND PEROXIDASE REAGENTS.

Under this head are given the names of the greater number of reagents which have been actually employed in the study of oxidases, peroxidases and other oxygen catalysts occurring in the living organism. As will be seen the greater number of these substances belong to the aromatic series, and on oxidation by the oxidase or peroxidase give rise to a colored substance; thus, guaiacum is converted into guaiacum blue, phenolphthalin into phenolphthalein, which is red in alkaline solution, the leuco-base of malachite green into malachite green, guaiacol into guaiacol-tetraquinone $[C_6H_3(OCH_3)O]_4$, which is red in color. No attempt has been made to classify these reagents either as to chemical constitution or according to the nature of the substance which they yield on oxidation by the oxidase or peroxidase.

Table of oxidase and peroxidase reagents.

Name of substance.	References.
Guaiacum	51, 74, 135, 136, 332, 377, 384, 417, 418, 419.
Guaiaconic acid	142, 362.
Guaiacol	75, 76.
Hydroquinone	48, 49, 76.
Aloin	238, 251, 363, 364.
Phenolphthalin	240, 247, 334, 335, 336.
Ethyl-phenolphthalin	238, 245.
Leuco-rosolic acid	245.
Leuco-base of malachite green	11, 104, 105, 131, 193.
Tyrosin	64, 77, 78.
Benzidin	11, 390.
Pyrogallol	30, 31.
Potassium iodide and starch	19, 377, 379, 414.
Tetramethyl-para-phenylene diamin	464.
Para-phenylene diamin	444.
Para-diethyl-para-phenylene diamin	437.
a-naphthol	77, 194.
a-naphthyl amin	390.
Vanillin	210, 266, 390, 453.
Phenol	11, 76.
Pyrocatechin	11.
Ortol	43.
Amidol	258.
Tannin	272.
Salicylic aldehyde	5, 225, 291, 292, 366.
Benzyl alcohol	366.
Formic aldehyde	329.
Arsenious acid	408.
Pyramidon	252.
a-naphthol and para-phenylene diamin in solution in sodium carbonate, and analogous reagents	351, 453.
Eugenol	76, 125.
Iso-eugenol	126.

Name of substance.	References.
Thymol	77, 124.
Ortho-toluidin	74, 76.
Para-toluidin	74, 76.
Meta-toluidin	76.
Ortho-, meta-, and para-cresols	76.
Xylidin	76.
Anilin	74, 76.
Ortho-, meta-, and para-xylenols	77.
Carvacrol	77.

Many of these reagents have found special application in the study of particular phases of biological oxidations. Thus, hydroquinone and guaiacum have been most extensively used in the investigation of laccase; tyrosin in the study of tyrosinase; and salicylic aldehyde in the study of aldehydase. So, in the same way, guaiacum, aloin, benzidin, the leuco-base of malachite green, and phenolphthalin have been most extensively used in testing for blood, whereas the potassium iodide-starch reagent (v. Storch ([414])), para-phenylene diamin, and guaiacum, have been most extensively employed in distinguishing between raw and boiled milk.

Various attempts have also been made to determine quantitatively the oxidizing power of these various catalysts by means of certain of these reagents. Thus, Laborde ([256]) has proposed a colorimetric method in which tincture of guaiacum is used for this purpose; similarly Slowtzoff([404]) has employed the indophenol reaction, Kastle([239, 240, 241]), the oxidation of phenolphthalin, and Czyhlarz and von Fürth ([131]) the oxidation of the leuco-base of malachite green, as the basis of colorimetric methods. Bach ([19]) has made use of the oxidation of hydriodic acid (potassium iodide and acetic acid), for quantitative purposes, and later Bach and Chodat ([30, 31]) have employed the change of pyrogallol to purpurogallin for the quantitative study of the peroxidase reaction. Herzog and Meier ([210]) have measured such oxidations quantitatively by means of vanillin, which is converted into dehydrovanillin, and Battelli and Stern ([35]) have employed the oxidation of formic acid. The conversion of tyrosin into melanin has also been studied quantitatively by von Fürth and Jerusalem ([178]), and by Bach ([24]). (See pp. 79–81.)

LACCASE.

This oxidase has been so fully considered in the historical development of the subject of the oxidases that but little additional need be said concerning it. As already pointed out in the foregoing, it rapidly blues guaiacum and oxidizes hydroquinone with the absorption of oxygen, and its activity is greatly augmented by the presence of very small amounts of manganese salts. Like other ferments, its

activity is lost on boiling. As is evident from the work of Planche [326, 327], Schoenbein, [377, 378, 383], and Bertrand [51], laccase or a guaiacum-bluing ferment of similar nature is almost universally distributed in the plant kingdom. Thus Bertrand found it in the tubers of the dahlia, in the potato, in the rhizome of the American reed, in the racines of the beet and turnip, in the stalk of the asparagus, and in apples, pears, etc. As a matter of fact, there are certainly but comparatively few of the higher fungi or of the chlorophyllous plants which do not blue guaiacum at least in certain of their tissues at certain stages of their development.

It was believed at one time by Rey-Pailhade [342, 344] that animal tissues have not the power to blue guaiacum and that therefore they contain no laccase. Subsequent investigations, however, have shown that this is much too sweeping a generalization. Thus Biedermann [67] observed that an aqueous extract of the middle intestine of the starving meal-worm gives an intense blue coloration with guaiacum. According to Giard [192], *Botrylloides cyanescens* and *Ascidia fumigata*, Grube, give an immediate and very intense blue color with tincture of guaiacum. The blood of the last-named ascidian was clear yellow when freshly drawn, becoming dark green on exposure to the air. Portier [330] has confirmed these observations on *Ascidia mentula*, and Abelous and Biarnes [6] have obtained similar results with the blood-plasma of the craw-fish. Similarly Pieri and Portier [324] have found a powerful oxidase in the gills and labial palps and blood of the acephalous molluscs, including the common oyster and the fresh-water species, *Anadonta cygnea;* this oxidase is said to give an intense blue coloration with guaiacum and to rapidly convert hydroquinone into quinone and quinhydrone. Hougonenq and Paviot [220] claim to have found that certain malignant tumors give the guaiacum reaction; Cavazzani [113] has made experiments indicating the presence of an oxidase in the cerebro-spinal fluid, which he has termed "Cerebro-spinase," and recently the brothers Lumière and Chevrotier [281] have prepared a protoplasmic extract of red blood corpuscles to which they have given the name "Hemoplase" and which they claim possesses the properties of an oxidase to a remarkable degree, as shown by its power to oxidize guaiacum, guaiacol, paraphenylene diamin, pyrogallol, and hydroquinone. Gessard [185] has found laccase in the ink-gland of the cuttle-fish.

While these observations are of interest as showing the occurrence of laccase or a similar guaiacum-bluing ferment in animal tissues and fluids, it is undoubtedly true that such ferments are of much rarer occurrence in animal than in plant tissues. At present the precise significance of this is only a matter of conjecture. It is interesting to note in this connection, however, that Ehrlich [157] found the tissues of the higher animals to possess powerful reducing properties, as indicated by their conduct toward methylene blue. It is quite con-

ceivable, therefore, that such oxidases as laccase are not really absent from animal tissues, but that their presence is merely obscured by the presence of powerful reducing substances, whose affinity for their oxygen is so great as to prevent the oxidation of the oxidase reagent.

Laccase is very soluble in water and aqueous solutions of the ferment are very readily obtained from such plants as the potato (*Solanum tuberosum*) (tuber), from the fruit of the egg plant (*Solanum melongena*), from the silk of the green corn (*Zea mays*), and from many varieties of fungi. In my own experience *Lactarius piperatus* and *Lepiota americana* afford excellent material from which to obtain the ferment. The former yields nearly colorless water-clear extracts of considerable oxidizing power, while the latter fungus affords extracts of remarkable oxidizing power. In this connection glycerin extracts of *Lactarius piperatus* and *Lactarius volumen*, which had been kept in the laboratory in glass stoppered bottles for a period of four years, still showed the laccase and tyrosinase reactions strongly. (See p. 56.) It not infrequently happens that laccase occurs in association with tyrosinase in the same plant or even animal tissues. See Gessard [185, 186], and Bertrand [54]. In the separation of the two oxidases advantage is taken of the greater stability of laccase toward heat and alcohol. Thus from *Russula delica* Bertrand separated the two ferments by precipitating the aqueous extract obtained by macerating the fungus with its own weight of chloroform water, with one and one-half times its volume of 95 per cent alcohol. The filtrate thus obtained after concentration at 50° C., still showed all of the reactions of laccase. It failed, however, to oxidize tyrosin. On the other hand, an aqueous extract of the alcoholic precipitate oxidized tyrosin, but failed to oxidize hydroquinone or pyrogallol to any appreciable extent.

Similarly it was found possible to destroy the tyrosinase and leave the laccase in the aqueous extract by heating to 70° C. (*See* Bach [23]). In the preparation of laccase no special precautions are necessary other than those which hold for the preparation of ferments in general. If tyrosinase is present along with the laccase, the extract is either heated to 70° C. for a short time to destroy the tyrosinase, or the latter is precipitated by the cautious addition of alcohol. The tyrosinase-free extract may then be concentrated by evaporation at low temperatures and the laccase finally precipitated by the addition of alcohol. Inasmuch as we have no criterion for judging of the absolute purity of a ferment, it is very doubtful whether much is gained by the attempt to isolate laccase and the other oxidases in pure condition, and it has been my own experience that we frequently lose in activity what we gain in the so-called purity of the enzyme by all attempts at its purification. The stability of laccase apparently depends upon the nature of the substances with which it finds itself in association or upon conditions which at present are altogether

unknown to us. Thus it is not an unusual thing to find that extracts of the peel of the potato lose their power to oxidize guaiacum after standing a few hours, (*see* Kastle and Shedd [247]), whereas aqueous extracts of *Lepiota americana* retain their activity for weeks or even months (*see* Kastle [238]), and glycerin extracts of *Lactarius piperatus* and *Lactarius volumen* were found by Kastle to blue guaiacum four years after they were first prepared.

Reference has already been made to the fact that aqueous extracts of laccase have been found to lose their activity on boiling. It has also been pointed out that laccase is less sensitive toward heat than tyrosinase. That it has a rather high thermal death point as compared with certain other ferments may be seen from the following observations bearing on this point. Kastle ([238]) found that exposure to a temperature of 80° C. or higher for a short time is sufficient to render the very active oxidases of *Lepiota americana* inert. On the other hand, at temperatures below 80° C. an hour's exposure was found to be insufficient to destroy the oxidizing power of these extracts toward guaiacum. Bertrand (cited by Green ([199]), p. 293) found laccase to be still active after heating to 70° C.

As already pointed out (see pp. 59–61), laccase is not specific in its action, but promotes the oxidation of a large number of easily oxidizable substances, such as guaiacum, guaiacol, phenolphthalin, hydroquinone, pyrogallol, adrenalin, and many other phenols and amino derivatives of the benzene series.

It has long been known that such substances as hydrocyanic acid and hydrogen sulfide destroy the activity of the oxidases and similar catalysts. Bouffard ([69, 70]) has pointed out that sulfurous acid prevents the action of oenoxydase. Up to the present, however, very little systematic work has been done on the effect of poisons on laccase. Kastle and Loevenhart ([244]) observed that the oxidizing power of aqueous extracts of the potato is destroyed by hydrocyanic acid, hydroxylamin, phenylhydrazin, sodium thiosulfate, and tenth-normal solutions of certain acids, such as hydrochloric, hydrobromic, benzene-sulfonic, para-nitro-toluene-sulfonic, oxalic, and salicylic, and that these substances also inhibited the oxidizing power of certain organic peroxides. Recently Bertrand ([61]) has found that most acids exert a poisonous or paralyzing action on laccase. On the other hand, there are some acids, such as carbonic, boric, and phosphoric, which are inactive at all concentrations.

THE PREPARATION OF LACCASE.

Slowtzoff ([404]), using the potato and cabbage as sources of the ferment, employed the following method for the preparation of what he calls pure laccase: One kilogram of fresh-washed potatoes were macerated to a paste and acetic acid added, so as to form a 0.5

to 1.0 per cent solution, in order to prevent the action of the oxidases on tyrosin and other chromogens contained in the potato. After standing twenty-four hours the mass is strained through a cloth and filtered. The clear reddish or yellowish liquid is then saturated with ammonium sulfate, and the precipitate, consisting of proteids, coloring matter, and ferments, is collected on a filter and washed with saturated ammonium sulfate solution, and finally dissolved in water. This salting out with ammonium sulfate and re-solution in water is repeated three or four times. The water solution finally obtained was then dialyzed against running water in pergament paper and then precipitated with 4 to 5 volumes of 95 per cent alcohol. The precipitate thus obtained was collected on a filter, washed with ether and dried over sulfuric acid. At the end of a week the yellowish-brown powder thus obtained was extracted with distilled water; a water-clear solution was thus obtained which, after saturation with chloroform and standing a month, gave only a slight percipitate (not weighable). In order now to obtain the pure ferment, the water extract thus obtained was precipitated with 5 or 6 volumes of ethyl alcohol, and the precipitate collected and dried over sulfuric acid in a desiccator. The yield of pure laccase was so small, however, that even after a year only about 1 gram of the material was obtained. The yield of laccase from cabbage was found to be even smaller.

The pure laccase obtained by Slowtzoff was found to give all of the reactions for protein and to contain 12.8 per cent nitrogen and 0.53 per cent of sulfur, and to be very poor in ash. According to this author, it belongs to the group of albumins and contains neither manganese nor phosphorus.

In order to determine the oxidizing power of his pure preparation, Slowtzoff made use of Röhmann's reagent, viz., a solution of paraphenylene diamin and meta-toluylene diamin in sodium carbonate solution, the quantity of coloring matter produced by the action of the ferment or other oxidizing agent, such as a ferric salt, being estimated colorimetrically. On the basis of these observations, he arrived at the following conclusions respecting the nature of laccase and its mode of action:

(1) Laccase belongs to the group of ferments for the reason—

(*a*) That it loses its activity at high temperatures. In this connection he found the thermal death point of the ferment to vary with the degree of purity of the preparation; thus his purest preparations lost their activity at as low a temperature as 50° C., whereas preparations richer in ash only lost their activity at temperatures of from 65° to 70° C.

(*b*) The amount of the substance oxidized is proportional to the square root of the quantity of laccase present.

(*c*) The quantity of product resulting from the action of the ferment is proportional to the quantity of the ferment, but not to the amount of substances being oxidized.

(2) The pure laccase preparations were found to act best in weakly alkaline solutions, as already observed by Bertrand and Bourquelot.
(3) Laccase belongs to the group of proteins. Its ash constituent is very small and without influence on its oxidizing power.
(4) Laccase is not destroyed by weak acids, nor by peptic or pancreatic digestion.

ANTI-LACCASE.

According to Gessard [191] it is possible to obtain a serum capable of retarding the action of laccase by the injection of preparations containing this oxidase subcutaneously into a rabbit. In order to obtain this anti-laccase serum Gessard adopted the following mode of procedure: A rabbit weighing about two kilograms received at intervals of five or six hours apart six injections, each of 1 gram of laccase powder in 10 c. c. of water. According to Bertrand [55] this represents about 0.15 gram of pure laccase. Two animals treated in this manner gave a serum of about the same potency. By means of guaiacum and guaiacol he found that the serum obtained from animals thus treated completely retards the action of laccase when two parts of the serum by volume are added to one part by volume of a 2 per cent solution of laccase. On the other hand normal serum and anti-tyrosinase serum were both found to be without effect on the action of laccase. Anti-laccase prepared by the use of the laccase from the juice of the lac tree was found to retard to a degree at least the color reactions produced by an extract of *Russula delica* on oxidase reagents. On the other hand Gessard [185, 186] observed that the anti-laccase obtained by the injection into rabbits of laccase from the lac tree is without retarding action on the laccase from the ink gland of the cuttle fish. He concludes therefore that the oxidases do not differ from other enzymes in their power to give rise to specific anti-bodies in the blood serum of animals, which have received a number of injections of the ferment.

According to Gessard the results of these researches on anti-laccase and anti-tyrosinase are sufficient to prove the individuality of these two oxidases, and serve to show that the oxidases do not differ from other enzymes in regard to their power of giving rise in the serum of animals to substances which oppose their action.

Czapek [129, 130] has also obtained evidence of the production of anti-oxidases in plants which hinder the oxidation of homogentisic acid, and which are produced in the growing ends of roots, special sense organs, and in fungi, as the result of irritation.

In the light of Bach and Chodat's views regarding the nature of the oxidases, the production of anti-bodies in the serum of animals as the result of the repeated injection of oxidases is probably due to the action of the peroxidase moiety of the oxidase and not to the oxygenase, since of these the former only seems to possess the properties of a ferment. Gessard's work is of such importance as to warrant

confirmation, especially in view of the failure of von Fürth and Jerusalem ([178]) to obtain evidence of anti-tyrosinase, and the whole subject of anti-oxidases should be reinvestigated by means of Bach's peroxidase.

TYROSINASE.

It has long been known that on exposure to the air certain of the higher fungi turn pink or red and finally black, whereas other species become blue. We have already seen that the latter change is due to an oxidizing ferment, laccase. That the reddening and final blackening of other species of mushrooms is due to the action of a specific oxidase was first suspected by Bourquelot and Bertrand ([89]), who in 1896 pointed out the existence in certain mushrooms, such as the *Rassula foetens* Pers., of a very active oxidizing ferment, probably different from laccase. They ([87, 88]) then showed that the blackening of *Russula nigricans* differs from the bluing of *Boletus cyanescens* by reason of the fact that the blackening of the crystalline chromogen contained in the former species is not accomplished by the laccase of the sap of the lac tree, whereas the blackening is undoubtedly due to an oxidation, as indicated by the fact that if one does not agitate the aqueous extract of the fungus the blackening takes place first in the upper layers of the liquid, and during the blackening oxygen is absorbed. Continuing these investigations Bertrand ([53]) proved the crystalline chromogen of *Russula nigricans* to be tyrosin. He also found that the beet root and the tubers of the dahlia and potato, like certain of the higher fungi, also redden and then turn black on exposure to the air. This change he now definitely proved to be an oxidation of tyrosin by atmospheric oxygen under the influence of a specific oxidizing ferment, to which he gave the name *tyrosinase*. From the roots and tubers of certain plants, such as the beet and dahlia, he was able to obtain in crystalline condition as much as 0.5 gram of tyrosin from one quart of the expressed juice, an amount about corresponding to the solubility of the compound in pure water. The tyrosin thus obtained was identified by Hoffmann's or Piria's reactions, and its composition determined by analysis. He also isolated tyrosin from *Russula nigricans*, and in this connection Bourquelot and Harlay ([90]) give a drawing of a transverse section through the stipe of *Russula nigricans*, showing the rosettes of tyrosin crystals distributed more or less regularly throughout the tissue of the fungus. Bertrand also showed that the blackening of tyrosin is due to an oxidase and that this oxidase differs from laccase. Thus when a small amount of the aqueous extract of the russula, prepared in the cold, and a solution of tyrosin were brought together, the mixture became red, then inky black, and finally deposited a black precipitate. He showed that oxygen was absorbed at the same time; this was proved by simply allowing the tube in which the reaction was being

carried out to remain quiet, whereby these color changes were first observed to occur on the upper surface of the liquid. In a vacuum or under a bell jar, resting in a watch glass on the surface of mercury, the mixture acquired a faint rose tint, since it was impossible to remove all traces of oxygen therefrom, but they showed no further deepening of color no matter what the duration of the experiment. With a boiled extract of russula no change of color was observed. A repetition of these experiments on the boiled juice of the beet, or with tyrosin of animal origin (from the horse), or of vegetable origin (from the dahlia or russula), or with the oxidase obtained from the beet or the dahlia, always led to the same results. Finally, in order to prove that the oxidation of tyrosin could not be accomplished by laccase, the following experiment was carried out: A certain amount of extract of russula was introduced into a vacuous flask, and then some tyrosin added. The flask containing these substances was then allowed to stand for twenty-four hours, at the end of which time no change of color had occurred. The contents of the flask were then heated to 100° C. for ten minutes in order to destroy all enzyme action. The flask was then opened and its contents exposed to the action of the air, but the tyrosin remained unaltered even after the addition of ordinary laccase. Hence the blackening of tyrosin is not due to the successive action of two ferments but solely to that of tyrosinase in the presence of oxygen. He then points out that independently of their special interest, these observations go to show that laccase is not the only oxidizing ferment existing in the vegetable world but that on the contrary, it should be regarded as a type of a series of analogous substances to which he had already given the generic name of *oxydases*. (See p. 54.)

While perhaps not so widely distributed in nature as laccase, tyrosinase has been found in a large number of plants and animal species. Bourquelot and Bertrand ([87, 88, 89]) found it in a large number of fungi, and also in phenogams. According to Lehmann ([260]) and Lehmann and Sano ([261]) tyrosinase is found in a number of species of bacteria, notably in *B. fluorescens nonliquefaciens*, and also in *B. phosphorescens*, *B. putridens*, and *Actinomyces chromogens*. As a general thing, wherever we find tyrosinase in plant tissues, we are apt to find laccase. The converse of this, however, does not hold generally—that is, we do not find tyrosinase wherever we find laccase. For example, in *Russula delica*, *Lactarius piperatus*, and in the tubers of the dahlia and potato, we find both tyrosinase and laccase, whereas in the silk of the green corn (*Zea mays*) we find laccase but no tyrosinase.

Tyrosinase is also widely distributed in the animal kingdom, where it plays an essential rôle in the formation of animal pigment (*melanogenesis*). The following are the more important investigations bearing on this point: The blackening (melanose) of the blood

(hemolymph) of certain insects was made the subject of an investigation by Krukenberg ([254]) and also by Fredericq ([176]) as early as 1881. According to Fredericq, oxygen was responsible for this change of color, whereas, according to Krukenberg, it was probably due to carbon dioxide. Fredericq also made the interesting observation that the blood obtained from insects which previous to bleeding had been heated to 50–55° C. for fifteen minutes, showed no blackening on exposure to the air. Dewitz ([138]) has also shown that an oxidase, the precise nature of which was not investigated, plays an essential rôle in the metamorphosis of certain insects. As is well known, the larvæ of the fly are white during the entire life of this phase of the insect, and only at the moment of the formation of the pupa does any change of color take place. This color change begins in the abdominal cavity as two large colored spots.

Biedermann ([67]) seems to have been the first to obtain evidence of tyrosinase of animal origin. In his experiments the middle intestine (*Mitteldärme*) of three or four starving meal worms (*Tenebria molitor*) were placed in chloroform water. The light yellowish solution thus obtained was divided into two portions. To one portion a few drops of a solution of tyrosin was added, and both were kept over night in open watch glasses in a moist chamber. The portion containing the tyrosin had become violet black in color, whereas the portion to which no tyrosin had been added was only slightly darkened.

Von Fürth and Schneider ([179]) have also made important contributions to our knowledge of animal tyrosinase and its relation to animal pigmentation. The pupae of the butterfly, chiefly of the species *Deiciphila elpenor* and *euphorbiae*, furnished the material for their investigations. By careful puncture and squeezing, about one cubic centimeter of a clear, bright greenish-colored fluid was obtained from each pupa. This was the hemolymph, called for the sake of brevity the *blood* of the insect. On standing a few moments in the air this fluid began to darken on the upper surface, and after a time a black clot separated. On the other hand, if the proteid of the fresh blood be removed by boiling, it shows none of these color changes on standing in the air. When dried in vacuo over sulfuric acid, no change of color in the blood was observed. When a few drops of the fresh blood was added to a few cubic centimeters of a fresh solution of tyrosin, there appeared on the upper surface of the solution a violet-colored ring; this color gradually spreads through the solution until the whole of it is colored a dark violet, and is turbid through the separation of a fine and coarse flocculent precipitate. On the other hand, two drops of the fresh blood added to water gave a solution which showed none of the color changes, but ultimately gave a precipitate scarcely darker in color than that of the fresh blood. The diluted blood gave with tincture of guaiacum a dirty, dark bluish-green color after long standing, and also a positive reaction

with Spitzer's para-phenylenediamin-α-naphthol reagent. It is therefore evident from these observations that the blood of these lepidoptera contains oxidases having the power of oxidizing guaiacum, Spitzer's reagent, tyrosin, and the chromogen of the blood itself. Attempts to purify the tyrosinase of the blood of lepidoptera by precipitation with uranyl acetate according to Jacoby ([225]) failed. Von Fürth and Schneider were able to separate it from the chromogen and from the crystalline substances of the blood by fractional precipitation with ammonium sulfate. The ferment thus purified gave with tyrosin a violet-colored solution, which after a short time became dark violet and finally black. It was also found to produce coloring matter with pyrocatechin (yellowish red) and hydroquinone (red solution becoming turbid and finally showing a considerable brown precipitate); a carmine-red solution of an iron compound of suprarenin, the blood-pressure-raising compound of the suprarenals, became dirty brown in color, whereas the control experiment showed no alteration. Oxyphenyl-ethyl amin (Emerson) became yellowish brown and gave finally an olive-colored precipitate. On the other hand, it was found to have no action upon casein, indicating that the ferment is powerless to act on the tyrosin residues contained in proteid molecule. The nature of the black substance produced by the action of the tyrosinase of lepidoptera on tyrosin was also studied. Ten to twenty c. c. of a freshly prepared solution of the ferment, obtained from the lymph of from twenty to forty Deiciphila pupae, was brought together with a few decigrams of finely pulverized tyrosin and shaken vigorously in a shaking machine for four to six hours. In a short time the characteristic violet coloration appeared in the solution, and after a time a considerable black precipitate separated, leaving the supernatant fluid clear and colorless. With fresh quantities of tyrosin this gave no further coloration. This material was then filtered off and washed with water, then with dilute hydrochloric acid, then with hot water until chlorine free, and finally with alcohol and ether and dried to constant weight at 110° C. This black substance was found to be insoluble in water, alcohol, ether, and the common organic solvents and in dilute alkalis at room temperatures and in strong boiling hydrochloric acid. A small quantity of it melted with the purest sodium hydroxide from sodium gave rise to an unmistakable odor of indol and skatol. On analysis it gave the following numbers:

	Per cent.
C	55. 44
H	4. 45
N	13. 74

corresponding to the following atomic ratios:

$$N:H:C=1:4.55:4.77,$$

hich is essentially the same as that obtained by Hofmeister for a hole series of pigments ordinarily called *Melanine*, to which belong he dark pigment of the hair, and of the skin, the choroid coat, melaotic tumors, sepia black, and certain split products of proteids, such s Schmiedeberg's melanine acid (melanin saüre). In its physical roperties, solubility, etc., and in its tendency to yield substances aving a skatol-like odor, on melting with alkali, it agrees closely with he melanins, and with a pigment obtained by Ducceschi [149] by xidizing tyrosin with potassium chlorate in hydrochloric acid soluion. For other analyses of the black pigment of hair and feathers, ee Hodgkinson and Sorby [213].

According to these authors (von Fürth and Schneider) tyrosinase n its occurrence in the animal kingdom is by no means limited to the nsects. They have also obtained it from the blood of the crawfish *Flusskrebse*), and by refined methods it would doubtless be possible to ecognize tyrosinase in the most widely differing classes of animals. Acting upon their suggestion, Przibram, of the zoological station at Trieste, found tyrosinase in the ink sac of the cuttlefish, *Sepia officinalis*. An extract of the washed epithelial lining of the sac gave with a solution of tyrosin first a very beautiful orange-yellow color, changing to sepia brown, and finally yielding a black precipitate. Von Fürth and Schneider are therefore of the opinion that probably wherever melanotic pigments occur in the living tissues of the lower and higher animals they originate as the result of the action of appropriate enzymes on substances of aromatic nature. They point out in this connection that Salkowski and Jacoby have shown independently that tyrosin results from the autolysis of various animal tissues. It would seem likely therefore that in the formation of melanotic pigments two ferments are jointly concerned, one, an autolytic ferment capable of splitting off tyrosin or a similar aromatic complex from the protein molecule, and the other tyrosinase, which transforms the tyrosin into melanin. To determine whether tyrosinase occurs in melanotic tumors offers an interesting and important field for further investigation.

Gessard [184] has obtained tyrosinase from the glands of the ink sac of the cuttlefish (*Seiches*) and calamary (*Calmar*), and in 1904 this same author [190] showed that the coloration of the integument of the green fly (*Lucilia Caesar* L.) is due to the action of tyrosinase. The larvæ of the fly are white, and from them this author was able to obtain tyrosin in crystalline condition, as well as to demonstrate the presence of tyrosinase. In the course of their metamorphoses these larvae exhibit a succession of colors similar to those shown by a solution of tyrosin when acted on by tyrosinase, until finally the insect attains the full iridescent green color of the fly. When the white pupae are kept in a vacuum the ferment is inactive and they remain

white. These facts serve to corroborate the hypothesis that tyrosinase is responsible for the production of cutaneous pigments in man and animals.

In order to demonstrate the presence of tyrosinase, the ink sac (glande du noir) of the cuttle fish was removed and macerated with chloroform water and filtered through a Chamberland filter. Under these conditions the fine granules of pigment are retained by the filter and a clear solution of the ferment is obtained which exhibits the same color changes with tyrosin as is shown by an extract of russula. Gessard was also able to demonstrate the presence of tyrosinase in the commercial product known as *sepia envessie*—crude sepia. This is simply the dried gland with its contents and is employed in the preparation of the refined coloring matter of the same name, sepia. In the course of this investigation he also showed that the anti-tyrosinase serum ([182]) obtained by the repeated injection of a rabbit with vegetable tyrosinase is powerless to hinder the action of the animal tyrosinase on tyrosin, indicating that, despite the similarity of the two varieties of tyrosinase in their action on tyrosin, they are not precisely alike in all respects. (See also Gessard, [183].)

Similarly in his studies on the formation of the melanotic pigments in tumors of the horse, this author ([184]) calls attention to the fact that a general chemical and physical relationship has long been recognized as existing between the black pigments of the eye and skin and that of the cuttle fish (Seich) and other molluscs. It is quite likely, therefore, that our knowledge of the formation of this pigment in the case of the cephalopods will hold equally well for its production in other animals. The abnormal production of melanotic pigments in healthy or diseased tissues of man is rare, but more common in those of the horse, in which case its production is of less formidable significance. Melanotic tumors are especially common on the white horse, and these furnished the material for Gessard's investigations. He has found that in the production of the melanotic pigment of such tumors the same agencies are at work as in its production in the ink sac of the cuttle fish, viz, a chromogen and an oxidizing ferment. From such tumors he was able to obtain tyrosin by appropriate methods, in crystalline condition, and aqueous extracts thereof were found to give with tyrosin the color changes characteristic of tyrosinase. The author concludes therefore that tyrosin is the chromogen whose oxidation by tyrosinase gives rise to the pigment in melanotic tumors and wherever else such pigments are met with in the animal economy. He is also of the opinion that the color of the negro is due to the reaction which gives rise to production of the ink of the cuttle fish (Seiche) and the black pigment of mushrooms. While such is doubtless the case, we are still a long way from an understanding of the physiological cause which gives rise to tyrosinase in the epidermal

sues of the negro and the lack of its general production in the idermal tissues of the white races under normal conditions. The ct, however, that melanotic pigments are formed over limited areas the dermal structures of white-skinned races indicates that the me causes of pigmentation may be operative in all races to a greater less degree and serves to emphasize the importance of further udies on the mode of action of tyrosinase and its mode of origin in imal tissues.

The occurrence of tyrosinase in the skins of certain pigmented rtebrates has been investigated by Miss Florence M. Durham (155). queous extracts of the skins of rabbits, rats, guinea pigs, and chickens the fœtal stage of development, containing small amounts of fer- us sulfate, were found to act upon tyrosin with the production of gments similar in tint to those characteristic of the coat of the ani- al. Thus black pigments were formed with extracts of the skins black-pigmented animals, and yellowish pigments with those of e skins of animals containing orange-colored pigments, and with tracts of the skins of white or albino animals no pigments were med by the action of an extract of the skin on tyrosin. The tyrosin- e present in the skins of animals was found to act most rapidly at ° C. It is destroyed by boiling and does not act in the cold. From e to ten days were required for the production of the pigment in tro. Another peculiarity of the tyrosinase contained in the skins animals is that it only acts upon tyrosin in the presence of small nounts of iron.

Phisalix (321) has obtained tyrosinase from the skin of the green g, and Gessard (188) has found it in the skins of frogs of other spe- s, such as the frog rousse and the common toad. More recently isalix (322) has investigated the color changes occurring in the rvæ of the cockroach (*Phyllodromia germanica*). Immediately ter hatching from the egg the integument of the larva is soft and ight white in color. In the course of a short time, however, it be- mes grey, then brown, and finally black, so that three hours after tching the little cockroach is entirely black. According to this thor this change of color is the result of the action of tyrosinase tyrosin, both of which occur in the embryo at the moment of its velopment; indeed, it is probable that they are both present in the g in which they are deposited at the time of ovagenesis.

Still more recently a very valuable contribution to our knowledge melanotic pigments and fermentative melanine formation has been ade by Von Fürth and Jerusalem (178). These authors have com- red hippomelanin (the black pigment produced in tumors of the rse) with other natural and artificial melanins, both as to mode formation and general physical and chemical properties. They und hippomelanin to be iron free and are inclined to look upon its

sulfur content as of accessory nature. In its physical properties, general chemical conduct, and decomposition products, hippomelanin shows a close resemblance to the artificial melanin produced by the action of tyrosinase on tyrosin, whereas it differs in certain respects from other melanins and epidermal pigments and the pigment phymatorhusin of malignant melanotic tumors. Up to the present, according to these authors, the chemical investigation of hippomelanin has disclosed no fact at variance with the well-established hypothesis of the fermentative origin of melanin as the result of the action of tyrosinase on the cyclic complexes resulting from the degradation of the protein molecule.

The principal sources of vegetable tyrosinase are certain of the higher fungi, especially many species of russula, such as the *Russula delica* (Bourquelot and Bertrand, [87, 88, 89]), *Agaricus melleus* and *Agaricus campestris* (Von Fürth and Jerusalem, [178]), and wheat bran (Bertrand and Mutermilch, [65]). The ink sac of the cuttle fish (*Sepia officinalis*) and the hemolymph of the pupæ of *Deiciphila euphorbiæ* ([178], p. 161) are the chief sources of animal tyrosinase. In order to obtain a solution of tyrosinase from *Russula delica* it is only necessary to macerate the fresh fungus with chloroform water or with glycerin, or, as recommended by Bach ([24]), the tyrosinase of *Russula delica* may be precipitated from the aqueous extract of the fungus by means of 96 per cent alcohol. Young, sound fungi are ground in a sausage machine and 300 c. c. of the clear expressed juice is poured into 1.5 liters of 96 per cent alcohol. The precipitate thus formed is filtered off by means of a filter pump, washed with alcohol, and dried in vacuo over calcium chloride. The dried precipitate is now mixed with 300 c. c. of water, whereby only a small amount of the material goes into solution. The mixture is then filtered, an entirely colorless solution of tyrosinase being obtained.

In this connection Bach ([24]) has observed that the activity of the tyrosinase solutions depends on the age and state of preservation of the fungus. Thus from three lots of the fungus (I, young, unblemished fungi; II, older, more or less injured fungi; and III, putrid fungi) he obtained aqueous extracts, equal amounts of which acting on the same quantities of tyrosin, required the following quantities of 0.002 N. permanganate:

I.	II.	III.
37.8 c. c.	13.6 c. c.	8.3 c. c.

The residue was then dried in vacuo or at once dissolved in 100 c. c. of chloroform water, in which case it was used at once, since aqueous solutions of the ferment are very unstable. By this mode of preparation the tyrosinase is separated from the catalase which the fresh aqueous extracts of this fungus contain. These authors have found

at tyrosinase is easily destroyed or rendered inactive by alcohol, d hence if the ferment is prepared by this method it is necessary filter off the alcohol as soon as practicable.

In order to prepare tyrosinase from the ink sac of the cuttle fish, ssard [184] macerated the ink sac with chloroform water and filtered rough a Chamberland filter. A clear solution was thus obtained ich gives with tyrosin the same color changes as those shown with aqueous extract of *Russula delica*. In the preparation of the animal rosinase employed in their latest investigations Von Fürth and rusalem [178] employed the hemolymph of the pupae of *Deiciphila phorbiae*. This was half-saturated with ammonium sulfate, and e washed and pressed precipitate thus obtained dissolved in 0.04 r cent soda solution. This solution exhibited strong tyrosinase actions.

In order to obtain the ferment from wheat bran, Bertrand and utermilch [65] recommend the following method: One part of wheat an is mixed with four parts of water and the mixture allowed to and for several hours. The mixture is then centrifugalized, and the lution thus obtained is mixed with three volumes of 95 per cent cohol and again centrifugalized. The precipitate is then washed th 80 per cent alcohol, mixed with distilled water, and again cen- fugalized. The solution thus obtained is then mixed with three to ur times its volume of alcohol, and the precipitate thus formed is llected, washed with strong alcohol, and dried in vacuo over sul- ric acid. The substance thus obtained, the yield of which is about 8 per cent, contains no laccase. On the other hand, when dissolved water and filtered through a Chamberland filter, a clear solution is tained which of itself undergoes no alteration on exposure to the r. On the addition of small amounts of tyrosin, however, it passes pidly through a succession of colors—rose, cherry red, and finally, ownish black. On the other hand, if all gaseous and dissolved ygen be removed, or if the filtered solution be heated to 100° C. r five minutes, no coloration with tyrosin is observed.

In the preparation of vegetable tyrosinase from *Agaricus melleus*, on Fürth and Jerusalem [178] rubbed up two kilograms of the fungus ith sand, and extracted the mass with two liters of chloroform water. fter two or three hours the supernatant liquid was poured off and ixed with twice its volume of 96 per cent alcohol. The precipitate as filtered off on raw silk.

All observers seem to be agreed that tyrosinase is a true enzyme. hus it appears to conform to Portier's [330] definition of an oxidizing rment, according to which, first, gaseous or dissolved oxygen is cessary for its action; second, its action is accompanied by a table absorption of oxygen; third, it is destroyed by heat; and, urth, it is nondialyzable.

Miss Durham (155) found that after filtering off the pigment produced by their action upon tyrosin, the preparations of animal tyrosinase could act upon fresh portions of tyrosin. According to Bertrand (59) tyrosinase is more easily destroyed by heat than laccase; thus he found the former to be destroyed in twelve minutes at 60–70° C., whereas laccase withstood this temperature for twenty hours. Gessard (182) found tyrosinase to be almost destroyed by heating to 65° C. for thirty seconds. Chodat and Staub (117) observed the activity of tyrosinase to increase with a rise of temperature to 61° C., and to become inactive at 61° C. Advantage has been taken of this difference in the stability of tyrosinase and laccase toward heat in the separation of the two enzymes, and tends to show that these two oxidases are different ferments. Thus *Russula delica* contains both tyrosinase and laccase. If now, according to Bertrand (54) the fungus be macerated with its own weight of chloroform water and the liquid thus obtained be treated with alcohol in the proportion of three volumes of alcohol to two volumes of extract, a precipitate is obtained which gives the reactions of tyrosinase. If now the filtrate be evaporated at 50° C. to one-tenth of its original volume, it will be found to act energetically on hydroquinone and pyrogallol, but to be without action on tyrosin. In other words, the filtrate that has been subjected to a temperature of 50° C. for some time still contains laccase, but no tyrosinase. Similarly Bach (25) has obtained laccase free from tyrosinase from the fungus *Lactarius vellereus* by heating to 75° C. Miss Willcock (451) has shown that tyrosinase is not killed by the radium emanation.

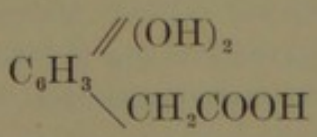
Kastle has observed that glycerin extracts of *Lactarius piperatus* and *Lactarius volumen* oxidize tyrosin strongly after having been kept four years in glass-stoppered bottles under ordinary laboratory conditions.

Like many other enzymes, tyrosinase is sensitive to the action of acids, alkalies, salts, and certain poisons like hydrocyanic acid. Thus Wolff (459) finds that the tyrosinase from *Russula delica* is most active on tyrosin when the solutions are neutral to phenolphthalein. Similarly Abderhalden and Guggenheim (1) find that N/100 hydrochloric acid inhibits the action of tyrosinase, and that N/100 sodium hydroxide retards it considerably. After treatment with acid or alkali, neutralization of the acid or alkali fails to restore it to its original activity, indicating that the ferment is actually destroyed under these conditions. Gessard (182) found that while salts of the metals promote the coagulation of the black pigment, they hinder the development of the initial coloration, the retardation being proportional to the quantity of salt present. Neutral salts were found to produce a retardation ranging from twenty-three minutes to nine days, and alkaline carbonates prevented the development of the

lor for seventeen days. Even egg albumin and blood serum were und to retard the action of the ferment.

The changes of color produced by the action of tyrosinase on rosin have been carefully studied by Gessard (183). According to is author, when tyrosinase is added to an aqueous solution of rosin, oxygen is absorbed and the liquid takes on a rose color which adually becomes reddish yellow, rapidly changing to mahogany d and then to garnet. He also observed the most marked colora- on in the upper layers of the liquid, due to immediate contact with ie air. Continuing these studies, Gessard (189) observed the colora- on of tyrosin by tyrosinase to consist of two distinct phases, the st of which alone, viz, the change to rose and then to red, is, accord- g to this author, attributable to the ferment. After a certain terval, depending on general conditions, the solutions acquire a iolet color, and finally yield a black precipitate (melanine), leaving ie supernatant fluid perfectly colorless. When, for example, the d liquid resulting from the action of tyrosinase upon tyrosin is xposed to a vacuum, it slowly becomes colorless. Now, on expo- ire to the air, the decolorized solution acquires a violet instead of a d color. Hence, a new substance has been produced by the duction of the first substance in the vacuum, from which the first ibstance apparently can not be regenerated. The substance thus otained by exposing the reddened solutions of tyrosin to a vacuum as been found to be very oxidizable and is characterized by its ielding a violet solution on oxidation, the coloring matter showing great tendency to separate from the solution in the form of a black recipitate. The production of the violet compound as the result f the action of tyrosinase on tyrosin is also facilitated by the presence f certain salts, a fact which would probably explain the production f the black compound under natural conditions.

Aside from the fact that melanines are formed by the oxidation of rosin through the agency of tyrosinase, but little is known as to the recise mechanism of the process. According to Gonnermann (195), omogentisic acid, hydroquinone-acetic acid—



the principal product resulting from the action of tyrosinase on yrosin in the presence of oxygen. According to this author, tyro- inase is not an oxidizing but a hydrolytic ferment, and his conception f the process is that the homogentisic acid produced by the action of yrosinase on tyrosin in the presence of air results from the spon- aneous oxidation of an unknown product of the hydrolysis of tyrosin y tyrosinase and not from the direct oxidation of the tyrosin, through

the agency of the ferment. This view of the mode of action of tyrosinase has recently been disproved by Bach ([25]), who has shown that tyrosinase does not produce black pigments from mixtures containing such substances as might be produced by the hydrolysis of tyrosin, such as phenol + d + l serin, hydroquinone + alanin, p-cresol + oxyamino-acetic acid, and p-oxy-benzyl alcohol + glycocoll.

Gonnermann's hypothesis respecting the mode of action of tyrosinase has also been refuted by Chodat and Staub ([117]). The results of their experiments in an atmosphere of carbon dioxide show clearly that oxygen is required for the process and that the action of the ferment is not simply the production of an easily oxidizable substance by the hydrolysis of tyrosin.

We have seen that according to Gessard ([181, 189]), the action of tyrosinase on tyrosin consists of two distinct processes, first, the oxidation of tyrosin to a red substance, and, second, the condensation of the red substance to a black product (melanine). The oxidation can be accomplished either by tyrosinase in the presence of air, or by a chemical oxidizing agent such as Millon's reagent, whereas, for the condensation of the red substance into the black pigment, the action of the mineral salts contained in the tyrosinase are necessary. This hypothesis respecting the mode of action of tyrosinase has recently been refuted by Bach ([25]). In order to throw further light on this subject he prepared an active solution of tyrosinase and heated it to boiling. After cooling, tyrosin was added to the solution, and 20 c. c. portions of it were placed in four reagent glasses. To the first of these there was then added 5 c. c. of water, to the second 2 c. c. of 1 per cent hydrogen peroxide and 3 c. c. of water, to the third 2 c. c. of 1 per cent hydrogen peroxide solution, 2 c. c. of a peroxidase solution, and 1 c. c. of water, and to the fourth a solution of an oxidase prepared by heating an extract of *Lactarius vellereus* to 75° C. to destroy the tyrosinase. These tests remained colorless for weeks, indicating that the oxidation of tyrosin by tyrosinase is not referable to the inorganic substances which it contains. Bach also sought in vain for a co-ferment of tyrosinase among plants. He concludes, therefore, that the oxidation of tyrosin only takes place through the action of peroxidases, hydrogen peroxide, plant juices, or ferment preparations when the preparation itself is active to tyrosin, that is, when it contains tyrosinase. Indeed, it would seem from his most recent utterances on the subject that Bach (ibid.) is inclined to look upon the action of tyrosinase as completely different from that of the common oxidases (peroxidase + hydrogen peroxide). Tyrosinase, he says, belongs to a peculiar class of oxidizing ferments, whose oxidizing action is exerted upon substances containing slightly labile hydrogen.

Formerly all observations upon the action of tyrosinase were qualitative in character, being confined to rather crude and indefinite colo

comparisons. Recently, however, three quantitative methods for determining the quantity of pigment resulting from the action of tyrosinase on tyrosin have been proposed. These are (1) a spectrophotometric method, (2) a sedimentation method, and (3) a volumetric method by means of a 0.002N solution of potassium permanganate. The first two methods were proposed by Von Fürth and Jerusalem [178]. For details concerning these methods and the use of the instrument, with which the writer is not familiar, the original article may be consulted. In this connection see also Mörner [307]. The second method of Von Fürth and Jerusalem [178] depends upon the sedimentation of the pigment by boiling with a small amount of calcium chloride. After boiling a short time the beakers or tubes containing the substances are allowed to stand, when the black pigment settles out, leaving the liquid clear. The clear supernatant liquid is then poured off and the residue washed with water and transferred to a graduated centrifuge tube by means of water, centrifuged and measured. The third method was first employed by Bach [23], and depends upon the fact that the brown pigment formed by the action of tyrosinase on tyrosin can be oxidized by a dilute acid solution of permanganate, 0.002N, to a colorless compound. Hence in order to determine the quantity of pigment produced in a given time by the action of tyrosinase on tyrosin, the black mixture is titrated with 0.002N permanganate, after the addition of sulfuric acid, until the color of the titer disappears.

Of the three methods, Bach's is the simplest and commends itself most strongly to chemists. It requires no special apparatus, and by its use he (Bach) obtained far more regular and generally concordant results than Von Fürth and Jerusalem were able to obtain with the spectrophotometric and sedimentation methods. (See Bach [24]).

THE KINETICS OF MELANINE FORMATION BY TYROSINASE.

The quantitative methods devised by Von Fürth and Jerusalem [178] and by Bach [23] have already been utilized by these observers and also by Chodat and Staub [117] in studying the kinetics of the tyrosinase process. According to the latter the velocity of the reaction at small concentrations is proportional to the quantity of ferment present. At greater concentrations the rate of the reaction has been found equal to the algebraic expression, $Kc + b$, in which c is the concentration, and K and b are constants. Von Fürth and Jerusalem (supra) have investigated the effect of temperature, quantity of ferment, and the influence of hydrogen peroxide, alkalinity, and inorganic catalyzers on the kinetics of melanine formation by vegetable and animal tyrosinase.

Von Fürth and Schneider [179] found the tyrosinase of insect blood to be exceedingly sensitive to the prolonged action of high tempera-

tures. Thus a long exposure to 30° C. is sufficient to inhibit the action of the ferment. In order to determine the effect of temperature on the action of vegetable tyrosinase (from *Agaricus melleus*) Von Fürth and Jerusalem prepared four tubes, each of which contained 4 c. c. of the tyrosinase solution, 60 c. c. of an alkaline solution of tyrosin, and 2 c. c. of 3 per cent hydrogen peroxide. These tubes were labeled *a*, *b*, *c*, and *d*; *a* was kept at 5°–7° C., *b* at room temperature, *c* at 40° C., and *d* at 55° C. At the end of half an hour *a* and *b* were not noticeably altered in appearance; *c* was darker, and *d* was the darkest in color. The next morning *a* and *b* were colored black, *c* was lighter, and *d* was the lightest in color. The quantities of melanine in the four tubes as determined by the spectrophotometric method were found to stand in the following ratio:

$$a = 1.29;\ b = 0.55;\ c = 0.42;\ \text{and}\ d = 0.22.$$

These authors conclude, therefore, that so far as the influence of temperature on the production of melanine by the action of tyrosinase is concerned, two opposing processes are at work, viz, the accelerating effect of temperature common to all chemical reactions and the destructive action of temperature on the labile ferment. The result is that the process reaches an equilibrium between 30° to 50° C., and that between 60° and 65° C. the ferment ceases to act on the tyrosin. The effect of small amounts of hydrogen peroxide is to increase the quantity of melanine produced by tyrosinase in a given time, whereas larger quantities of the peroxide exert a retarding effect on the process. Roughly, the quantity of melanine formed by the action of a given amount of the ferment acting under the same conditions was found to be proportional to the concentration of the tyrosin. Within narrow limits of concentration (0 to 10 c. c. of 0.04 per cent sodium carbonate solution in a total dilution of 27 c. c.) the effect of alkali was found to be practically negligible. Within certain limits the quantity of melanine formed increases with increase in the concentration of the fungus tyrosinase. Thus the effect of doubling the quantity of ferment was to cause an increase in the production of melanine from 1.0 to 1.4. On the other hand, the addition of further amounts of the ferment caused no increase in the production of melanine; in fact, a slight diminution in the quantity of melanine occurred, indicating that increasing quantities of the ferment above certain limits cause a retardation of the process.

With animal tyrosinase (from the hemolymph of *Deiciphila euphorbiae*) the rate of melanine formation was considerably increased by an increase in the quantity of hydrogen peroxide present, but in the end very nearly the same amounts of melanine were produced in all cases. Acids, even the weakest, were found to prevent the action

of animal tyrosinase, while the effect of the addition of small amounts of alkali is to cause a distinct increase in the activity of the ferment. Of the metallic catalyzers tested, viz, 1 per cent solutions of the sulphates of manganese, iron (ferrous), copper, and nickel, only manganese was found to increase the rate of production of the melanine by animal tyrosinase. With the animal tyrosinase much greater increases in the quantities of melanine produced resulted from increases in the quantities of ferment; in other words, while the further addition of animal tyrosinase over and above a certain amount caused no corresponding increase in the quantity of melanine produced, no distinct hindrance of the process, such as that brought about by large amounts of vegetable tyrosinase, was observed.

As already indicated in the above, Bach's [23] permanganate method for the determination of melanine has yielded more concordant results in the study of the kinetics of melanine formation by tyrosinase than any that have been employed up to the present time. Without going into details, this author [24] has shown that in the production of melanine from tyrosin, tyrosinase undoubtedly obeys the law of mass action, the departures therefrom observed during the later phases of the reaction being due to the fact that the activity of the ferment becomes more or less exhausted during the course of the reaction, this exhaustion being the more rapid the greater the concentration of the ferment or substrat; that is, the greater the velocity of the reaction.

THE ACTION OF TYROSINASE ON VARIOUS AMINO COMPOUNDS, ESPECIALLY THE PRODUCTS OF PROTEIN DEGRADATION.

It has been pointed out that laccase is not specific as an oxygen carrier, but that it can effect the oxidation of various easily oxidizable substances such as guaiacum, guaiacol, hydroquinone, phenolphthalin, etc. The question naturally suggests itself in this connection, Is the action of tyrosinase confined to tyrosin or can it likewise accomplish the oxidation of other aromatic amino compounds? The earlier workers in this field were doubtless inclined to look upon it as specific in its action on tyrosin. Thus vegetable tyrosinase (from *Russula delica*) was employed by Bougault [72] as a reagent for the detection of tyrosin in various animal products, and by Harlay [206] for the detection of tyrosin in the products of the pancreatic digestion of fibrin, and also in the products of the proteolysis in germinating grain. With the products of the pancreatic digestion of fibrin, tyrosinase gives a reddish brown color (tyrosin); with the products resulting from the peptic digestion of fibrin, Harlay found the extract of russula to give a green color. This indicates, of course, the absence of tyrosin, but it also indicates that tyrosinase can act

upon substances other than tyrosin. During the last few years this conduct of tyrosinase toward a large number of aromatic amino compounds has been studied, including the optical isomers of tyrosin, and also the effect of various amino compounds and other substances on the action of tyrosinase on tyrosin. Thus it has been found by Bertrand and Rosenblatt ([66]) to act equally well upon racemic and laevo-tyrosin. They found the tyrosinase from *Russula queletti* Fr., to give equal amounts of melanine with (d+l) and l-tyrosin, in a given time, without any separation of the racemic compound into its optically active components. According to Chodat and Staub ([117]) albumoses do not give a red color with tyrosinase. Such a coloration is produced by the action of tyrosinase on glycyl-tyrosin anhydride, indicating that possibly other peptids may give the reaction. In a continuation of their researches on the action of tyrosinase on the products of protein degradation, these authors ([118]) have observed that the oxidation of tyrosin by tyrosinase is diminished by certain amino acids, such as glycin, leucin, and alanin. They have found tyrosinase to act upon certain dipeptids, such as tyrosin anhydride, and glycyl-tyrosin anhydride, giving rise to yellow substances which do not become black, as does tyrosin itself. When, however, an amino acid, such as glycin, leucin, or alanin, is present, a red coloration similar to that resulting from tyrosin is obtained. Thus, a mixture of glycyl-tyrosin anhydride with glycin gives with tyrosinase a rose color, changing to bluish green; with alanin it gives a deeper red, and with leucin a deep brown color. Phenyl-alanin is not acted on by tyrosinase. These observers conclude, therefore, that the action of the ferment does not depend altogether upon the presence of a benzene nucleus in an amino acid. They have also found that tyrosinase acts readily on p-cresol, less readily on m-cresol, and still less readily on o-cresol. As a rule they have observed tyrosinase to act most readily on the homologues of phenol, in which the side chains occupy the para- position, and in this respect the ferment seems to differ essentially from Millon's reagent, which is apparently specific for benzene compounds containing one hydroxyl group, especially meta derivatives. According to Chodat and Staub (supra) the action of tyrosinase on p-cresol serves to distinguish the ferment from laccase. The addition of glycin or another amino acid greatly increases the rapidity of its action on p-cresol, giving rise to violet color which ultimately becomes blue with a reddish fluorescence. These authors conclude that tyrosinase may be employed as a reagent for tyrosin and that with the addition of amino acids, the ferment may also be employed to detect peptids containing tyrosin residues in the products of the digestion of protein.

Bertrand ([62]) in his recent researches on melanogenesis, has also studied the action of tyrosinase from wheat bran on various com-

pounds analogous to tyrosin. With phenyl-alanin, phenyl-ethyl-amin, phenyl-methyl-amin, phenyl-amino-acetic acid, phenyl-propionic acid, phenyl-acetic acid, alanin and glycocoll, no coloration was observed. On the other hand, compounds containing phenolic hydroxyl were oxidized with the production of characteristic colors, as may be seen from the following table:

Name of compound.	Color produced with tyrosinase.
Tyrosin	Grenadine-red; then inky black.
p-hydroxy-phenyl-ethyl-amin	Grenadine-red; then olive-black.
p-hydroxy-phenyl-methyl-amin	Orange-yellow, orange-red, clear maroon.
p-hydroxy-phenyl-amin	Orange, mahogany-red, brown.
p-hydroxy-phenyl-propionic acid	Orange-yellow, grenadine-red, brown.
p-hydroxy-phenyl-acetic acid	Yellow, orange-yellow, brown.
p-hydroxy-benzoic acid	Rose, orange, yellow.
p-cresol	Yellow, orange, red.
phenol	Yellow, orange, red, brown.

He concludes, therefore, that only substances containing phenolic hydroxyl are oxidized by tyrosinase. He found that polypeptids are not colored exactly as is tyrosin, but become first yellow, then orange, and then mahogany-red, without the production of any precipitate. He concludes, therefore, that if in these polypeptids there were previous splitting into tyrosin and other products, one should obtain the same coloration as with tyrosin, since glycocoll in the proportion ordinarily found would not modify the action of the tyrosinase on tyrosin. In his opinion, it is necessary to separate the chromogen in pure condition in order to properly identify it by means of tyrosinase.

The action of tyrosinase from *Russula delica* on tyrosin, tyrosin-containing polypeptids, and certain other compounds, under various conditions, has also been studied by Abderhalden and Guggenheim.(1) According to these authors glycocoll, d-alanin, d-valin, l-prolin, d-serin, d, l-iso-serin, l-phenyl-alanin, l-aspartic acid, and d-glutaminic acid are without effect on the action of tyrosinase on tyrosin, except in so far as they influence the rapidity of the development of the color. Aspartic and glutaminic acids were found to inhibit the action, as did also the other amino acids, especially if present in strong solution. The action of tyrosinase was also tried on the following substances: l- and d-tyrosin, di-iodotyrosin, l-phenyl-alanin, homogentisic acid, l-tryptophane, skatol, indol, l-prolin, and cystine. Of these, homogentisic acid and tryptophane were the only substances except tyrosin to show a color change with the ferment. On the other hand, polypeptids containing tyrosin residues were colored by tyrosinase, the color being modified to some extent by the nature of the amino acid combined with the tyrosin in the polypeptid. Halo-

gen derivatives of the polypeptids were not acted upon. The action of tyrosinase on a polypeptid containing tyrosin was modified to some extent by various amino acids. Thus the action was greatly accelerated by l-prolin, whereas it was retarded by aspartic and glutaminic acids. Prolin was found to act especially energetically in augmenting the action of tyrosinase on glycyl-l-tyrosin anhydride. They also found tyrosinase to act on phenol, giving a brown color, and here again the color produced by the action of tyrosinase was modified by amino acids. Thus glycocoll and phenol gave a cochineal color, and prolin and phenol gave a violet reaction. These authors conclude that the character of the pigment resulting from the action of tyrosinase on tyrosin is dependent upon the combination in which the tyrosin exists. In the free state it is colored differently from what it is when in the anhydride or in the polypeptids. The amino acids when present apparently take part in the production of the pigment. In a later communication ([2]) these authors point out that tyrosinase acts rapidly on d-alanyl-l-tyrosin, and on l-leucyl-l-tyrosin. They also found it to act on adrenalin with the rapid production of a red color and ultimately dark red flocculi. It was also found to act on the three optical isomers of adrenalin with equal rapidity.

ON THE NATURE OF TYROSINASE.

Bach and Chodat ([28]) (see p. 118–120) have shown that laccase is composed of two distinct substances, an oxygenase—that is, a substance which forms a peroxide by taking up of oxygen and which is replaceable by hydrogen peroxide,—and a peroxidase, which activates this peroxide or the hydrogen peroxide added. According to this conception, the system, peroxidase + hydro-peroxide, is to all intents and purposes identical with the oxidases in its general behavior toward readily oxidizable substances. The question, therefore, naturally suggests itself in this connection, Is tyrosinase similarly constituted? In other words, Is this oxidase composed of a specific peroxidase and an oxygenase, and can other peroxides, such as hydrogen peroxide, take the part of the oxygenase in tyrosinase oxidations? Bach ([21]) has attempted to answer these questions. According to this observer, tyrosinase contains a peroxidase and an oxygenase, and it is to the former that it owes its specific power to oxidize tyrosin and similarly constituted substances, since hydrogen peroxide may be employed in the place of the oxygenase contained in tyrosinase in accomplishing the oxidation of tyrosin. Thus he observed that a fresh aqueous extract of young potato tubers rapidly oxidizes and blackens a solution of tyrosin, whereas if the expressed juice of finely ground new potatoes be allowed to stand for twenty-

four hours with one-tenth of its volume of strong alcohol in order to remove mucilaginous substances, and the filtrate therefrom be mixed with four times its volume of absolute alcohol, there is obtained, after filtering and drying in vacuo over calcium chloride a dark-grayish mass, which on treatment with water dissolves only in part. After treatment with water and filtering, a perfectly clear and colorless solution is obtained, which shows strong peroxidase reactions, but weak oxygenase reactions, and which only acts upon tyrosin after standing from thirty-six to forty-eight hours. In other words, by the action of alcohol, the activity of the potato tyrosinase has been greatly weakened, a fact which is in harmony with Bertrand's earlier observations on the tyrosinase contained in *Russula delica*. According to Bach, it is weakened for the reason that the oxygenase moiety of the ferment has been destroyed by the alcohol. He therefore sought to restore it to its original activity by the addition of small amounts of hydrogen peroxide. As a matter of fact, the weak tyrosinase solutions which only oxidize the tyrosin after thirty-six to forty-eight hours, become dark brown in one hour after the addition of small amounts of hydrogen peroxide. Bach concludes, therefore, that the specific character of tyrosinase lies in the specific nature of its peroxidase.

On the other hand, R. Chodat and Staub ([118]) found that hydrogen peroxide not only did not accelerate the action of tyrosinase on tyrosin, but actually retarded it. As Bach pointed out in his first communication on the subject ([21]), however, tyrosinase is very sensitive to the action of hydrogen peroxide, and it is necessary to work with very dilute hydrogen peroxide in order to demonstrate the accelerating effect. Von Fürth and Jerusalem ([178]) have also observed that small amounts of hydrogen peroxide materially accelerate the action of tyrosinase, whereas with larger amounts of the peroxide the reaction is retarded.

More recently Bach ([23]) has been able to accelerate the oxidation of tyrosin by weak tyrosinase from *Russula delica* by means of hydrogen peroxide. As already pointed out under the preparation of tyrosinase (see p. 74), he prepared aqueous extracts of the ferment from three lots of the fungus (I, from fresh, unblemished fungi; II, from older, more or less damaged fungi, and III, from putrid fungi). Portions of these original extracts were diluted ten times with water, and 10 c. c. of the diluted extracts were mixed with 10 c. c. of a tyrosin solution containing 0.05 per cent of tyrosin and 0.04 per cent of sodium carbonate, and 30 c. c. of water added. After standing twenty-four hours, 1. c. c. of 10 per cent sulfuric acid was added, and each solution was titrated with 0.002 N potassium permanganate to complete decolorization. The quantities of permanganate required

to decolorize, and the original colors resulting from the action of the tyrosinase, were as follows:

	Number of extract.		
	I.	II.	III.
Appearance before titration	Deep black; black sediment.	Violet black.	Dark brown.
Permanganate required.....c. c.	37.8	13.6	8.3

In order now to determine the effect of hydrogen peroxide on each of these extracts, similar experiments were carried out, except that to each solution 1 c. c. of 0.05 per cent solution of hydrogen peroxide was added to each of the tests. After standing twenty-four hours the three solutions were titrated with 0.002 N permanganate, with the following results:

	Number of extract.		
	I.	II.	III.
Permanganate required.....c. c.	37.3	26.7	23.2

It is evident from these results that the weaker the tyrosinase the greater the accelerating effect of hydrogen peroxide on its activity. After a few days extract No. I had become acid in reaction, and was slimy and brown and not in a condition to be filtered. In order, therefore, to neutralize the acid and coagulate the slimy material, the extract was treated with 10 grams of magnesium carbonate and filtered. The residue was then treated with 30 c. c. of water, in which, however, but little of the residue dissolved. Ten cubic centimeters of this solution were then mixed with an equal volume of the tyrosin solution. After twenty-four hours the mixture was entirely colorless. When, however, 0.5 c. c. of a 0.05 per cent solution of hydrogen peroxide was added, a tolerably rapid oxidation of the tyrosin occurred, and in the course of ten hours the mixture was colored black. It would seem, therefore, that by shaking the extract of the fungus with magnesium carbonate the peroxidase of tyrosinase can be separated from its oxygenase. A partial separation of the constituents of tyrosinase can also be accomplished by means of methyl alcohol. Thus, according to Bach (ibid.), 100 c. c. of the fungus extract were poured into 500 c. c. of strong methyl alcohol. The resulting precipitate was rapidly filtered, washed with methyl alcohol, and dried in vacuo over calcium chloride. The dry residue was rubbed up in a mortar with 100 c. c. of water, whereby only a

mall amount of the residue passed into solution. This mixture was hen filtered and the filtrate tested toward tyrosin with and without ydrogen peroxide. While the solution containing hydrogen peroxide showed the characteristic blackening with tyrosin after twelve ours and required 17.6 c. c. of 0.002 N permanganate to decolorize [a] the test without hydrogen peroxide remained colorless for two whole days. Bach concludes, therefore, that at ordinary dilutions ydrogen peroxide exerts no influence on fresh, normal tyrosinase. As the result of certain changes in the tyrosinase, however, which may be brought about naturally or by artificial means, whereby the ferment becomes greatly weakened, its activity toward tyrosin may be greatly increased by dilute hydrogen peroxide. He is therefore of the opinion that the simplest view to take of the weakening of tyrosinase and its partial restoration by hydrogen peroxide is to refer it to the destruction of its unstable oxygenase.

In a more recent communication Bach (25) shows that it is impossible to oxidize tyrosin by the oxidase of *Lactarius vellereus*, and also that it is impossible to oxidize it by this peroxidase in the presence of hydrogen peroxide and a preparation of tyrosinase which had previously been destroyed by boiling.

It has also been shown by Chodat (116a) and also by Bach (21, 22) that ordinary peroxidase and hydrogen peroxide are without action on tyrosin. It would seem, therefore, that tyrosinase presents us with a case of specific ferment action connected in some way with the chemical constitution of the substance oxidized, and that while the two oxidases, laccase and tyrosinase, are similarly constituted in the sense that each contains a peroxidase and an oxygenase, and that in each case the oxygenase component may be replaced by hydrogen peroxide, the two enzymes differ in the specific character of their peroxidase constituent. The peroxidase of laccase is specific in the sense that while it can activate hydrogen peroxide toward guaiacum, hydroquinone, pyrogallol, phenolphthalin, etc., it can not activate it toward tyrosin, and while the peroxidase of tyrosinase can activate hydrogen peroxide toward tyrosin, and certain other amino compounds, it can not activate it toward the laccase reagents.

ANTI-TYROSINASE.

According to Gessard (182), the blood serum of a rabbit which has been inoculated with vegetable tyrosinase at successive intervals, retards the action of the ferment on tyrosin to a considerable extent, so that a long interval is required for the solution of the ferment and

[a] The excess of hydrogen peroxide remaining at the end of these experiments was in all cases removed by the addition of 1 c. c. of catalase solution. This quantity of catalase solution was sufficient to decompose 10 c. c. of a 1 per cent solution of hydrogen peroxide in one minute.

tyrosin to exhibit the succession of color changes ordinarily shown by a solution containing tyrosin and tyrosinase. This author reached the conclusion, therefore, that by the repeated injection of tyrosinase into an animal, there is developed in the blood of the animal receiving the injection an anti-body, to which he gave the name *anti-tyrosinase* (183). He observed further that as the result of the repeated injection of animal tyrosinase (from the ink sac of the cuttle fish) into an animal, such as the rabbit, the blood serum of the animal receiving such injections acquires the property of retarding the action of tyrosinase of the same origin. On the other hand, such serum was found to be without effect on vegetable tyrosinase. On the other hand, Von Fürth and Jerusalem (178) were unable to obtain any evidence of the formation of an anti-tyrosinase in the blood serum of rabbits following the injection of tyrosinase from the hemolymph of certain lepidoptera.

OXIDASES FROM VARIOUS SOURCES.

Since the earlier work of Bertrand and Bourquelot on laccase and tyrosinase, a number of oxidases have been obtained by various observers from different sources, to which special names ending in *-ase* have been given, usually to indicate something pertaining to the particular occurrence of the ferment, but in some cases referring to some peculiar chemical transformation which they can effect. Among these may be mentioned the following:

ŒNOXIDASE (OXIDASE OF WINE).

According to Cazeneuve (114, 115) and other French investigators, this is the oxidase which is responsible for the disease of wine known to French wine makers as *La Casse* or *Cassure*, as the result of which a red wine loses its characteristic color, due to the oxidation and sedimentation of its characteristic coloring matter. According to Martinand (287), this oxidase is present in the ripe grapes, whereas, according to Laborde (256), it is produced by a fungus, *Botrytis cinerea*, which grows freely on grapes and on wine must and is known commonly as the "sweet rot." It has also been found by Martinand (287) in other fruits beside the grape, such as the plum, pear, and apple. It has also been shown that under ordinary conditions the greater quantity of the ferment normally present in the fresh juice of the grape is lost or disappears during fermentation. In all probability it is carried down by the precipitates which go to form the wine stone. According to Martinand, it is destroyed in four minutes at 72° C., or even at 55° C., after an exposure of one and one-half hours. Bouffard and Semichon (71) have found it to be destroyed by very dilute sulfurous acid, 0.02 gram of sulfur dioxide per liter of enzyme. In many respects

œnoxidase resembles laccase. It is worthy of note in this connection, however, that, according to Legatu [259], the disease of wine ordinarily ascribed to œnoxidase is in reality due primarily to the presence of ferrous salts in quantities above the normal. These are oxidized to ferric salts and then precipitated by the tannin, the precipitate carrying down the coloring matter of the wine. According to this author the only part played by the œnoxidase is that it assists in the rapid oxidation of the ferrous salts, and hence may participate to that extent in accomplishing the changes already described.

MALOXIDASE (APPLE OXIDASE).

The color changes occurring in fruits like the apple, pear, peach, etc., as a result of a wound or cut in the fruit or an abrasion of the skin are familiar to everyone. It is also known that no such changes of color occur if the fruit be previously heated to about the temperature of boiling water. The effect of heat in preventing these changes is also seen to good advantage in various sorts of artificially and naturally dried fruits, especially dried apples. Apples which have been dried in the sun by natural processes are brown in color in consequence of the oxidation of tannin by an oxidizing ferment, maloxidase, contained in the fruit (*see* Lindet [271, 272]). On the other hand, evaporated apples which have been dried artificially at higher temperatures, and in some instances even exposed to the action of sulfur dioxide, are white, for the reason that the oxidase has been destroyed. The juice of the apple has been found to blue guaiacum and to oxidize hydroquinone and pyrogallol. In this connection it has been pointed out by Kastle and Shedd [247] that those vegetable tissues which readily oxidize guaiacum and phenolphthalin rapidly turn brown or reddish in color when their freshly cut surfaces are exposed to the air, whereas the tissues of those plants which do not oxidize these reagents do not turn brown or red on exposure to the air. In other words, the oxidases present in the raw fruit are responsible for both phenomena.

According to Lindet [271, 272] the oxidase of the apple and the tannin upon which it acts are stored in different cells, and hence it is only when these are brought together by actual rupture of the cells that we have those color changes occurring which are characteristic of the bruised or macerated fruit. On the other hand, Kastle and Loevenhart [244] have pointed out that, on the assumption that the oxidizing ferments are of the nature of peroxides, the oxidase (peroxide) is not present as such in the intact cell, but only its precursor, viz., an autoxidizable substance which, when it comes into contact with the air through the rupture of the tissue or cell, unites with the oxygen to produce the peroxide or the so-called oxidizing ferment,

or according to the views of Bach and Chodat [25] the precursor of the oxygenase portion of the ferment. Hence the tannin and the substance destined to become the oxidase or a part thereof as the result of the action of oxygen could exist together in the same cell, or, for that matter, even in the same solution, and yet no oxidation or no coloring take place until the cell had been ruptured and air admitted. It seems highly probable that the so-called maloxidase and Bertrand's laccase are the same.

SPERMASE.

This oxidase has been detected by Grüss [201] in the embryo of the barley by means of Wurster's reagent (tetramethyl-para-phenylene diamin). If a grain of barley be cut in two along the greater length of the grain and the cut surface pressed against moist "tetra" paper, a violet color develops on the exposed surface of the embryo, while the endosperm remains colorless. On the other hand, if the grain be heated to 55° C. for fifteen minutes the embryo shows no color with "tetra" paper, indicating that the ferment has been destroyed. The ferment scarcely colors guaiacum, and hence, according to Grüss, should not be confused with laccase. He is of the opinion that it plays a part in the morphological and physiological changes which accompany the production of malt. Thus, he has shown that in the kiln-drying of very moist malt, without ventilation, the oxidase is destroyed [201].

OXIDIN (*Boutroux*).

According to Mégé-Mourier [293, 294, 295], the characteristic color of brown bread is produced during panification by a substance of ferment-like nature, to which he gave the name "*Cerealin.*" Afterwards Boutroux [92] showed that these changes of color result from the action of an oxidase (laccase), to which, for reasons already indicated, he gave the name "*Oxidin.*" Quite recently Bertrand and Mutermilch [65] have obtained tyrosinase from wheat bran and have reached the conclusion that the changes of color observed in bread during baking are due, in part at least, to the action of this ferment. It is quite likely, therefore, that Mégé-Mourier's *Cerealin* and Boutroux's *Oxidin* are merely mixtures of laccase and tyrosinase.

SCHINOXIDASE.

Sarthou [357, 359] obtained this oxidase from the latex of *Schinus molle* by the usual method. An aqueous extract of the latex of this plant blues guaiacum and oxidizes the phenols, such as hydroquinone, resorcin, and pyrogallol, and transforms potassium ferrocyanide into potassium ferricyanide with the absorption of oxygen. The ferment

iffers from Bertrand's preparations of laccase chiefly by the fact hat the ash is rich in iron, but contains no manganese. According o Sarthou (358), this iron exists in the active ferment in organic com- ination, and to this the ferment owes its oxidizing power.

OLEASE.

Tolomei (424) has observed that fresh olives gradually undergo fer- nentation when exposed to the air, with the production of acetic and ther fatty acids and the evolution of carbon dioxide. According to his author these changes are brought about by the action of an oxidase to which he has given the name "Olease." This ferment has lso been found by Tolomei in the olive oil itself, and to its action are lue those changes which take place when the oil becomes rancid. The acids produced by its action serve to gradually weaken the erment.

PURPURASE.

One of the most interesting and picturesque changes attributable o oxidizing ferments is that described by Dubois (148), which results n the production of a purple pigment in the mollusc, *Murex brandaris*. According to this author the pigment glands of these gasteropods are analogous to the photogenic (luminous) organs of *Pholas dactylus*, the principal difference being that while the fixation of oxygen in the latter gives out its energy in the form of light, the latter absorbs luminous vibrations with the production of a pigment. In other words, the changes occurring in the production of the pigments of molluscs are also photochemical. Dubois observed that on extract- ing the pigment gland with absolute alcohol the chromogen contained in the gland passed into solution. The alcoholic solution of the chromogenic substance was concentrated on the water bath and the concentrated solution used to impregnate test papers for the purpose of experimentation. The residue of the gland remaining after extrac- tion with cold absolute alcohol was extracted first with chloroform water, and the residue was finally macerated with glycerin, all of these operations being carried out in the dark or in the feeble light of the dark room. On adding a drop of the glycerin extract of the gland to the test paper containing the chromogenic substance of the gland and moistening with water and exposing to sunlight, a purple color gradually developed on the paper, the intensity of which de- pended on the time of exposure and the intensity of the light. The glycerin extract alone is not colored on exposure to the light, and it loses its activity when heated to 120° C. in the autoclave. Three factors are therefore concerned in the development of the purple pig- ment in molluscs, (1) a chromogenic substance soluble in alcohol, (2) a ferment, and (3) light. According to Dubois, the chromogenic

substances (*les substances chromogeniques*) concerned in these changes consist of a definite, crystallizable substance, the chromogen proper, which has been described by Letellier (268) (*see* footnote to Dubois's article), and a ferment. To the former Dubois gave the name "purpurin," and to the latter "purpurase." No special evidence has been brought forward by Dubois, or by anyone else for that matter, to indicate that purpurase is an oxidizing ferment. Indeed, its conduct toward the ordinary oxidase reagents seems never to have been investigated. However, the changes resulting in the production of the pigment in the pigment gland of *Murex brandaris*, in which it evidently participates, present many close analogies to those oxidations which are accomplished by oxidases, and hence certain authors have been led to put purpurase in the class of oxidizing ferments.

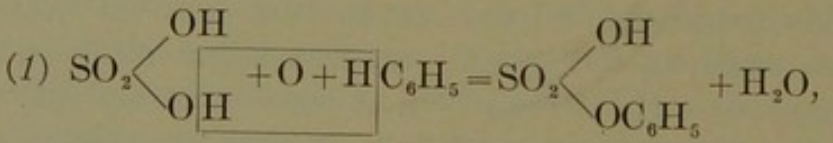
LUCIFERASE.

The production of light by animals and plants has been the subject of many researches. In this connection it was long ago pointed out by Dubois (145, 147) that the active agent of light production in animals and plants is a substance having the general properties of a ferment. This enzyme he has called "luciferase." According to Dubois (146), the transformation resulting in the production of light in animals and plants takes place under the influence of life, water, and a suitable temperature. From his earlier researches on the subject he concluded that physiological light is not the result of a combustion, nor even of a slow oxidation, but a direct chemical act (*mais directe*). In his later investigations, however, he claims to have shown that the fixation of oxygen is necessary, the oxidation taking place not directly but indirectly, and for the most part, at least, through the action of luciferase as an intermediary, which thus conducts itself like an oxidizing ferment. In this connection he observed that the luminous organs of the *Lampyridæ* and also the eggs contained in the ovaries of the females give a beautiful blue color with tincture of guaiacum. So, also, the filtered extract of the luminous mucus from the body of a dead fish, prepared with chloroform water, gave a similar reaction. Dubois (147) concludes, therefore, that the photogenic substances concerned in the production of physiological light are "luciferin" and "luciferase." In other words, the light generated by various life forms is the result of a chemical change, probably an oxidation, brought about by the action of luciferase on luciferin. My own experience with the firefly native to the Central and Middle Western States, *Luciola pennsylvanica*, is that the aqueous extracts of the luminous organs do not show the guaiacum reaction directly, but only after the addition of hydrogen peroxide. The whole subject requires further investigation.

ALDEHYDASE (SALICYLASE).

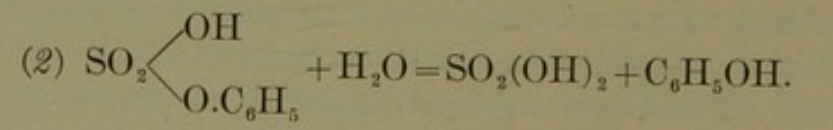
We have seen that Schoenbein (384), especially as the result of his work on the oxygen-carrying power of blood, recognized the importance of oxygen carriers for oxidation processes occurring in the animal organism. Traube (427) also believed that a complete analogy exists between the respiratory changes occurring in muscle and the process of slow combustion. His theory of muscular activity was based upon the assumption that the muscle fiber contains a vital, oxidizing (*verwesungs*) ferment (*see* Traube (425), p. 107), which carries oxygen from the blood to the oxidizable substances contained in the muscle fluids, without the ferment itself suffering any destruction. These or similar views were also shared by other distinguished observers. Thus, according to Claud Bernard (47) the respiration of tissue is not a direct combustion, but an indirect oxidation accomplished by chemical agents of the nature of ferments. Some few observations were made by Schützenberger (393) on the oxidation of certain substances in the animal organism. It remained for Schmiedeberg to greatly extend our earlier knowledge of such processes. Thus, as the result of their investigation of the formation of hippuric acid in the animal organism, Bunge and Schmiedeberg (106) came to the conclusion that the red blood corpuscles play an essential rôle in the formation of hippuric acid in the living kidney, for the reason, possibly, as these observers surmised, that it plays the part of an oxygen carrier. From these observations Schmiedeberg himself (366) was naturally led to the study of those oxidations which in the animal organism might result in the formation of hippuric acid. He arrived at the conclusion that the principal changes occurring in the animal organism are splittings (hydrolysis), oxidation, and synthesis, and, as he proved in these researches, the two latter processes frequently go hand in hand. It was shown that benzyl alcohol was oxidized by blood to only a slight extent, and almost equally well by a solution of sodium carbonate, but not at all in pure water. Similar results were obtained with salicylic aldehyde. On the other hand, by the action of oxygenated blood in the tissue of the kidney and lung both of these compounds are oxidized a thousand times more rapidly than they are by blood alone or by a solution of sodium carbonate. From this it follows that conditions exist in the tissues whereby an increased activity is conferred on the oxygen of the blood, in that it is rendered active or gotten into the nascent state, or some change is effected in the oxidizable substance, whereby it becomes more easily oxidizable. This increased activity on the part of the oxygen may be explained as a result of the action of (1) an exciter analogous to platinum-black, or (2) of readily combustible substances in the tissues which have the power of

decomposing the oxygen molecule, as a result of which not only would they themselves become oxidized, but other substances as well. This last was an application of Hoppe-Seyler's theory of oxidation (1878). On the other hand, Schmiedeberg observed that these aromatic compounds are more rapidly oxidized than phosphorus. This, he says, is evident from the fact that in phosphorus poisoning in man following the administration of 0.1 to 0.2 gram of the substance, some of the phosphorus apparently remained in the body in unaltered condition after death. Hence he distinguishes between synthetic oxidations and those like the oxidation of phorphorus, and points out that it is only substances containing hydrogen that lend themselves to such oxidations as the former. He concludes also that in the apparent activation of oxygen in such synthetic oxidations the living tissue acts not upon the oxygen molecule, but upon the oxidizable substance; otherwise it would oxidize the phosphorus as readily as the benzyl alcohol or salicylic aldehyde. Essentially similar views have recently been advanced by Mathews [289] in order to account for the oxidation of the sugars (see footnote, p. 56). Schmiedeberg also pointed out that all of these oxidation processes have this in common, viz., that the final product of the oxidation occurs in the urine in the form of a conjugated compound in which it is paired with sulfuric or glycuronic acid, or with glycol. Hence the production of phenol in the organism following the administration of benzene may result from the following changes:



(1) $SO_2(OH)(OH) + O + H C_6H_5 = SO_2(OH)(OC_6H_5) + H_2O,$

and



(2) $SO_2(OH)(O.C_6H_5) + H_2O = SO_2(OH)_2 + C_6H_5OH.$

See also Baumann and Herter [40].

As a matter of fact, Schmiedeberg found that a dog which had received 24 grams of benzene, in eight doses in twenty-four hours, excreted 1.6907 grams of phenol, of which 1.1005 grams were found in combination with sulfuric acid.

Several years later this subject was reinvestigated by Jacquet [222], who showed, first, that the blood alone does not possess the power of accomplishing the oxidation of such substances as benzyl alcohol and salicylic aldehyde; second, that certain animal tissues or cell-free extracts thereof in contact with blood or atmospheric oxygen have the power of accomplishing the oxidation of these substances; third, that while such extracts lose their oxidizing power on boiling, their oxidizing powers are not destroyed by carbolic acid, quinine,

by freezing, or by precipitation with alcohol and re-solution in er. He arrived at the conclusion, therefore, that oxidations in animal organism are brought about by ferments or enzymes. Further studies on the oxidizing ferments were made by Pohl [329] h the view of determining whether the several oxidizing fer- nts described by various authors as occurring in animal and plant sues are the same or different enzymes. He reached the conclu- n that two essentially different kinds of oxidations occur in liv- tissues and that these are brought about by at least two dis- ctly different ferments, one of which accelerates the oxidation fatty (formic) and aromatic aldehydes, and the second brings out such oxidations as the production of indophenol-blue from alkaline solution of *a*-naphthol and para-phenylene diamin. He und further that certain plant extracts have the power of accom- ishing the indophenol reaction, but are powerless to oxidize formic salicylic aldehyde or mannite. An aqueous solution of tannin edles proved to be especially active. In this connection he also owed that amygdalin gives the indophenol reaction apparently dependently of the action of any ferment.

On the other hand, Spitzer [406] arrived at the conclusion that lycolysis (the disappearance of sugar in the blood) is only a special se of oxidation by blood and animal tissues, and that the glycolytic rment of Lepine is probably identical with the oxidizing ferments tissues capable of oxidizing aromatic alcohols and aldehydes. In upport of these conclusions he cited, first, an observation by Sal- owski [356] to the effect that under certain conditions blood can xidize salicylic aldehyde, and, secondly, some observations of his wn to the effect that those tissues which can rapidly oxidize alco- ols and aldehydes can also destroy glucose. The results obtained n subsequent researches tend to confirm him (*see* Spitzer [407]) in the belief that the oxidizing ferments, especially of the animal organism, are substances of the same nature, viz., nucleoproteids, and that cer- tain of these owe their activity as oxygen-exciters to the organic iron which they contain.

On the other hand, Abelous and Biarnes [7, 8] observed that cer- tain globulins have the power to oxidize guaiacum, but are without action on salicylic aldehyde, and, as already pointed out, Raudnitz [338] proved that the substance in milk which causes the bluing of guaiacum in the presence of hydrogen peroxide is not identical with that which catalyzes the hydrogen peroxide. It was also pointed out by Lepine [262] and also by Jacoby [224] that the glycolytic ferment differs from the oxidizing ferment oxidizing salicylic aldehyde in several respects, especially in regard to the effect of temperature on the two ferments, the glycolytic ferment being destroyed at 58° C., whereas the oxidizing ferment under consideration is only destroyed

at 100° C. This last observation was confirmed by Jacoby [224]. From these and other observations Jacoby [225] came to the conclusion that several oxidizing ferments occur in the liver, so that according to this author it seemed no longer advisable to refer to the ferment capable of oxidizing salicylic aldehyde merely as an oxidation ferment. He therefore adopted Bertrand's nomenclature in the naming of these substances, designating as *aldehydase* the ferment or ferments of the liver found to be capable of oxidizing aldehydes, and giving the name *salicylase* to the particular ferment concerned in the oxidation of salicylic aldehyde (*see* Jacoby [225]).

In order to obtain a water-clear solution of the ferment having powerful oxidizing powers, Jacoby [225] recommends the following method of preparation: Fresh beef liver is put through a sausage machine and then rubbed up with quartz sand, and the paste thus obtained mixed with some distilled water. Toluene is added and the mixture allowed to stand several hours with vigorous shaking; the mass is then filtered. The dark, clear liquid thus obtained is then saturated with ammonium sulfate to the extent of 25 per cent and the fluid rendered weakly alkaline with sodium carbonate, so that after saturation with ammonium sulfate the liquid generally smells of ammonia. In about twenty-four hours a small amount of precipitate is produced in the liquid, which is then filtered off. The filtrate is then saturated to the extent of 33⅓ per cent with common salt, and after twenty-four hours the precipitate is again removed by filtration. The clear, dark-colored liquid is then saturated to the extent of 60 per cent with ammonium sulfate, with the result that a heavy precipitate is obtained which requires about twenty-four hours to settle out completely. This precipitate, which contains the aldehydase, is then filtered off, washed with 60 per cent ammonium sulfate solution, and then taken up with distilled water, in which it only partially dissolves. After several hours the aqueous solution is filtered, and 95 per cent alcohol gradually added to the clear filtrate, until a good filterable precipitate is obtained. As a general thing sufficient 95 per cent alcohol has to be added to bring the concentration of the alcohol in the whole liquid up to about 30 per cent. The solution containing the precipitate is then filtered. The precipitate is then extracted five or six times with distilled water containing a few drops of dilute soda solution, and the extracts united. As a rule the ferment is most completely extracted by allowing the finely divided precipitate to stand over night with water. The bright clear liquid thus obtained is still found to contain protein. It is then made faintly alkaline with soda and uranyl acetate added until a filterable precipitate is obtained, which is then handled in precisely the same way as the alcoholic precipitate. There is thus obtained

finally a water-clear liquid which vigorously oxidizes salicylic aldehyde and which now gives none of the protein reactions.

The ferment thus obtained is soluble in water and, as has been pointed out, can be salted out with ammonium sulfate. It is practically nondialyzable. In spite of its great solubility in water, the ferment is not removed from the liver by intensive washing out of the circulatory system with 0.7 per cent salt solution. At a pressure of six atmospheres the ferment passes through the Chamberland filter. The aldehydase was found by Jacoby to be perceptibly soluble in 20 per cent alcohol, but insoluble in concentrations equal to or greater than 30 per cent. It is also precipitated from its solutions by tannin. With dilute solutions of the ferment the Millon and biuret tests were found to be negative. Aldehydase was found to lose its oxidizing power on boiling or by treatment with small amounts of free acid or alkali. Toward salts it conducts itself much like a globulin, and yet at the concentration at which it vigorously oxidizes salicylic aldehyde it does not give the protein reactions.

That aldehydase is a ferment is indicated by the fact that it is not consumed in the oxidations which it can accomplish, but can react with fresh quantities of the oxidizable substance. Thus Jacoby allowed a clear extract of liver to act upon salicylic aldehyde for forty-eight hours, at the end of which time small portions of the liquid gave an excellent test for salicylic acid. The main portion of the liquid was then dialyzed against water for three days and the ferment was then salted out with ammonium sulfate and the precipitate dissolved in water. Two-thirds of this solution alone gave no test for salicylic acid after seventy-two hours' digestion, whereas another portion digested with salicylic aldehyde gave an abundant test for salicylic acid.

In addition to the above-mentioned researches, aldehydase has formed the subject of numerous investigations during the past fifteen years, among which may be mentioned those by Salkowski and his coworkers, Abelous and Biarnes, Medwedew, and others. The occurrence and distribution of the ferment in various animal tissues has been studied by Salkowski and by Salkowski and Yamagiwa, and also by Abelous and Biarnes. In oxidizing power toward salicylic aldehyde, Salkowski and Yamagiwa ([355]) found certain animal organs to stand in the following order:

Liver = 100.0
Spleen = 80.4
Kidney = 15.5
Pancreas = 2.0
Muscle = 1.0,

whereas Abelous and Biarnes (5) arranged the tissues in the following order with regard to their activity toward salicylic aldehyde:

1. Spleen.
2. Lung.
3. Liver.
4. Thyroid.
5. Kidney.
6. Thymus.
7. Suprarenal.
8. Testicle.

According to these authors muscle, pancreas, and brain are incapable of oxidizing salicylic aldehyde. Medwedew (291) studied the kinetics of the oxidation of salicylic aldehyde by extracts of various organs and has found that the quantity of salicylic aldehyde oxidized is proportional to the square of the concentration of the ferment, and inversely proportional to the square root of the concentration of the aldehyde.

One of the most interesting facts disclosed by these investigations is that the oxidation of the aldehyde by aldehydase is independent of the presence of atmospheric oxygen. Thus in certain of his experiments Medwedew (292) found the following amounts of salicylic acid to be produced (1) during aeration, and (2) in the absence of air:

No. of experiment.	Amount of salicylic acid found, in milligrams.	
	(1) Aerated.	(2) In absence of air.
1.....	85	83
2.....	118	115
3.....	80	78

It will be observed that the differences between the several amounts of salicylic acid formed in the two cases are very small and are probably within the limits of experimental error, and yet in experiment No. 3 (1) was subject to a continuous current of air for 16 hours, whereas (2) remained without aeration, and portion (2) of experiment No. 2 was kept in a hermetically sealed vessel. Similarly it has been shown by Abelous and Aloy (4) that the oxidation of salicylic acid by various organs proceeds more rapidly in a vacuum than in the presence of air. In fact, according to these authors, liberal supplies of free oxygen diminish the rate of oxidation very considerably and may even suppress it altogether. These authors are of the opinion, therefore, that in such oxidations as take place in a vacuum the oxygen necessary for the oxidation is furnished by certain oxygen compounds which are dissociated by the oxidizing ferment. In other words, such processes

as the oxidation of salicylic aldehyde under the influence of aldehydase partake of the nature of anaerobic fermentations, and, as is well known, the oxygen required for the latter processes is furnished by various oxygen-containing substances participating in the fermentation. In this connection it is interesting to recall that Traube provided for such cases in his general theory of fermentation. According to this distinguished observer the oxygen of water might under certain conditions be transferred to the oxidizable substance by means of a ferment, and in the fermentation of a compound like sugar the oxygen present in one part of the molecule might be transferred to another part of the molecule, thereby giving rise, after splitting, to one substance richer in oxygen than the original, and to another poorer in this element. Such a change is met with in the breaking up of glucose into carbon dioxide and alcohol by yeast. It is interesting to note in this connection that similar changes have been met with among inorganic substances; thus, when an aqueous solution of phosphorous acid is heated, phosphoric acid and phosphine are produced—

$$4H_3PO_3 = 3H_3PO_4 + PH_3$$

Whether water actually participates in such changes is at present unknown. In my opinion these simple processes would warrant further investigation from the point of view of anaerobic fermentation.

In this connection Abelous and Gerard ([9,10]) have shown that extracts of various animal tissues have the power of reducing nitrates to nitrites. So also it was afterwards found by Abelous ([3]) that the juice of the potato can also reduce nitrates, but is incapable of oxidizing salicylic aldehyde. On adding a small amount of potassium chlorate, however, the aldehyde is oxidized at the same time that the chlorate is reduced. This property of the juice is not lost on boiling. In some respects these reactions are similar to those investigated by Kastle and Elvove ([243]). These authors also observed that the aqueous extract of the potato has the power of reducing nitrates to nitrites and that in the presence of certain readily oxidizable organic compounds, such as formic aldehyde and benzyl alcohol, the quantities of nitrite formed are greatly increased. On boiling, the aqueous extract of the potato loses its power to reduce nitrates even in the presence of readily oxidizable substances. All of these facts point to the presence in the potato and in animal tissues of certain ferments which have the power of effecting the transfer of oxygen from a substance rich in this element, such as the chlorate or nitrate, to an easily oxidizable substance. According to Abelous ([3]) these are the oxido-reducing ferments.

Dony-Hénault and Mlle. J. van Duuren ([144]) have recently investigated the oxidation of salicylic aldehyde by means of aqueous extracts of calf's liver. According to these observers the methods

for the estimation of salicylic acid are far from exact. In their own work they adopted the method of Elion of estimating the salicylic acid formed during the oxidation by means of bromine. They have also arrived at the conclusion that the salicylic acid appears to combine in part at least with certain substances present in the extracts and hence would be likely to escape detection in most of the methods. They agree with Abelous and Aloy (4) that the acid is only produced rapidly in the absence of oxygen. The quantity of salicylic acid produced in a given time also varies with the concentration of the aldehyde and the amount of extract employed, the former being the preponderating factor in determining the velocity of the reaction. They observed, further, that the oxidizing power of extracts of the liver diminishes spontaneously when such extracts are left to themselves, especially at higher temperatures. According to these authors, the results thus far reached in the study of this oxidation do not warrant the conclusion that the oxidation in question is accomplished by a ferment acting in the absence of air. About all that can be said is that a certain amount of oxidizing material is present, but this being limited the amount of salicylic aldehyde oxidized by a given quantity of extract is also limited, while the oxidase is a true catalyst, probably of a mineral nature, and as such ought to be able to accomplish the oxidation of practically unlimited quantities of material. According to these authors the presence of such substances has not been proven in such animal extracts as those under immediate consideration, nor is it necessary to assume their presence in order to account for the phenomena observed.

THE INDOPHENOL OXIDASE; THE RÖHMANN-SPITZER REAGENT.

In his study of the oxygen requirements of the organism it was shown by Ehrlich(157), in 1885, that the intravenous injection of α-naphthol and para-phenylene diamin results in the formation of indophenol in the tissues of the animal. So also Würster (466) showed that di- and tetramethyl-para-phenylene diamin are reagents for active oxygen, and that in the animal organism these substances are converted into blue coloring matters. Acting upon these suggestions, Röhmann and Spitzer (351) have made use of a reagent consisting of equal amounts of α-naphthol and para-phenylene diamin, in a dilute solution of sodium carbonate (three molecules of sodium carbonate to one molecule of each of the organic compounds), for the investigation of the oxidizing power of the various animal tissues. Various other related substances can be employed in the preparation of the reagent; thus dimethyl-para-phenylene diamin(453) can be used instead of para-phenylene diamin. They also showed that other coloring matters result from the oxidation of suitable mixtures by the action of various animal tissues. Thus they succeeded in show-

ing that certain indamines like toluene-blue result from the oxidation of meta-toluene diamin and para-phenylene diamin, and Bindschedler's green from dimethyl-para-phenylene diamin and dimethyl-anilin, and that the synthesis of various eurhodins may be accomplished in the same manner. An easy method of demonstrating such reactions consists in placing a small amount of an animal tissue, such as the liver, on a test paper which has been prepared with the two chromogenic components of the dye. If, for example, a small amount of fresh liver be placed upon a test paper which has been saturated with a sodium carbonate solution of *a*-naphthol and para-phenylene diamin, the place on the paper occupied by the tissue is soon deeply colored, whereas the other portions of the paper show only a slight coloration. According to these authors, the change in question is represented by the following equations:

1. $$C_6H_4(NH_2)_2 + C_{10}H_7OH + O = NH\begin{matrix} C_6H_4NH_2 \\ C_{10}H_6OH \end{matrix} + H_2O,$$

and

2. $$NH\begin{matrix} C_6H_4NH_2 \\ C_{10}H_6OH \end{matrix} + O = N\begin{matrix} C_6H_4NH_2 \\ C_{10}H_6O \end{matrix} + H_2O.$$

Thus one atom of oxygen goes to effect the synthesis of the leuco compound and a second atom to effect the oxidation of the leuco compound to the coloring matter. When the solution of the chromogens is allowed to stand in contact with atmospheric oxygen, the coloring matter is formed only slowly in the presence of alkalies; when the animal tissue is added the process is greatly accelerated. It was shown by these authors that these oxidations are most readily accomplished by extracts of cells, the body fluids and secretions being comparatively inactive. In this respect the change is analogous to the destruction of glucose, a change which is readily accomplished by extracts of red and white blood cells in physiological salt solution, but not by blood serum. They reached the conclusion, therefore, that the oxygen exciters responsible for these oxidations are contained only in the cells. That such is the case is evident also from the conduct of such extracts and secretions toward guaiacum and hydrogen peroxide. Extracts of red blood cells blue a mixture of guaiacum and hydrogen peroxide instantaneously, whereas the blood serum causes no change of color, or if it acts at all it does so very feebly. While they knew nothing of the particular cell components responsible for these changes, they seemed to think that their observations furnished evidence, hitherto wanting, of a single oxidizing ferment. By precipitating the macerates of fresh organs with alcohol and washing the precipitate thus obtained with alcohol and ether, they obtained dry powders which retained their oxidizing powers for a

year, whereas the alcohol extracts were found to be inactive. A comparison of the different organs showed that they activated oxygen toward these reagents with different degrees of intensity. As a rule they were found to be energetic oxygen carriers, accomplishing these oxidations more rapidly than they were brought about by palladium foil or palladium-hydrogen. Finally, these authors called attention to the general similarity between the synthesis of indophenol and similar dyes with that of bromphenol cystein, which, according to Baumann, results from the administration of brom-benzene to dogs. They arrived at the conclusion therefore that the cells of animal tissues contain substances which have the power of activating molecular oxygen, whereby it can accomplish the oxidation of substances not ordinarily directly oxidizable (*dysoxydabler Stoffe*). They protest, however, against the supposition that the oxidation of all difficultly oxidizable substances in the organism depends on the action of oxygen-carriers.

The indophenol oxidase is by no means confined to animal tissues. Mention has already been made of the work of Pohl ([329]) on this subject. This author found that many plant tissues react strongly to the Röhmann-Spitzer reagent. Especially is this the case with tannin needles. He also pointed out that the reaction is also brought about by amygdalin, apparently altogether independently of the action of an oxidase. It is possible, of course, that his amygdalin may have been contaminated with a vegetable oxidase. Rey-Pailhade ([342]) also found the indophenol oxidase to be widely distributed among plants, and according to this author yeast reacts feebly to the Röhmann-Spitzer reagent. Abelous and Biarnes ([5]) have determined the oxidizing power of various tissues from the same and different animal species, colorimetrically, by means of the Röhmann-Spitzer reagent. According to these authors, the tissues of the frog and rabbit, the latter killed by bleeding, stand in the following order with regard to oxidizing power:

Frog.	Rabbit.
Lung.	Spleen.
Liver.	Lung and thyroid.
Kidney.	Liver and kidney.
Testicle.	Pancreas and suprarenals.
Brain.	Ovary.
Muscle.	Brain.
	Muscle.

It will be observed that there is general agreement between these findings and those of the same authors and also of Salkowski with regard to the relative oxidizing power of the various animal tissues toward salicylic aldehyde (see pp. 97–98).

One of the most interesting facts revealed by these determinations is the slight oxidizing power of muscle and nerve tissue as compared with that of other animal tissues, and, as pointed out by Abelous

and Biarnes (5), this is all the more remarkable when it is borne in mind that the muscular system is the seat of very powerful exothermic reactions. In this connection it has been observed by Kastle (240) that extracts of muscle and brain greatly retard the oxidation of an alkaline solution of phenolphthalin by blood, and it may be, as suggested by him in explanation of his own results, that muscle and nerve tissues may be unusually rich in powerful reducing agents which would interfere with the oxidation of all extraneous oxidizable material. The occurrence and distribution of various intra-cellular ferments, including the indophenol oxidase, has been studied by Rosell (352) with the following results. In the table the presence of the ferment is indicated by the sign (+) and its absence by the sign (−).

Intra-cellular ferments in beef tissues (Rosell).

Organ.	Aldehydase.	Indophenol oxidase.	Catalase.	Trypsin.	Pepsin.
Pancreas	−	+	+	+	−
Salivary glands	+	+	+	+	−
Lymph glands	+	−	+	+	−
Spleen	+	+	+	+	+
Bone marrow	−	+	+	+	+
Thymus	+	+	+	+	−
Lacteal glands	−	−	+	+	−
Muscle	−	−	+	+	−
Lung	+	−	+	−	+
Brain	+	−	+	−	−
Suprarenals	+	−	+	+	−
Testicle	+	−	+	+	−
Kidney	+	−	+	−	−

The presence of the guaiacum-oxidase could not be established with certainty.

THE PURIN OXIDASES (XANTHIN-OXIDASE).

It has long been known that on a flesh diet alone the ratio of urea to uric acid, $\frac{\text{urea}}{\text{uric acid}}$, in the urine is fairly constant, varying from 45 to 65. On the other hand it was pointed out by von Noorden (312) that on different diets it is subject to enormous variations, even in the same individual. Thus in some of his cases it ranged from 23.2 to 122.4. So also by feeding milk, peptone, and vegetable proteid, Camerer (108) was able to double the total nitrogen secreted without causing any increase in the output of uric acid. On the other hand it was pointed out by Kossel (253) that those foodstuffs which cause an increase in the excretion of uric acid contain much larger amounts of hypoxanthin than human muscles. Thus the muscles of the hen and pigeon contained over 0.1 of hypoxanthin per 100 grams of moist muscle, whereas the muscles of man contained

only 0.039 to 0.048 gram. It was afterwards shown by Weintraud (445) that following a diet rich in nucleins, as for example, the calf's thymus, the uric acid in the urine is considerably increased, whereas according to Hess and Schmoll (211) a similar increase in the uric acid does not follow a nuclein-free proteid diet.

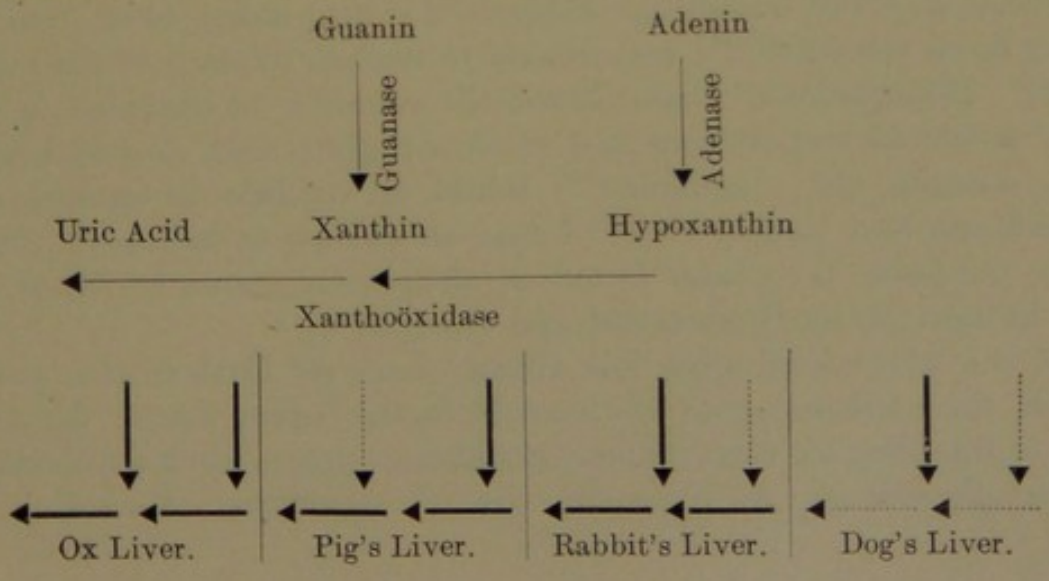
On the other hand it is of interest to note in this connection that uric acid itself can be destroyed to a certain extent in the organism, and that following the administration of uric acid by the mouth in men and dogs only a small part of it is recoverable from the urine (*see* Minkowski, 302). It has also been shown by Minkowski that hypoxanthin causes a distinct increase in the output of uric acid, and that meat extract which contains considerable amounts of this substance has a similar influence on the uric acid excretion. That certain purin bases are formed by the decomposition (autolysis) of the nucleins was shown by Horbaczewski (218, 219). This author allowed a mixture of spleen pulp and water to stand at 50° C. From this slightly putrid mixture he was able to isolate large amounts of xanthin and hypoxanthin, whereas from the fresh extract of spleen only traces of these purins were obtainable. After shaking the slightly putrid mixture with air he obtained no xanthin or hypoxanthin, but only uric acid. Similar results were obtained by adding blood or hydrogen peroxide to the slightly putrid spleen pulp. It is evident from these results that the nucleins of the spleen are the precursors of the purins.

On repeating Horbaczewski's work, Spitzer (409) showed that uric acid is formed in aqueous extracts of spleen pulp through which is passing a current of air and in which putrefaction is prevented by chloroform or thymol, and that contrary to the opinion of Horbaczewski, xanthin and hypoxanthin can be readily converted into uric acid by the action of a current of air on an aqueous extract of spleen containing these substances at 50° C. Similar results were obtained with the liver, but other organs, such as the kidney, pancreas, and thymus, were found to be much less active in their power to accomplish this oxidation. Similar results were obtained by Wilner (452), who arrived at the conclusion that in the liver, not only of birds but also of certain mammals, there are produced large amounts of uric acid. He also showed that the addition of hypoxanthin to liver extracts causes a great increase in the quantity of uric acid obtainable from the extract.

Evidently, therefore, the oxidation of xanthin and hypoxanthin is accomplished *in vivo* and *in vitro* through the instrumentality of an oxidizing ferment. This ferment has been made the subject of an exhaustive investigation by Burian (107) and named by him *xanthin-oxidase*. By following Spitzer's method and using one part of macerated liver to two parts of chloroform water he was able to obtain clear extracts of xanthin-oxidase containing only very

small amounts of purin bases. On digestion in a current of air these extracts alone gave practically no uric acid. They were found, however, to oxidize xanthin and hypoxanthin rapidly in a current of air. By means of these preparations of xanthin-oxidase he was able to confirm the previous observations of Spitzer and Wilner and to show that, as is the case with many ferments, the conversion of xanthin and hypoxanthin under the influence of the ferment is a reaction of the first order. He showed also that the xanthin-oxidase is not consumed in the process, but is capable of oxidizing fresh quantities of xanthin and hypoxanthin in the presence of oxygen. Hence it would seem to be a true ferment. This is evident from the fact that no noteworthy falling off in the coefficient of the velocity of the reaction was observable.

On the other hand, the reverse process, viz, the reduction of uric acid to other purin bodies does not take place to any noticeable extent. He is of the opinion therefore that xanthin-oxidase is responsible for the production of uric acid from purin bases in the living animal. Contrary to the observations of Wilner ([452]) he found that in the absence of purin bases, tartronic and dialuric acids cause no production of uric acid with his extracts of xanthin-oxidase. He concludes therefore that these substances can act only by accelerating the action of the xanthin-oxidase on purin bodies already present in the liver extracts. Neither can xanthin-oxidase convert guanin or adenin directly into uric acid, but, as has been found by Jones and Austrian ([230, 231]), these substances must first be desamidized by the action of guanase and adenase, whereby they are converted into xanthin and hypoxanthin, respectively, before their oxidation into uric acid can occur. The occurrence and distribution of these ferments have been exhaustively studied by Jones and Austrian ([230, 231]) and also by Schittenhelm ([365]) and others. A very good idea of their distribution in the liver of various animals is afforded by the following diagram, which is given in certain of Jones's original contributions to the subject:



In the above diagram the solid black lines indicate the presence of the particular ferment to which it appertains, whereas the dotted lines indicate the absence of the ferment; the first diagram explains the four that follow.

It is evident from these results that the production of uric acid from xanthin or hypoxanthin takes place directly as the result of the combined action of the xanthin-oxidase and molecular oxygen, whereas it can only be formed from guanin and adenin by previous desamidization through the action of guanase and adenase, respectively, which belong to the group of hydrolytic ferments.

Reference has been made repeatedly to the wide distribution in nature, especially in the plant kingdom, of oxidases of the type of laccase. Whether these represent merely special occurrences of laccase or whether they are really distinctly different oxidases can not be determined in the present state of our knowledge. So far as one can judge from the experimental evidence now at hand it would seem probable, however, that all of these guaiacum-bluing vegetable ferments are pratically indentical with laccase, and it is doubtful if there is much to be gained by the introduction of these new names, which after all only serve to tell something with regard to a special occurrence of the ferment without throwing any light on differences in properties, if such exist. On the other hand, Sarthou ([357, 359]) is inclined to the opinion that the oxidases are distinct substances which can not replace one another in the changes which they bring about.

Irrespective of these differences of opinion, however, the following additional facts pertaining to their occurrence are of interest.

Aso found oxidases in tea ([15]) and in kaki fruit ([14]); Loew ([277]) found them in tobacco, and Carles ([110]) in valerian; they have been found in the grapevine by Cornu ([123]), in plants of the genus *Spiræa* by Beijerinck ([41]), and in the vanilla bean by Lecomte ([257]). According to Breaudat ([95]) indigo-producing plants contain two ferments, one a hydrolytic enzyme and the other an oxidase; the latter in the presence of alkali transforms indigo-white into indigo-blue. On the other hand Bergtheil ([46]) was unable to find an oxidase in the indigo plant. Bourquelot ([86]) has proved the presence of oxidases in certain medicinal preparations and in certain gums such as gum arabic, gum senegal, etc. Lepinois ([264]) found an oxidase in aconite and belladonna, and Vadam ([438, 439]) found an oxidase in hellebore. Similarly oxidases have been found in oleaginous grains by Mazé ([290]) and in digitalis by Brissemoret and Joanne ([98]).

As is evident from what has already been set forth in this monograph, the oxidases occur abundantly in the higher fungi. In addition to this they have been found in different yeasts, such as *Saccharomyces ellipsoideus*, *S. cerevisiæ*, and *S. apicultus*. According to

Tolomei ([423]), Effront ([156]), and Buchner ([103]) the oxidases of these yeasts oxidize guaiacum and are concerned in the production of the bouquet of certain wines. To these also are attributable the darkening in color of zymase (expressed yeast juice) on exposure to the air, and also the rise in temperature produced when large amounts of yeast cells are exposed to the air. These observations by Tolomei and Effront on the occurrence of oxidases in yeasts have been confirmed by Grüss ([202]), only this author found these oxidases to be without action on guaiacum or hydroquinone. On the other hand, like his spermase, they were found to be capable of oxidizing tetra-methyl-para-phenylene diamin, and other amino compounds, such as phenyl-hydrazin. Hence according to Grüss the yeast oxidase belongs to the class of amino-oxidases.

Issajew ([221]) has also investigated the oxidases of yeast and has confirmed the work of the earlier observers mentioned. He found that the oxidase in yeast oxidizes both the easily oxidizable substances which the yeast contains and also other easily oxidizable substances, such as the polyphenols, and that the upper yeast (*Oberhefe*) contains, as one might naturally expect, more oxidase than the under yeast (*Unterhefe*).

Various bacteria, such as the bacillus of malignant pustules (Dietrich and Liebermeister ([139]), and the colon bacillus (Roux ([353])), have been found to contain oxidases. The oxidase of the latter was found to oxidize hydroquinone, gelatin, peptone, etc., with the production of a brown pigment, and to be active only in the presence of oxygen.

Chapter III.

THE PEROXIDASES AND CATALASES.

In addition to the oxidases, two other classes of enzymes are more or less immediately concerned in the oxidation processes occurring in the plant and animal. These are the peroxidases and catalases. These have been found to have even a wider distribution among living cells and tissues than the oxidases. They were called by Bourquelot (83) the "indirect oxidizing ferments." It was known to Schoenbein (383) that many substances of animal and vegetable origin which in themselves are incapable of bluing guaiacum, acquire this property when mixed with hydrogen peroxide. In other words, just as certain extracts and tissues have the power of rendering active the oxygen of the air, so certain others possess the property of rendering active the oxygen of hydrogen peroxide, thereby enabling this compound to effect certain oxidations which ordinarily it is incapable of bringing about. Thus on adding a small amount of extract of malt to a mixture of guaiacum and hydrogen peroxide the mixture rapidly becomes blue in color. He also made the interesting observation that many plant and animal tissues and the extracts thereof and many hydrolytic ferments have the power of effecting the decomposition of hydrogen peroxide into water and oxygen. He seems to have regarded the power to render active the oxygen of hydrogen peroxide and the power to catalyze this substance not as specific activities of any given substances but as properties pertaining to the ferments as a class. In other words, the power to oxidize guaiacum by means of hydrogen peroxide and the power to decompose the latter substance into water and oxygen were looked upon by him simply as properties of diastase, emulsin, myrosin, and other ferments. This view prevailed for a long time and was shared by others. Thus, according to Spitzer (407), the power to decompose hydrogen peroxide is a measure of the oxidizing power of various animal tissues. It was afterwards shown by Raudnitz[a] (338, 339), however, that the substance in milk which gives the guaiacum reaction with hydrogen peroxide is really different from the other substances present in milk, in that it conducts itself differently toward various precipitants; and Jacobson (223) pointed out that the power of any given

[a] Raudnitz (339) called the catalase of milk a superoxydase, and the guaiacum-bluing ferment a globulin-oxydase.

tissue or ferment to decompose hydrogen peroxide can be destroyed by certain degrees of heat and by certain poisons which have no action on the other ferments present in the preparations. Similar conclusions were also reached by Loew (278) in his work on catalase. (*See also* Bourquelot (83).) Hence we have come to regard the peroxidases and catalases as distinctly specific enzymes. Apparently, therefore, three distinctly different sets of substances are concerned in vital oxidations: First, the oxidases, by means of which the oxygen of the air is rendered sufficiently active to effect the oxidation of guaiacum and other oxidizable substances directly; second, the peroxidases which render active the oxygen of hydrogen peroxide and other peroxides (including the oxygenases); and third, the catalases, which decompose hydrogen peroxide into water and molecular oxygen, without, apparently, being able to activate the oxygen of the peroxide toward oxidizable substances.

Schoenbein (383) included the peroxidases and catalases among his oxygen-carriers and catalysts (*Sauerstofferreger* and *Sauerstoffueberträger*) without apparently assigning particular names to these particular groups of carriers. Later, those substances which induce the oxidation of guaiacum and similar reagents through the agency of hydrogen peroxide and which lose this property on boiling, were called by Bourquelot (83) "indirect oxidizing ferments" (*ferments oxydants indirect*), and in 1898 Linossier (273) gave them the name of peroxidases as signifying those substances whose function it is to decompose hydrogen peroxide and other analogous peroxides and thereby induce oxidations by means of these peroxides. The term "catalase" was proposed by Loew (278) for those ferments which decompose hydrogen peroxide into water and molecular oxygen without apparently being able to activate the oxygen of the peroxide toward readily oxidizable substances.

The peroxidases and catalases seem to be even more widely distributed in various living tissues of the plant and animal than the oxidases. To such an extent is this the case that the properties of these substances might almost be turned to account as a general chemical test for vital activity. It can certainly be said of any living tissue or organ that it is dead when it fails to show the reactions of the peroxidases and catalases. Thus Brocq-Rosseu and Gain (100), in their observations on the duration of the peroxidases in grains, found that all seeds which retained their germinating power contained peroxidases, and in corn peroxidases were recognized by these observers (99) in samples of the grain over two hundred years old. On the other hand, it was observed by these authors that the peroxidase activity outlasts the power to germinate by a hundred years. In other words, it would appear that peroxidase activity is one of the most characteristic and persistent properties of living material.

SOURCES OF PEROXIDASE AND METHODS FOR ITS PREPARATION.

Among plants the peroxidases are almost universally distributed. There are but few, if any, plant tissues which do not give peroxidase reactions at some stage of their growth and development. So, likewise, they are widely distributed in animal tissues and secretions; saliva, milk, pus, etc., show typical peroxidase reactions. By means of certain of the very sensitive peroxidase reagents employed by Kastle and Porch ([246]) in their study of the peroxidase reaction of milk, Doctor Roberts, working in this laboratory, has been able to stain a large number of the leucocytes in fresh blood smears, indicating that in the blood the peroxidase proper is confined to the white cells. Thus with a reagent containing cresol, para-phenylene diamin and hydrogen peroxide, the leucocytes are stained blue.

By precipitation with alcohol, Linossier ([273]) prepared a peroxidase, free from oxidase, from pus. In many instances plant peroxidases may be obtained free from oxidases by heating the aqueous extract to 70° C., at which temperature the oxidase is destroyed. Aso ([16]) has separated plant peroxidases from oxidases by fractional precipitation with alcohol, in which solvent the oxidases are soluble to a considerable extent.

As sources of peroxidase, Bach and Chodat ([27]) have employed the pumpkin (*Kürbisfrüchten*) and the root of the horse-radish (*Meerrettigwurzel*). In order to obtain the peroxidase, these authors proceed as follows: Two kilograms of finely grated horse-radish root are allowed to stand several hours at ordinary temperature in order to completely hydrolyze all glucosides that may be present. The mass is then digested for four to five days with 80 per cent alcohol in order to dissolve the essential oils. The reddish alcoholic extract is then poured off, the residue is washed with 80 per cent alcohol, pressed, and extracted with about 8 liters of 40 per cent alcohol. The 40 per cent alcoholic extract, which shows strong peroxidase reactions, is then reduced to small volume at 30° C. in vacuo, filtered, and absolute alcohol added until a turbidity is produced. The white precipitate thus produced is dissolved in a small amount of water, again precipitated with absolute alcohol, and dried in vacuo. In this way a yellowish white gummy mass is obtained which is very soluble in water and readily soluble in 40 per cent alcohol. As ordinarily obtained these peroxidase preparations strongly reduce Fehling's solution. By re-solution in water and re-precipitation with alcohol, peroxidase preparations may be obtained which no longer reduce Fehling's solution. The purest peroxidase preparations obtained by this method give ammonia and substances having the odor of pyridin on heating with caustic soda, but failed to show the ordinary reactions for proteid. On heating to boiling, solutions of the peroxidase were found to lose their activity; on

standing, however, such boiled solutions regain their activity. That such is the case was first observed by Woods (463) in his study of the peroxidase of tobacco, and has been accounted for by Woods, and also by Aso (16), upon the supposition that the peroxidase is regenerated from zymogens which are more stable towards heat and various reagents than the ferment itself. Bach and Chodat (27) find that a second heating destroys the peroxidase altogether; so also an alcoholic solution of the ferment is destroyed by heating to the temperature of boiling alcohol. The interval required for the destruction of the enzyme has been found to vary with the concentration of its solution. Thus when diluted with twenty times its volume of water a given specimen which in its original dilution required eighteen minutes in boiling water for complete destruction, required but three minutes at this temperature when thus diluted. According to Bach (19) the peroxidase is a single enzyme, whose function is to activate hydrogen peroxide in the oxidation of substances containing labile hydrogen.

Bach and Tschermiack (32) have given detailed directions for the purification of peroxidase. No essential differences have been observed between the conduct of crude peroxidase and that of the purest preparations. The pure preparations were found to contain 6 per cent of ash; this ash was found to be iron-free, but to contain aluminium and manganese. Bach and Chodat (27) found that peroxidase, the chief constituent of oxidase, contains nitrogen and gives the pyrrol reaction. They conclude, however, that it is not a proteid since neither the raw product nor the purified enzyme show the protein reactions.

According to Bach and Chodat (27) the action of peroxidase is specific in that it strongly activates hydrogen peroxide and other peroxides in a large number of oxidation processes, such as the oxidation of pyrogallol, gallic acid, anilin, dimethylanilin, etc. They observed further that while it strongly activates small amounts of hydrogen peroxide, it is destroyed by larger amounts of the peroxide (*see also* Schoenbein (370), p. 474), and also that in the absence of hydrogen peroxide or a similar substance the peroxidase has no oxidizing power (*see also* Linossier (273)). Decidedly the most interesting observations recorded by these observers is that the peroxidase not only activates hydrogen peroxide and the peroxides resulting from the slow oxidation of various organic compounds such as ether, alcohol, and the essential oils, but that it also has the power of increasing the oxidizing power of the oxidases. As elsewhere pointed out, Moore and Whitley (306) argue from this that the oxidase is nothing more than a mixture of peroxidase and an unstable, naturally occurring peroxide (*see also* Bach and Chodat (28)).

Quantitative studies of the oxidation of pyrogallol by hydrogen peroxide and a peroxidase have also been made by Bach and Chodat [31] with the following results:

(1)

[Pyrogallol, 1 gram; hydrogen peroxide, 0.1 gram; peroxidase, from 0.01 to 0.1 gram, in 50 c. c.]

Peroxidase	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08
Purpurogallin	0.021	0.042	0.066	0.083	0.102	0.123	0.145	0.166

(2)

[Pyrogallol, 1 gram; peroxidase, 0.1 gram; hydrogen peroxide, from 0.01 to 0.1 gram.]

Peroxide	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08
Purpurogallin	0.020	0.042	0.060	0.078	0.099	0.121	0.142	0.168

(3)

[Peroxidase, 0.1 gram; hydrogen peroxide, 0.1 gram; pyrogallol, 1 to 4 grams.]

Pyrogallol	1.0	1.5	2.0	3.0	4.0
Purpurogallin	0.168	0.205	0.203	0.208	0.202

It would seem therefore that the amount of pyrogallol oxidized under these conditions is proportional to both the quantity of the peroxidase and of the hydrogen peroxide, but independent of the quantity of pyrogallol present, provided that the latter is in excess of the quantity capable of being oxidized within the given time by the several amounts of the peroxidase and hydrogen peroxide employed.

NATURE AND MODE OF ACTION OF THE OXIDASES, PEROXIDASES AND CATALASES, AND THE CHEMICAL RELATIONSHIPS EXISTING AMONG THESE SUBSTANCES.

The question still remains to be considered as to how these substances act as oxygen carriers. We have seen that according to Schoenbein they react by ozonizing the oxygen of the air, and in the case of the peroxidases, by converting hydrogen peroxide, an antozonide, into an ozonide, after the manner of lead acetate. On the other hand, according to Bach [18] the oxidases are substances having a special aptitude for forming peroxides. Similar views have been advanced by Kastle and Loevenhart [244]. Thus according to the peroxide theory of oxidation, when molecular oxygen finds itself in contact with the complex autoxidizable substances contained in the plant cell, it combines with the same in much the same way that it unites with rubidium or benzaldehyde. There is produced under these circumstances a complex unstable peroxide which, in turn, can give up a part, if not all of its oxygen, to any oxidizable substance or acceptor that may happen to be present, or in the event that no other oxidizable substance is present, it may oxidize a part of itself.

In this manner we account for the oxidation of guaiacum, potassium iodide, and hydroquinon by atmospheric oxygen through the intermediate action of the vegetable oxidase. This theory also enables us to understand the instability of these substances.

Diagrammatically, these changes may be represented as follows:

$$(1) \qquad A + O_2 \xrightarrow{\text{(Oxidase)}} AO_2,$$

and

$$(2) \qquad AO_2 + B \xrightarrow{\text{(Oxidase)}} AO + BO,$$

or

$$(3) \qquad AO_2 + 2B = A + 2BO,$$

and

$$(4) \qquad AO_2 + A = 2AO.$$

In this connection see also Engler and Weisberg ([164]), supra.

In the above equations A is the autoxidizable substance or substances contained in the plant or animal which is the immediate precursor of the oxidase. AO_2 is the oxidase resulting from the union of A with molecular oxygen, and B is the substance oxidized by the oxidase, as, for example, guaiacum, phenolphthalin, or hydroquinon. If the change proceeds as indicated in equation (2), both the precursor of the oxidase and the second oxidizable substance (the oxidase reagent) will be completly oxidized, and the oxidase will not be regenerated as the result of the oxidation, but will be destroyed. On the other hand, if the oxidation proceeds according to equation (3), the precursor of the oxidase will be regenerated, and at the end of the process will be in a condition to again enter into the cycle of changes indicated in equations (1) and (3). Finally, it is conceivable that the oxidase may oxidize a second part of A, in which case, as indicated by equation (4), both the precursor of the oxidase and the oxidase itself will be destroyed. As a matter of fact, it is known that solutions of oxidases gradually lose their oxidizing powers; this is readily intelligible in the light of equations (1), (2), and (4), especially in view of the fact that all living tissues and extracts contain powerful reducing substances which in the processes under consideration would function as B in equation (2).

In support of the view that the oxidases are really peroxides, we may cite the following observations of Bach and Chodat([26]): The fresh sap of the *Lathræa squamaria*, which contains an oxidase, was subjected to the action of a current of air and a 1 per cent solution of barium hydroxide was added drop by drop. A precipitate is formed under these circumstances which is found to contain barium, and when decomposed by sulfuric acid the resulting solution gave

a blue coloration with potassium iodide and starch, but no reaction with titanic acid. Nitrites were also shown to be absent. Hence, according to these authors, the separation of the iodine from the potassium iodide could only have been brought about by an acyl-hydroperoxide. A similar experiment carried out on a specimen of the *Lathræa squamaria* sap which had lost its power to blue guaiacum gave a completely negative result. Hence the peroxide formation with the active sap depends on the presence of the oxidase, and leads to the belief that the oxidase itself is of a peroxide nature, or that it at least contains a peroxide as one of its constituents.

Schoenbein, in his paper on the catalytic action of organic materials (peroxidases and catalases) and their distribution in the plant and animal kingdoms ([383]), explains the activating and catalyzing power of such substances on the supposition that, like lead acetate, they convert hydrogen peroxide (an antozonide) into an ozonide (like lead peroxide), and that under the influence of the latter the former is actively decomposed with the production of water and molecular oxygen. Thus he proved that when lead acetate is added to a solution of hydrogen peroxide, lead peroxide (an ozonide) is formed, under the influence of which the hydrogen peroxide is actively decomposed; and that if lead acetate be added to hydrogen peroxide solution containing guaiacum, the latter is oxidized at the same time that a part of the hydrogen peroxide is decomposed, for the reason that the lead peroxide oxidizes the guaiacum at the same time that it decomposes the hydrogen peroxide. According to Schoenbein, therefore, what we now know as the peroxidases are those substances occurring in the secretions and tissues of animals and plants which have the power of ozonizing hydrogen peroxide or converting it into an ozonide. The catalysis or decomposition of the hydrogen peroxide he looked upon as a secondary phenomenon resulting from the action of the ozonide thus formed upon the hydrogen peroxide remaining unchanged. Lepinois ([263]) conceives that the decomposition of hydrogen peroxide takes place in such a way that where only a part of the oxygen is set free it is fixed by the guaiacum or guaiacol.

From their study of the hydrogen peroxide-guaiacum reaction, Kastle and Loevenhart ([244]) arrived at the conclusion that the peroxidases are substances which are capable of reacting with hydrogen peroxide to form peroxides, which are more vigorous oxidizing agents than hydrogen peroxide itself. This view regarding the nature of these substances has been concurred in by Bach ([20]). The decomposition of hydrogen peroxide and the mechanism of oxidations by means of this substance has formed the subject of a still further investigation by Loevenhart and Kastle ([275]). It is now known that hydrogen peroxide undergoes spontaneous decomposition into water and molecular oxygen; it is also known that it can

effect the oxidation of many substances directly, such as formic aldehyde and sulfurous acid, without the intervention of an oxygen carrier. It is also a matter of common observation that its decomposition into water and molecular oxygen is greatly accelerated by various catalysts, such as spongy platinum, lead peroxide, manganese dioxide, etc., and also that its oxidizing powers are greatly increased by these same substances. It has also been established through the researches of Schoenbein ([383]) and through some observations of Loevenhart and Kastle ([275]) that, with but few exceptions, those substances which can bring about the decomposition of hydrogen peroxide catalytically can also greatly increase its oxidizing power; and in proportion as a substance is able to decompose the peroxide, so also can it accelerate oxidations by this substance to a corresponding degree. A good catalyst has been found to be a good oxygen carrier and, vice versa, a poor catalyst is a poor carrier. Finely divided platinum decomposes hydrogen peroxide very rapidly; it has been found also to be a powerful oxygen carrier. On the other hand, sugar charcoal decomposes hydrogen peroxide very slowly; it is a very poor oxygen carrier. It is evident, therefore, that oxidation by hydrogen peroxide in the presence of a carrier and its catalytic decomposition go often, if not always, hand in hand, so that the latter process, as has been pointed out by Spitzer ([407]), is often a measure of the former. It would seem, therefore, that all of these phenomena, widely differing as they appear at first sight, are in reality closely correlated and referable to a common cause, namely, the tendency on the part of the hydrogen peroxide to unite directly with oxidizable substances, forming thereby either a peroxide or some other complex unstable holoxide derivative which tends to part with its oxygen more easily than the hydrogen peroxide itself. This derivative, therefore, readily oxidizes some other substance to which one of its compounds stands in the relation of an oxygen carrier, or it gives up molecular oxygen, or both.

It is important for this assumption, of course, that such higher oxides and unstable addition-products of hydrogen peroxide with other substances should actually exist. In reality, we have abundant proof of their existence. In fact, as may be seen from the following, many such compounds are known. Thus in 1878, Schöne ([388], [389]) isolated a number of compounds of hydrogen peroxide with the alkalis and alkaline earths, such as the following:

$BaO_2.H_2O_2$, or H_2BaO_4;
$K_2O_2.2H_2O_2$, or $H_4K_2O_6$;
$Na_2O_2.2H_2O_2$, or $H_4Na_2O_6$.

All of these compounds were found by this observer to be very unstable and are obtainable in a state of purity only at very low

temperatures. He therefore explained the catalytic decomposition of hydrogen peroxide by alkalis on the assumption that these hydrogen peroxide addition-products with the alkalis are first produced, thus:

$$2ROH + 3H_2O_2 = H_4R_2O_6 + 2H_2O,$$

and that these substances then undergo the following transformations:

(1) $$R_2H_4O_6 = R_2O_4 + 2H_2O;$$

(2) $$R_2O_4 + H_2O = 2ROH + O_3.$$

He was led to assume the intermediate production of such peroxides as R_2O_4 for the reason that the compounds such as $H_4K_2O_6$ turned yellow during decomposition. The work of Schöne on these complex addition-products of hydrogen peroxide has been confirmed by the later researches of Forcrand (173), who has obtained evidence not only of the existence of such compounds as H_2BaO_4 and H_2CaO_4, but also of still more complex derivatives such as $CaO_2 10H_2O_2$, and Moissan (304) from his investigation of the unstable blue compound of chromic anhydride and hydrogen peroxide, reached the conclusion that it must be represented by the formula H_2CrO_5, or $CrO_3.H_2O_2$.

Similarly, in order to explain the great acceleration caused by small amounts of molybdic and tungstic acids in the oxidation of hydriodic acid by hydrogen peroxide, Brode (101) has been led to assume the formation of permolybdic and pertungstic acids as the result of the action of the peroxide on the catalyzer, while nine years previously Cammerer (109) had obtained permolybdic acid, $2MoO_3.H_2O.H_2O_2$, and pertungstic acid, $WO_3.H_2O.H_2O_2$, by boiling the ordinary acids with hydrogen peroxide, and still more recently Pissarjewsky and Mellikoff (325) and also Muthmann and Nagel (308) have obtained similar acids and their salts, some of the latter being very unstable and even explosive. According to Job (226) cerous salts act as oxygen-carriers in the presence of hydrogen peroxide, probably through the alternate formation and decomposition of cerium peroxide.

Petrenko (319) also, while studying the action of hydrogen peroxide on sodium arsenate, obtained a compound having the composition $Na_9As_3O_{17}.21H_2O$. This he found to be a hydrogen peroxide derivative having the following constitution: $3Na_3AsO_4.5H_2O_2.16H_2O$. So also Kazanesky (248) obtained the compounds $K_2CO_3.2H_2O_2.12H_2O$, and $K_2CO_3.3H_2O_2$, and similarly Staedel (410) found that on adding a 90 to 95 per cent solution of aqueous hydrogen peroxide to a concentrated solution of cadmium chloride, a compound was obtained which crystallized in silky needles containing 23 per cent of hydrogen peroxide, the theory for $CdCl_2.2H_2O_2$ being 27.09 per cent.

Jones and his co-workers ([232], [233]) have recently investigated the lowering of the freezing point of certain salts and acids in aqueous hydrogen peroxide as a solvent. In discussing the abnormal lowering of the freezing point of certain of these salts, such as potassium nitrate, in this solvent, these observers make this significant statement: "The most probable explanation of the above results, all things considered, seems to be that there is union between the molecules of the salts investigated and the molecule of hydrogen peroxide."

According to Manchot ([284]) the apparent reductions by means of hydrogen peroxide are in reality due to the formation of more highly oxidized primary products, which ultimately break down into final products which are less highly oxidized than the hydrogen peroxide itself. (*See also* Kastle and Loevenhart.)

There seems to be abundant evidence, therefore, that hydrogen peroxide is not only able to form peroxides by double decomposition, but that it also frequently combines directly with various substances to form complex unstable addition products, and that oxidations by means of this substance are often greatly accelerated and its decomposition greatly hastened by the formation and subsequent decomposition of these complex substances. It wouid seem logical, therefore, to look upon the peroxidases as substances capable of forming unstable peroxides from hydrogen peroxide by double decomposition, or of combining directly with hydrogen peroxide to form unstable holoxide (Traube) or moloxide (Engler) derivatives, possessing greater powers of oxidation than hydrogen peroxide.

Thus, if we designate the peroxidase by P, and the second oxidizable substance by B, the oxidations effected by the peroxidase might be represented diagrammatically as follows:

$$P + H_2O_2 = PO_2 + H_2O,$$
$$\text{and } PO_2 + B = PO + BO,$$
$$\text{or } PO_2 + 2B = P + 2BO,$$

$$\text{or } P + H_2O_2 = H_2PO_2,$$
$$\text{and } H_2PO_2 + B = P + BO + H_2O.$$

As already pointed out, these views respecting the mode of action of the peroxidases are shared by Bach, who has observed that when a mixture of a peroxidase and hydrogen peroxide is allowed to stand for some time both disappear from the solution, a fact which indicates the mutual interaction of these two substances. According to Bach and Chodat ([27]) the peroxidases exert not the slightest oxidizing action in the absence of the peroxide. On the other hand, they found that it can activate not only hydrogen peroxide but also organic peroxides, such as ethyl-hydroperoxide, $C_2H_5O_2H$. Further, they have shown that the system (peroxidase + peroxide) accomplishes precisely the

same kind of oxidations as are accomplished by the oxidases, and that between the two sorts of oxidizing agents there is the closest sort of agreement, and in this connection they have made a number of interesting observations which would seem to indicate that the vegetable oxidases are not single enzymes but mixtures of peroxidases and peroxide-forming substances, to which they have given the name "oxygenase."

THE OXIDASE A MIXTURE OF PEROXIDASE AND OXYGENASE.

As early as 1898, Linossier ([273]), in discussing the function of the peroxidases, stated that the oxidases fix the oxygen of the air, forming peroxides which are destroyed by the peroxidases as fast as they are formed.

In the course of their investigations on the "Function of Peroxides in the Living Cell" Bach and Chodat ([27]) found that by the fractional precipitation of an aqueous solution of the oxidase of a fungus, *Lactarius vellereus*, by alcohol, two fractions could be obtained possessing markedly different properties. The first of these is almost insoluble in 40 per cent alcohol and was found to have the properties of a weak oxidase. The second substance is soluble in 40 per cent alcohol but insoluble in pure alcohol, and has no oxidizing powers. This second substance, however, was found to impart great activity to hydrogen peroxide as an oxidizing agent, and not only has it the power of conferring activity upon hydrogen peroxide, but also upon organic peroxides, such as ethyl hydro-peroxide, etc., and what is even more interesting, it was also found to impart activity to the weak oxidase composing the first fraction obtained in the precipitation of the *Lactarius* extract. In a recent paper these authors ([31]) give the following interesting results of their experiments with these substances:

Pyrogallol.	Horse-radish peroxidase solution.	Lactarius peroxidase solution.	Lactarius oxygenase	Oxygen absorbed.	Carbon dioxide evolved.
	c. c.	*c. c.*	*Gram.*	*c. c.*	*c. c.*
1 gram.	15	.....	.....	0.5	0.1
1 gram.	.....	.....	0.059	3.1	1.1
1 gram.	15	.....	0.059	9.9	5.5
1 gram.	.....	15	.....	0.2	0.0
1 gram.	.....	.....	0.05	3.1	1.1
1 gram.	.....	15	0.05	11.0	6.8

These results show conclusively that the oxidizing power of the oxygenases is greatly increased by the peroxidases in general, and still more greatly increased by the peroxidase from the same source as the oxygenase itself.

According to Bach and Chodat [28], therefore, the oxygenases are of the nature of substituted peroxides. They are exceedingly unstable and have, as compared with the peroxidases, only a limited distribution in the vegetable kingdom. On the other hand, the peroxidases are characterized by great stability. Thus the peroxidase of the horse-radish root is not completely destroyed by a single boiling of its solution. The peroxidases occur in practically every plant thus far examined for them, and they have likewise been found widely distributed in the tissues and secretions of animals, such as the leucocytes, milk, saliva, etc. Like the oxidases, the peroxidases have been found to contain manganese, and to this element Bach and Chodat [31] ascribe their activity. They are also disposed to regard them as true ferments, although they gradually lose their activity and ultimately disappear as the result of the oxidations which they bring about.

It would seem, therefore, that it is not the oxidase but rather the peroxidase which is the most important agent in plant and animal oxidations, inasmuch as it would render active the oxygen occurring in any peroxide combination whatsoever, whether it be hydrogen peroxide, an organic peroxide, or an oxygenase.

Indeed, according to Moore and Whitley [306] the peroxidases are the only true ferments participating in biological oxidations. According to these authors, Bach's so-called oxygenase is merely an unstable peroxide resulting from the action of the oxygen of the air on some readily oxidizable substance contained in the plant or animal tissue. From their standpoint the only essential differences between a tissue showing oxidase reactions and one showing only peroxidase reactions is that the former contains, in addition to the peroxidase, a store of naturally formed peroxides, which are very unstable toward heat, whereas the latter contains only the ferment (peroxidase). In this connection it was long ago pointed out by Bach [18], and also by Kastle and Loevenhart [244], that the oxidizing ferments (oxidases) were not true ferments for the reason that they present many close resemblances to the organic peroxides, and for the further reason that they are not true oxygen-catalysts in the sense of being unable to accomplish the oxidation of practically unlimited amounts of oxidizable material. In the writer's opinion, therefore, the objections which have been recently urged against the prevailing conceptions regarding the oxygenases are well taken. It should be borne in mind, however, that the precursors of the oxygenases are unstable toward heat and hence possess, to some degree at least, the characteristics of biologically active substances.

THE OXYGEN-CATALYSTS OF THE BLOOD.

Certain oxidases, such as aldehydase and the glycolytic enzyme, peroxidases and catalases have been found in the blood, and also certain more stable oxygen-catalysts, such as hemoglobin and its iron-containing derivatives, and in the case of certain of the invertebrates, hemocyanin, the oxidizing powers of which are not destroyed upon boiling. In this connection it is interesting to note that all of the oxygen carriers of the blood of whatever nature are contained in the formed elements and not in the plasma or serum. Even the cellular elements exhibit well marked and characteristic differences in their conduct toward oxidase and peroxidase reagents. Furthermore, various observers do not seem to be agreed with regard to the oxidizing properties shown by the formed elements of the blood. Thus it was shown by Klebs in 1868 (250) that pus has the power of bluing guaiacum directly. On the other hand, according to Linossier (273), if one uses a freshly prepared tincture of guaiacum the superficial portion of the resin having been previously removed by washing with alcohol, the blue color is not produced upon addition of pus, but only after the addition of hydrogen peroxide. Hence, according to Linossier, pus (or white blood corpuscles) contains a peroxidase, but no oxidase. More recently, however, Meyer (300) has shown that in the perfectly fresh state the thick leucocyte layer obtained by centrifugalizing the blood of patients suffering with myelogenous leucemia, gives no reaction with guaiacum. If, however, water be added and the mixture shaken a few moments, a very intense blue color develops. On the other hand, if the corpuscles be shaken with serum or with an isotonic salt solution no blue color develops. According to Meyer this shows that the leucocytes contain an oxidizing ferment (oxidase) which first becomes liberated or activated by the solution of the cells in water. Similarly a few drops of leucemic blood diluted with such a large amount of water that scarcely any blood color was visible gave with guaiacum a deep blue color. He also observed that fresh untreated pus gives no guaiacum reaction, but that when shaken with distilled water it gives the guaiacum reaction at once. He also found that these aqueous extracts of pus and leucocytes from myelogenous leucemia could oxidize phenolphthalin. He concludes, therefore, that the polynuclear and probably also the mononuclear, neutrophile cells contain a substance extractable with water which blues guaiacum without the addition of hydrogen peroxide, or in other words, that it contains an intracellular oxidizing ferment killed by boiling, but not at 73° C., and which, as found for other oxidases by Bach and Chodat, contains an oxygenase and a peroxidase.

Reference has already been made to the fact that A. and L. Lumière and Chevrotier (281) have prepared a protoplasmic extract of blood

corpuscles which exhibits the properties of an oxidase to a marked degree, as shown by its power to oxidize guaiacum, guaiacol, hydroquinon, etc.

In the light of these facts there can scarcely be any doubt that certain of the leucocytes contain both oxidases (see also Portier [331, 332,]) and peroxidases.

Dr. Norman Roberts, working in the Hygienic Laboratory on the peroxidase activity of the urine in health and disease, has examined the urines of 175 diseased persons and also the urines of a considerable number of normal individuals for peroxidases. As nearly as he has been able to discover, the peroxidase reaction of urine is not specifically characteristic of any of the diseases studied, except such as involve an active inflammation of the genito-urinary tract. It does seem to be constant, however, for active inflammations of this character and is due to the leucocytes and possible in some instances to certain epithelial cells which these urines contain. He has also shown that in fresh blood smears and fresh sections of highly vascular tissues the majority of the leucocytes are stained blue on treatment with the peroxidase reagent (a solution containing cresol, para-phenylene diamin, and hydrogen peroxide), whereas the red cells of the blood and the cells characteristic of the tissues examined remained unstained. (See also Winkler[453].) As is well known, pus also actively decomposes hydrogen peroxide.

In the light of these facts there can scarcely be any doubt that certain of the leucocytes contain oxidases, peroxidases, and catalases.

We have seen further that Senter [400] has prepared a very active catalase from defibrinated blood, free from hemoglobin and from any oxidase or peroxidase. While, therefore, the blood undoubtedly contains oxidases, peroxidases, and catalases, it has gradually come to be recognized that its oxygen-carrying power can not be due entirely to oxidases and peroxidases, for the reason that this oxygen-carrying power persists after boiling, and after treatment with acids and alkalis. Indeed it seems to persist as long as the blood pigments are not deprived of their iron. Thus according to Moitessier [305] the so-called peroxidase reaction of the blood, upon which is based most of the chemical tests for blood, is not really due to a peroxidase but to hemoglobin and hematin. According to this author the non-ferruginous blood pigments, such as hematoporphyrin, do not exhibit such reactions. Czyhlarz and von Fürth [131] have also arrived at the conclusion that the so-called peroxidase reaction of the blood is due to hematin and not to a peroxidase. Lesser [267] also obtained the guaiacum reaction with blood which had been boiled. He also is of the opinion that the reaction is due to the blood pigment and that the iron-free derivatives of hemoglobin do not give it. Whitney [450] also concludes that it is the iron of the hemoglobin and its iron-

containing derivatives which are responsible for the guaiacum test and similar reactions. Buckmaster [104, 105] also regards the oxidizing power of blood in the presence of hydrogen peroxide as due to a pseudo-peroxidase and in some way dependent on the part played by iron in the hemoglobin molecule, although the precise way in which the iron acts is still obscure. Studies on the oxygen-carrying power of blood toward solutions containing hydrogen peroxide, phenolphthalin, and alkali have also been made by Kastle and Amoss [241] and later by Kastle [240], who has recently recommended this reaction as a chemical test for blood. It has been shown that the amount of phenolphthalin oxidized both in the presence and absence of hydrogen peroxide is proportional to the quantity of hemoglobin present, and that while the oxygen-carrying power of the blood is somewhat diminished, it is by no means entirely destroyed even after repeated boiling. The formed elements of the blood therefore contain oxidases, peroxidases, catalases, and a more stable oxygen-carrier, viz, hemoglobin, or, in certain of the lower animals, hemocyanin. To the catalases we owe the active decomposition of hydrogen peroxide into water and molecular oxygen, as shown by blood and pus. To the oxidases we owe the oxidation of guaiacum, phenolphthalin, and similar substances, as shown by aqueous solutions of the leucocytes. To the peroxidases we owe those oxidations which take place only in the presence of hydrogen peroxide or a similar compound, as also shown by the leucocytes. These properties are all lost on boiling. On the other hand, certain oxidations by means of hydrogen peroxide are induced by hemoglobin and its iron-containing derivatives, and hence we find that the blood can still induce certain oxidations by hydrogen peroxide even after it has been boiled. The fact that these carriers become inactive with the splitting off of the iron which they contain is a matter of great interest in view of the part played by iron and manganese in the activation of the oxidases and in view of the relation of iron to active nucleo-proteids.

The fact that hemoglobin and its iron-containing derivatives oxidize various chromogenic substances in the presence of hydrogen peroxide has been turned to practical account in hematological and forensic investigations. This subject has recently been discussed at length by Kastle [240] in a communication entitled "Chemical Tests for Blood," in which there is given a reasonably complete bibliography of the extensive literature of this subject.

IRON, COPPER, AND MANGANESE IN THEIR RELATION TO THE OXIDIZING FERMENTS. ARTIFICIAL OXIDASES, PEROXIDASE ACCELERATORS, AND AUXILIARY OXYGEN CARRIERS.

According to Spitzer [407] the oxidizing power of animal tissues is referable to certain nucleo-proteids which they contain, and the oxidizing power of these substances is in turn referable to combined

iron. In this connection it is of interest to note that so far as is known the most stable and most perfect oxygen catalysts occurring in the animal organism contain either copper or iron. According to Wells (verbal communication) the blood of certain marine forms has been found to contain zinc. The fact that hemoglobin and its iron-containing derivatives are all capable of inducing the oxidation of such substances as guaiacum, phenolphthalin, etc., by means of hydrogen peroxide or other compounds of this nature, whereas the iron-free blood pigments can not accomplish such oxidations, speaks strongly in favor of the assumption that the activity of such oxygen carriers is dependent on iron in organic combination. (*See* Kastle [240]), "Chemical Tests for Blood," Bulletin No. 51, Hygienic Laboratory.) In this connection it is also of interest to note that Floyd [171] found nearly twice as much ash in the skin of the negro as in that of whites, and nearly twice as much iron in the ash of the negro's skin as in the ash of whites. According to this author the pigment appears to originate in the outer layer of the true skin, and in all probability is the product of the alteration of the red coloring matter of the blood. It has also been shown experimentally that certain iron salts accelerate biological oxidations. Thus Battelli and Stern [36] have found that the quantity of carbon dioxide produced by the action of an extract of the muscle of the horse or dog on calcium lactate is considerably increased by the presence of small amounts of ferrous sulfate.

It has been shown by Bertrand [56, 57, 58] that the oxygen-carrying power of laccase is in some way associated with the presence of manganese. By fractional precipitation with alcohol he was able to resolve a certain specimen of laccase into two portions, one of which was poorer and the other richer in manganese than the original sample. The oxidizing powers of these three specimens were proportional to their manganese content, as may be seen from the following table:

No. of specimen.	Quantity of manganese in ash.	Oxygen absorbed in 1½ hours by 50 c. c. of a 2 per cent solution of hydroquinon in the presence of 0.2 gram of the specimens.
	Per cent.	c. c.
1	0.159	19.1
2	.126	15.5
3	.098	10.6

He also succeeded in showing that the laccase contained in lucerne is poor in manganese and comparatively inactive toward hydroquinon. In the presence of a small amount of manganese (1 milligram in the form of the sulfate) the oxidizing power of lucerne laccase is greatly increased. That such is the case is evident from the quantities of oxygen absorbed by a solution of hydroquinon in the presence

of manganese alone, lucerne laccase alone, and a mixture of manganese and laccase, thus:

	Oxygen absorbed.
	c. c.
1. Manganese alone	0.3
2. Lucerne laccase alone	.2
3. Laccase + manganese	6.3

In other words, the effect of the manganese on laccase poor in this constituent was to increase the oxygen absorption twenty-five times. Other metals, such as iron, aluminium, cerium, zinc, copper, calcium, magnesium, and potassium, were found to be incapable of increasing the oxidizing power of laccase.

He therefore arrived at the conclusion that the oxidizing ferments as we ordinarily recognize them consist in reality of two portions, one organic and very unstable with which we associate those properties usually characteristic of the ferments as a class, and a second portion, which might be called the co-ferment, mineral or organic in its nature, and which with the first substance forms the really active system. Bertrand [59] therefore is disposed to regard manganese as the co-ferment of laccase, just as calcium is the co-ferment of pectase, and hydrochloric acid the co-ferment of pepsin.

In a later communication Bertrand [56, 57] proved that various manganese salts can bring about the oxidation of various oxidizable substances, such as hydroquinon, etc., in the air, and that the quantity of oxygen absorbed varies with the nature of the manganese salt employed, being greater with the manganese salts of the organic acids. The several amounts of oxygen absorbed by hydroquinon in the presence of various manganese salts are shown in the following table:

Salt of manganese.	Oxygen absorbed.	Salt of manganese.	Oxygen absorbed.
	c. c.		c. c.
Nitrate	1.5	Acetate	15.7
Sulfate	1.6	Salicylate	16.3
Chloride	1.8	Lactate	17.6
Formate	7.4	Gluconate	21.6
Benzoate	15.3	Succinate	22.1

It would seem, therefore, that those manganese salts which are most easily hydrolyzable are the most efficient oxygen carriers. According to Bertrand, therefore, when a manganese salt finds itself in contact with water it hydrolyzes in the sense of the equation—

$$R''\mathrm{Mn} + \mathrm{H_2O} = R''\mathrm{H_2} + \mathrm{MnO}.$$

As is well known, however, manganous oxide is spontaneously oxidizable, and in the course of this oxidation molecular oxygen is split into two atoms, one of which combines with the manganous oxide to form the peroxide, the other going to oxidize the hydroquinon or other oxidizable substance, which ordinary molecular oxygen is powerless to oxidize. Thus

$$MnO + O_2 = MnO_2 + O,$$

and

$$C_6H_4(OH)_2 + O = C_6H_4O_2 + H_2O.$$

In the presence of an acid, $R''H_2$, and a substance like hydroquinon the latter is oxidized. Thus a further amount of hydroquinon is oxidized and the manganese salt regenerated, thus:

$$R''H_2 + MnO_2 = R''Mn + H_2O + O,$$

and

$$C_6H_4(OH)_2 + O = C_6H_4O_2 + H_2O.$$

In harmony with this view Bertrand proved that while manganese peroxide is stable in dilute acids it is reduced on adding hydroquinon, with the production of quinon and a manganous salt. He was therefore led to consider the oxidases whose oxidizing powers are so greatly intensified by a manganese salt as special combinations of manganese and an acid radicle, the latter probably of a proteid nature and partaking of the nature of a ferment and having just the affinity necessary to maintain the metal in solution. According to this conception the manganese would be the really active element of the oxidase, so far as the activation and transfer of oxygen is concerned, whereas the acid albuminoid radicle would impart to the ferment its other properties, such as its conduct toward heat, solubility, etc. According to Rey-Pailhade (343) the reducing ferment which he has described under the name of "philothion" possesses the properties of the acid albuminoid radicle of the oxidases.

Other observers have found manganese in oxidases. Thus Lepinois (264) found both manganese and iron in the oxidase of the roots and leaves of aconite and belladonna. Carles (110) found manganese in quantity in the oxidase of valerian root. According to Vitali (440) the oxidase of pus contains a trace of manganese, and to this it owes its oxidizing properties. Aso (15) likewise found both manganese and iron in the ash of the oxidase of tea leaves. Portier (332) has also shown that the activity of the oxidase of the branchies of *Artemis exoleta* is generally increased by the addition of small amounts of sulfate of manganese.

According to Bach and Chodat (31) manganese is also the active agent of the peroxidases. Kelley (249) has recently shown that pineapple plants containing a high percentage of manganese contain a more

active oxidase than plants which have only small percentages of this element.

On the other hand, oxidases have been obtained which contain no manganese. Thus Sarthou ([357, 359]) found the oxidase of *Schinus molle* to contain iron, calcium, and sodium, but no manganese; according to this author the oxidizing power of this oxidase is proportional to its iron content. Vadam ([438, 439]) found iron but no manganese in the ash of an oxidase from hellebore. According to Stoecklin ([412]), manganese is not present in the ash of the oxidase from horseradish.

It is evident, therefore, that certain metals, such as iron, manganese, and copper, one or more of them, enter into the composition of the oxygen carriers present in the living organism as essential ingredients. In this connection it is interesting to note that small amounts of manganese greatly accelerate a number of chemical processes. Thus according to Villiers ([441]) if one boils equal volumes of a saturated solution of oxalic acid and hydrochloric acid containing 25 per cent of HCl, and nitric acid equally dilute, there is not produced any disengagement of gas, even after a long time; on the other hand, if one adds to the solution a trace of a manganese salt the action develops instantly and there is produced a regular disengagement of carbon dioxide and nitrogen. This author has compared the action of a manganese salt to that of a mineral ferment (*ferments mineraux*). In another communication he ([442]) describes the use of small amounts of a manganese salt in the destruction of organic matter in toxicological analysis. Gigon and Rosenberg ([193]) have observed that the presence of small amounts of manganous or ferrous sulfate increases very markedly the digestion of starch by the diastatic blood ferment and by the pancreatic diastase.

Attempts have been made, therefore, to prepare artificial oxidases by means of certain manganese and iron compounds, and some observers have pointed out various analogies existing between certain metallic salts and the oxidases. Thus according to Trillat ([436]) when a small quantity of manganese chloride is added to a dilute solution of fresh egg albumin (3 parts to 100 of water) and the solution made faintly alkaline with caustic soda or potash there is produced a solution which in the raw state rapidly turns brown in the air, the change of color beginning on the upper surface of the liquid. In contact with the air this solution has been found to blue guaiacum and to oxidize hydroquinone with the production of quinone, and pyrogallic acid to purpurogallin. Thus with a solution of alkali alone a given quantity of pyrogallic acid gave 0.102 gram of purpurogallin, whereas under the same conditions the albuminous solution containing manganese gave 0.617 gram of purpurogallin. It was observed, further,

hat the oxidation of the phenols is accompanied by the evolution of arbon dioxide. With certain colloids the artificial oxidase resulting from admixture with the manganese salt could be precipitated with alcohol and the precipitate redissolved with water without losing its oxidizing powers. For the most part heat destroys the oxidizing power of such solutions. Thus when heated to 105° C. for twenty minutes the solution no longer oxidizes guaiacum nor certain diphenylmethane derivatives. The effect of heat is also seen in the several amounts of oxygen absorbed by pyrogallic acid under the influence of the fresh and heated solutions; thus:

	Absorption in cubic centimeters.		
1. With the fresh unboiled solution the following amounts of oxygen were absorbed	45.0	38.0	47.0
2. With the boiled solution	0.0	5.0	3.0

t is evident, therefore, that colloidal solutions of manganese hydroxide obtained by the action of caustic soda on manganese salts in the presence of albumin and similar colloidal substances exhibit properties closely resembling those of the natural oxidases.

Garrigou (180) has found certain metals to exist in a colloidal condition in mineral waters. According to this author, therefore, such substances possess the properties of natural oxidases, and to them he ascribes the beneficial results which attend the drinking of such waters and which are the result of more vigorous oxidations in the tissues. A. and L. Lumiére and Chevrotier (280) have confirmed the work of Trillat (436) on the oxidizing power of colloidal solutions of manganese and the general analogy of such solutions to the oxidases. According to these authors, colloidal solutions of any metal capable of existing in two or more states of oxidation should exhibit properties similar to those shown by the oxidases, the state of division of the colloidal substance also determining the oxidizing power of the substance. As a matter of fact, they found that emulsions of iron and cerium in albumin, gelatin, or gum, have the power to blue guaiacum, oxidize hydroquinon, pyrogallol, guaiacol, and para-phenylene diamin, but less actively than emulsions of manganese. These authors also ought to utilize these properties in destroying the bacterial poisons, since it is known that such poisons are destroyed by powerful oxidizing agents. As a matter of fact, they found that the death of an animal by tetanus toxin was greatly delayed by the administration of these artificial oxidases, and that with a slightly acid oxidase and tetanus toxin the animal did not show the slightest sign of intoxication.

Robin and Bordet (350) have also investigated the action of artificial oxidases in infectious diseases and have arrived at the conclusion that the colloidal metals, especially manganese, promote oxidation in the tissues and intensify metabolism.[a] They claim also to have confirmed the work of A. and L. Lumiére and Chevrotier (supra) on the curative action of such substances. Fouard (174) has observed that the halides of the alkalis and the alkaline earths exercise a catalytic action in the fixation of oxygen by the polyphenols, and that the chlorides of the rare earths act even more vigorously as oxygen carriers (175). In the title of the latter article, (175), he refers to these reactions as of the oxidase type, and yet there is nothing to indicate that the activity of these carriers is destroyed by heat, so that, to my mind, the analogy is only the product of a very active imagination.

Martinand (288) has also pointed out that the oxides of the alkalis and alkaline earths which form soluble peroxides and percarbonates, give reactions similar to organic peroxidases. He calls attention to certain analogies existing between the oxides of the alkalis and the alkaline earths and the organic oxidases on the one hand and to those existing between certain metallic salts and the peroxidases on the other. He is of the opinion that these inorganic oxidases can be considered as similar to the organic oxidases in that the former are made up of a peroxidase, which is a salt of the metal, and an oxygenase, which is the peroxide formed by the action of the air on the salt. Like the organic oxygenases, the inorganic oxygenase can be replaced by hydrogen peroxide. These analogies fall short, however, in that they fail to take into account the destructive action of heat on the oxidases and peroxidases. Wolff (455) has shown that certain mineral salts can play the part of peroxidases. Thus, if a trace of ferrous sulfate (less than one part per million) be added to an old tincture of guaiacum, or to a fresh tincture containing a trace of hydrogen peroxide, a very intense blue coloration is obtained. On heating the solution containing ferrous sulfate and hydrogen peroxide, it loses its power to blue guaiacum, and traces of mineral acids prevent the bluing of guaiacum by the ferrous sulfate and hydrogen peroxide just as they prevent the action of the peroxidases, the dosage required to inhibit the reaction being the same in both cases. According to this author these reactions closely resemble those produced with plant extracts containing peroxidase, such as extract of malt, etc., and he is of the opinion that special interest attaches to these observations in view of the fact that salts of iron, like the peroxidases, are very widely distributed among living things. He has shown (456) further that certain colloidal compounds of iron are practically identical with the peroxydiastases (a name recently proposed by

[a] In this connection see also Sée (398) and Schade (360).

Bertrand for the peroxidases). Thus with infinitesimal amounts of colloidal ferrocyanide of iron he claims to have been able to reproduce all of the reactions of the peroxidases. Thus it is filterable without loss of activity, its activity is weakened after one minute's boiling, and traces of mineral acids reduce its activity. It is also sensitive to an excess of hydrogen peroxide, as has been observed by Bach and Chodat ([27]) for the plant peroxidases. Other compounds of iron and cyanogen exhibit similar properties, although less active than the ferrocyanide. In another communication, entitled "Artificial Peroxydiastases," Wolff ([457]) points out that ferrous ferrocyanide acts in all respects like a natural enzyme. On the other hand, Wolff and Stoecklin ([460]) have found that while colloidal ferrous ferrocyanide acts like a peroxidase toward phenols, it fails to accelerate the oxidation of hydriodic acid by hydrogen peroxide. They conclude, therefore, that the catalytic action of vegetable peroxidases, such as extract of malt, on this reaction, is due to a special enzyme which may be eliminated by careful purification of the peroxidases. Continuing these investigations, Wolff ([458]) has shown that a feebly alkaline solution of ferrous ferrocyanide acts as an oxidase toward hydroquinone. He also points out that the alkalinity of the liquid plays a principal rôle in such oxidation phenomena. Thus the manganous salts employed by Bertrand ([56, 57, 58]) in his studies of the effect of manganous salts on the oxidation of hydroquinon, were always found to be alkaline toward alizarin sulfate, helianthin, and even in certain instances to turmerol. It was also shown by Wolff that the activity of neutral manganous salts, such as the sulfate is considerably increased by the addition of traces of pyridin, which does not precipitate manganese from its solutions.

Euler and Bolin ([168]) have also studied the oxidation of hydroquinone in alkaline solution under the influence of manganese salts. The relationship of the manganous salt and alkali is compared to that of the enzyme and co-enzyme. These authors have also shown that laccase has no action on hydroquinone in the absence of manganous salts, thereby confirming the work of Bertrand ([57, 58]). They showed further that laccase is not of the enzyme type, since solutions of it could be boiled for three minutes without destroying their activity. It was also found that salts of the hydroxy-acids, such as Rochelle salts, sodium citrate, calcium glucinate, and sodium mucate, accelerate the oxidation of hydroquinone in a marked manner when manganese salts are present, and these authors have suggested that laccase owes its activity to the presence of such salts.

According to Stoecklin ([413]), iron tannate can act as a peroxidase, causing the oxidation of guaiacol and tyrosin and the conversion of alcohol into aldehyde by hydrogen peroxide.

During the last two or three years Dony-Henault [143] and his co-workers have carried out a number of investigations on the subject of the oxidases. According to this author the oxidizing action of laccase is not really due to a specific enzyme but to the presence of manganese salts, the activity of which is stimulated by hydroxyl ions. He is inclined to the opinion that too much stress has been laid on the inhibition of catalytic processes by heat, as indicative of the action of enzymes. On the other hand he is of the opinion that in the presence of colloids, manganese salts would be decomposed in aqueous solution by heating to 100° C., and thereby lose their oxidizing power. Artificial laccase has been prepared by this author [144] by adding iron formate to blood serum and twice precipitating with alcohol. The substance thus obtained was found to show the reactions characteristic of natural laccase, but much less intensely. Still other artificial laccases were prepared by precipitating gum arabic with alcohol in the presence of manganous and other salts. A very active preparation was obtained by precipitating a solution containing 10 grams of gum arabic, 1 gram of manganese formate, and 0.4 gram of crystallized sodium carbonate in 50 c. c. of water, with alcohol. The precipitate was filtered off, redissolved in water, filtered, and reprecipitated. This second precipitate when washed and dried constituted the active, artificial laccase. The fact that artificial laccases can be obtained without resorting to the use of albuminous substances in these preparations, as was done by Trillat [436], indicates that Bertrand's idea that laccase contains an acid proteid radical is incorrect. Dony-Henault is of the opinion, therefore, that laccase as a distinct oxidizing enzyme does not exist. He explains the hardening and blackening of the juice of the lac-tree as due to the action of manganese in the presence of alkalis.

This author has also criticised all previous work on the oxidases on the ground that whereas in the study of the digestive ferments we have employed for the investigation the true substratum upon which they normally act, thus for diastase we use starch and for invertase, cane sugar, the action of the oxidases has been studied upon artificial or fictitious substrata, such as guaiacol, hydroquinone, etc. He therefore distinguishes between diastatic or enzymic action and catalytic action, the former being specific whereas the latter is variable or general, and he concludes that for the reason that the known oxidizing ferments act upon a number of oxidizable substances, they can not be true enzymes. He is of the opinion, therefore, that the belief in the existence of oxidases proper does not rest upon a sufficiently rigorous experimental basis. On the other hand it would seem preposterous to assume the existence of several dozen or a hundred distinct lipo-

lytic ferments in the liver for the reason that an aqueous extract of this organ can hydrolyze a large number of ethereal salts, and it would indeed be surprising to find that an oxidase, such as laccase, could oxidize but one substance, viz, laccol, and not other phenols, especially in view of the close analogy existing between this compound and the phenols as to constitution and properties. On the other hand certain of the oxidases are apparently as specific in their effects as any other ferment; thus tyrosinase has the power of bringing about the oxidation of tyrosin, whereas the latter is not acted upon by laccase. Effront (156) is of the opinion that the individuality of tyrosinase and laccase has been established definitely, so that conclusions of this kind are open to considerable objection.

Sjolleman (403) states that by adding a solution of Seignette salt to a solution of manganous sulfate or acetate, and then hydrogen peroxide and then sodium hydroxide, one obtains a dark brown solution of manganous oxide, which shows itself to be colloidal on dialysis and toward electrolytes, and which retains its dark brown color for several weeks. This solution has been found to give characteristic reactions for the oxidases, and to decompose hydrogen peroxide like potassium permanganate. The author calls attention to the occurrence of manganese in plants as of interest in this connection, and states that manganese probably plays an important rôle in the oxidations occurring in the soil. (*See* Schreiner and Reed (390).

PEROXIDASE ACCELERATORS AND AUXILIARY OXYGEN CARRIERS.

In his investigation of the ozonic reactions of certain mushrooms it was shown by Schoenbein (378) that guaiacum blue (believed by him to be an ozonid), which results from the action of the juices of such fungi on tincture of guaiacum, can in turn oxidize other oxidizable substances, as a result of which action the guaiacum blue is reduced and decolorized. It has been recently shown by Kastle (239) that peroxidase oxidations also are in many cases greatly accelerated by the action of certain phenols. Thus it was observed by Kastle and Porch (246) that the oxidation of guaiacum, paraphenylen diamin, and phenolphthalin by hydrogen peroxide under the influence of the peroxidase of milk is greatly accelerated by phenol, the cresols, and by beta-naphthol, and similar results have been obtained by Kastle (239) with peroxidases from a number of different sources. According to this author the peroxidase accelerators probably act in the capacity of auxiliary oxygen carriers and are themselves more or less completely oxidized in such processes. Thus if P represents the peroxidase, A the auxiliary oxygen carrier, and B the peroxidase reagent, that is, the substances whose oxidation is really under obser-

vation, the changes taking place might be represented graphically as follows:

(1) $$P + H_2O_2 \rightarrow PO_2;$$

and

(2) $$PO_2 + A = P + AO_2;$$

or

(3) $$2PO_2 + A = 2PO + AO_2,$$

and

(4) $$AO_2 + B = AO + BO,$$

and since, as has been shown by Bach and Chodat [27] the oxidases consist of an oxygenase (an organic peroxide) and a peroxidase, it is readily conceivable that oxidations accomplished by the oxidases might also be accelerated by certain oxidizable substances. Thus laccol itself might function as a peroxidase accelerator and in its oxidation by laccase it might accomplish the oxidation of other less readily oxidizable substances contained in the juice of the lac-tree.

CATALASES.

One of the most characteristic properties of hydrogen peroxide is the ease with which it is decomposed into water and oxygen. Thus in the earlier researches on this substance by Thénard [420, 421] it was observed that this decomposition can be effected by finely divided metals, by the oxides and peroxides of the heavy metals, and also by substances of animal origin such as fibrin. We have already seen that these observations were greatly extended by Schoenbein [383]. He proved that certain plant juices blued guaiacum directly; after standing for a short time, however, they lose this property but still retain the power to blue guaiacum in the presence of hydrogen peroxide, although this property is also lost after a time. Such extracts and juices were also found to have the power of decomposing hydrogen peroxide with the evolution of oxygen gas, and as a general thing he observed that extracts and tissues having the power to decompose hydrogen peroxide have also the power to blue guaiacum if hydrogen peroxide is present. Yeast and rennin, however, proved to be exceptional in their conduct in this regard. Both of these substances were found by Schoenbein to decompose hydrogen peroxide, yet neither had the power to blue guaiacum in the presence of hydrogen peroxide. According to Schoenbein the power to decompose hydrogen peroxide and to blue guaiacum containing small amounts of the peroxide were not specific properties of any particular class of substances but rather general properties of all soluble ferments, since the soluble ferments exhibit these changes and since the loss of its specific activity on the part of any particular soluble ferment by the action of heat or otherwise is attended with the loss of

its activities toward hydrogen peroxide and a mixture of this substance with guaiacum. As a matter of fact, he was led to regard the decomposition of hydrogen peroxide into water and oxygen as accomplished by these various agents as the prototype of all fermentation ([383]; page 335). These erroneous ideas prevailed for a number of years. Thus, according to Flügge ([172]) cited by Loew ([278]), all enzymes can decompose hydrogen peroxide, and in 1900 Babcock and Russell ([17]) attempted to measure the activity of galactase, the proteolytic ferment of milk, by the activity of an aqueous extract of the separator slime toward paraphenylen-diamin and hydrogen peroxide (von Storch's reagent).

Gradually, however, facts accumulated in the literature tending to show that the power to decompose hydrogen peroxide and to blue guaiacum by means thereof were not general properties of all soluble ferments, nor were the two properties necessarily correlated, but that they were specific properties of distinct sets of substances. Thus Bergengrün ([45]) in 1888 observed that the fibrin ferment does not decompose hydrogen peroxide. Similarly Jacobson ([223]) demonstrated that the property of certain soluble ferments to decompose hydrogen peroxide could be destroyed by heating to certain temperatures and by the action of acids and alkalis without in any way injuring the specific activity of the particular ferment. So also Raudnitz ([338]) from his studies on the oxidases of milk reached the conclusion that the substance in milk which decomposes hydrogen peroxide is essentially different from that which gives the guaiacum reaction. Lepinois ([265]) also pointed out as the result of his studies on the ferments decomposing hydrogen peroxide, that there is not always a parallelism between the quantities of oxygen liberated and the intensity of other reactions, such as the bluing of guaiacum, the reddening of guaiacol, etc. Finally, in the course of the examination of a number of samples of tobacco for oxidases, Loew ([278]) observed that aqueous extracts of certain of the samples of cured tobacco gave an abundant evolution of oxygen on the addition of hydrogen peroxide, without giving a blue color with guaiacum. He proved the absence of diastase and emulsin in these samples of tobacco, so that evidently the power of such extracts to actively decompose hydrogen peroxide is not due to either of these ferments. He showed further that active preparations of certain of the ferments, such as diastase, emulsin, and papayotin, from other sources, are without action on the peroxide, so that evidently the power to decompose hydrogen peroxide is not a general property of enzymes. He found further that certain liquids and extracts have the power of bluing guaiacum in the presence of hydrogen peroxide, which have not the slightest power of decomposing the peroxide into water and oxygen, and conversely, certain kinds of animal and vegetable matters have the power of vigorously decomposing hydrogen peroxide

without giving the peroxidase reaction with guaiacum. Thus it often happens that samples of cured tobacco have the power of decomposing hydrogen peroxide, ofttimes very actively, and yet have no power to blue guaiacum either alone or in the presence of hydrogen peroxide. On the other hand, an aqueous extract of the fresh green leaves of tobacco, containing one-fifth of its volume of absolute alcohol, showed both the oxidase and peroxidase reactions with guaiacum, whereas such extracts had no power to decompose hydrogen peroxide. It would seem, therefore, that the power to decompose hydrogen peroxide and the oxidase and peroxidase reactions ordinarily exhibited by plant and animal tissues and secretions are distinctly different properties. According to Loew ([278]), therefore, the decomposition of hydrogen peroxide by plant and animal tissues is due to a special enzyme, to which he gave the name "catalase." [a]

Still other observations point to the specific nature of catalase. Thus in 1902, Pozzi-Escot ([333]) observed that catalase did not give the guaiacum reaction nor oxidize hydroquinon. Somewhat later

[a] The name "catalase" (spelled by these authors "katalase"), together with the names "oxygenase" and "peroxidase," has recently been objected to by Moore and Whitley ([306]) as being ill chosen. According to these authors "there is no reliable evidence of this destruction of hydrogen peroxide being due to an enzyme at all." They point out that it is not specific, and that the decomposition can be accomplished by every ferment solution of whatever type, by nearly all animal and vegetable fluids, and by means of numberless inorganic catalysts. "In any case it is absurd," according to these authors, "to give it a name which belongs to or includes the whole vast range of catalytic actions." Every true enzyme is a catalase in the sense that it acts catalytically, and why a catalytic agent which happens to act upon hydrogen peroxide and which furthermore has never been shown to be a specific enzyme should be dignified by the name "catalase" is difficult to conceive. Every investigator in this field will no doubt appreciate the force of some of these objections. At the same time there are doubtless many who would be inclined to question the exclusion of catalase from the group of soluble ferments. The mere fact that innumerable substances decompose hydrogen peroxide has nothing to do with the question. One might as well object to our looking upon invertase as an enzyme for the reason that innumerable acids can hydrolyze cane sugar, and while many names in science are ofttimes nothing more than apt and striking catchwords, they have taken such a firm hold in the minds of those dealing with the subject that it is practically impossible to replace them. An instance of this kind is met with in the term "catalysis" itself, which in its original meaning simply begs the question as to the inherent causes of all phenomena of this kind. To say that an agent simply acts by its presence is merely emphasizing the most obvious phase of the whole phenomenon, and conveys no insight into the causes thereof, which after all are the things which we are chiefly concerned in discovering, and yet it is quite likely, from the present drift of things, that the term "catalysis" will remain long after contemporary chemists have ceased to have a voice in the shaping of chemical thought. If a committee of chemists, philologists, and advocates of the simple spelling were all to get together with the express purpose of introducing new names for the oxidases, peroxidases, and catalases it is more than likely that the result would be something far less euphonious and suggestive and of less real meaning than the names which these substances now bear.

Senter (400) isolated from the blood a catalase of remarkable activity, which he called "hemase," and which was without action upon guaiacum even in the presence of hydrogen peroxide. More recently L. Liebermann (269) has shown that an aqueous extract of the mesenteric fat of the hog and rabbit decomposes hydrogen peroxide energetically, without being able to oxidize guaiacum. According to Neumann-Wender (449) yeast, by heating to 45° to 50° C., loses its power to induce alcoholic fermentation, whereas the yeast catalase is only destroyed at 68° to 70° C. He also showed that yeast in which the maltase has been destroyed still retained its catalytic power. L. and P. Liebermann (270) have also shown that the catalase of malt is destroyed by heating extract of malt to 80° C., or by shaking it with mercuric oxide and magnesia, whereas the solution can still exhibit the guaiacum reaction, especially if oil of turpentine be added. Hence they conclude that only the peroxidase is concerned in the oxidation of the guaiacum. Pure hemoglobin is powerless to effect the decomposition of hydrogen peroxide, and yet it acts as a powerful oxygen carrier in a mixture of hydrogen peroxide and guaiacum. The general drift of modern opinion seems to be, therefore, that the catalases and peroxidases are distinctly different ferments.

So far as its occurrence is concerned, catalase seems to be one of the most widely distributed of any of the enzymes. Thus from his own observations Loew (278) arrived at the conclusion that there does not exist a group of organisms, or any organ, or even a single vegetable or animal cell, that does not contain some catalase.

According to Loew (278), vegetable catalase exists in two forms, one insoluble and the other soluble in water. These he designated "α-" and "β-catalase," respectively. According to him, α-catalase is probably a compound of β-catalase with a nucleo-proteid, while the β-catalase is an albumose, and can be liberated from the insoluble form (α-catalase) by means of a dilute alkali. He found that cured tobacco contains but little soluble catalase (β-catalase), whereas well-sweated tobacco contains a great deal. Water alone dissolves only traces of α-catalase, while the prolonged action of water at moderately high temperatures gradually splits off β-catalase, especially if a small amount of sodium carbonate be added. Whether the vegetable catalases obtained from different sources are identical in composition and nature, and whether they are identical with the animal catalases, is as yet unknown. By most observers the catalases are regarded as enzymes. They may be obtained in perfectly clear solution; thus Senter (400) found that the catalase of blood passes through the Berkefeld filter. They are destroyed by heat; Loew (278) found that vegetable catalase is destroyed at 80° C.; according to Senter (400), hemase is destroyed at 65° C.; Neumann Wender (449)

found yeast catalase to lose its activity at 70° C., although the dry yeast catalase could be heated to 100° C. without being destroyed.

The kinetics of the decomposition of hydrogen peroxide by catalase (hemase) and various inorganic catalysts, especially the colloidal metals (inorganic ferments), has been exhaustively studied by Senter [400] and also by Bredig [96, 97]. The decomposition of hydrogen peroxide both by catalase (hemase) and by the colloidal metals has been shown to conform to the law of an irreversible monomolecular process. With constant quantities of the catalyst the velocity of the decomposition has been found to be directly porportional to the concentration of the peroxide within certain limits. At small concentrations, however, both with the ferment and inorganic catalysts, the decomposition is proportionately slightly greater than with greater concentrations. In other words, the hydrogen peroxide itself, or some impurity which it contains, slightly inhibits the decomposition. With constant amounts of the hydrogen peroxide, especially at small concentrations, the decomposition has been found to be proportional to the quantities of catalase (hemase) or inorganic catalyst present. With more concentrated solutions of the catalase, however, both with the hemase and colloidal metal, the velocity of the reaction increases more rapidly than the increase in the concentration of the catalyst. A rise of temperature of 10° C. increases the velocity of the decomposition of hydrogen peroxide by platinum 1.7, and by hemase 1.5, whereas, according to van't Hoff [216] for most chemical processes the velocity is doubled by such an increase in temperature.

The power to decompose hydrogen peroxide as shown both by hemase and the inorganic catalysts is greatly inhibited by certain poisons and foreign substances, such as hydrocyanic acid, hydrogen sulfide, hydroxylamin, metallic nitrates, etc., at very great dilutions; and in this connection Loevenhart and Kastle [275] made the interesting observation that the activity of the catalase of hog liver was greatly inhibited by ammonium sulfocyanide, whereas it was rendered even more active by thiourea.

Since the recognition of catalase as a specific enzyme its occurrence and distribution in various animal and vegetable tissues has been investigated by a number of observers. We have seen that Spitzer [407] measured the catalytic power of various animal tissues toward hydrogen peroxide with the view of determining their relative oxidizing power. The various animal tissues have been found to vary greatly in catalytic power. Thus, according to Battelli and Stern [35] the liver contains the most and the brain the least amount of catalase of any of the tissues examined. They found, further, that the tissues of the guinea pig contain more catalase than those of the frog. Similarly Jolles and Oppenheim [229] found that the tissues of warm-

blooded animals have a greater catalytic activity than those of cold-blooded animals, such as the fish. In 1899 Carrière [112] examined a number of normal and pathological secretions for indirect oxidases (peroxidases and catalases). He obtained no evidence of catalase in the urine of 10 normal persons, nor in the urines of persons suffering from diabetes, hysteria, or chlorosis. On the other hand, in nephritis (Bright's disease), tuberculosis, and in acute inflammation of the lungs, the urine was found to decompose hydrogen peroxide. With other normal and pathological liquids his results are not sufficiently numerous to permit of any general conclusions.

The catalytic power of the blood toward hydrogen peroxide in normal and diseased conditions has also been investigated by Jolles [227,228] and later by Jolles and Oppenheim [229]. According to these observers the catalase of blood is contained exclusively in the formed elements, and is roughly proportional to the hemoglobin. They have determined the quantity of hydrogen peroxide in grams which is decomposed by 1 cubic centimeter of blood in normal and diseased conditions; this they have designated as the "catalase value." For normal blood this was found to be 23, whereas for blood in certain diseased conditions the catalase value was considerably less than for normal blood; thus in tuberculosis it ranged from 10 to 13, and in nephritis from 8 to 13, the most interesting and remarkable decrease occurring in carcinomatous conditions, in which condition the catalase values of the blood fall to 1.3 to 2.1. The blood of the two sexes and venous and arterial blood exhibited no differences in catalytic power.

Still more recently Winternitz and Meloy [454] have studied the occurrence of catalase in the human blood and tissues and its variation in certain diseases. These authors have reached the conclusion that there is no marked variation in the catalytic activity of human tissues due to the age of the individual. In this same connection, Mendel and Leavenworth [298] observed that catalase does not seem to be less abundant in very young embryos than in the adult. Winternitz and Meloy (supra) found the catalytic activity of the tissues to vary greatly in certain diseased conditions; thus in nephritis the activity fell off, especially in the kidney itself. In two cases of eclampsia the catalytic activity of the blood was not reduced. In pneumonia, the lung, in the stage of red hepatization, was found to have an increased catalytic activity, due in all probability to the increased number of red blood cells. In tuberculosis a decrease in the catalytic power of the various tissues was observed, but there was no reduction in diabetes mellitus nor in jaundice. The tissues in one case of congenital syphilis showed a marked lowering in catalytic activity, as did also the blood in one case of asphyxiation by illuminating gas.

THE FUNCTION AND NATURE OF CATALASE.

Very little is known regarding the function of catalase or of its mode of action on hydrogen peroxide. We have seen that according to Schoenbein's theory of oxygen-activation the inactive oxygen of the air is decomposed during processes of autoxidation, the negatively polarized atom uniting with molecular oxygen to form ozone and the positively polarized atom combining with water to form hydrogen peroxide (an antozonide), and, further, that through the action of certain carriers, such as finely divided platinum and the red coloring matter of the blood and what are now known as the peroxidases, the relatively inactive oxygen of the hydrogen peroxide is ozonized. This ozonized product can then accomplish the oxidation of easily oxidizable substances, such as guaiacum, etc., or it can react with another portion of the hydrogen peroxide, forming water and molecular oxygen after the manner of ozone itself, or of an ozonide, such as lead peroxide. In other words, the catalysis of hydrogen peroxide was of the nature of a secondary process in the sequence of changes occurring in the formation of ozone and ozonides as the result of the autoxidation of easily oxidizable substances. The occurrence of catalase in the organic world is, according to Loew, too general and widespread to be accidental, and hence the enzyme must have a certain significance. In considering its possible significance he pointed out that hydrogen peroxide results as either a primary or secondary product in the autoxidation of many readily oxidizable organic substances. Hence he regarded it as conceivable at least, that hydrogen peroxide might also be produced in the living cell as the result of the respiratory process. The accumulation of such a substance as hydrogen peroxide would undoubtedly prove harmful to the life of the organism, and hence he conceived the function of catalase to be to destroy the hydrogen peroxide as fast as formed. The ferment would thus afford an important protection against the accumulation of this poisonous substance, and the oxygen thus liberated could be again utilized for a continuance of the respiratory process. He also advanced the idea that just as catalase loosens the affinities of oxygen in hydrogen peroxide, so also it might loosen the affinities in certain other compounds, as the result of which they might be more easily decomposed or oxidized by the protoplasm.

Herlitzka ([209]) has found that within certain limits the greater the concentration of catalase the greater the concentration of peroxidase required to produce oxidation. This points to a protective action on the part of catalase against the peroxides of the organism. Shaffer ([401]) is also of the opinion that through the decomposition of hydrogen peroxide by catalase into water and molecular (inactive) oxygen, the tissues are protected against injurious oxidation. In

like manner Battelli and Stern [37] claim to have shown that catalase inhibits to some extent the oxidations produced by ferrous sulfate in the presence of animal tissues. Hence they, too, are of the opinion that the function of catalase may be to prevent the excessive oxidation of organic substances in the living cell. According to Jolles and Oppenheim [227, 229] the catalases of the blood eliminate oxygen from oxyhemoglobin, and this oxygen is then transferred to the tissues by the oxidases.

On the other hand the fact that catalase can not decompose either the substituted organic peroxides, such as ethyl hydroperoxide, or the oxygenases (Bach and Chodat,[29]), both of which are probably more powerful oxidizing agents than hydrogen peroxide, is difficult to reconcile with the view that the function of catalase is to protect the organism against excessive oxidation. As pointed out by Chodat [116], the only property of the catalases of which we have any certain knowledge at present is their power to decompose hydrogen peroxide into water and molecular oxygen. In this respect they differ from all other known catalysts. Thus all other catalysts of this kind can not only decompose hydrogen peroxide but they also have the power of rendering active the oxygen thereof. Thus finely divided platinum actively decomposes hydrogen peroxide. It also gives the guaiacum reaction both with hydrogen peroxide and with molecular oxygen. As shown by Kastle and Clarke [242] potassium iodide actively decomposes hydrogen peroxide at 100° C.; it was also found to greatly accelerate the oxidation of formic acid by hydrogen peroxide at 60° C., whereas potassium chloride neither actively decomposes hydrogen peroxide nor does it increase its oxidizing powers. If, therefore, the catalases are really exceptional in this regard, they certainly afford a very remarkable class of exceptions, and of such a nature, indeed, as to be altogether inexplicable at the present time. On the other hand the thought naturally suggests itself that they are not exceptional at all, but that they simply represent special cases under the general rule governing the conduct of such substances toward hydrogen peroxide. Thus it is readily conceivable that the catalases, like the peroxidases, combine with hydrogen peroxide to form an unstable holoxide derivative, thus:

$$K + H_2O_2 = H_2KO_2.$$

This might prove to be so unstable, however, that it would decompose in the sense of the equation—

$$H_2KO_2 = K + H_2O + O,$$

before it would have a chance to effect the oxidation of any oxidizable substance at hand; or in the event that oxidations occurred, it

is conceivable that the catalase itself, K, might be more readily oxidizable than guaiacum or any of the peroxidase reagents, in which event we would have—

$$H_2KO_2 = H_2O + KO.$$

This would explain the fact that while powerful catalysts, the catalases are not unlimited in their power to effect the decomposition of hydrogen peroxide. In this connection it is of interest to note that Senter ([400]) found blood-catalase (hemase) to be oxidized at all temperatures above 0° C. It is easier, however, and more in harmony with what we know regarding the conduct of other catalysts, to suppose that both of these changes would occur simultaneously, viz,

$$H_2KO_2 = K + H_2O + O,$$

and

$$H_2KO_2 + \text{Guaiacum} = K + H_2O + \text{Guaiacum-blue},$$

in which event the given substances would exhibit the properties of both a catalase and a peroxidase; and it may be after all, that when examined more closely, the catalases will show peroxidase reactions. As it is, the two sets of substances, if they are really distinct, are certainly found in the closest and most intimate association in both plant and animal tissues.

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LIST OF HYGIENIC LABORATORY BULLETINS OF THE PUBLIC HEALTH AND MARINE-HOSPITAL SERVICE.

The Hygienic Laboratory was established in New York, at the Marine Hospital on Staten Island, August, 1887. It was transferred to Washington, with quarters in the Butler Building, June 11, 1891, and a new laboratory building, located in Washington, was authorized by act of Congress, March 3, 1901.

The following *bulletins* [Bulls., Nos. 1–7, 1900 to 1902, Hyg. Lab., U. S. Mar.-Hosp. Serv., Wash.] have been issued:

*No. 1.—Preliminary note on the viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 2.—Formalin disinfection of baggage without apparatus. By M. J. Rosenau.

*No. 3.—Sulphur dioxid as a germicidal agent. By H. D. Geddings.

*No. 4.—Viability of the *Bacillus pestis*. By M. J. Rosenau.

No. 5.—An investigation of a pathogenic microbe (*B. typhi murium* Danyz) applied to the destruction of rats. By M. J. Rosenau.

*No. 6.—Disinfection against mosquitoes with formaldehyd and sulphur dioxid. By M. J. Rosenau.

No. 7.—Laboratory technique: Ring test for indol, by S. B. Grubbs and Edward Francis; Collodium sacs, by S. B. Grubbs and Edward Francis; Microphotography with simple apparatus, by H. B. Parker.

By act of Congress approved July 1, 1902, the name of the "United States Marine-Hospital Service" was changed to the "Public Health and Marine-Hospital Service of the United States," and three new divisions were added to the Hygienic Laboratory.

Since the change of name of the Service the bulletins of the Hygienic Laboratory have been continued in the same numerical order, as follows:

*No. 8.—Laboratory course in pathology and bacteriology. By M. J. Rosenau. (Revised edition, March, 1904.)

*No. 9.—Presence of tetanus in commercial gelatin. By John F. Anderson.

No. 10.—Report upon the prevalence and geographic distribution of hookworm disease (uncinariasis or anchylostomiasis) in the United States. By Ch. Wardell Stiles.

*No. 11.—An experimental investigation of *Trypanosoma lewisi*. By Edward Francis.

*No. 12.—The bacteriological impurities of vaccine virus; an experimental study. By M. J. Rosenau.

*No. 13.—A statistical study of the intestinal parasites of 500 white male patients at the United States Government Hospital for the Insane; by Philip E. Garrison, Brayton H. Ransom, and Earle C. Stevenson. A parasitic roundworm (*Agamomermis culicis* n. g., n. sp.) in American mosquitoes (*Culex sollicitans*); by Ch. Wardell Stiles. The type species of the cestode genus *Hymenolepis;* by Ch. Wardell Stiles.

No. 14.—Spotted fever (tick fever) of the Rocky Mountains; a new disease. By John F. Anderson.

No. 15.—Inefficiency of ferrous sulphate as an antiseptic and germicide. By Allan J. McLaughlin.

*No. 16.—The antiseptic and germicidal properties of glycerin. By M. J. Rosenau.

No. 17.—Illustrated key to the trematode parasites of man. By Ch. Wardell Stiles.

*No. 18.—An account of the tapeworms of the genus *Hymenolepis* parasitic in man, including reports of several new cases of the dwarf tapeworm (*H. nana*) in the United States. By Brayton H. Ransom.

*No. 19.—A method for inoculating animals with precise amounts. By M. J. Rosenau.

*No. 20.—A zoological investigation into the cause, transmission, and source of Rocky Mountain "spotted fever." By Ch. Wardell Stiles.

No. 21.—The immunity unit for standardizing diphtheria antitoxin (based on Ehrlich's normal serum). Official standard prepared under the act approved July 1, 1902. By M. J. Rosenau.

*No. 22.—Chloride of zinc as a deodorant, antiseptic, and germicide. By T. B. McClintic.

*No. 23.—Changes in the Pharmacopœia of the United States of America. Eighth Decennial Revision. By Reid Hunt and Murray Galt Motter.

No. 24.—The International Code of Zoological Nomenclature as applied to medicine. By Ch. Wardell Stiles.

No. 25.—Illustrated key to the cestode parasites of man. By Ch. Wardell Stiles.

No. 26.—On the stability of the oxidases and their conduct toward various reagents. The conduct of phenolphthalein in the animal organism. A test for saccharin, and a simple method of distinguishing between cumarin and vanillin. The toxicity of ozone and other oxidizing agents to lipase. The influence of chemical constitution on the lipolytic hydrolysis of ethereal salts. By J. H. Kastle.

No. 27.—The limitations of formaldehyde gas as a disinfectant with special reference to car sanitation. By Thomas B. McClintic.

*No. 28.—A statistical study of the prevalence of intestinal worms in man. By Ch. Wardell Stiles and Philip E. Garrison.

*No. 29.—A study of the cause of sudden death following the injection of horse serum. By M. J. Rosenau and John F. Anderson.

No. 30.—I. Maternal transmission of immunity to diphtheria toxine. II. Maternal transmission of immunity to diphtheria toxine and hypersusceptibility to horse serum in the same animal. By John F. Anderson.

No. 31.—Variations in the peroxidase activity of the blood in health and disease. By Joseph H. Kastle and Harold L. Amoss.

No. 32.—A stomach lesion in guinea pigs caused by diphtheria toxine and its bearing upon experimental gastric ulcer. By M. J. Rosenau and John F. Anderson.

No. 33.—Studies in experimental alcoholism. By Reid Hunt.

No. 34.—I. *Agamofilaria georgiana* n. sp., an apparently new roundworm parasite from the ankle of a negress. II. The zoological characters of the roundworm genus *Filaria* Mueller, 1787. III. Three new American cases of infection of man with horsehair worms (species Paragordius varius), with summary of all cases reported to date. By Ch. Wardell Stiles.

*No. 35.—Report on the origin and prevalence of typhoid fever in the District of Columbia. By M. J. Rosenau, L. L. Lumsden, and Joseph H. Kastle. (Including articles contributed by Ch. Wardell Stiles, Joseph Goldberger, and A. M. Stimson.)

No. 36.—Further studies upon hypersusceptibility and immunity. By M. J. Rosenau and John F. Anderson.

No. 37.—Index-catalogue of medical and veterinary zoology. Subjects: Trematoda and trematode diseases. By Ch. Wardell Stiles and Albert Hassall.

No. 38.—The influence of antitoxin upon post-diphtheritic paralysis. M. J. Rosenau and John F. Anderson.

No. 39.—The antiseptic and germicidal properties of solutions of formaldehyde and their action upon toxines. By John F. Anderson.

No. 40.—1. The occurrence of a proliferating cestode larva (*Sparganum proliferum*) in man in Florida, by Ch. Wardell Stiles. 2. A reexamination of the type specimen of *Filaria restiformis* Leidy, 1880=*Agamomermis restiformis*, by Ch. Wardell Stiles. 3. Observations on two new parasitic trematode worms: *Homalogaster philippinensis* n. sp., *Agamodistomum nanus* n. sp., by Ch. Wardell Stiles and Joseph Goldberger. 4. A reexamination of the original specimen of *Tænia saginata abietina* (Weinland, 1858), by Ch. Wardell Stiles and Joseph Goldberger.

*No. 41.—Milk and its relation to the public health. By various authors.

No. 42.—The thermal death points of pathogenic micro-organisms in milk. By M. J. Rosenau.

No. 43.—The standardization of tetanus antitoxin (an American unit established under authority of the act of July 1, 1902). By M. J. Rosenau and John F. Anderson.

No. 44.—Report No. 2 on the origin and prevalence of typhoid fever in the District of Columbia, 1907. By M. J. Rosenau, L. L. Lumsden, and Joseph H. Kastle.

No. 45.—Further studies upon anaphylaxis. By M. J. Rosenau and John F. Anderson.

No. 46.—*Hepatozoon perniciosum* (n. g. n. sp.); a haemogregarine pathogenic for white rats; with a description of the sexual cycle in the intermediate host, a mite (*Lelaps echidninus*). By W. W. Miller.

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No. 50.—Further studies upon the phenomenon of anaphylaxis. By M. J. Rosenau and John F. Anderson.

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