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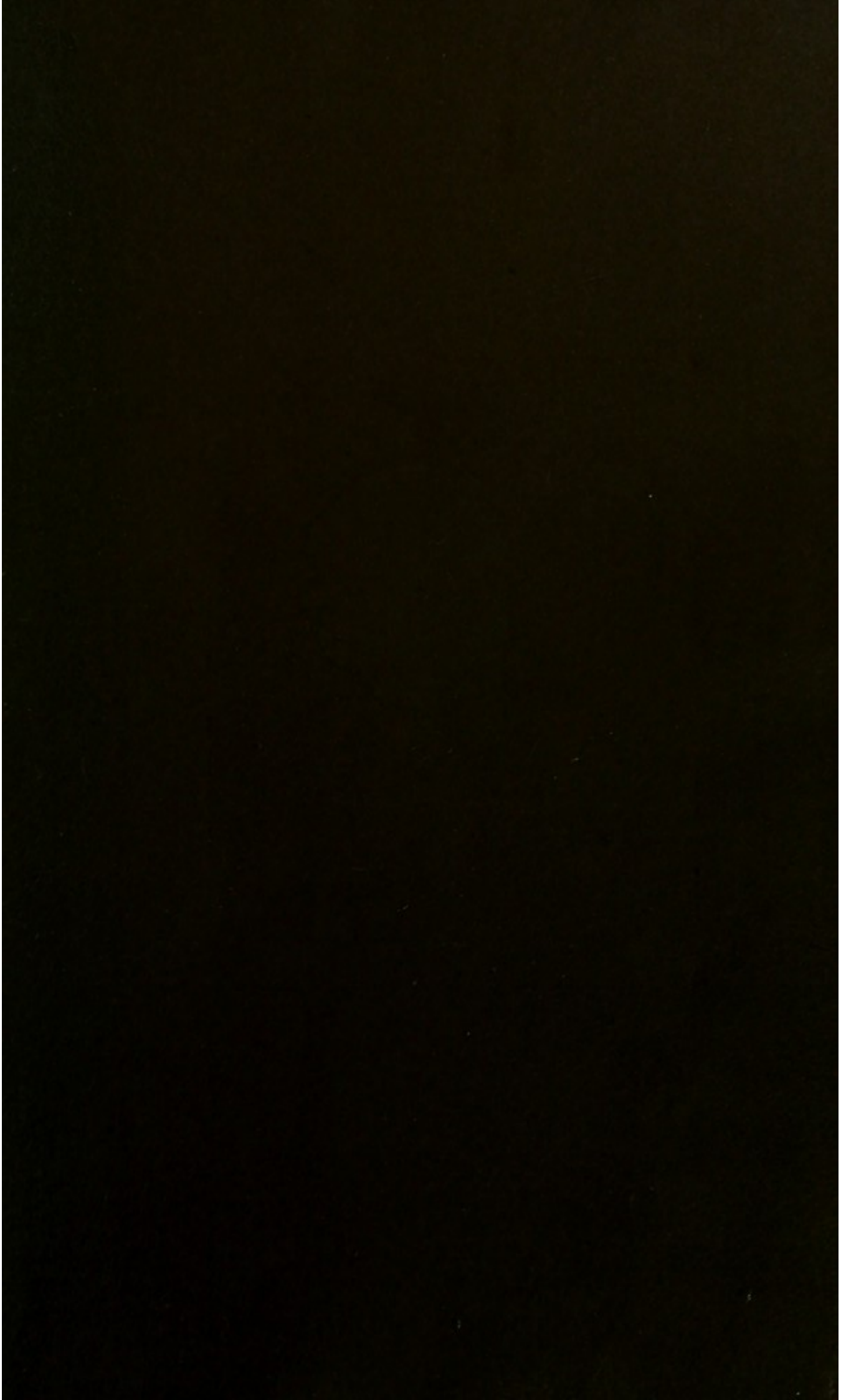

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ENZYMES

AND THEIR APPLICATIONS.

BY

DR. JEAN EFFRONT,

PROFESSOR IN THE NEW UNIVERSITY IN BRUSSELS AND
DIRECTOR OF THE FERMENTATION INSTITUTE.

ENGLISH TRANSLATION

BY

SAMUEL C. PRESCOTT, S.B.,

INSTRUCTOR IN INDUSTRIAL BIOLOGY,
MASSACHUSETTS INSTITUTE OF TECHNOLOGY, BOSTON.

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THE ENZYMES OF THE CARBOHYDRATES.

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AUTHOR'S PREFACE.

THE study of chemical ferments affords the double advantage of presenting a broad scientific interest and having at the same time numerous industrial applications. The phenomena of assimilation and respiration which take place in the interior of the living cell are in direct relation to the diastatic secretions, the study of which is consequently of as great importance to physiologists as to botanists and bacteriologists. A knowledge of the reactions caused by the diastases is also of first importance to chemists, for whom these physiological agents may become reagents of an exceptional sensitiveness. The science of chemical ferments comprises also the knowledge of certain microbial poisons, which, by their properties, are singularly allied to ordinary diastases. To study these poisons from the point of view of their diffusion, conservation, and destruction in the organism, one must also possess an accurate knowledge of enzymes. Finally, a whole class of soluble ferments have found, at the present time, industrial application, and undoubtedly the future will add many others; here, then, is a further interest which attaches to the study of enzymes.

The present work, which is a summary of the course given at the Institute of Fermentations of the new University of Brussels, is designed both for persons who give themselves up to purely scientific studies and for those who are occupied particularly in fermentation industries. So, while reserving the largest place for theoretical questions, we have

not neglected the practical results. Our work is divided into two parts. In the first, which constitutes the present volume, we deal with the enzymes of carbohydrates and with the oxidases, as well as with their industrial applications. In the second part, now in preparation, we shall study the proteolytic enzymes and the toxins.

We have personally verified the greater part of the experimental data which this first volume contains, in which the reader will find a certain number of hitherto unpublished experiments, methods of preparation, methods of analysis, and technical processes.

BRUSSELS, 1898.

TRANSLATOR'S NOTE.

UP to the present time very few works upon Enzymes have appeared in our language. In the translation of Professor Effront's book I have been actuated by the desire to make available in English the valuable material contained in the original French edition. I have endeavored to reproduce the author's ideas with clearness and simplicity, without at the same time deviating too much from his own form of expression. I have made no additions or changes, and the book is therefore presented in a form as nearly as possible like that in which it came from the pen of the author.

I wish to acknowledge my indebtedness to my friend, Mr. Percy G. Stiles of Johns Hopkins University, for much assistance in proof revision.

SAMUEL C. PRESCOTT.

MASSACHUSETTS INSTITUTE OF TECHNOLOGY,
December, 1901.

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THE ENZYMES AND THEIR APPLICATIONS.

CHAPTER I.

GENERAL REMARKS.

Synthetical and analytical work of the living cell.—Synchronism of the two phenomena.—Difference between the chemical work and the physiological work.—Chemical agents and physiological agents.—Intervention of vital energy.—Necessity of studying the physical and chemical conditions of the media.—Definition of enzymes.—Their part in assimilation and disassimilation.—Enzymes as producers of heat.

THE activity of living cells gives rise to a series of chemical reactions very complex and various. A limited observation of phenomena will show that, aside from purely synthetical activity, the cell always carries on analytical work: in other words, that the organic substance, in the presence of the living cell, is built up and broken down. The synthetical activity is more apparent in the transformation of substances which are not very complicated, which, submitted to the action of living cells, become hydrated, oxidized and transformed into complex chemical compounds. In the transformation of complex substances, on the contrary, it is the analytical work which predominates, and the complex substance is reduced to more and more simple bodies. For example, if one submits a sweetened must, containing nitrates or some ammoniacal salts, to the action of yeasts, one notices the appearance of new cells and consequently the formation

of protoplasmic substances, which are, from a chemical point of view, very complex substances.

When, on the contrary, one subjects albuminous matters to the action of certain ferments, they undergo a putrefactive fermentation, and it is seen that they pass through a series of transformations.

The albuminous matters change first into proteoses, then into peptones, into amides, and finally into ammonia, hydrogen sulphide, oxalic and carbonic acids.

In the first of the two examples which we have just given, we apparently see a work exclusively synthetic, characterized by the formation of protoplasm at the expense of sugars and nitrates. In the second example an activity absolutely opposed to the preceding one seems to be exhibited.

However, the phenomena are much more complex. In the first example the formation of new cells of yeast is accompanied by that of a protein matter, the protoplasm; but that substance does not persist in an unchangeable state: on the contrary, it is constantly destroyed, hydrated, and transformed by the living cells. When, therefore, new cells are formed, organized matter is decomposed. In the second example, the ferments which decompose the albuminoid matters and transform them into various products of cleavage, multiply, grow, and thus carry on an extensive synthetical process accompanying the process of decomposition.

We may conclude from these observations that the living cell works constantly in two different ways, analytically and synthetically, and one of these actions is more apparent than the other according to the particular case.

Since Wöhler accomplished the synthesis of urea, it has been possible to produce artificially a great number of organic substances. Emil Fischer has shown us the course to follow for carbohydrates, and it has been possible to reproduce artificially almost all the natural sugars which are found in plants, according to the methods which he has indicated.

While we do not actually know how to reproduce albu-

minoid matters synthetically, the works of Schützenberger have at least made known the manner of decomposition of all these substances, as well as their cleavage products.

Although these different works have made possible many syntheses, and have indicated the course to follow to accomplish others, one must admit that there is a great difference between the chemical work and the physiological work of cells.

In order to promote chemical reactions, very violent means are often employed in the laboratory. Strongly acid or alkaline solutions are used; pressure or high temperatures are employed. To produce, for example, the phenomena of oxidation, use is made of reagents such as nitric acid, chromic acid, or permanganates. As means of dehydrating are used concentrated sulphuric acid, anhydrous phosphoric acid, zinc chloride, etc., substances which destroy cells. In the case of living cells, on the contrary, the reactions occur in a medium either neutral or weakly acid or alkaline; the temperature is always very moderate and almost constant. The difference between these two conditions is striking. In the living cell bodies react which, according to our ideas, have only very feeble affinities; one observes at the same time that substances which we regard as very stable are readily decomposed in the interior of cells. The affinity of chemical bodies appears then stronger when they are in the presence of living matter, and seems to diminish when the cells are destroyed.

The increase of potential of the molecules in the interior of living cells is generally explained by the intervention of vital energy. The reactions, it is said, are produced more easily, owing to the intervention of a special force, the vital energy, which increases the interior energy as well as the aptitude for combinations and decompositions, as do electricity, magnetism, light, etc. The explanation of the phenomena by vital energy throws little enough light on the subject. In brief, it reduces itself to saying that reactions

are favored in living cells by physical and chemical conditions peculiar to the medium. This proposition cannot be considered as an explanation of intercellular phenomena. We cannot really understand them except by a thorough study of the media in which they are produced. A careful study of these conditions shows that the ready occurrence of all intercellular reactions is not to be ascribed to a single cause; that the affinity is sometimes favored by a purely physical condition and sometimes by a purely chemical one. Certainly, we are far from knowing all the conditions which favor intercellular reactions, but it has been possible to study some among them, and from the acquired knowledge, one may conclude that whenever one observes an increase of cellular energy, this is produced, not by a single condition common to all such phenomena, but by a cause strictly determinable and differing in different cases.

We know certain reactions in which chemical affinity increases because of purely physical conditions, such as osmosis, which continually occurs through cell membranes. In other cases we find that reactions are favored in the cells by the presence of mineral substances.

The decomposition of sodium chloride, for example, and the formation of hydrochloric acid in certain cells is one of those phenomena which do not accord at all with the general ideas we have of the stability of certain substances. We know, in fact, that sodium chloride is a very stable substance, and that its decomposition in the cold, in a medium slightly acidified by weak acids, is impossible. Thus the decomposition of sodium chloride in the cells was formerly explained by the intervention of vital energy, which was said to render the body less stable and more readily decomposed. To-day a more rational explanation is given to the phenomenon: we recognize that the decomposition of sodium chloride is caused simply by osmosis and independently of vital energy, because the salt, in a very dilute solution, is dissociated. In the cells an analogous phenomenon of dis-

sociation must be produced, and, by osmosis, the acid must pass through the cell membrane and accumulate in a certain quantity. Thus the acidity is seen to be a result of the dissociation of the very dilute salt solution and of its passage, by osmosis, through the cell membrane. This is a very striking example of a physical condition favoring reaction.

The most convincing example of the intervention of mineral substances is furnished by the results obtained in agriculture by the use of chemical fertilizers. When one places at the disposal of the cells relatively small quantities of phosphate, the quantity of protein matter produced in the plants increases considerably. The plant-cell needs, then, to carry on its synthetic work, the presence of mineral substances, which form organo-metallic combinations; combinations which, having more affinity than the organic substance uncombined with the mineral substance, enter more easily into reaction.

But we also know another extensive series of bio-chemical reactions which are produced without intervention of physical factors or mineral substances, and are due to the presence of chemical substances of a particular nature which we call enzymes. The study of these substances, of their manner of secretion, and of their method of action will form the object of the present work.

The enzymes, soluble ferments, zymases, or diastases are active organic substances, secreted by cells, and have the property, under certain conditions, of facilitating chemical reactions between certain bodies, without entering into the composition of the definite products which result. These substances play a very important part in the phenomena of assimilation and of disassimilation of foods. In fact, most of the foods which occur in nature at the disposition of men, lower animals, or plants, are not directly assimilable; they require the intervention of a diastase in order to be transformed into substances assimilable and suitable for the formation of new tissues.

Starch, which serves in the nutrition of almost all living creatures, is not directly assimilable, and in the higher organisms it undergoes various transformations before it can be absorbed. First of all, it encounters the enzymes in the saliva, then others in the pancreatic juice, and thus it is transformed into maltose and glucose, foods directly suitable for the construction of tissues. Meat, milk, and white of egg must also be transformed under the influence of the diastases before becoming assimilable. These substances find the enzymes which can act upon them in the gastric and pancreatic juices.

These phenomena which are observed in the higher organisms are also met with in the vegetable kingdom. During germination and florescence, the reserve substances, like starch, cellulose, fatty substances, and proteid matter, are in part consumed by the developing plant. But this utilization of reserve food is not done directly: these substances must be previously transformed by the diastases into assimilable products. Let us examine, for example the phenomenon of germination. A grain of barley left for ten or fifteen days in darkness loses thirty to forty per cent of its weight. If one determines the hydrogen and oxygen in the grain before and after germination, one finds that the loss of these two elements is in the ratio of one to eight. One may conclude from this that oxygen has combined with hydrogen to form water. On the other hand, if one determines the quantity of carbonic acid formed, it is found that it corresponds almost exactly to the quantity of carbon that has disappeared. There would be, then, combustion of carbon and formation of carbonic acid on one hand, formation of water on the other hand, and the phenomenon would appear to be a simple oxidation. If one analyzes the reactions more closely, it is seen that germination is not a phenomenon of simple oxidation, and that during its course there occurs a series of secondary reactions. First of all, there appear in the grain diastases which act on the starch and the cellulose in such a way that

little by little these two substances change their nature as well as their chemical composition. The cellulose is dissolved, the starch is transformed into maltose, part of which is oxidized, and part changed into cane-sugar by the tissue of the seed. All these transformations, as well as the oxidation itself, are produced by the diastases secreted during germination.

One can follow the course of most of these transformations; for example, the solution and the transformation of starch. For this purpose an embryo is separated from the grain and made to develop on a gelatinized must in which starch has been placed in suspension.

By observing the phenomenon very closely and by examining the starch under the microscope, one can see that the grain of starch loses its original form, that it is corroded in several places, that it then liquefies and disappears. In the culture liquid one finds substances which did not exist before: a sugar, and a nitrogenous substance, the diastase, which is soluble, capable of precipitation by alcohol, and can itself produce a transformation of starch.

In the assimilation of albuminoid matter by cells, there occurs a phenomenon quite analogous to the assimilation of carbohydrates. The albuminoid substances are gradually transformed by the active substances of the cells into proteids, peptones, and finally into amides.

We have said above that the diastases play an extremely important part in the phenomena of disassimilation. The molecules of albuminoid substances, hydrated, decomposed, and transformed by the enzymes, are regenerated in the presence of the protoplasm of the cells, by a process of dehydration and molecular condensation. The reconstructed molecules undergo new changes; they are again hydrated, decomposed, and at the same time gradually oxidized. In this phase of the transformation the albuminoid molecule is decomposed into urea, glycogen, fatty, substances, and amides. These transformations are also in great part due to the active substances secreted by the cells.

Finally, the enzymes are powerful producers of heat; the reactions caused by the diastases are exothermic reactions.

Thus a molecule of urea transformed into ammonium carbonate yields 8 calories. A molecule of glucose, in being transformed into carbonic acid and alcohol, sets free 71 calories. Tripalmitin, in splitting into fatty acid and glycerin, yields 30 calories. A gram of albuminoid matter transformed into urea furnishes 4.6 Calories.

It is seen that the rôle of the enzymes as producers of heat in living organisms is of considerable importance. This heat, set free by exothermic reactions, is then utilized by the cells for maintenance as well as for the construction of their new tissues.

When yeast is put in a solution of saccharose, there is first secreted a diastase which makes the medium assimilable, because the saccharose cannot be directly assimilated by the yeast. There is produced in the yeast a sucrase which transforms the sugar into invert-sugar, that is to say, into dextrose and levulose. The cell is then in a medium favorable for its development: it can utilize the nutritive substances and transform them into tissues, but this transformation involves an absorption of energy.

On the one hand then, the yeast has need of energy for the maintenance of its tissues; on the other, the heat set free by the transformation of cane-sugar into invert-sugar is not very considerable and entirely insufficient to produce the requisite energy. The yeast-cell, therefore, secretes a second diastase acting on the invert-sugar much more powerfully, and transforming it into alcohol and carbon dioxide. These two substances, the alcohol and the carbon dioxide, are not of use to the yeast; but the transformation which has produced them is an exothermic reaction which furnishes to the cell the energy which it needs for its maintenance.

An example, perhaps still more striking, is the transformation of urea into carbonate of ammonium by special ferments.

If these ferments are cultivated in a medium containing urea and peptones, it is found that the cells select the peptones as tissue-building materials; at the same time the urea is attacked and transformed into ammonium carbonate. The aim of this second transformation is to furnish the energy necessary to the cells for the construction and the maintenance of their tissues.

We observe again the same phenomenon in the vegetable kingdom. In the green parts of plants, under the influence of the sun's rays, the carbonic acid is constantly decomposed; formic aldehyde is produced, which is polymerized, and transformed into different carbohydrates. Through diffusion, these carbohydrates are distributed to different parts of the plants, where they undergo the action of diastases which hydrate them and decompose them. The carbohydrates are then sources of heat, which is set free by their decomposition. The products of decomposition or hydration are returned by diffusion to the green parts, where they can again become synthesized and consequently store up heat.

Thus are explained the migration, hydrations, and dehydrations of the carbohydrates which one observes in these plants.

We may conclude from all these facts that the diastases are substances absolutely indispensable to the life of organisms, for they make possible the construction of the cellular tissues, by rendering the materials assimilable and by furnishing the necessary energy.

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CHAPTER II.

GENERAL PROPERTIES.

History of the knowledge of enzymes.—Works of Réaumur and Spallanzani, Kirchoff, Dubrunfaut and Payen.—General properties of diastases.—Means of distinguishing a diastatic action from a purely chemical action.—Test by tincture of guaiacum.—Law of proportionality in diastatic action.—Means of distinguishing the work of organized ferments from diastatic action.—Means of isolating the diastase from the medium which contains it.—Chemical composition of enzymes.—Zymogenesis.—Method of action of diastases.

The first ideas concerning the existence, as well as the action, of enzymes belong to a very remote period. As early as the beginning of the sixteenth century the phenomena of digestion claimed much attention from students. Opinion on this subject was very much divided; some held that digestion was a purely mechanical work, a trituration of substances by the walls of the stomach; others, on the contrary, explained digestion as due to a dissolving and transforming activity of the juices of the stomach.

Réaumur and the Abbé Spallanzani upheld the second hypothesis and performed very conclusive experiments to confirm their theory. Réaumur, in order to take account of the influence of secretions of the stomach, caused hawks to swallow little metallic tubes pierced with holes and filled with meat, grain, or albumen. He examined the contents of the tubes after these had been cast out and found that the albuminoid substances alone were liquefied and transformed by the gastric juice, while the starchy substances had not undergone any change.

Abbé Spallanzani devoted himself to the study of the gas-

tric juice and its action. To procure active secretions he made birds of prey swallow little sponges attached to strings. He then drew back the sponges saturated with gastric juice. Spallanzani first succeeded in producing artificial digestion by placing meat in contact with the liquid squeezed from the sponges. He found that it became liquefied and changed.

These conclusive experiments which throw so much light on the phenomena of digestion, as well as on the rôle of the diastases, unfortunately were not appreciated at their true value. They did not succeed in convincing the scientific world, and at the beginning of the nineteenth century the phenomena of digestion were still interpreted in different ways. Certain scholars maintained that the gastric juice had no such constant character, and that the nature and properties of the secretion depended especially on the foods absorbed. These differences in the interpretation of the phenomena of digestion retarded the study of enzymes, although it was already greatly advanced by the works of Réaumur and Spallanzani. It was not until nearly two centuries after their publication that the question of active substances secreted by the cells again became prominent.

It is very curious to find that it is the study of brewing which has led to the greatest discoveries of this century. It was by the study of the beer-yeasts that Pasteur established definite arguments against the theory of spontaneous generation. It is also by the study of the raw materials of the brewery, notably malt, that Dubrunfaut laid the foundation for the study of enzymes.

The work of Dubrunfaut is connected with an observation made by Kirchoff in 1814. This distinguished scholar, who was the first to study the transformation of starch by acids, had noticed that fresh gluten can act under certain conditions on starch, dissolve it, and transform it into a saccharine substance. This experiment was taken up again by Dubrunfaut, who, in a long and masterly study, demonstrated that the activity of gluten is due to the presence of a

small quantity of active substance originating in the raw grain. He demonstrated that this diastase is soluble in water, that the ungerminated grain contains very little of it, and that germination develops it. He explained the mode of action of this substance and the conditions under which the maximum effect is produced; he proved finally that the sugar prepared from starch by the aid of this substance is not identical with the glucose which Kirchoff obtained by the action of the acid. It is in the works of Dubrunfaut, published in 1822, that we find for the first time a scientific study of the diastases as well as precise data concerning their mode of action.

Payen took up again the work of Dubrunfaut, from whom he unjustly withheld the credit for the discovery of the diastases of malt. In studying the properties of an infusion of malt, Payen recognized that the active substance can be precipitated from its solution by alcohol, and the precipitate thus obtained exhibits all the properties which the liquid itself possessed. This experiment has played a considerable part in the discovery of enzymes, because Payen had thus found, at once, a general property of diastases and a general means of isolating them.

By this method have been isolated the active substances of the gastric juice and of the pancreatic juice, as well as the enzymes acting on fatty substances and glucosides.

GENERAL PROPERTIES OF ENZYMES.

We have just seen that the enzymes are precipitated from their solutions by alcohol, but it is necessary to add that, while all the enzymes are precipitated by very concentrated alcohol, they are more or less soluble in dilute alcohol. Since the discoveries of Payen, there have been recognized a certain number of other properties more or less characteristic of enzymes. Enzymes are soluble in water, are thrown down from their solutions by indifferent precipitates, become

fixed on different substances as silk and fibrin, and are somewhat resistant to poisonous substancès. Enzymes lose their activity at a temperature in the neighborhood of 100°. The greater part of them decompose hydrogen peroxide. They are also characterized by the fact that, under given conditions, the action which they produce is proportional to their quantity. All these properties are, however, far from being distinctive; many other substances than diastases possess one or another of them. The most characteristic property of an enzyme is its special mode of operation.

Let us consider each of the properties which we have just cited. We have seen, first of all, that enzymes are precipitated by alcohol; as they are more or less soluble in dilute alcohol, the quantity of alcohol which is necessary to precipitate the enzymes from an aqueous solution will not always be the same. For certain diastases, the fibrin ferment for example, it will be sufficient to put in the solution 10 to 15 per cent of alcohol, which constitutes a minimum proportion. For others, as the coagulative ferment of milk, it is necessary to add a large proportion of alcohol, in order to have a liquid containing 80 to 90 per cent.

But if all the enzymes are precipitated by alcohol, they are likewise all destroyed by this same agent. By a prolonged contact of the diastase with the alcohol, the active substance is transformed, becomes insoluble and inactive. So, if one precipitates an enzyme by concentrated alcohol, the action must be stopped as quickly as possible.

Enzymes, from the point of view of solubility in water, present noteworthy differences. We know of diastases which dissolve very easily and of others, on the contrary, which require for their solution a large quantity of water. Moreover, remembering that active substances are fixed with ease on different bodies, it is easy to understand that the same substance can be presented in soluble or insoluble form.

The precipitation of enzymes by "dragging down" (mechanical precipitation) can be easily effected. One adds

to a filtered and clear infusion of malt a very dilute solution of sodium phosphate, then a solution of a calcium salt; there is produced in the liquid a precipitate of calcium phosphate which finally settles at the bottom of the vessel; the clear liquid is decanted, the precipitate placed on a filter, washed with a little water, and a powder is obtained which possesses all the properties of the infusion of malt. This powder, for instance, acts on starch and produces maltose, as does the malt from which it has been extracted. This method makes it possible to obtain all the enzymes contained in an infusion. Only one condition is necessary,—that the substances employed for the precipitation shall be harmless to the diastase. Good results are obtained, for instance, in making use of magnesium carbonate or aluminium hydrate.

We have seen that the diastases become fixed on different substances. Thus a piece of fibrin placed in a solution of gastric juice becomes impregnated with the active substance in such a way that the diastase cannot any longer be removed by washing.

If, after having withdrawn this fibrin from the infusion and washed it to remove as far as possible every trace of active substance, it is placed in water at a suitable temperature, the piece of fibrin dissolves. It is evident that this transformation of the protein matter is due to the fact that the active substance is fixed on the fibrin like a dye. It is not moreover necessary for the diastase to act on a substance to become fixed on it. For example, if one places some pieces of silk in gastric juice, they are impregnated with active substance, although the diastase does not act at all on the silk.

Most diastases are insensible to the action of certain substances, such as hydrocyanic acid and chloroform, which paralyze the vital activity of cells. If, for example, yeast is put in a solution of cane-sugar in the presence of chloroform, the yeast remains quiescent and does not reproduce; however, the cane-sugar is still transformed into invert-sugar. The diastase continues, then, to be secreted by the cells and

to do chemical work, while the cellular activity, properly speaking, is paralyzed by the chloroform.

From these experiments it appears that enzymes are not sensitive, either to the action of antiseptics, or to that of substances which are opposed to the vital action. But the rule is not universal. We know, in fact, of several enzymes which are extremely sensitive to chloroform, ether, and thymol, as well as to hydrocyanic acid.

In reality, the various diastases differ considerably among themselves as to their nature, and as to their sensitiveness towards the different reagents.

The diastases of malt as well as the active substances of the yeasts transforming the cane-sugar into invert-sugar, are very resistant enzymes, and much less sensitive than the cells which elaborate them.

These same diastases sometimes occur in other more resistant living cells, because there exists among cells, as among these diastases, considerable differences in sensitiveness to reagents. There are, then, cases where antiseptics attack enzymes before acting on the cells, and other cases where the reverse is the case. The yeast which we have just cited furnishes an example of the relative sensitiveness of diastases to antiseptics. As a matter of fact it is known that beer-yeast contains, in addition to the diastase changing cane-sugar into invert-sugar, a second enzyme which transforms the invert-sugar into alcohol. The absence of fermentation in presence of chloroform proves that, of the two diastases contained in the yeast, one is destroyed by the antiseptic while the other resists it.

The greater or less sensitiveness of diastases to the action of antiseptics, and to those substances paralyzing the vital activity, can be utilized to exclude the activity of micro-organisms during diastatic action.

When one studies, for example, the saccharification of starch or the transformation of meats by enzymes, one may often be led into error by the invasion of organisms which

produce the same effect as the diastase whose action is being studied. In this case, a small amount of antiseptic, for example, a few drops of chloroform, is put in the liquid which is kept under observation, and interference from ferments is prevented. Only, in the case of certain enzymes more sensitive than others, it is necessary to use other means for preventing the action of organized ferments, because the enzyme itself would be destroyed by the antiseptic. This precaution is especially necessary when one is studying the action of enzymes yet unknown; in this case a negative result may be due to the presence of an antiseptic.

The action of heat on the enzymes is an extremely important point and one which, better than any other, may serve to characterize a diastatic action. In general, with a certain number of exceptions, enzymes exert their action slowly at a temperature of 0°; often at this temperature the effect they produce is imperceptible. If one gradually increases the temperature to 40° the reaction is intensified; from 40° to 50° there is a marked increase in intensity,—it is generally at this temperature that the diastase attains its maximum activity,—above 50° the activity diminishes; at 80° a considerable weakening is produced, and finally, above 90° the diastase is wholly destroyed. The different diastases are characterized by their optimum temperatures, that is, by the temperature at which they give their maximum of action. This temperature varies quite considerably in different enzymes, and this variation constitutes a property which admits of differentiation.

But the property of enzymes which is most useful in studying them, is the facility with which they are destroyed from 90° to 100° in the presence of water. Some diastases, when they are in a completely dry state, can stand a temperature of 90° and even more; but all enzymes, without exception, lose their activity when their aqueous solution is brought to the neighborhood of 100°. This property is utilized for distinguishing diastatic from purely chemical action.

When one puts an infusion of yeast in a solution of saccharose, the transformation of saccharose into invert-sugar takes place. But it is not to be inferred that this is necessarily diastatic action, for the transformation may be due, either to the acidity of the must, or to some other chemical agent.

To prove that there exists in a yeast an active substance, a double experiment is necessary. One must treat equal quantities of sugar equally diluted, during the same length of time and at the same temperature, on the one hand with a certain quantity of infusion of yeast, and on the other hand with an equal quantity of the same infusion, which has been previously heated to 100° for several minutes, and then cooled again.

If the same result is obtained in the two experiments, one may conclude that the transformation is not due to an active substance contained in the infusion under examination. On the contrary, the activity of a diastase becomes evident, if in the experiment with the heated infusion inversion is not obtained, while from the action of the infusion not heated a transformation is observed.

The fact that diastases are destroyed at 100° relates them in a striking manner to living organic matter.

We have said above that when an enzyme is put in solution in the presence of hydrogen peroxide, the latter is decomposed. To demonstrate this reaction, one makes use of an alcoholic solution of guaiacum. Generally 2 or 3 cubic centimetres of tincture of guaiacum are taken; a few drops of hydrogen peroxide are added and then, drop by drop, the liquid in which an enzyme is supposed to exist. In the presence of an enzyme the red liquid becomes a very intense blue.

This coloring is due to the transformation of the guaiaconic acids into guaiacosonide, a dye. The reaction with tincture of guaiacum is extremely sensitive: one can produce it with exceedingly small quantities of active substance.

It must not be forgotten that tincture of guaiacum loses

in time the property of giving color, and that the best way is to make the tincture before the experiment by grinding powder of guaiacum with alcohol; moreover, the use of this reagent always offers certain difficulties: it must be noted that the coloring matter formed is not very stable, that it is decomposed by heat as well as by different chemical reagents. A slight alkalinity or even a slight acidity is sufficient to prevent the production of coloration, and it follows that it is necessary to take some precautions when using this reagent. It is well first to neutralize exactly the hydrogen peroxide used, for this is generally very acid; it is also well to measure the degree of acidity or alkalinity of the liquid containing the diastase to be studied, then to neutralize it when it is clearly alkaline or clearly acid.

The coloration produced by guaiaconic acid is not destroyed by acetic acid and it is often of advantage, when working with an absolutely neutral solution, to acidify it with a drop of dilute acetic acid.

The reaction of the tincture of guaiacum is of much use in the investigation of enzymes. However, this reagent is not absolutely reliable, for the coloration observed in a solution may be due to other bodies than enzymes. Moreover, if a reaction is not obtained, one must not conclude that the liquid does not contain an active substance, for the coloration may be prevented by different substances which may be present with the enzymes in the liquid tested.

Moreover, enzymes are known which do not give coloration with guaiacum and, on the other hand, diastases which, after having been submitted to certain influences, lose this property without always losing their activity. Thus, at a high temperature, certain enzymes no longer give coloration with guaiacum, although the active substance is not destroyed. With other diastases the property of coloring tincture of guaiacum disappears in a prolonged contact with hydrogen peroxide, contact which has no influence on the activity of the diastase. However, no enzymes are known

which, after having lost their activity by the action of chemical or physical agents, still give a coloration with tincture of guaiacum.

It follows that, to use the reaction of guaiacum, one must, as with experiments on the effect of heat, make double experiments with the fresh infusion on the one hand, and with the infusion heated to 100° on the other hand. When the fresh infusion produces a coloration and the infusion after being heated does not, one may be confident of the presence of a diastase.

Further, tincture of guaiacum gives a blue coloration, with a whole group of diastases, without hydrogen peroxide. In this case, the guaiacum justifies not only the inference that a diastase is present in a solution, but gives further information; the reaction of guaiacum without hydrogen peroxide being possible only with an oxidizing enzyme.

Tincture of guaiacum can also be of great service when plant enzymes are being studied.

It often happens that diastases contained in vegetable cells are changed or destroyed as the result of maceration in water, on account of the dissolving out, from the cells, of extractive substances which destroy the enzymes. In this case one must look for the diastases, not in the solution, but in the cells themselves.

For this, very small sections are made which are introduced either in the pure tincture of guaiacum or in a solution of guaiacum added to hydrogen peroxide. The cells containing the active substance are colored blue.

It is often very difficult to distinguish a diastatic from a strictly cellular action. If it is noticed that a certain liquid is capable of producing chemical changes in certain substances, one is led to think that an enzyme is present if the same liquid after boiling has not the same power. But in reality there is nothing to prove in this case that the action observed is really a strict diastatic phenomenon, for certain organized ferments may have caused it.

To determine exactly whether organized or soluble ferments are present, recourse may be had in certain cases to a filtration by means of a porous filter which is capable of retaining the organisms. If the filtered liquid is still active, one may conclude that the transformations noticed are really diastatic phenomena. But the contrary does not prove the absence of an enzyme in the solution, for all enzymes are more or less retained by the porous substance of the filter, and certain among them do not pass through at all.

It is in the proportionality which exists between the quantity of diastases employed and the quantity of substance transformed by these diastases that a certain proof is found of the existence of a diastase. The law of proportionality is not, however, an absolute law. With an infinitely small quantity of enzyme one can transform a very considerable quantity of substance on condition that the action is allowed to be continued for a long time under such conditions that the enzyme is not destroyed by the physical and chemical agents of the medium. However, at the beginning of the action, especially if one employs a very small quantity of active substance and a large quantity of passive substance, one notes a fixed ratio between the quantities of enzyme employed and of substance transformed.

It is under these conditions only that the law of proportionality can be verified. If one adds, for example, to 100 cubic centimetres of a ten per cent sugar solution a slight quantity of sucrase, for example a cubic centimetre of an infusion of yeast, and if the action is stopped after one hour, it is found that a part of the sugar has been transformed. If in a similar liquid under the same conditions of dilution and temperature, $\frac{1}{2}$ cubic centimetre of the same solution of sucrase be added, one finds that the quantity of sugar inverted is very nearly half of the quantity transformed in the preceding experiment.

If instead of diastases one employs organized ferments capable of effecting the same transformation, one never ob-

serves a proportionality between the quantity employed and the result obtained. A double quantity of organized ferments does not transform twice as much sugar. There is evidently in the second case a larger quantity of sugar inverted, but this quantity is not double. The proportionality between the quantities of diastase employed and of substance transformed is of great use, especially when one suspects the presence of organized ferments in an active liquid.

Chemical Composition of Enzymes.—Now that we know the means of recognizing the presence of diastases in a liquid, let us study closely the chemical composition of enzymes.

The elementary analysis of enzymes gives discordant figures for the different known kinds, and sometimes even for the same diastase different authors have found very different results. This fact may arise because the materials submitted to analysis are never pure, but mixtures of different substances. It may be also that the enzymes really differ in their composition, and this should not surprise us since they are bodies which act in various ways and upon very different substances. The composition of some enzymes is here given:

	Carbon.	Hydrogen	Nitrogen.	Sulphur.	Ash.	Experimenters.
Diastase from malt	45.68	6.9	4.57	0	6.08	Krauch.
	47.57	6.49	5.14	.....	3.16	Zulkowski.
	46.66	7.35	10.41	.....	4.79	Lintner.
	.....	.....	16.53	.....	.....	Wroblewski.
Ptyalin	43.1	7.8	11.86	.....	6.1	Hüfner.
Invertin	.....	.....	4.30	.....	.....	Mayer.
	43.90	8.4	6.	0.63	.....	Brauth.
	40.50	6.9	9.30	.....	.....	Donath.
Emulsin	43.06	7.2	11.52	1.25	.....	Brucklau.
	48.80	7.1	14.20	1.3	.....	Schmid.
Pancreatin	43.6	6.5	13.81	0.88	7.04	Hüfner.
Trypsin	52.75	7.5	16.55	.....	17.7	Loenid.
Pepsin	53.2	6.7	17.8	.....	.....	Schmid.
	Albuminoid Substances.					
White of egg uncoagulated	53.7	7.1	15.8	1.8	.....	Dumas.

By examining the percentage of nitrogen of the several enzymes whose composition we give in the table above, we notice that certain diastases, as pepsin, contain great quantities, and approach albuminoid substances in composition. We see, on the contrary, that other enzymes as invertin have a much smaller nitrogen content. In the group of the oxidases there are also enzymes, quite recently discovered, which appear to be absolutely lacking in nitrogen. These latter substances are analogous rather to the gums.

We have just said that the non-agreement of results may be due to impurity of substances analyzed. In reality, the methods which are followed for separating the enzymes from the media which contain them cannot furnish pure substances.

Usually the diastase is obtained from the cells by extracting with water and then precipitating with alcohol the infusion obtained. In liquids which have been in the presence of protoplasmic substances, there is always a large quantity of matter which can be precipitated by alcohol, and the results obtained are of necessity mixtures of different bodies. When it is proposed to purify the precipitates by dissolving them and reprecipitating, one obtains many bodies of a stable composition, but almost entirely destitute of all active power.

In enzymes one always finds a great quantity of inorganic salts, particularly calcium phosphate, in very varied proportions. If the method of mechanical precipitation is employed to isolate the diastase, the result is the same: after the precipitation bodies containing many impurities are found. Furthermore, in precipitating a diastase in an active liquid, one always runs the risk of obtaining a mixture of different diastases and not one alone. It then becomes absolutely impossible to separate them from each other, because their insolubility in alcohol is not such that they can be separated by precipitation. Thus, when barley-malt is steeped in water, there is obtained in the liquid a whole series

of active substances which are precipitated together by alcohol or by other substances which can drag them down.

The diastases which, according to the analyses, most nearly approach proteids in composition, still differ considerably from these substances.

Enzymes do not give all the color reactions of proteids. Bodies of this class cannot diffuse through a parchment membrane, while diastases are capable of doing it, although with some difficulty. Diastases act differently from proteids. These latter bodies can be assimilated by cells, while the diastases cannot be. The salivary and pancreatic enzymes never serve as reserve substances. Though stored in the cells during the period of normal nutrition, these substances are rejected in time of starvation. According to Beijerinck, amylase cannot replace in a nutritive medium either carbohydrates or nitrogenous substances, the yeasts and the bacteria absolutely refusing to be nourished by it.

Zymogenesis.—Enzymes are produced by certain special cells. According to Hüfner they are formed by the oxidation of albuminoid substances. This theory is attacked by Wroblewsky, who considers diastases as proteoses.

There are as yet very few data on the manner of formation of enzymes. In most cases one can only observe their presence when they have acquired all their properties; in some isolated instances the presence of a non-active substance capable of becoming a ferment by suitable treatment has been observed.

Thus the gastric mucous membrane, when soaked in water, yields a liquid which does not coagulate milk; but this liquid acquires that property when one adds 1 per cent of hydrochloric acid, and preserves its activity even after neutralization. Fresh pancreatic tissue yields in water a substance acting very slowly in the presence of a slight quantity of acid. The activity of this liquid can be accelerated by passing through it a current of oxygen, or by introducing into it hydrogen peroxide.

These substances, capable of becoming active, are called zymogens (proferments, proenzymases), and the transformation of the zymogen into the ferment is called according to Arthus: zymogenesis. It is very probable that most enzymes come from zymogens, and that the phenomena of zymogenesis are as frequent as the phenomena of destruction of the diastase, called zymolysis.

Manner of Action of Diastases.—The chemical analysis of an enzyme is not sufficient to characterize it. To determine exactly the characteristics belonging to a diastase, one must observe its manner of action, the chemical change it can produce, and especially the substances on which it can act.

Diastases can induce, according to their nature, very different chemical reactions. Some have a hydrating action, that is to say, they can cause one or more molecules of water to unite with the substances on which they act. We can cite for example the transformation of saccharose into dextrose and levulose.

$$\underset{\text{Saccharose.}}{C_{12}H_{22}O_{10}} + \underset{\text{Water.}}{H_2O} = \underset{\text{Dextrose.}}{C_6H_{12}O_6} + \underset{\text{Levulose.}}{C_6H_{12}O_6}.$$

Another series of enzymes acts on the contrary like oxidizing agents. An example of this class is the transformation of hydroquinone into quinone.

$$C_6H_4\begin{matrix}OH\\OH\end{matrix} + O = H_2O + C_6H_4\begin{matrix}O\\|\\O\end{matrix}.$$

Finally, other enzymes act only on the molecules by decomposing them without producing hydration or oxidation, only causing a molecular change in the substance. It is thus that the diastase of yeast which produces alcoholic fermentation gives rise to a simple molecular decomposition without hydration.

$$\underset{\text{Glucose.}}{C_6H_{12}O_6} = 2\underset{\text{Carbonic anhydride.}}{CO_2} + 2\underset{\text{Alcohol.}}{C_2H_5OH}.$$

Thus, in the example which we have cited of the transformation of saccharose into glucose and levulose, the mole-

cule of cane-sugar is found to be hydrated and decomposed.

This decomposition of the molecules with hydration also takes place in the transformation of glucosides by diastases. The complex molecule of the glucosides, becoming hydrated, divides into two parts, yielding glucose and the body with which it was combined.

One observes the same phenomenon in the action of diastases on fatty matters. The diastases acting on the proteid substances also produce a decomposition and simultaneously a hydration, although in this case it would be difficult to demonstrate the reaction.

The molecules of albuminoid substances are excessively complex; it is generally assumed that they have a molecular weight of about 5500, and as certain products of cleavage have molecular weights of 2800, of 1400, and of 400, it can be seen that diastatic action causes a diminution of molecular weight.

Enzymes hydrating or decomposing the molecules may give rise to two different substances, as saccharose yields dextrose and levulose.

In the inversion of lactose, one observes the same phenomenon; the two portions into which the molecules are decomposed are different, dextrose and galactose.

There are also cases of cleavage in which two molecules of identical chemical configuration are produced. Thus the diastase found by Cusenier, glucase, acts on maltose, giving two molecules of dextrose.

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CHAPTER III.

MANNER OF ACTION OF DIASTASES.

Manner of action of diastases.—Different opinions on this subject.—The diastatic property and the diastase itself.—Works of Bunzen, Hüfner, Naegeli, Wittich and Fick, de Jäger, Arthus.—Analogy between organized ferments and soluble ferments.—Hypothesis of Armand Gautier on the nature of enzymes.

We have seen above that enzymes can produce, according to their nature, a molecular change, a hydration or an oxidation. We have also seen that diastatic actions are characterized by the disproportion existing between the results produced and the weight of the active substance. This disproportion between cause and effect proves that the active substances do not enter into the definite composition of the products whose formation they induce. Enzymes appear to us to play in these transformations the part of intermediaries, provided with the property of increasing the interior energy of the substances on which they act and rendering them more liable to decompositions or combinations.

Berzelius has compared diastatic reactions to the phenomena called catalytic phenomena which were formerly explained simply by the effect of contact or the presence of a body. This investigator had noticed an analogy between the action produced by an enzyme and the decomposition of hydrogen peroxide by spongy platinum.

The comparison of Berzelius is not a happy one. Hydrogen peroxide is a very unstable substance, and porous bodies, as powdered charcoal, and metals in finely divided condition, cause the decomposition of certain bodies on account of their

extreme porosity. Now, it is evident that enzymes do not act in this way, and the relation imagined by Berzelius is still more strange in that there is on one hand a reaction between a liquid and a solid, and on the other an action which takes place exclusively between bodies in solution. But if the example cited by Berzelius is ill-chosen, it must still be acknowledged that enzymes appear, at first sight, to act by simple contract, and that, in fact, there exists a striking analogy between catalytic reactions and diastatic actions. In the two cases one finds, at the end of the reaction, the body acting in the same state as at the beginning, and one observes that the quantity of excitant allowed to act has no influence on the results obtained. There is known in inorganic and in organic chemistry a whole series of reactions of that nature: the decomposition of calcium hypochlorite by cobaltic oxide, of hydrogen peroxide by potassium bichromate, the combination of benzene with methyl chloride in presence of aluminium chloride, etc. In all these, the acting substance remains at the end of the reaction. It is phenomena of this kind that were formerly considered as catalytic reactions, but the real workings of which are now known.

It is thus that the decomposition of calcium hypochlorite by certain metallic oxides, by cobaltic oxide, for example, appears to be brought about by the simple presence of the cobaltic oxide, because this body is found again intact and appears to have undergone no change during the reaction. But in reality its part is not one of complete indifference: there is formed during the reaction cobaltous oxide, which is then oxidized and which can act anew on the hypochlorite.

(1) $(ClO)_2Ca + 2Co_2O_3 = CaCl_2 + 2O_2 + 4CoO.$

Hypochlorite of calcium. Cobaltic oxide. Cobaltous oxide.

(2) $4CoO + O_2 = 2Co_2O_3.$

When hydrogen peroxide has been decomposed by potassium bichromate, we have an analogous process. Potassium

bichromate possesses the property of decomposing little by little an unlimited amount of hydrogen peroxide, being itself unchanged at the end of the reaction.

Berthelot explains this phenomenon by the formation of an intermediate compound, repeatedly destroyed and renewed, and whose destruction and recomposition are carried on until the moment when all the hydrogen peroxide is decomposed.

Berthelot, by adding ammonia to a mixture of hydrogen peroxide and dissolved bichromate, obtained, when the oxygen was liberated, a precipitate composed of hydrogen peroxide, and chromic oxide, and ammonium chromate.

It is the compound of hydrogen peroxide with chromic oxide which, during the reaction, is again transformed into chromic acid and water. The reaction probably takes place according to the formula:

$$6H_2O_2 + 2CrO_3 = Cr_2O_3 \cdot 3H_2O_2 + 3H_2O + 3O_2.$$
$$(Cr_2O_3 \cdot 3H_2O_2) = 2CrO_3 + 3H_2O.$$

In organic chemistry one can obtain reactions entirely analogous. It is thus that, in the reaction of Friedel and Crafts, metallic salts favor the substitution of monatomic groupings for atoms of hydrogen in the benzene series.

Benzol, C_6H_6, and methyl chloride, CH_3Cl, do not act upon each other under ordinary conditions; but in the presence of a metallic salt, such as aluminium chloride, there is formed toluol, $C_6H_5\text{-}CH_3$, and hydrochloric acid, HCl. The function of the salt is to form an intermediate combination which facilitates the reaction.

$$C_6H_6 + Al_2Cl_6 = C_6H_5Al_2Cl_5 + HCl.$$
$$C_6H_5Al_2Cl_5 + CH_3Cl = C_6H_5\text{-}CH_3 + Al_2Cl_6.$$

On the whole, all these reactions resemble each other in principle; and the formation of ether by the action of sulphuric acid upon alcohol may serve as a characteristic example of this kind of reaction. The transformation of alcohol

is produced in two phases: in the first stage the alcohol combines with the sulphuric acid to form ethyl sulphuric acid; in the second stage the product formed acts again on the alcohol: ether is formed and the sulphuric acid is regenerated.

It is very probable that the molecules of enzymes form with substances subject to their action combinations which are not permanent, unstable, and which are easily decomposed, either by water or by oxygen. This theory may be presented in the following manner. Two bodies having feeble affinities, as for example starch and water, are made to react by the aid of a third substance, for example malt diastase. A molecular combination of the starch with the diastase is thus secured. This combination has no longer the properties of the bodies which have entered into its composition; it is a substance much less stable which is decomposed by water. Following this decomposition the diastase reappears in its original state, the water remaining combined with the starch molecule, and this hydration transforms the starch into sugar. This theory, due to Bunzen and to Hüfner, is unfortunately based, not on precise facts, but on analogies, and this circumstance renders the theory open to question.

Würtz attempted to prove this hypothesis by studying papaïn, which is known to be an enzyme acting on albuminoid substances. He observed that fibrin immersed in a solution of this enzyme united with the active substance in such a way that the fibrin could be washed without losing it. The albuminoid substance thus impregnated was transformed, liquefied, and peptonized as soon as it was brought to a temperature favorable to diastatic action. As the same phenomenon is observed with pepsin, Würtz considered that albuminoid substances form with enzymes insoluble combinations, and that these combinations are the intermediate stages analogous to those found in all catalytic reactions.

Unfortunately this explanation is far from being accurate,

for the diastases are fixed not only on the bodies which they are capable of transforming, but also on bodies upon which they have no action, as silk. From another point of view albuminoid substances form combinations analogous to those which have just been cited, not only with the diastases which are able to act upon them, but also with other enzymes incapable of transforming them. Thus, the experiments of Würtz do not at all prove the formation of intermediate bodies. Still his theory of the manner of action of diastases finds support in the experiments of Schoenbein, Schaer, and Büchner relative to the action of hydrocyanic acid on active substances.

Hydrocyanic acid added to an aqueous solution of diastase prevents the latter from decomposing hydrogen peroxide and from transforming bodies on which the diastase can act. Still hydrocyanic acid does not destroy the diastase; in fact, when a current of air has been passed through the inactive solution, the activity of the enzyme reappears. One may conclude that the enzymes form with the hydrocyanic acid an unstable combination which is destroyed by the passage of a current of air.

These views are very favorable to the theory of Bunzen. If it is once established that the enzymes can form intermediate combinations with hydrocyanic acid, it may also be admitted that they react with the substances which they affect, and form combinations of the same nature. These intermediate substances furnished by the hydrocyanic acid unfortunately cannot be isolated in a pure state, and the hypothesis of Würtz, although probable, is not, however, based upon rigidly demonstrated facts. It is then quite natural to seek to explain the manner of action of enzymes by other hypotheses.

Naegeli explains the action of enzymes in an entirely different manner; he does not consider the action of diastases as a purely chemical phenomenon, but as being, at least partially, of a physical nature. This investigator considered

that the molecules of the enzymes are animated by special vibrations capable of producing in the fermentable substance molecular vibrations which can break down the molecules. As may be seen, this theory greatly resembles the old theory of fermentation of Liebig, according to whom the phenomena of fermentation in general are brought about by substances in process of decomposition, which communicate to other bodies in contact with them the same molecular movement.

This hypothesis, based on very speculative considerations, was finally taken up again by de Jäger. He carried his conclusions much further, and thought to bring out facts demonstrating that enzymes act, not as substances, but rather as forces. The experiments on which de Jäger based his views are due to Wittich and Fick.

Fick placed a solution of rennin in glycerin in a long tube, then carefully filled it with milk. As the rennin did not diffuse, de Jäger concluded that the coagulation was produced, not by the rennin, but by a property inherent in that substance.

Wittich placed in a dialyser furnished at its lower base with a parchment membrane, some pepsin dissolved in a certain quantity of water. He then introduced this dialyser into a larger basin containing water and flakes of fibrin. He did not detect any dialysis of the pepsin, yet the flakes of fibrin liquefied and became peptonized as if they had been in contact with the enzyme.

It is very evident that if these experiments were exact, the theory that enzymes act as chemical substances would be discredited, and it would then appear that the action of enzymes must be purely physical. But other investigators who have repeated these experiments have never been able to reach the results described.

The opinion of de Jäger has been quite recently taken up by Arthus who, while recognizing the inaccuracy of the experiments of Fick and Wittich, still inclines to support the theory of enzyme-properties.

Arthus has been no more successful than his predecessors in performing experiments which decisively favor his theory, but he shows effectively the weaknesses of the theory which considers enzymes as substances. He first notes that the percentage analysis is not enough to characterize enzymes. He emphasizes, as we have done above, the disagreement existing between the analyses of enzymes made by different authors; he shows also that diastases cannot be classed in any definite chemical group, for they are neither albuminoid substances nor gums. He is especially struck with the fact that each author claims to have prepared a pure enzyme by successive precipitations, without one of them being able to say by what characteristics a pure enzyme is to be recognized. He has also remarked that enzymes appear to vary in composition and properties according to the manner in which they have been prepared. He then cites the opinions of various authors on the impurity of diastatic precipitates, and concludes that all diastases which have been analyzed up to the present time were much mixed with foreign substances. He shows himself equally opposed to the theory which seeks to show an analogy between the manner of formation of ether by sulphuric acid and the action of enzymes. He bases this opinion on the difference between the quantity of sulphuric acid necessary to transform the alcohol into ether, and the quantity of enzyme which must be used to work a diastatic transformation. In fact, sulphuric acid etherizes only 25 to 30 times its weight of alcohol, while a certain quantity of diastase transforms quantities of matter infinitely great in relation to itself. Rennin, for example, can coagulate 250,000 times its weight of casein. He shows finally that the properties of enzymes do not at all necessitate that these latter shall be chemical substances, but that they may be imponderable agents, like heat, electricity, etc.

To demonstrate this, Arthus takes up the properties of diastases one by one, and attempts to match with them analogous phenomena of light, heat, and electricity.

Enzymes bring about chemical transformations; but light, heat, and electricity also bring them about: of this, electrolysis is a striking example. Enzymes are destroyed by heat; in like manner, a magnetized bar loses its magnetic property when it is heated red-hot. Enzymes are soluble in water and glycerin; but when a warm body is plunged in any liquid whatever, the latter is heated, although the body may not dissolve. Enzymes are thrown down from their solutions by alcohol or by mechanical precipitants; but sodium chloride precipitated by alcohol also stores up a certain quantity of heat which reappears when it is redissolved in water. In the same way, diastatic precipitates thrown down by alcohol enclose a certain quantity of the enzyme, and this enzyme reappears when placed in a suitable medium. Enzymes are retained by fresh fibrin; but electric accumulators retain electricity, and certain bodies, as barium sulphide, absorb rays of light. Certain substances, under the action of chemical agents, acquire a diastatic power; so the oxides of phosphorus are known to diffuse light. Enzymes are destroyed by certain agents; the magnetism of a magnetized bar disappears when the bar is dissolved in hydrochloric acid. The action of diastases is hindered by certain bodies and facilitated by others; in an electric current, if a resistance is interposed, the intensity of the current diminishes, and if, on the contrary, this resistance is removed, the current increases in intensity. Diastatic action is generally produced on certain bodies to the exclusion of others; iron and steel alone can fix the magnetic property.

Arthus concludes from all these comparisons that enzymes are not substances but properties of substances. He admits that his theory has not been demonstrated, but on the other hand, he asserts that the theory of enzyme-substance has not received any clearer demonstration.

On the whole, we recognize the existence of two theories. The one assumes that enzymes act chemically and that they have a definite chemical composition, the other considers the

enzyme as a property and not as a substance. The arguments which Arthus brings up against the theory of enzyme-substances are not of a nature to overthrow that theory. The disagreement between the analyses of the same diastase may very well be due to the manner of preparation and purification of that substance. If it could be proved, on the other hand, that diastases cannot be classified in any chemical group actually known, that would not disprove the existence of diastases as substances. In fact, at the present time we are still far from knowing all the chemical combinations, and it is more than probable that there exists a great number of bodies about which we know nothing. The fact that enzymes act in infinitely small amounts is not at all of a nature to invalidate the hypothesis of enzyme-substance. In the action of strychnin, aconite, and many other alkaloids, there is seen also an enormous disproportion between the result produced and the weight of the acting substance. The action of musk is indisputably much more sensitive than the action of enzymes: reactions can be obtained on the olfactory nerve-endings with exceedingly small amounts, and this remarkable property is entirely due to the chemical constitution of these bodies.

The parallel between the phenomenon of fermentation and physical phenomena is very attractive; but, on the whole, the hypothesis of enzymes, as properties, is much less probable than that of enzymes as substances. One always finds the capacity for action incorporated in a material substance, and one has never succeeded in separating the property from the substance. There is nothing to justify a belief that material substance plays no part in the diastatic phenomenon.

Enzymes present various resemblances to living protoplasm.

The enzyme, like the living organic substance, is extremely sensitive to chemical agents, such as acids and alkalies. These two classes of substances are destroyed at a tem-

perature of 100°, and they have the property of exciting chemical reactions in the surrounding medium.

Most enzymes have a chemical composition very similar to that of protoplasm, and both these materials furnish some reactions common to albuminoid substances. The analogy becomes still more striking when one studies the composition of the mineral substances which evidently enter into the chemical composition of protoplasm and soluble ferments. Both contain phosphates of calcium, potassium, and magnesium, and alkaline chlorides and sulphates. The mineral and organic elements which are favorable to the living cell are also exciting agents for certain diastases, as has been demonstrated for asparagin and phosphates. Enzymes, like protoplasmic substance, are diffusible with difficulty, and in many cases do not even pass through a porcelain filter. One may then consider that diastases are not even soluble bodies, properly speaking, and that when they are in contact with water they go only into a state of extreme tenuity, as do colloid bodies such as starch-powder.

This analogy between organic substance and enzymes has led Armand Gautier to suppose that chemical ferments resemble in their constitution the cells from which they are derived. He considers further, that enzymes have an organization analogous or very similar to that of protoplasm, and he credits them with the fundamental properties of the living cell, which are assimilation and reproduction. According to this investigator, enzymes can transform certain substances into bodies like themselves. On the strength of this bold hypothesis, he cites a single experiment, made with pepsin, and to which we shall have occasion to return when we come to study the action of enzymes upon proteid substances.

For the present we may say that the experiment cited by Gautier does not at all confirm his theory, and we are rather inclined to regard enzymes as chemical bodies of a particular nature and a definite constitution. And in reality,

in proportion as our knowledge of diastases increases, the theory of enzyme-substance seems more and more probable. At present we possess an array of facts which indicate that we are really dealing with bodies and not with properties. We know, for example, that amylase procured from various media, from raw grain, from malted grain, from the saliva, from the pancreatic juice, from bacteria and moulds, always shows the same chemical composition and always gives the reaction of a proteose.

The chemical nature of enzymes is still further confirmed by the color reactions which they show with certain reagents. As Guignard has shown, emulsin gives a violet color with orcin and a red color with Millon's reagent. Another enzyme, myrosin, gives a violet tint in hydrochloric acid.

In special cases it has been possible to cause one enzyme to react upon another. This action is very characteristic and affords data on the chemical nature of enzymes. According to Naegeli and Kühne pepsin, for example, acts upon trypsin as upon an albuminoid substance. Chittenden and Griswold have observed the same phenomenon with ptyalin: this enzyme is also attacked by pepsin. The zymase, or enzyme producing the alcoholic fermentation, is destroyed, according to Büchner, in the presence of trypsin. In the action of one enzyme upon another, one of the active substances is always hydrated and chemically changed, with the complete suppression of its activity.

As pepsin and trypsin act exclusively upon albuminoid substances, one may conclude that ptyalin and trypsin as well as zymase belong to that class of substances.

The existence of enzymes containing little or no nitrogen cannot, however, constitute an argument for the support of the theory of enzyme-properties. Different diastases act on different bodies, and cause very varied reactions. It is then very evident that all active substances cannot belong to the same class of bodies and that, in all probability, the enzymes will present wide differences in composition and structure.

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CHAPTER IV.

INDIVIDUALITY OF ENZYMES.

Difficulties encountered in proving the individuality of enzymes.—Influence of the nutrition of cells upon the nature of enzymes which they secrete.—Direct proofs of the individuality of enzymes.—Relation among the diastases; chemical constitution and structure of the bodies upon which they act.—Nomenclature of enzymes.—Classification.

When one studies the active substances secreted by living cells with reference to their chemical action, one finds generally that this action is very complex, that different substances are affected, and that very varied products arise. Thus, an infusion of malt acts upon starch, cellulose, pectin, trehalose, and caroubin. Furthermore, the results obtained with these various substances are very different: The diastase in acting on starch gives maltose and dextrins, it liquefies the cellulose, transforms the pectic materials into a gelatinous substance, causes the trehalose to pass into monosaccharid, and changes the caroubin into another monosaccharid.

We observe the same phenomenon in studying the properties of an aqueous extract of beer-yeast. This infusion acts on cane-sugar, maltose, and the glucosides, and gives in every case a specific product.

These facts lead us to inquire whether living cells secrete a single active substance having the power of acting on different chemical combinations, or if, on the contrary, they give rise to a mixture of many enzymes, each adapted to produce a special action. The same question arises for enzymes precipitated from their solutions, for they also generally give very varied results.

It is difficult to answer this question clearly in each particular case; but in general the individuality of enzymes cannot be denied.

This individuality becomes evident in many cases, when we compare the actions of the products secreted by cells of a special kind placed under different nutritive conditions.

Duclaux has found that by cultivating *Penicillium glaucum* on starchy substances, there is produced in the culture medium a complex substance acting on cane-sugar as well as on starch. It is very difficult to give a direct proof of the existence in this medium of two different enzymes, one acting on saccharose, the other on starch, for in isolating the active substances from this medium, either by mechanical precipitation or by alcohol, one obtains still another substance acting upon different carbohydrates and giving different products.

But the question may be answered by making a second culture of *Penicillium glaucum*, and in the culture medium replacing the starch by calcium lactate. The active substance which is formed this time acts very strongly on the cane-sugar but not upon starch. We may then conclude that in the first culture there were present two enzymes, while in cultivating the mould on calcium lactate only one of these was obtained.

Our conclusion will be strengthened if we can find other examples in which an active substance will produce an action exclusively on saccharose or on starch. These examples are very numerous. Thus it is that an infusion of barley acts on starch and not on cane-sugar, and that the amylase obtained from the rennet of sheep acts in a similar manner. One may also find present in the saliva a ferment acting on the starch without causing any transformation of saccharose, especially if the saliva does not contain any organized ferments. An infusion of yeast can act on sugar and leave starch unaffected.

In certain cases the individuality of enzymes can be con-

trolled by different means from those which we have just set forth. If, for example, we leave beer-yeast in prolonged contact with water with the addition of ether or thymol, we find that the solution acts at the same time on cane-sugar and on maltose. We may then ask if the diastase which transforms the maltose does not also act on cane-sugar. It is easy to demonstrate here the existence of two ferments. It is only necessary to leave the yeast in contact with water for a very short time in order to find in the liquid a substance acting on cane-sugar without having the least action on maltose. In this case we have actually separated the two enzymes, owing to the fact that one of them is not firmly held by the cells which secrete it, while the other escapes from the cells with much difficulty.

We have seen that malt diastase acts on starch, giving maltose and dextrins; it also acts on trehalose, which it transforms into a monosaccharid. Some authors have concluded from this that there is present a single substance. But the amylase which is taken from the saliva acts upon starch in exactly the same way as the amylase from malt, while it has no action on trehalose. It seems to us that in this case also it is more logical to assume the presence of two different enzymes in the infusion of malt, than to explain the phenomenon by supposing that there exists in the saliva and in the malt infusion two different diastases, both acting in the same manner on the starch and differing in their action on trehalose.

Emulsin acts on glucosides, but at the same time this enzyme will transform sugar of milk into dextrose and galactose. In emulsin the presence of two diastases becomes evident, in our opinion, if one considers that the active substances secreted by certain yeasts have the power of transforming lactose without acting in any way on glucosides.

From many facts of this kind we are justified in concluding that in a majority of cases cellular secretions are composed of different active substances, and that the chemical

action of each of these enzymes is limited to a certain number of bodies. These facts are sufficiently numerous for us to draw a general conclusion in favor of the individuality of enzymes. And as a matter of fact, it is difficult to believe that the same active substance can in one special case act on two or three chemical substances, while in another case its action is limited to a single one of these substances.

As we have just seen, a diastase having a hydrating or oxidizing function does not act on all the substances capable of being hydrated or oxidized; the diastatic agent differs completely from a chemical agent having a definite function and exercising it independently of the constitution of the bodies on which it acts. For example, by the action of a mineral acid is obtained the splitting of saccharose, the saponification of fatty matters, the decomposition of glucosides, the peptonization of albuminoid substances, in a word all the phenomena which we meet in diastatic hydrations. Among diastases, on the contrary, decompositions and hydrations are caused by numerous agents, each capable of a special diastatic work and acting upon only a very limited number of substances. The action of acids is, then, up to a certain point, independent of the constitution of the bodies on which they act, while diastases exercise their hydrating or oxidizing action only on bodies of a strictly definite structure.

A hydrating enzyme may sometimes exert its action on different bodies, but only when the chemical constitution of these bodies is very much like that of the diastase, and when they can furnish the same products of decomposition. It is thus that amylase acts on starch, glycogen, and dextrin, giving always the same end-product, maltose.

Pepsin acts on a great number of bodies, for example, on all albuminoid substances.

Now all these bodies resemble each other and have a very similar structure, since their products of decomposition by the diastase are always the same: proteoses and peptones.

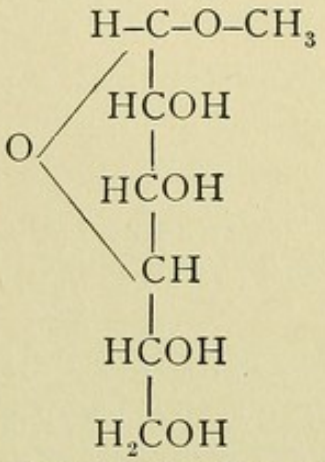
The enzymes of glucosides appear capable, at first sight,

of a more energetic action, and one extending to chemically different bodies, but this anomaly is only apparent: emulsin, which acts on very complex bodies, affects only that part common to all the molecules of glucosides. The action of emulsin is due to the affinity it has for glucose, and, as Emil Fischer has demonstrated, this affinity is explained by the stereochemical structure of the carbohydrate molecules. Emulsin acts, not only on natural glucosides, but also on artificial ethers, which are obtained with glucose.

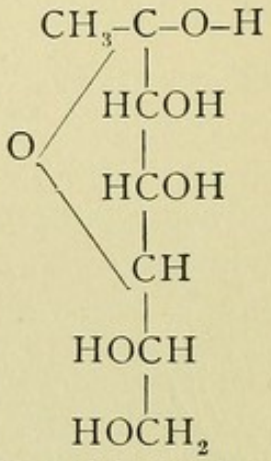
In studying the action of enzymes on artificial ethers, Emil Fischer has found this very interesting fact: that the action or inaction of an enzyme depends, not only on the composition of the substance on which it is made to act, but also on its configuration. By treating glucose with methyl alcohol in the presence of hydrochloric acid one obtains two isomeric ethers, differing in their geometrical structure on account of the asymmetrical carbon atoms of the glucoside chain.

The formation of two isomeric ethers is easy to explain: the aldehydic glucose group disappears by the action of the alcohol in presence of hydrochloric acid, and dehydration is produced in the glucose chain, giving rise to an intramolecular ethereal group. The carbon of the aldehydic group thus becomes asymmetrical, and in consequence the appearance of two stereo-isomers becomes comprehensible.

The two isomeric glucosides



α. Methyl dextroglucoside.



β. Methyl dextroglucoside.

behave differently under the action of enzymes.

Emulsin, which acts on certain derivatives of dextrose and galactose, acts also on the β-methyl glucoside, but has no action on the isomer α.

In the beer-yeasts is found another soluble ferment which acts on natural glucosides, but this ferment has absolutely no effect on the β-methyl glucoside, while it acts on the isomer α.

This example is another proof of the individuality of enzymes and shows in a striking way the influence which the chemical structure of bodies has on the diastatic action.

Emil Fischer has evolved the hypothesis that a diastatic action cannot be produced except on condition that there shall be a stereochemical relation between the acting substance and the body acted upon.

According to him, it is necessary that the ferments and the substances they act upon shall have a like geometrical structure, or at least a certain structural resemblance.

We believe that this hypothesis will explain very happily the development of different diastases from cells which have been fed with different substances.

A cell nourished by starch will secrete an enzyme having the stereochemical structure of starch, while if the cell is nourished by cane-sugar, the diastase which it will form will have the geometrical constitution of cane-sugar.

Our knowledge of oxidizing enzymes is much less extensive than that which we possess of hydrating enzymes. But the facts hitherto observed demonstrate unquestionably that in this case, as in the preceding, there are found to be present different specific ferments, all acting like oxidizing agents, but, individually, on different materials. For this class of bodies also, it is evident that the arrangement of the various chemical groups in the molecules of the oxidizable substances considerably influences the activity of the enzymes. Examples are known of oxidizing enzymes acting on an entire series of homologous bodies, whose action is still possible, if one group is substituted for another, while the action of these

same enzymes ceases when the arrangement of the groups is changed. Thus it is that laccase, which oxidizes diphenol, its homologues and the products of substitution of these substances, exercises its action on all their derivatives in which the two hydroxyl groups are found in the ortho position, while the same diastase does not act on the isomeric products in which these groups occupy the meta position.

Classification of Enzymes.—Now that we have acquired some general knowledge of enzymes and their mode of action, we can turn our attention to the individual properties of each known enzyme. But before attempting this description, it is necessary to agree upon a nomenclature and a classification of diastases. The chemists who discovered the first diastases designated them by very different names, according to their different points of view. As long as the study of enzymes was confined to a small number of substances, the drawbacks of this nomenclature were not very great. But at the present time a considerable number of diastases are known, and this number is certain to increase. Under these conditions it would be desirable to have a logical nomenclature seeking to designate a ferment by a name giving a clear idea of its own characteristics.

Recognizing this need, Duclaux has suggested a rational nomenclature, by designating an enzyme by the name of the body on which its action was first observed; and in order to distinguish the substance on which the diastase performs its action from the enzyme itself, he has proposed to add to the root of the word the termination *ase*. Thus the diastase acting on casein would be called *caséase*, and the diastase which transforms starch (amylum) becomes *amylase*.

Unfortunately, the nomenclature of Duclaux has not been adopted by all workers, and a certain number of new diastases have received from their discoverers a name having, to be sure, the termination *ase*, but whose root is not derived from the name of the substance on which the diastase acts, but from that of its product. Thus the glucase of Cuse-

nier is not a diastase acting on glucose, but an active substance transforming starch and maltose into glucose. This new nomenclature has the great disadvantage of bringing about confusion, and it would have been better to have adhered to the nomenclature of Duclaux, although that was not all that could be desired. It is not well to take as the root of the name that of the product formed, because different diastases may, at the end of the reaction, produce identical results, while acting on very different bodies. Thus we know, in addition to glucase, many ferments which transform certain carbohydrates into glucose. It is true that the nomenclature of Duclaux also gives rise to confusion. Thus, the action of glucase was first observed on starch; one should then designate this enzyme by the name of amylase, a name applied to the diastase from malt. It is then necessary to take account, not only of the substance on which the diastase acts, but also of the substance produced by the diastase. From this point of view one would name the glucase of Cuisenier amylo-glucase, that is to say, indicating that it is a diastase acting on starch and producing glucose. The diastase of malt, on the contrary, ought to be called amylo-maltase, because the product finally resulting from the action of this diastase on starch is maltose.

However, in the present work we shall retain the old nomenclature and give to the diastases the names which are generally met in the literature. The reason for this method is that we know very well that every change of nomenclature, although aiming to simplify matters, only adds new complications, and on the whole produces a result contrary to that which was intended. The most rational classification of enzymes consists in distinguishing them according to the chemical work which they produce. We already know that diastases can produce hydration, oxidation, or molecular transformation. We shall then describe the diastases, grouping them according to the chemical character of their action. The study of the diastases of proteid matters will be the sub-

ject of the second volume of the present work. In the first we shall only occupy ourselves with the diastases producing either hydration, oxidation, or molecular change.

Hydrating diastases act on carbohydrates, fatty substances, glucosides, proteins, and urea.

Oxidases act on bodies of very diverse nature: alcohols, phenols, amides, fatty substances, etc.

Enzymes causing molecular transformations are so few that not many bodies susceptible to their action can be named.

CLASSIFICATION OF SOLUBLE FERMENTS.

A. Soluble Hydrating Ferments.

1st. Soluble Ferments of Carbohydrates.

Names of the Enzymes.	Substances on which the Enzyme Acts.	Products of the Reaction.
Invertin or sucrase.	Cane-sugar.	Invert-sugar.
Amylase or diastase.	Starch and dextrin.	Maltose.
Glucase or maltase.	Dextrin and maltose.	Dextrose.
Lactase.	Lactose.	Dextrose and galactose.
Trehalase.	Trehalose.	Glucose.
Inulase.	Inulin.	Fructose, levulose.
Cytase.	Cellulose.	Sugars.
Pectase.	Pectin.	Pectates and sugars.
Caroubinase.	Caroubin.	Caroubinose.

2d. Soluble Ferments of Glucosides.

Emulsin.	Amygdalin and other glucosides.	Glucose, oil of bitter almonds, and hydrocyanic acid.
Myrosin.	Potassium myronate.	Glucose and allyl isosulphocyanate.
Betulase.	Gaultherin.	Oil of wintergreen.
Rhamnase.	Xanthorhamnin.	Glucose. Rhamnetine, isodulcite.

3d. Soluble Ferments of Fatty Substances.

Steapsin. Lipase.	Fatty substances.	Glycerin and fatty acids.

4th. Soluble Ferments of Proteins.

Rennet.	Caseinogen. (Casein, Hammarsten.)	Casein. (Para casein.)
Plasmase.	Fibrinogen.	Fibrin.
Casease.	Casein.	
Pepsin.	Albuminoid substances.	Proteoses, peptones.
Trypsin. Papäin.	" "	Proteoses, peptones, amides.

5th. Ferments of Urea.

Urease.	Urea.	Ammonium carbonate.

B. Soluble Oxidizing Ferments.

Laccase.	Uruschic acid.	Oxyuruschic acid.
	Tannin, anilin, etc.	Products of oxidation.
Oxidin.	Coloring matters of cereals.	" " "
Malase.	Coloring matters of fruits.	" " "
Olease.	Olive oil.	Products of oxidation.
Tyrosinase.	Tyrosin.	" " "
Oenoxidase.	Coloring matter of wine.	" " "

C. Ferment Causing Molecular Decomposition.

Zymase or alcoholic diastase.	Various sugars.	Alcohol and carbonic acid.

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CHAPTER V.

SUCRASE.

Extraction of sucrase from yeast.—Secretion by *Aspergillus niger*.—Preparation of sucrase in the dry state.—Influence of the quantity and of time.—Influence of temperature.—Difference between the properties of sucrase of different origin.—Effect of the acidity or alkalinity of the medium.—Action of oxygen and of light.—Action of chemical substances.—Mode of secretion of sucrase in the cells.—Measurement of sucrase.—Method of Fernbach.—Method of Effront.

SUCRASE is a diastase capable of transforming cane-sugar into invert-sugar. Saccharose, under the action of sucrase, is decomposed with addition of a molecule of water, giving two monosaccharids: dextrose and levulose,

$$\underset{\text{Saccharose.}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Dextrose.}}{C_6H_{12}O_6} + \underset{\text{Levulose.}}{C_6H_{12}O_6}.$$

Sucrase is very widely distributed in nature. For example, we find its existence in the saliva, in the gastric juice, and in the small intestine.

Cane-sugar, retained for some time in the mouth, is transformed by the action of the saliva into invert-sugar. However, this transformation is not due to the action of a secretion of the salivary glands, but rather to the sucrase evolved by the numerous bacteria which are found in the saliva, for the active substance which occurs in the mouth transforms only very limited quantities of cane-sugar.

The diastases of the gastric juice are endowed with much stronger inverting power. However, in spite of this energy, the inversion of saccharose does not proceed actively in the

stomach. A considerable part of the cane-sugar absorbed reaches the circulation without having undergone the action of the diastases, and it is only in the small intestine that the transformation becomes complete. In the blood the presence of active substances capable of transforming saccharose has not been observed.

Sugar injected in the veins or in the cellular tissue of an animal is eliminated in the urine; but this elimination does not occur when sugar is injected in the portal vein. It then traverses the liver and there undergoes a strong diastatic action which completely transforms it.

Sucrase is also of very common occurrence in the vegetable kingdom: it is found in buds and flowers, as well as the leaves of a very great number of plants. Furthermore, numerous moulds, such as *Aspergillus niger*, *Mucor racemosus*, *Penicillium glaucum*, *Penicillium Duclauxi*, *Aspergillus oryzae*, yeasts, and many other ferments, also effect the inversion of saccharose.

As a general rule, a cell nourished by sugar must necessarily contain a sucrase. This rule has, however, been opposed by Hansen, who has called attention to the fact that the mould called *Monilia candida*, while nourished by saccharose, does not secrete sucrase. This assertion has been successfully refuted by E. Fischer, who in a more thorough study of this mould discovered that it really contains a sucrase, but that the enzyme is retained in the cells, and is difficult to isolate.

In the literature sucrase is designated under different names: it is called glucose ferment, cytozymase, zymase, and invertin. This enzyme was discovered by Döbereiner and Mitscherlich. These investigators first found that beer-yeast inverted saccharose. They also remarked that this active substance can be extracted from the yeast by washing with water. Berthelot first succeeded in isolating the diastase in the solid form by precipitating from yeast extract by alcohol.

Mode of Preparation.—Several different methods of preparation of sucrase may be employed. The enzyme can easily be obtained by putting some beer-yeast in water with the addition of a few drops of chloroform; after a time the active substance is dissolved in the water. Then the liquid is filtered to remove the suspended yeast-cells. The solution thus obtained is necessarily far from being composed solely of sucrase, the yeast containing, besides the sucrase, other extractive matters which enter into solution at the same time. Notwithstanding this, the infusion is very active, and may very well serve for the study of sucrase.

A better method of preparation of this enzyme consists in the extraction of a culture of *Aspergillus niger* in Raulin's solution. Yet, the extraction of the diastase from *Aspergillus niger* demands, in order to give sufficient quantities of enzyme, the maintenance of certain conditions without which the results would not be satisfactory. The best method of procedure has been suggested by Duclaux. He advises allowing a culture of *Aspergillus niger* to develop on a large surface of Raulin's solution for about four days, and when the moulds formed have taken on a green or light brown color, drawing off the liquid and replacing it by pure water or water containing sugar. On this new liquid one allows the mould to grow further for two or three days up to the complete exhaustion of the nutritive medium. Then the enzymes secreted by the plants enter into solution and it only remains to filter the liquid to free it from the fragments of mould which may be found in suspension there. The solution of sucrase prepared in this way is very active and contains relatively few impurities. To prevent the liquid changing during the growth of the plant, one may add a few drops of mustard oil which, acting as an antiseptic, preserves the medium from the invasion of bacteria without destroying the diastase. However, it is better to cultivate the mould in sterilized liquid, and to inoculate this liquid with a pure culture of *Aspergillus niger*. When the plant has sufficiently developed

on the Raulin's solution, this is replaced by sterilized distilled water.

To obtain sucrase in a dry state, E. Donath recommends the following method: extract the beer-yeast for some time in absolute alcohol; then decant the alcohol, filter, and dry by exposing to the air. In this way a brittle mass is obtained which is pulverized and treated with distilled water. This infusion is filtered to remove the yeast-cells which are present. However, as the cells easily pass through the filter, one must make sure by a microscopic examination that they have all disappeared from the liquid. If cells are still present, the solution must be filtered several times through a double filter. When the liquid is free from cells, ether is added and it is shaken. A viscid substance appears which remains in suspension in the upper part of the liquid and which must be separated from the rest of the infusion. This substance is then treated with distilled water and dropped slowly into absolute alcohol, where a pulverulent precipitate is produced. This precipitate, separated from the liquid, is washed with alcohol and dried *in vacuo*. This procedure furnishes a white powder, swelling in water and dissolving in it with great difficulty. It can be kept for a long time and possesses great diastatic power. It appears beyond doubt, however, that a considerable part of the active substance must be coagulated by the treatments with alcohol and ether, and consequently rendered inactive.

The rapidity of inversion of saccharose by sucrase depends upon the quantity of active substance employed, as well as upon the physical and chemical conditions of the medium in which the transformation takes place. The study of special conditions which favor or retard diastatic action is still more interesting in that it furnishes valuable data from the theoretical as well as from the practical point of view.

We shall, therefore, accord to this question the development which it merits. We shall first study the influence exerted on the rate of inversion by the quantity of sucrase,

and the temperature at which it works. We shall next determine the time-factor in the inversion, as well as the influence of the acidity or alkalinity of the medium. Finally, we shall see how light, oxygen, and certain other chemical substances influence the rate of the transformation.

Influence of Quantity and of Time.—When sucrase acts on a solution of saccharose, the results obtained are very different according to the quantity of active substance employed.

If one holds to definite conditions one may observe an almost constant relation between the quantity of sucrase employed and the quantity of invert-sugar. This proportion is, up to a certain point, independent of the concentration of the sugar solution in which the diastase works. If, for example, 1 and 2 cubic centimetres of sucrase are made to act during the same time and at the same temperature upon equal quantities of saccharose, it is found that with 2 cubic centimetres of sucrase twice as much invert-sugar is obtained as with 1 cubic centimetre.

However, this proportionality between the quantity of acting substance and the quantity of product formed is not always constant. Duclaux has observed that the law of proportionality is true only when the sucrase is used in very small amounts, and if the inversion is arrested at an early stage. The ratio holds good until 10 to 20 per cent of the sugar is inverted, after which it fails. When one studies the influence of time on the action of sucrase, the same rule holds good.

Sucrase is a very energetic enzyme. According to Duclaux, 1 gram of active substance transforms 4000 times its weight of sugar. However, while very energetic, the action of this diastase is relatively slow. One hundred cubic centimetres of 10 per cent solution of saccharose to which 1 cubic centimetre of sucrase was added, when kept at 50°, yielded the following results:

After	1	hour.....	.20	gr.	Invert-sugar.
"	2	hours.....	.41	"	" "
"	3	"	.60	"	" "
"	4	"	.80	"	" "
"	5	"	.97	"	" "

This experiment illustrates the remarkable slowness with which inversion is produced. It is noteworthy in this connection that the amount of invert-sugar increases proportionally to the duration of the action. After two hours, one finds about twice as much invert-sugar as after one hour, and after five hours almost five times as much. But from this time on the proportion ceases to exist. If we continue in fact to follow the action of sucrase in the preceding experiment, we obtain:

After	10	hours.....	1.72	gr.	Invert-sugar.
"	20	"	3.12	"	" "

If the transformation had continued with the same speed as at the beginning of the action, we should have had:

After	10	hours.....	2.00	gr.	Invert-sugar.
"	20	"	4.00	"	" "

The retardation which we observe commences when about 20 per cent of sugar has been transformed, and in proportion as the inversion proceeds, the abatement continues to be marked. The irregular course which we observe in the action of sucrase has been the subject of different researches and has occupied many investigators. It has given rise to various hypotheses which we will examine later. It is sufficient now to note the fact before we pass to the action of temperature.

Influence of Temperature.—Temperature plays a very important part in the inversion of saccharose, and exerts a considerable effect on the degree of activity of sucrase. At 0° sucrase exercises only a very feeble action, but it increases considerably with increase in temperature. This in-

crease is gradual between 5° and 30°. Above this temperature, from 30° to 50°, diastatic activity increases rapidly.

By allowing sucrase from yeast to act for an hour upon a 20 per cent solution of sugar, we have obtained with the same quantity of sucrase at different temperatures the following figures:

Temperature, Degrees Centigrade.	Gr. Invert-sugar formed per 100 c.c. solution.
0°	0
5°	0.05
10°	0.11
15°	0.18
20°	0.35
30°	0.40
40°	1.65
50°	2.20
60°	2.10

The temperature at which inversion proceeds with the greatest rapidity is, according to Kjeldahl, 52.5°; beyond that the diastase becomes more and more weakened.

In trying to determine the temperature at which sucrase is destroyed, it is important to have conditions absolutely constant, because the concentration of the liquid and the reaction, as well as the other special qualities of the medium, have a considerable influence on the activity of the diastase.

Sucrase from yeast, much diluted, can be maintained for an hour at 52° without losing its inverting power; on the contrary, the more concentrated solutions of sucrase weaken very perceptibly when they are kept at that temperature even for a short time. When yeast is placed for an hour in water at 65°, its diastase is completely destroyed; while at the same temperature a part of the active substance remains unchanged, when a very dilute solution of sucrase is used. The reason for this difference of resistance is that other bodies unfavorable to the action of sucrase are found with it in the

extract, and the retarding action of these substances evidently diminishes with the degree of dilution of the solution.

The presence of sugar in the liquid containing sucrase perceptibly increases the power of the enzyme to resist heat.

On the whole, the variations observed between the optimum temperature and the destructive temperature are quite considerable. The optimum temperature is found, according to different authors, between 50° and 56°, and the destructive temperature between 65° and 70°. But the activity of the sucrase is weakened as the destructive temperature is approached.

Sucrases of Different Origin.—Kjeldahl has observed that sucrase extracted from bottom yeasts possesses an optimum temperature different from that of the active substances of top yeasts. For the latter he has found that the optimum temperature is 3.5° higher than that of bottom yeasts. It is not only the optimum temperature which varies with the origin of the sucrase: most of the properties of the enzyme depend upon its origin as well as upon the mode of preparation. Thus, sucrase extracted from yeasts can be filtered by the Chamberland filter, while the active substance of *Aspergillus niger* is completely held back by the filter.

In beer-yeast sucrase is found in an uncombined state and can easily be extracted by water; in *Monilia candida*, on the contrary, the enzyme is retained in the cells, where it is found combined with other substances which render it insoluble. Sucrases obtained from different yeasts may also differ by their greater or less sensitiveness to chemical reagents. Fernbach has found, for example, that the enzyme of the yeast of Tantonville is fifty times more sensitive than sucrase extracted from other kinds which had been given him to study.

These differences of properties, found in sucrase, are not confined to it. We shall meet with similar facts when we study pepsin as well as many other soluble ferments. These differences may be explained by the presence of various foreign substances having the property of lowering the opti-

mum temperature and the destructive temperature of changing the solubility of enzymes, and of influencing their sensitiveness towards physical and chemical agents.

This explanation leads to the conclusion that the enzyme has in itself constant properties, and that if two sucrases, for example, show different characteristics and act in different ways, one must simply seek the cause in the conditions of the medium,—in the presence of substances endowed with an accelerating or retarding power.

But all authors do not concur in this opinion. The difference which exists between the properties of two enzymes of the same nature, but of different origin, has sometimes been interpreted quite otherwise. One may suppose, for example, that the medium in which the enzyme is secreted influences not only the mode of action, but also the very composition of the diastase.

By this hypothesis, the difference existing between the modes of action of various enzymes must be regarded as resulting from a series of changes in the composition or in the structure of the diastase, whereby one simply is dealing with various modifications of the same enzyme.

We have just said that sucrase of top yeasts produces its maximum effect at a higher temperature than the optimum temperature of low yeasts. This difference may be attributed to a phenomenon of adaptation of the yeast to the medium in which it works, adaptation having as its consequence the formation of different diastases at the different temperatures. This adaptation to the medium is manifested still more clearly when one studies the action of gastric juice. The pepsin of warm-blooded animals does not act at 0°, and its maximum effect is produced at 50°, while the gastric juice of cold-blooded animals produces a manifest action at 0°, and has an optimum temperature of 40°.

Many facts analogous to the preceding are known which support the hypothesis of the adaptation of diastases to their environment.

But the existence of different varieties of the same enzyme is very difficult to demonstrate exactly, because we always have to deal with mixtures of enzymes and more or less clearly recognized foreign substances. However, we are the more led to deny the existence of different varieties of the same enzyme, because the variations in the properties of the same enzyme are generally not very pronounced, and are susceptible of being artifically reproduced by starting with a definite diastase, and simply changing the conditions of the medium. We think it more logical to assume, until the contrary is proved, that the variations observed in enzymes of different origin are due to the presence of foreign substances.

We shall have occasion to return to this question repeatedly in studying the enzymes individually.

Part Played by the Acidity or Alkalinity of the Medium.—The acidity or alkalinity of the medium has considerable influence on the sucrase. Kjeldahl has demonstrated that a slight acidity is favorable to its action, while large amounts of acid or alkali diminish its diastatic power.

In a very comprehensive work, Fernbach has studied a sucrase derived from *Aspergillus niger* and has examined with great care the influence of the medium. His study has given valuable information on the question which we have to consider. Fernbach found that the solution of sucrase extracted from *Aspergillus niger* always possesses an acid reaction due to the oxalic acid elaborated, in greater or less quantity, by the mould. While this acid reaction is in reality very weak, the diastatic solution may have dilute soda added in considerable amount before it will turn litmus paper blue. The sucrase still shows itself very sensitive to the action of quantities of alkali too weak to be revealed by litmus paper and other indicators of alkalinity. Fernbach made the following experiment to show this sensitiveness to the acids and alkalies of the medium:

Into each of eight test-tubes he poured two cubic centimetres of an infusion of sucrase, added to each of them a

different quantity of a solution of soda (1:15000), then brought the volume of liquid in each tube up to 10 cubic centimetres by the addition of sugar solution. At the end of an hour of action at 56°, he determined the quantity of invert-sugar formed in each of the tubes and obtained the following results:

Numbers of the tubes.	Quantity of soda added.	Invert-sugar formed.
1	0 cc.	35 mgr.
2	0.5	31
3	1	25
4	1.5	17
5	2	12
6	2.5	7
7	3	5
8	3.4	3

The liquid in the tubes 1, 2, 3, 4, showed an acid reaction; after tube 4, to tube 7, the solution was neutral; it gave a slightly alkaline reaction in tubes 7 and 8.

We notice in this table that the invert-sugar formed diminishes in proportion as the quantity of soda in the solution increases. When soda is not added 35 milligrams of invert-sugar are obtained, while the addition of only 1.5 cubic centimetres of soda (1:15000), an amount hardly sufficient to neutralize the solution, reduces the quantity of invert-sugar to 17 milligrams. This diminution of about 50 per cent is here effected by the action of a quantity of soda equivalent to about 1 gram per hundred litres.

This extreme sensitiveness of the diastase to the alkalinity or acidity of the medium suggests one of the causes of the non-proportionality between the quantity of active substance employed in an inversion and the quantity of invert-sugar which results. In fact, when the sucrase is neutral and used in small amount, the quantity of matter transformed is, as we have seen, proportional to the quantity of active sub-

stance allowed to act, but this proportion ceases to exist when the experiments are made with an acid or slightly alkaline solution of sucrase. It is evident that by using these increasing quantities of sucrase, there are at the same time introduced increasing quantities of acid or alkali, which influence more and more strongly the degree of the inversion and change the ratio.

Fernbach has determined, in his work, the optimum amount of different acids for the greatest activity of the diastase. To this end he first neutralized, as exactly as possible, a sucrase solution, and then acidified it, using increasing quantities of different acids. He obtained the results shown in the following table:

Acids.	Optimum quantity (No. of grams per litre).	Inhibiting quantity (No. of grams per litre).
Sulphuric acid	0.025	0.2
Tartaric "	1	2
Oxalic "	0.066	0.1
Succinic "	2	4
Lactic "	5	10
Acetic "	10	50

It is seen that the optimum amount depends upon the nature of the acid employed.

The activity of the enzyme increases in the presence of small amounts of acid up to the moment when the optimum amount is reached; but this once passed, the presence of the acid becomes destructive to the diastatic action, which decreases perceptibly.

The amount of oxalic acid producing the greatest effect on the inversion does not, by itself, possess inverting power at 56°; but the other acids, by their own action, invert a certain amount of sugar. The invert-sugar formed in the presence of acids results then from the combined actions of the acid and of the diastase. From this it results that by employing different acids, each in its own optimum amount, there will of necessity be obtained different quantities of in-

vert-sugar with the same amount of sucrase. This difference is due to the action of the acid alone and not to that of the sucrase, for the latter is always influenced in the same degree by the different acids.

Fernbach made a series of comparative experiments for studying the combined actions of acid and sucrase. He performed, on different sugar solutions, two experiments, A and B, for each acid. In experiment A he used the optimum amount of acid with the addition of a certain amount of sucrase; in experiment B, he allowed the acid to act alone. By afterwards determining the amount of invert-sugar formed in each experiment, he has been able by subtraction to determine the amount of invert-sugar which may be attributed to the special action of the diastase. These experiments, carried out with different acids, have given him the following results:

Quantities of Acid per litre.	Sugar Inverted by Acid and Diastase.	Sugar Inverted by Acid.	Difference or Sugar Inverted by Diastase.
Sulphuric acid, 0.05 gr.	31.3	0.7	30.5
Oxalic " 0.066	30	0	30
Tartaric " 1	40	8.6	31.4
Succinic " 2	34.2	3.7	30.5
Lactic " 5	41.5	12.2	29.3
Acetic " 10	37.9	7.2	30.7

It will be seen that the figures of the last column, which designate the results of the diastatic action, properly speaking, are the same for all the acids, for the slight differences observed may be attributed to errors in measurement. This experiment demonstrates then the fact that we have stated above, namely, that the diastase is always influenced in the same degree by the different acids. However, the data furnished by Fernbach upon the influence of the medium apply exclusively to sucrase secreted by *Aspergillus niger* cultivated on Raulin's solution. It is probable that the same mould cultivated in other media would furnish sucrase solutions not having the same sensitiveness to reagents. Furthermore,

the determination of the amounts of acid checking or favoring diastatic action, has always been made by him at a temperature of 56°; it is, therefore, presumable that the figures which he has calculated are only correct for that temperature.

In reality, from 30° to 40°, the quantities of acid which correspond to the maximum of result are entirely different from the amounts necessary at a temperature of 56°. At these temperatures the amounts of acid must be multiplied by 5 to produce the same result as at 65°. According to O'Sullivan and Tompson, the maximum acid amount depends also upon the quantity of sucrase used, for they have found that with increased amounts of sucrase, increasing amounts of acid must also be used.

On the whole, the influence of the reaction of the medium on the rate of inversion is not a simple one.

Sucrase derived from yeasts differs from that obtained from *Aspergillus niger* in the resistance to the action of acids. The solution of sucrase which is obtained by extracting yeast with cold water is generally more sensitive to varying reactions of the medium than the diastatic solution extracted from *Aspergillus niger*. The sensitiveness of the sucrase of yeasts varies, furthermore, with the nature of the yeast used, and, for the same yeast, with the nutrition to which it has been subjected.

Fernbach has determined the amount of acid which is most favorable to the action of the sucrase in three kinds of yeasts (see the table on page 64). It is seen by an inspection of this table that the maximum amount of acid is about 0.2 cubic centimetres for the champagne yeast, about 0.5 cubic centimetres for *Saccharomyces Pastorianus* and the yeast of pale ale, while sucrase extracted from *Aspergillus niger* gives a maximum result only when much larger quantities of acid are present.

The considerable influence which the content of alkali in the medium exercises on the course of transformation has caused it to be supposed that the accelerating action of the

acid arises from a modification in the nature of the enzyme brought about by the action of this chemical agent.

Quantity of Acetic Acid per litre.	Champagne Yeast.	Saccharomyces Pastorianus.	Yeast of Pale Ale.
0	38.3	29.7	18.8
0.02	38.7	31.9	19.8
0.05	63.9	32.4	22.3
0.1	74.3	32.4	25.5
0.2	79.4	32.9	28.3
0.5	78.4	33	29.4
1	7.5	31.3	28.9
2	71.9	29.6	27.6
5			
10	50.4		

But this transformation in the nature of the diastase is very difficult to prove, and in any case appears to be slight.

The quantity of alkali clearly checking the inversion does not really cause an appreciable change in the active substance. The diminution of activity is due to the abnormal conditions of the medium, rendered refractory to the action of the enzymes by the addition of alkali. But the active substance evidently remains unchanged because it is only necessary to neutralize the liquid again to have the diastatic work resumed with all its initial intensity. It is only by increasing the amount of alkali to very large proportions that the diastase is destroyed, just as albuminoid substances are destroyed with the same amounts of the same agents.

Action of Oxygen and of Light.—Duclaux was the first to find that air exercised a very appreciable action on sucrase. He observed that a solution of sucrase in ordinary water changed color in contact with air and became inactive as a result of oxidation.

This oxidation of sucrase is influenced to a very great degree by the presence or absence of light, as well as by the acidity or alkalinity of the medium.

Sheltered from light and in a slightly alkaline medium, de-

composition by the oxygen of air is produced very rapidly; it is less pronounced in a neutral medium, and is manifested slowly in the presence of an acid. By exposing a solution of sucrase to the action of the air at 35°, 50 per cent of the active substance is destroyed in about 48 hours; at a temperature of 50° oxidation is more rapid and the same degree of change is reached after 4 or 5 hours exposure to oxygen. Light alone, in the absence of oxygen, is without action on sucrase. Fernbach has shown this by exposing to sunlight exhausted tubes containing sucrase. The sucrase remained unchanged for several months.

We have just seen that in darkness acids give to the diastase a considerable resistance to the action of air. When sucrase is not protected from light, this ceases to be the case and there are alkalies which become capable of protecting the enzyme against oxidation. By leaving in contact with air and light two solutions of sucrase, one slightly acid and the other slightly alkaline, it is found that the acid liquid undergoes a very rapid alteration, while the alkaline solution is preserved for a long time. This fact has been observed by Fernbach, who, by exposing three sugar solutions of different reactions to the action of the air and sunlight for 48 hours, found that they possessed at the end of that time the following diastatic powers:

Slightly acid solution	3.7
Neutral "	6.6
Slightly alkaline "	7.4

The favorable or unfavorable influence of the acidity or alkalinity of the medium on the oxidation of the sucrase has been very well shown by the following experiment:

Five solutions of sucrase showing a diastatic power of 18, some acid, others alkaline, in different degrees, were subjected, in darkness, to the action of the air at a temperature of 35° for 48 hours. Determination of diastatic powers at the end of the experiment gave the following results:

Numbers of the experiments.	Quantity of acid in millionths.	Diastatic power.
1	420	18
2	270	18
3	Neutral	17
4	75 soda	14.6
5	150 soda	10.6

We clearly see from this table the preservative action which acids exercise and also the destructive influence of alkalies.

The study of the effects of light and oxygen on inversion of saccharose leads us to a practical conclusion relative to the preservation of the diastase.

To preserve a solution of sucrase it is of foremost importance to avoid oxidation and consequently contact with air. For this purpose a vacuum is made in the partially filled flask, or else the diastatic solution is covered with a layer of oil. I have found in my experiments that a solution of sucrase prepared in this way still possesses all its energy after three months of preservation.

Action of Chemical Substances.—Sucrase is very sensitive to different chemical reagents. Duclaux has completely elucidated this question and has given figures which, without being absolute, are sufficiently accurate.

Calcium chloride markedly suppresses the action of sucrase and its retarding influence increases with the amount.

Chlorides of sodium and potassium, after having produced a favorable effect in slight quantities, such as 0.4 per cent, lessen the diastatic action when the quantity is increased.

Ammonium chloride, according to Nasse, acts very favorably in 10 per cent solution, and in small amounts is indifferent.

The action of alkaline salts and of bases is, according to Duclaux, retarding and destructive in the following amounts:

Salts.	0.1%	0.2%	0.4%	0.5%	0.8%
Sodium arseniate.....	4	.....	.....	7.2	
Sodium borate.....	1.4	25	5.6	.....	9.3
Sodium salicylate.....	.....	1	1.3		

Sodium salicylate up to 0.2 per cent seems to be without effect; with 0.4 per cent, the diastatic action is reduced, for we have to employ 1.3 of the diastase instead of 1.0 to obtain the same effect. Sodium borate and sodium arseniate retard hydration in a marked degree. The presence of 0.1 per cent reduces the efficiency of diastase to one-fourth the normal. Antiseptics affect very differently the diastatic power of sucrase.

Chloroform, ether, and oil of wintergreen, when in excess, reduce the activity of sucrase by about ten per cent. Toxic substances have also an inhibiting action, as appears from the following table (Duclaux):

Salts.	Per cent.				
	0.01%	0.02%	0.04%	0.1%	0.2%
Mercuric chloride...	.....	1.03	1.04	1.25	1.40
Silver nitrate.....	1.26	1.30	1.25	0.70	
Potassium cyanide..	.....	16.30	44	62	

Mercuric chloride has then a very slight retarding influence; in the presence of 0.1 per cent, the diastase is but little weakened.

Potassium cyanide is a very strong retarding agent; in the presence of 0.02 per cent, the power of the ferment is reduced to one-sixteenth the original. Silver nitrate first checks, then accelerates the transformation, due, according to Duclaux, to the acidity which it produces in the sugar solution.

Finally, 10 per cent alcohol produces a retardation expressed by the figure 1.3. Oil of garlic and other essential oils produce only inappreciable effects on the degree of inversion.

Formation of Sucrase in Living Cells.—We have seen above that all cells which are nourished with cane-sugar secrete sucrase. We will now discuss the most favorable conditions for the secretion of this enzyme by living cells.

In the cells of beer-yeast cultivated in a nutrient solution containing saccharose, sucrase appears. To explain this phenomenon, it must be stated that the cells found in the presence of non-assimilable substances produce a secretion capable of transforming these substances into assimilable materials. But on studying the phenomenon more closely, it is found that the secretion of sucrase does not strictly depend on the manner of nutrition of the cell; that it seems rather to be intimately allied to the nature of the organism, and that it is produced independently of the real needs of the cell. If, for example, in the nutrient solution cane-sugar is replaced by directly assimilable carbohydrate the secretion of sucrase continues. In this case, however, the nutrition does not at all necessitate the presence of this enzyme. Though the nature of the sugar does not possess any influence on the secretion of sucrase, one must not conclude that, as a general rule, the secretion of diastase is independent of the mode of nutrition of the cell. Experiment has shown, on the contrary, that the diastatic secretion is directly allied to the nature of the food, while being independent of the carbohydrate employed. Yeasts cultivated in beer-wort secrete much more sucrase than yeasts cultivated simply in sugar solutions; the secretion of sucrase is favored in this case by the nitrogenous materials of the malt. Experiment has shown, for example, that the addition of peptones increases the quantity of sucrase in the culture medium.

The substances most favorable for the growth of yeast are not always those which most favor the formation of sucrase Phosphates, for example, which influence yeast very favorably are, on the contrary, unfavorable to the formation of sucrase. Nitrogenous materials are not then the only ones having an influence on the secretion of sucrase. Unfortu-

nately, the conditions which favor the formation of diastase are imperfectly known. They merit thorough study, for they are of a nature to afford very interesting information from a theoretical point of view.

If the conditions favorable to the formation of sucrase are little known, we are much better informed as to the manner of diffusion of sucrase through the cells.

To study the mode of formation of sucrase in *Aspergillus niger*, Fernbach proceeded in the following manner: He sowed a certain number of dishes containing equal parts of Raulin's solution with a definite number of spores, all coming from the same culture of *Aspergillus*. He then subjected the liquid thus sown to a constant temperature of 35°.

He determined daily in one of his experiments the weight of the plants, the sugar remaining, and the acidity, as well as the amount of sucrase produced.

Each dish contained 400 cubic centimetres of Raulin's solution, 17.6 gr. of saccharose, and 0.72 gr. of free tartaric acid.

The results which he obtained are tabulated below:

	Saccharose remaining.	Invert-sugar.	Sugar assimilated.	Acidity. grams.	Sucrase.	Weight of the plant.	Ash.
After 2 days....	4.4	8.3	4.9	1.16	0	3.105	0.116
" 3 "	0.3	4.5	12.8	0.74	50	6.200	0.171
" 4 "	0	0	17.6	0.076	67	7.835	0.191
" 6 "	0	0	.....	0.038	104	6.870	0.200
" 8 "	0	0	.....	traces	285	5.580	0.198

By following in this table the figures placed under the heading "Sucrase," it is seen that at the beginning of the development of the young plant, when it is using great quantities of sugar, the sucrase does not appear in the culture liquid, and that one cannot detect its appearance until the sugar is exhausted and inversion no longer takes place.

This fact is of great interest; it shows us that inversion is not produced in the liquid which surrounds the mould. The presence in the liquid of 8.3 grams of invert-sugar after two days tends to confirm the opinion that the transformation takes place inside the cell. If we accept this hypothesis, we must at the same time assume that sucrase exists in the cell from the beginning, and the diffusion noted is produced as the result of a modification of the cell contents.

In fact, Fernbach, in looking for sucrase in the plant, found that the greatest quantity of diastase secreted by the cells appeared at the beginning of its development, and that the moment of its appearance outside coincided with the instant when the plant had already caused the greatest quantity of sugar to disappear.

	Saccharose remaining.	Invert-sugar.	Sugar consumed.	Acidity.	Sucrase of the liquid.	Sucrase of the cells.	Weight of the plant.
After 1 day.....	1.36	2.36	0.92	0.293	2	58	0.65
" 2 days....	0.22	1.65	2.57	0.368	3	47	1.265
" 3 " ...	0	0.7	3.74	0.267	5	45	1.78
" 4 "	0	0	4.44	0.143	10	44	1.65
" 5 "	0	0	.....	0.135	13	35	1.61

The diffusion of the sucrase at the moment of the disappearance of the sugar may then be considered as a consequence of the disintegration of the plant. When we reflect a little, we realize that, in cells well nourished and fortified with reserve food, the diffusion must be accomplished with great difficulty.

With the disappearance of the invert-sugar in the liquid, the cells begin to consume their reserve food; vacuoles are formed and filled with water, which certainly facilitates diffusion.

Moreover, there are some very conclusive experiments which prove that the diffusion of diastases characterizes a pathological state of the cells:

Place two young and identical cultures of *Aspergillus niger*, one in water, the other in a rich nutritive medium. After 48 hours examine the two liquids and it will be found that the first medium contains a great quantity of active enzymes, while the second has no trace of any. Denutrition then favors the secretion of sucrase. One may, moreover, take a culture of *Aspergillus niger* and exclude it from the action of the air. Thus fructification is prevented and this abnormal condition causes, like inanition, an abundant diffusion of the diastase in the culture medium. Finally, beer-yeast suspended in water may be heated for several seconds at 100°. Thus the active substance is completely destroyed as well as a majority of the yeast cells. On allowing the liquid to cool one notes, after a while, the appearance of sucrase. The secretion of the enzyme may be attributed to the cells which have escaped the destructive action of the heat, although greatly injured by the high temperature to which they have been subjected. They are then in a certain pathological state and diffuse with ease the active substance they contain.

As we see by these experiments, the lack of sugar or oxygen, the elevation of the temperature, etc., may equally favor the diffusion of the sucrase secreted by the cells.

Measurement of Sucrase.—We may easily ascertain the transformation of saccharose into invert-sugar by the aid of Fehling's solution. Cane-sugar does not reduce this solution, while 0.4941 gr. of invert-sugar reduce 100 cubic centimetres of Fehling's solution.

The transformation of saccharose into invert-sugar may likewise be ascertained by the change of optical activity that accompanies the transformation. Cane-sugar rotates to the right and the mixture produced by hydrolysis on the contrary, rotates to the left. Saccharose gives a rotation to the right of $\alpha j + 73.8$, and invert-sugar a rotation to the left of -44. The measurement of sucrase requires, first, the determination of the quantity of invert-sugar. Now, the same

quantity of sucrase may furnish greater or less quantities of invert-sugar. These variations are due to the different factors which we have indicated; the reaction of the medium, temperature, duration of the action, etc. It must not be forgotten that the ratio between the quantities of ferment employed and of invert-sugar obtained only exists in early stages of the action, and before 20 per cent of the total amount of sugar submitted to the action has been transformed. Hence, it is absolutely indispensable, in order to be able to compare two diastatic products, to place them in identical conditions. To avoid errors arising from the acidity, care must be taken to neutralize the liquid as exactly as possible, then to acidulate with 1 per cent of acetic acid. The choice of acetic acid is not arbitrary; it is due to the following reasons: acetic acid can be used in considerable, and, consequently, easily measureable quantities. It does not displace the other organic acids of the solution, and, finally, it has little influence on the sucrase. In the measurement, the greatest care should be taken to prevent oxidation of the sucrase, and to this end the analysis should be made as rapidly as possible. Generally one should let the sucrase act only for one hour.

To avoid the errors which may come from the deviations from the law of proportionality between the quantity of enzyme used and the quantity of sugar inverted, one must seek the quantity of sucrase capable of transforming a certain quantity of cane-sugar, and not the amount of sugar which a given quantity of sucrase can invert.

In the method of measurement suggested by Fernbach, one takes as the unit the quantity of sucrase capable of inverting 20 centigrams of saccharose in one hour at a temperature of 56° in the presence of 1 per cent of acetic acid. To perform this measurement the solution of sucrase is previously neutralized, then in a series of reaction tubes, each containing 4 cubic centimetres of a 50 per cent solution of saccharose, is added 1, 2, 3, 4, 5, cubic centimetres of the

sucrase solution to be analyzed; one cubic centimetre of acetic acid (1 :10) is added; the volume in each tube is brought up to 10 cubic centimetres. The tubes are left for one hour at a temperature of 56°, then quickly cooled; and several drops of a soda solution are added to arrest inversion, and the quantity of invert-sugar formed in each is estimated by the use of Fehling's solution. It can thus be seen in which of the tubes the 20 centigrams of sugar have been inverted.

Let us suppose that this result was obtained in the tube containing 5 cubic centimetres of sucrase: one then finds present a solution containing only traces of saccharose.

Since the amount of acetic acid used in the experiment might itself have inverted some centigrams of sugar, it may be that the whole of the inversion is due to foreign substances and not to the diastase.

In order to be certain that the transformation of saccharose is due to the effect of a diastase, the experiment must be made once without heating, and again with a solution heated to 100°, to see if the results are the same.

In case 1, 2 or even 3 cubic centimetres of solution are sufficient to obtain the transformation of 20 centigrams of sugar, the inverting power of the solution is considerable, and by repeating the experiment with $1\frac{1}{2}$, $1\frac{3}{4}$, 2, $2\frac{1}{4}$, etc., cubic centimetres of the solution experimented with, a very accurate measurement of the diastatic activity may be obtained.

In case $1\frac{1}{2}$ cubic centimetres of solution must be used to obtain 20 centigrams of invert-sugar, we say the unit amount of sucrase is found in $1\frac{1}{2}$ cubic centimetres and, consequently, that the solution possesses two-thirds of the diastatic power of the standard solution.

The method of Fernbach gives fairly accurate results, but it demands many trials and a long series of measurements which take a great deal of time.

When it is a question of a qualitative rather than quanti-

tative estimate, the measurement of the sugar may be completely omitted.

In order to test for sucrase in liquids we make use of a very expeditious method requiring only half an hour, and in which the inversion is ascertained by the color which the inverted solution gives with soda.

For this kind of experiment we make use of a 10 per cent solution of sugar. The liquid in which the sucrase is measured is neutralized as accurately as possible with soda (1 : 1000). In two test tubes, A and B, are poured 10 cubic centimetres of sugar solution; to A is added one cubic centimetre of diastatic solution and to B one cubic centimetre of the same solution previously heated for several minutes to 100°. The two tubes are left for thirty minutes at 50°. One cubic centimetre of ordinary soda is added to each of the tubes and heated 5 minutes at 98°. If a solution of sucrase is present, tube A takes a much deeper color than tube B.

It is possible to use this procedure as a colorimetric method.

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CHAPTER VI.

SUCRASE (*Continued*).

Retarding factors and their explanation.—Deterioration and alteration of sucrase.—Experiments of Effront on the influence exerted by invert-sugar in the medium in which the inversion is produced.—Hypothesis of O'Sullivan and Tompson.—Arguments for and against this hypothesis.—Theory of Effront on the decomposition of cane-sugar, and experiments on the manner of action of acids in the inversion of saccharose.

Factors Retarding Inversion and their Explanation.—When we examined the course of the transformation of saccharose by sucrase, we noted that the quantity of invert-sugar formed during a given time diminished constantly with the progress of the inversion. This diminution is produced in such a manner that the last portions of saccharose which remain in the solution are transformed very slowly, whereas at the beginning of the transformation the inversion is accomplished much more rapidly.

Various hypotheses have been put forth for explaining the irregularities which are observed in the hydration of cane-sugar. Certain authors attribute the observed retardation to a deterioration or alteration of the sucrase, a change taking place in proportion as the work of hydration progresses. According to other authorities the retardation in the inversion proceeds from the disappearance of cane-sugar, whose presence favors diastatic action. Finally, the hypothesis that the products of transformation accumulated in the liquid check diastatic action may give a probable explanation

of the irregularity with which the transformation proceeds. In this last hypothesis, the efficiency of the enzyme would be hindered by the invert-sugar formed during the action.

Let us see on what facts these different hypotheses rest, and seek a rational interpretation of the irregular course of the inversion.

Deterioration and Alteration of the Diastase.—The hypothesis explaining the retarding of the inversion by a deterioration of the active substance during its work does not appear to us to merit a serious discussion. The ratio observed at the beginning between the duration of the action and the quantity of sugar transformed affords a conclusive proof of the permanence of the diastase. In fact, if after the second hour of action we can ascertain that the quantity of sugar transformed is double that which we have found after the first hour, it is very evident that the diastase has acted during the second period of operation with the same energy as during the first. The work done during the first hour has not then caused any destruction of the active substance, and it appears difficult to us to believe that weakening, which is not found at the beginning, can be produced during the course of the operation.

Moreover, the mode of action of enzymes excludes all idea of deterioration of the active substance in the course of transformation.

In studying the mode of action of amylase, we have had occasion to make evident by direct experiments the enduring character of the diastase during activity, and we believe that the explanation which we have given of this phenomenon can be generalized and extended to all analogous phenomena, for the slackening has identical characteristics in a great number of diastatic actions.

The hypothesis of the alteration of the diastase during its activity appears to be very probable. In fact, numerous chemical agents, as well as various physical conditions, influence the sucrase differently and to a very great degree.

In the experiments cited in the preceding chapter, for example, the slackening in the transformation must unquestionably be attributed to the combined action of oxygen and light.

Still, we cannot attribute the irregularity in inversion entirely to physical or chemical causes, for, even when avoiding the action of light and oxygen, it is still found that irregularity occurs.

Furthermore, the alteration of sucrase caused by oxygen does not become appreciable till after a prolonged contact with the air, while the ratio soon ceases to hold when the diastatic action takes place in a very dilute solution of sugar, or for that matter, when large quantities of sucrase are put in action.

We have seen that in placing, under certain fixed conditions, a mass of sucrase in the presence of any quantity of sugar it is found that just as much invert-sugar is formed during the second hour as during the first.

If, under the same conditions, ten times as much sucrase is employed, this equality of work during the first and second hours of the action no longer holds good, but the proportionality will be observed if quantities of invert-sugar are compared after 10 and 20 minutes of work.

If the amount of sucrase is again augmented, the ratio is found to hold during the first part of the action, but to cease after ten minutes.

As we see, the retarding force may appear in the liquid at different times according to the amount of sucrase used. If then we accept the hypothesis attributing the slackening to an alteration of the active substance, we must assume at the same time that the same sucrase may alter very rapidly or very slowly according as it is used in a large or small amount.

Finally, as the proportionality ceases at different times for the same quantity of sucrase put in sugar solutions of different densities, it must be assumed that the quickness of the alteration depends, not only on the amount of enzyme

used, but also on the concentration of the sugar solution. The improbability of this theory can be seen.

It results from the facts which we have just presented that neither wearing out by working nor alteration by physical or chemical agents can be the true cause of the slackening of the diastatic action.

Experiments on the Influence of the Invert-sugar. —Most authors have attributed the failure of the proportionality during the course of the transformation to the invert-sugar formed, which, according to them, checks the diastatic action.

We have sought to verify by a direct experiment this retarding action of the invert-sugar.

To this end two solutions, A and B, were made, each containing 100 cubic centimetres of water, 5 grams of saccharose, 1 cubic centimetre of acetic acid, and 10 cubic centimetres of yeast sucrase. In solution B was added 2 grams of invert-sugar. These solutions were left in a water-bath, and from time to time samples were taken in which the quantity of reducing sugar formed was determined.

Minutes.	Solution A. Reducing sugar formed.	Solution B. Reducing sugar formed.
15	0.26	0.25
30	0.51	0.52
45	0.79	0.74
60	0.9	1.11
90	1.2	1.2
120	1.4	1.32
180	1.75	1.89

It is seen from this table that the proportionality ceased after 45 minutes of action in solution A, which contained only cane-sugar, and that the weakening of the diastatic power began when about one-twentieth of the total sugar

contained in the solution had been transformed. In solution B, which contained 40 per cent of invert-sugar at the beginning of the action, inversion was not at all retarded during the first 45 minutes. On the contrary, the transformation appeared to conform very nearly to the law of ratio, and the slackening in the transformation was not manifested until after an hour of action.

By comparing the quantities of sugar inverted during the first hour in experiments A and B, we find that only 18 per cent of saccharose had been transformed in the experiment made with pure sugar, and 22 per cent in the experiment made with a mixture of saccharose and invert-sugar. The slackening in the action of the diastases must not then be attributed to the presence of the products of transformation in the medium where the diastatic work is performed.

Hypothesis of O'Sullivan and Tompson.—O'Sullivan and Tompson have put forth the hypothesis that the effect produced by sucrase is constantly proportional to the weight of the cane-sugar present in the liquid at the time of action. Starting from that, they attribute the retardation produced in the transformation to the diminution of the quantity of saccharose as the inversion proceeds. According to this view, the sucrase would act in the same way and with the same energy from the beginning to the end of the action, and the slackening would be exclusively due to the reduction of the supply of saccharose.

Then, if we invert a solution containing 10 grams of sugar by the aid of a quantity of sucrase able to produce a gram of invert-sugar in the first 10 minutes, we may expect to have produced during each of the following ten minutes a hydration corresponding to one-tenth of the total quantity of cane-sugar contained in the solution at that time.

According to this theory, the mode of action of sucrase would not change during the work; the course of the inversion would be on the whole regular and the slackening observed would be the direct and inevitable consequence of the

regularity of the phenomenon itself. For, if after the first 10 minutes of the action we have found the production of one gram of invert-sugar, after the following 10 minutes, we shall have obtained only 0.9 grams, inasmuch as the action is produced in this case no longer upon 10 grams, but only upon 9 grams of cane-sugar. After 20 minutes, there will remain in the solution 8.1 grams of saccharose and, by acting always under the same conditions, the sucrase will invert during the following 10 minutes 10 per cent of the sugar remaining, or 0.81 grams.

This hypothesis is, these authors say, fully confirmed by the measurement of the quantities of sugar inverted at the end of periods varying in arithmetical progression.

Arguments for and Against this Hypothesis.—The theory of O'Sullivan and Tompson is very attractive; it has not, however, found many adherents, and various objections have been raised against it.

First, it has been objected that the experimental proofs which they bring forward in favor of their theory do not at all prove that the slackening comes from the diminution of the quantity of cane-sugar. In fact, the results of their experiments may equally well be explained by the gradual increase, during the action of the sucrase, of the quantity of invert-sugar.

Our experiments, cited above, on the influence of the invert-sugar, show the invalidity of this argument.

But, still another objection may be raised to the theory of O'Sullivan and Tompson. If the gradual disappearance of cane-sugar is really the retarding cause, the quantity of sugar inverted by any amount of sucrase will be in direct relation with the weight of the cane-sugar present in the liquid. Increase in the amount of cane-sugar will then cause a corresponding increase in the amount of sugar inverted. We already know that these expectations are not always realized, and that the same quantity of sucrase produces the same amount of invert-sugar, independently of the concentration

of the sugar solution. There, then, is a serious argument against the hypothesis that we are considering, but it is none the less true that the quantity of sugar contained in the medium is not without influence on the slackening. By studying the phenomenon more closely, we find that the degree of hydration depends upon two factors.

At the beginning of the action it is the quantity of sucrase used which plays a predominant part, and the quantity of invert-sugar formed is proportional to the quantity of active substance used. When the inversion is more advanced, the influence of the quantity of sucrase becomes less. The transformation becomes directly proportional to the sugar content.

The successive influence of the two factors may be shown by the following experiment:

To 100 cubic centimetres of three liquids, A, B, and C, containing respectively 5, 10, and 20 grams of saccharose, add the same quantity of sucrase. Then leave these solutions in a water-bath at a temperature of 50°. From time to time, take samples, and determine the saccharose remaining in them and, when in solution A 15 per cent of saccharose has been transformed, begin to measure the invert-sugar in the two other specimens. There is then obtained the following figures:

Invert-sugar at the end of	A	B	C
2 hours.....	0.75	0.74	0.78
4 "	1.1	1.4	1.6

One finds then at the beginning of the action almost the same quantities of sugar transformed in the three liquids, A, B, and C, but at the end of four hours the conditions change and there is found in the solution containing 20 per cent of sugar 1.6 gr. of transformed sugar, while the 5 per cent liquid affords only 1.1 gr. of invert-sugar.

The concentration of the sugar solution, then, influences

the action of the sucrase up to a certain point. The course of the hydration of the sugar in solutions of different concentrations is shown to be rather favorable to the theory of O'Sullivan and Tompson, especially if the beginning of the transformation is excluded.

Still this theory does not appear to us to be based on very well established data.

By noting the transformation of the sugar at different periods we have, indeed, found that the slackening in the inversion increases in proportion as the action advances, but we have never been able to see the regularity which the authors of the hypothesis claim, and which is the very basis of their theory.

Even admitting that it can be demonstrated experimentally that the decrease in the inversion varies in geometric progression, this demonstration would show the way in which the retardation takes place, but it would not at all reveal the real cause. By making the same quantity of sucrase act on sugar solutions of different concentration, it is seen that the retarding force is manifested very differently. In a dilute solution, the proportionality between the duration of the action and the quantity of sugar formed is lost at the end of a relatively short time. In a concentrated solution, on the contrary, it persists for a longer time.

These great differences in the action of sucrase are easily explained if one determines in liquids of different concentration the quantitative relation which exists between the sugar inverted and that which is not yet inverted.

By studying the variations of this relation for a dilute solution and a concentrated solution, it is found that the slackening of hydration does not become really appreciable until the sugar solutions contain about 15 parts of invert-sugar to 85 parts of unchanged saccharose.

It being granted that sucrase produces, at the beginning of the transformation a hydrating effect proportional to its quantity, it is quite evident that in the dilute solution the ratio

$\frac{15}{85}$ will be reached much more quickly than in the concentrated solution.

In other words, when the slackening of hydration is first observed, the concentrated solution contains more invert-sugar than the dilute solution, although the quantity of sucrase put in action would be the same in the two solutions.

The retardation in the inversion depends directly upon the composition of the liquid in which the diastase acts. It is not caused by the diminution of the quantity of cane-sugar contained in the solution, and neither does it arise from the increase of the quantity of invert-sugar. It is rather caused by the combination of the two circumstances.

Theory of Inversion of Cane-sugar.—We must, we believe, seek in the structure of the molecules of saccharose the real origin of the retarding force. It is generally supposed that the action of the sucrase is manifested by the successive hydration of molecules of sugar with which it comes in contact. It is, however, probable that the mechanism of inversion does not possess this simple character.

It is more probable that the sucrase acts from the beginning on the whole mass of sugar with which it comes in contact, and that, in the transformation of cane-sugar into invert-sugar, it produces a series of modifications. It is easily conceived that, by successive hydrations, there may be formed, besides invert-sugar, a series of substances very similar to saccharose, but which may have a different degree of sensitiveness to the sucrase. Such are the intermediate substances produced by hydration which afterwards appear more or less suitable for the transformation, and it is from this greater or less susceptibility to hydration that the checking of the inversion comes.

It may be, too, that the changes undergone by the saccharose consist in changes in the configuration of the molecules, and that stereochemical isomers are formed in the solution.

Still it is impossible for us to bring forward convincing

facts in favor of our hypothesis. Having predicted the formation, in the course of inversion, of products intermediate between the saccharose and the invert-sugar, we have sought to isolate these products, or at least to characterize them. But the various experiments attempted with this in view have remained without results. Still the hypothesis, as we shall see, finds support in the process of hydration by acids.

Experiments on Transformation by Acids.—By studying the inversion of sugar in the presence of increasing amounts of acid, we have been able to ascertain that there is produced at certain periods a noticeable retardation in the progress of hydration. The slackening takes place at times which always coincide with certain stages in the hydration of saccharose. Thus there is a striking analogy between the action of acids and that of sucrase. The retarding force is found in both cases, in the action of the acids as well as in the diastatic action, and the moment when the slackening begins corresponds to the instant when the relation between the quantities of invert-sugar and of non-transformed sugar reaches a certain value.

This resemblance between the mode of action of chemical and of physical agents proves that the retarding force does not come from the sucrase, and that the origin of the retardation is not necessarily to be attributed to the manner of decomposition of the cane-sugar and to the formation of transitory products which resist differently the agents of transformation.

Some details of the experiments which we have made are here given:

Dissolve a gram of cane-sugar in distilled water, add 2 cubic centimetres of $\frac{n}{10}$ sulphuric acid, and bring the volume up to 100 cubic centimetres. Leave in water-bath at 60°, for one hour, then neutralize accurately with normal soda, and determine the quantity of invert-sugar formed by the action

of the acid. This experiment is then repeated with 4, 6, 8, 10, etc., cubic centimetres of $\frac{n}{10}$ sulphuric acid, and the following results are obtained:

Cubic centimetres of acid.	Percentage of invert-sugar.	Increase.
2	5.71	5.71
4	11.36	5.65
6	15.29	3.93
8	22.12	6.83
10	26.34	4.22
12	32.00	5.16
14	37.14	5.14
16	46.76	9.62
18	51.36	4.60
20	53.33	1.97
22	52.00	1.33
44	65.20	1.20

The heading " Percentage of invert-sugar " indicates the quantity of sugar transformed during the experiment.

Under the heading " Increase " we have written the increase in the quantity of sugar transformed by each addition of two cubic centimetres of acid.

By following, in the table, the progress of the hydration in the presence of increasing amounts of acid, it is found that the ratio between the quantities of acid and invert-sugar formed is not at all constant. This ratio exists in the first experiments and disappears completely in those where 50 per cent of cane-sugar is transformed. Thus 2 cubic centimetres of acid have formed 5.71 centigrams of invert-sugar; with a double amount, 11.36 cg. of invert-sugar are formed, or practically double the preceding quantity. If we increase the amount of acid and use 20 cubic centimetres we cause a hydration of 53 per cent of sugar; but beyond this amount of acid the hydration slackens and 44 cubic cen-

timetres of acid hydrate only 65 per cent of the cane-sugar present in the liquid. If the ratio really existed, with this amount of acid a complete inversion of all the saccharose contained in the liquid would be obtained.

The action of increasing amounts of acid is still better shown when the heading "Increase" is followed. In the first experiments the increase gradually falls from 5.71 to 3.93, but in the following ones, when nearly a quarter of the whole quantity of cane-sugar has been transformed, a complete change is found in the progress of the inversion. The increase rises to 6.83 to fall again to 5.14.

The increase is again augmented when half the cane-sugar has been transformed, then it undergoes another decline, and is represented by the number 1.2 in the presence of 65 per cent of invert-sugar.

The course of hydration by acids is then far from being regular. A great number of analogous experiments, made under the same conditions, have always confirmed in our work the non-existence of a ratio between the quantities of acid used and invert-sugar formed. We have always found a slackening in hydration, which coincides with the appearance of a definite ratio between the quantities of sugar inverted and of non-transformed sugar in the liquid.

The action of acids is then, in the main, identical with that of the diastase, and the retardation observed in hydration by sucrase must rather be attributed to a structural transformation of the saccharose molecule.

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CHAPTER VII.

FERMENTATION OF MOLASSES.

Industrial application of sucrase.—Fermentation of molasses.

THE enzyme producing the inversion of saccharose does not constitute a commercial article. It has a very limited manufacture and serves exclusively for study and for laboratory experiments. But, if specially prepared sucrase is not used in industrial operations, this diastase nevertheless plays an important part in fermentations, and especially in the manufacture of alcohol from molasses.

The fermentation of molasses, a substance which contains nearly 50 per cent of saccharose, is a relatively simple operation. It is effected in the following manner:

The molasses is first diluted with water acidified with sulphuric acid, and brought to from 9° to 12° Beaumé.

Thus a mash is formed ready to undergo the action of the beer-yeast which inverts the saccharose and causes the invert-sugar produced to ferment. The transformation of molasses into alcohol appears then to be a simple industrial operation. The apparatus required is, in fact, less complex than that of a grain distillery. Moreover, the working of the molasses requires relatively little supervision, and much less practical knowledge on the part of the operators than does the distillation of grains. Yet there are few manufactories which utilize rationally the former materials and secure results even approximating the theoretical yield.

Manufacturers attribute the difficulties they encounter

either to the quality of molasses used, or to the inefficiency of yeasts, or to infection by foreign ferments, and they seek to remedy these imperfect conditions by a strong acidification of the mash. They sometimes try also to regulate the work by a preliminary heating of the molasses in order to eliminate the volatile organic acids. These acids are freed by the addition of sulphuric acid to the mash at the time of acidification.

The causes which occasion these troubles in the working of molasses are very numerous. We cannot here enter upon a thorough study of this question, but, nevertheless, we think we ought to call the attention of manufacturers who are occupied with fermentations to some of the very frequent causes of the smallness of the output, especially the insufficiency of inversion.

In the practice of distilling molasses this point is wholly neglected. Although it is known that non-inverted sugar is not fermentable, little importance is attached to the inversion of the saccharose of molasses, because of the current opinion that inversion is very easily accomplished owing to various conditions of the medium.

If the question is studied more closely, it is seen that, on the contrary, inversion is very slow, and that in most cases it is not completed at the end of the fermentation.

The reason that in practice little attention is paid to hydration is that during fermentation two factors are generally counted on: first, the sulphuric acid which has been introduced into the molasses and which is considered sufficient in itself to produce inversion; second, the yeast which is supposed to be an inexhaustible source of sucrase.

Let us see to what extent each of these two factors contributes to the inversion, and let us first study the part played by the acid.

By the addition of acid to mashes of molasses there is secured in practice an acidity corresponding to from 1 to 2.5 grams per litre of sulphuric acid.

The acidification of the musts is done at a low or high temperature according to the distillery.

To give an idea of the inverting power which these amounts of acid possess, let us add to a certain number of 100-gram specimens of a 10 per cent solution of cane-sugar different amounts of sulphuric acid and let us submit these experiments for 24 hours to a temperature of 30°.

Numbers of the specimens.	Number of grams of sulphuric acid per litre.	Grams of invert-sugar.
1	2.5	1
2	5	1.8
3	10	3.3
4	25	6.7

Thus when we submit our experiment to the action of 2.5 grams of sulphuric acid, the greatest amount used in manufacture, we obtain at the end of 24 hours only 10 per cent of invert-sugar, and in order to obtain 67 per cent, we must use 25 grams of acid, or a quantity 10 times greater.

The action of the cold acid is not then an important factor in inversion. The results obtained by boiling the sugar solutions appear quite different, it is true.

If, to ascertain the influence of high temperatures, we repeat the preceding experiments at 90°, we shall observe that with the smallest quantity of acid (0.5 gr. per litre) we produce a complete inversion of the saccharose.

We may conclude from this, that heating the molasses with slight amounts of acid present is very important from the point of view of inversion. But molasses does not act towards the different factors in the same manner as the solutions of pure sugar. In fact, the acidity found in mashes of molasses comes, not from mineral acids which have been added to them, but rather from organic acids which have been freed by the sulphuric acid, and which act on the saccharose with much less energy than inorganic acids. More-

over, the presence of salts in the molasses weakens the action of the acids.

The effect produced practically by heating acid molasses may be shown by the following experiments: 100 grams of molasses are diluted in 400 grams of water. Different specimens of this must are taken; they are acidified with varying amounts of sulphuric acid; they are kept boiling for some time, then cooled and brought up to their original volume. By examining the rotatory power of these specimens, one may study the course of the transformation in the presence of different amounts of acid. The solution before inversion gives a rotation of 38° to the right, and after complete inversion a rotation of 8½° to the left. Moreover here are the intermediate results:

Numbers of the specimens.	Grams of sulphuric acid per litre.	Rotation to the right.
1	1.25	37°
2	2.5	36
3	5	35
4	10	24
5	12.5	3.6

With the amount of acid used in manufacture, or two grams and a half, inversion is then trifling; rotation decreases only from 38° to 36°. We find, indeed, that by using five times as much acid, we are still far from obtaining complete inversion; sulphuric acid used in an amount of 12.5 gr. still gives us a rotation *to the right* of 3.6°, while complete inversion would have given a rotation *to the left* of 8.5°.

In many manufactories it is the custom to boil acidified molasses after diluting it with water. It is seen that in this case practically no inversion takes place. We have been able, however, to ascertain that the inversion is hastened when the acid molasses is heated before dilution.

In the practice of distilling molasses it is the sucrase of

the yeasts and not the acids used which produces hydration of the saccharose, and the course of the fermentation depends, in very large measure, on the manner in which the secretion of diastases by the cells is accomplished. Now, the action of the sucrase is considerably influenced by the saline substances contained in the molasses. The following experiment is well calculated to demonstrate this fact:

' Acidify a sugar solution at 12° Balling with sulphuric acid in the proportion of 0.5 grams per litre, and take from this must two specimens, A and B. To specimen A, which is the control specimen, add 10 cubic centimetres of yeast sucrase. The second specimen receives the same quantity of sucrase, then the accurately neutralized ash of 100 cubic centimetres of a must of molasses of 12°, Ball. The two specimens are then left in a water-bath at 30°.

Here is the comparative progress of inversion in the two specimens:

Minutes.	A. Invert-sugar.	B. Invert-sugar.
40 min.	4.7%	2.4%
2 h.	5.79	2.9
3 h.	7.0	3.2
4 h.	9.2	4.6

These figures prove in a conclusive manner that the mineral substances of the molasses retard inversion considerably. After four hours of action, there is found in the control solution 9.2% of invert-sugar, while in the solution to which ash of molasses has been added there is found only 4.6%. These data show the nature of the difficulties encountered in the fermentation of molasses.

Still it may be objected that in the fermentation industries, a solution of diastase is not used: but that inversion is accomplished by living cells. It must then be admitted that the conditions of transformation are completely different; that since inversion by yeasts can be accomplished inside the

cells, the composition of the exterior liquid naturally has a much smaller influence on that account.

To answer this objection we have made the following experiments:

To a 10 per cent solution of cane-sugar is added yeast ash. This solution serves as the basis of two specimens, A and B, each of 500 cubic centimetres. In specimen A there are introduced the neutralized ash of 50 grams of molasses and 5 grams of yeast. Specimen B is submitted to the influence of the same quantity of yeast, but without the addition of salts.

Here is the comparative course of the fermentation of the two specimens:

		A	B
After 6 hours	Invert-sugar	0.5%	1.8%
	Alcohol	0.4	0.65
After 12 hours	Invert-sugar	0.2	3
	Alcohol	1.5	2.6
After 24 hours	Invert-sugar	0.5	0.2
	Alcohol	3	5.9

By comparing the quantity of invert-sugar after six hours in the two solutions, we see that inversion proceeds much more slowly in solution A, treated with the salts contained in the molasses. It is true that after 24 hours we find a greater quantity of invert-sugar in solution A than in solution B, but if we take into account the quantity of alcohol present at this time in the two solutions, it becomes evident that hydration has followed a much more regular course in B than in A.

Phenomena of this nature—slowness in fermentation, irregularities in the progress of the transformation—are often found in molasses distilleries and are generally attributed to the degeneracy of the yeasts.

This opinion is absolutely erroneous; yeast does not generally degenerate in molasses mashes; on the contrary, it re-

produces abundantly and the cells formed under these conditions generally have great activity. These yeasts cause a very rapid fermentation of grain mashes, but the quantity of sucrase which they secrete diminishes.

It is to this diastatic weakening that we must attribute the difficulties encountered in fermenting unchanged saccharose with yeasts cultivated in molasses.

Not all the beer-yeasts contain the same quantity of sucrase; the quantity of the enzyme secreted varies with the special variety. In the choice of a yeast for molasses, one should first take account of the inverting power, as well as of the degree of resistance of the active substance which it contains. Generally the distiller seeks to substitute quantity for quality in the yeasts. This practice is far from rational. The expense in yeast is thus rendered quite large and the alcoholic yield is diminished, for the yeast consumes a part of the carbohydrate for the construction of its tissues and for their maintenance.

To test a yeast with a view to its action on molasses it is not enough to determine its inverting power in a solution of pure saccharose. It is better to make the experiment with saline substances present. The advantage of this method is that it gives more certain results, because it approximates actual industrial conditions. We have had occasion to make experiments with yeasts from different sources, and these experiments have shown us that the degree of resistance of the sucrase contained in the cells differs much according to the variety. These experiments have proved to us, moreover, that the resistance of the enzyme plays a very important part as influencing the yield.

Compressed yeasts as well as beer-yeasts have been replaced in the fermentation of molasses by leaven. The distiller cultivates his yeasts himself and for this purpose uses mashes of grain prepared either by sulphuric acid or by malt.

There is generally used for the preparation of the mash

leaven 3 to 5 kilograms of grain for 100 kilograms of molasses. In many distilleries this amount of grain is increased. Sometimes it is thought best to add to the molasses or to the yeast mash a certain amount of nutritive nitrogenous materials, such as rootlets of malt, amides, and peptones.

Undoubtedly the use of grains and nitrogenous materials furnishes appreciable results with certain varieties of yeasts which demand a special medium to acquire their inverting power. It must, however, be recognized that the very principle of this practice is wholly false and that the results obtained are far from being satisfactory from an economic point of view. Molasses contains all the nutritive substances necessary to feed the yeast-cells abundantly.

If a variety of yeast cannot become accustomed to acting upon molasses, if a special manner of nutrition must be adopted so that it can live in this medium, this yeast should be given up and another less delicate kind should be used.

While visiting the molasses distilleries at Breslau, Leipzig, Darmstadt, etc., in 1895, we found out that the leaven used for the fermentation of molasses in these manufactories cost from 8 to 10 francs per hundred litres of alcohol manufactured. This expense, a total loss, arose from the fact that the distilleries used, for the making of their leaven, malt and grains without which their yeasts did not work well. We advised them to take a yeast suitable for action upon molasses, and we have since had the satisfaction of learning of the almost complete suppression of the use of grain for leaven in those places.

The leaven is at present made with pure molasses and the yield in alcohol is unquestionably greater.

Most kinds of beer-yeast furnish a sucrase of little resistance, but their greater or less alterability depends especially on the culture medium in which they are developed. Sucrase secreted by yeasts cultivated in molasses possesses a resist-

ance inferior to that of the same yeasts cultivated in a must of grains or malt.

This weakening of the resistance is due neither to the nature of the enzyme secreted nor to the prolonged contact with saline substances; its true cause is rather the sudden passage of the cells from one medium to another.

We have found that yeasts capable of producing the fermentation of dilute molasses can be brought to effect the complete fermentation of very concentrated mashes by acclimating these yeasts to the new medium by gradually furnishing them with solutions of increasing concentration.

The changes which are produced in the resistance of a sucrase by acclimatization to the medium may be demonstrated by the following experiments:

. Sucrase is extracted from yeasts in different stages of habituation and tried simultaneously on a solution of pure sugar, and on a solution of sugar to which ash of molasses has been added. It is thus found that the yeast has acquired new properties and furnishes an enzyme which is little changed when saline substances are present. These new properties acquired by the yeasts are, however, transitory properties. *

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* These experiments throw a special light on the mechanism of acclimatization as well as on the individuality of diastases.

By studying the sensitiveness of beer-yeasts to the action of different antiseptics, we have established the fact that one can acclimatize the yeasts to relatively large amounts of these agents. Thus, a beer-yeast which shows itself very sensitive to the action of 10 milligrams of hydrofluoric acid and which no longer gives any fermentation in the nutritive mash can be made to reproduce in the presence of 30 times as much of this acid, and to give rise to very active fermentation. This acclimatization requires that one accustom the yeast to increasing amounts of this antiseptic.

The cells thus obtained acquire characteristic properties:

The fermenting power is considerably augmented, while the power of multiplication is reduced to its lowest limits.

Yeast acclimated to antiseptics preserves the characteristic property of resisting them for several months, even when it is daily cultivated in solutions free from the agent to which they have become accustomed.

The difficulty of inversion in the fermentation of molasses may also arise from some other cause than the insufficiency of the sucrase of the yeast. Thus, strong acidity of the musts or a great concentration of the sugar may produce a slackening in the fermentation.

The excess of sugar acts unfavorably on account of the accumulation of alcohol in the musts. Even with 5 per cent of alcohol present, the diastatic force is influenced, and with 10 per cent the inversion proceeds very slowly indeed.

The acidity of the musts, such as is found in practice, does not act directly on the sucrase and the retardation observed must rather be attributed to foreign ferments, which, favored by the acidity, develop in the mash.

From molasses which is fermentable with difficulty we have isolated bacteria which produced a slight acidity in

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Acclimatization has then produced a profound change in the cells, and one which is transmitted from one generation to another.

Quite a different thing is observed in the action of saline substances on yeast.

The resistance which sucrase acquires by acclimatization commences to be weakened as soon as the yeast is again found in a medium free from salts.

The properties of sucrase are here then closely related to the composition of the medium. This fact contradicts the hypothesis of the existence of different enzymes acting on the same body and producing the same chemical reactions.

According to this hypothesis, indeed, there exist different sucrases. The sucrase of *Aspergillus niger*, for example, would be a different substance from the sucrase of yeast, etc. One would also have to distinguish between the sucrases of different yeasts, because these are not equally sensitive to the temperature and to the reaction of the medium. The variable resistance of sucrase to chemical agents would evidently lead to new distinctions, but these latter distinctions would be absolutely illusory.

The difference in the manner of action in the presence of chemical substances arises, not from a change in the nature of the sucrase, but rather from difference in the external media.

The same thing must occur with sucrases of different origin, showing different properties.

In fact, the diastase is accompanied in the cells which secrete it by different substances which modify its properties.

sugar solutions. Alcoholic fermentation is manifestly arrested by such ferments. These micro-organisms act also on yeast in grain mashes, but they are shown to be specially dangerous in the fermentation of saccharose.

In the presence of these organisms the fermentation of the sugar solutions is arrested when the must has still only a slight acidity and a small quantity of sugar has as yet been transformed.

The difficulties in fermentation of molasses caused by lack of sucrase are accompanied by the following symptoms:

The fermentation begins regularly, but when about 50 per cent of the sugar is transformed, that is, before the end of the principal fermentation, there is suddenly found a noticeable slackening, then a stop which is prolonged for several hours. The yeast is deposited slowly; there is produced little by little new amounts of sucrase and the fermentation recommences, often with great energy. Then a second stop occurs which is generally final. The mash in fermentation often contains at this period considerable quantities of non-inverted sugar. Such is the course of events when the work is carried on in the presence of antiseptics.

In the opposite case, the fermentation has an entirely different aspect.

When the slackening occurs, the mash, invaded by bacteria, becomes decidedly acid. The yeast degenerates and the fermentation, once stopped, does not recommence, or at least, does so only very feebly.

From the practical point of view, it is well for the distiller of molasses to pay more attention than he usually does to the manner of inversion of cane-sugar during fermentation. It is especially necessary to exercise great care in the choice of yeasts. It is then indispensable to protect the mashes by antiseptics against foreign ferments. It is also well to filter or decant the molasses mashes after their acidification. Not all the ferments are destroyed by simply boiling. The bacteria found in difficultly fermentable molasses are only

destroyed at a temperature of 110°, though they may be easily removed either by filtration or by decantation.

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CHAPTER VIII.

AMYLASE.

Presence of amylase in vegetable and animal cells.—Preparation.—Cohnheim's method.—Lintner's method.—Effront's method.—Wroblewsky's method.—Properties.—Influence of quantity, time, and temperature.—Influence of chemical agents; acids, alkalies, salts.—Substances which accelerate diastatic action.

The enzyme called amylase, or simply diastase, is a soluble ferment hydrating starch and transforming it into maltose and dextrins.

The existence of this enzyme was first observed by Kirchoff, in 1814, in gluten. Dubrunfaut, Payen, and Persoz have since studied this substance thoroughly.

Amylase is widely distributed in nature. It is found in barley, oats, rice, maize, and in general, in all cereals.

The raw grains have little amylase, the enzyme being formed especially in the course of germination.

The presence of amylase has been observed in the tubers of potatoes, as well as in the leaves and shoots of different plants.

The transformation of starch into carbohydrates assimilable by living cells being generally accompanied by the action of amylase, it may be believed that this substance plays a very important part in the formation of vegetable tissues. Yet the transformation of starch is not always produced by the aid of amylase, and because a cell brings about the transformation of amylaceous materials does not at all prove that it secretes this diastase.

We shall see later that other enzymes exist which act on

starch and render it assimilable and suitable for the construction of tissues.

Wortmann asserted that the assimilation of starch is not always accompanied by the action of enzymes, and he believed that he demonstrated that protoplasm alone can by itself produce a hydrating and dissolving action on starch. This view may be seriously questioned, however. It is true that in the leaves of plants where a very active transformation of starch is brought about, there are generally found quantities of diastase small in comparison with the work observed. It is equally true that often the stalks and stems do not secrete active substances, while in these organs is found an energetic assimilation of starch. But these facts are not sufficient to prove the direct action of protoplasm in the hydration of starch. The fact that amylase has not been found in the cells may simply arise from difficulties like those which occur in the study of sucrase; in other words, amylase may be more or less retained within the cells, or it may enter into combination with other substances and thus become more or less soluble.

As we have found for tannin, amylase is sometimes present in an inactive form, because the environmental conditions under which it is found are unfavorable for its action. In this case, it again acquires its normal properties as soon as it is placed under favorable conditions. We know, moreover, that the effect produced by an enzyme depends especially upon the conditions of the medium, and we have reason to believe that in living cells the action of diastases is more energetic than in our experiments. It is probable, moreover, that with a very small quantity of active substance one could obtain marked results if the conditions of the medium are favorable. If, then, amylase has not been found in the various vegetable organs examined, these negative results may be attributed to the different circumstances which we have just enumerated. Then it is not at all established that protoplasm would be capable, without the inter-

vention of enzymes, of hydrating starch, and all the data which we possess on enzymes tends rather to prove that diastatic action takes place here also.

Again, amylase is found, according to some authors, in moulds. Thus, *Aspergillus niger*, or *Penicillium glaucum*, if cultivated under certain conditions, would secrete a certain quantity of amylase; the presence of this diastase in moulds is very rare, however. The diastase which hydrates starch is met with, not only in the vegetable kingdom, but also in animal secretions. It is present in the saliva, the pancreatic juice, and the liver. The constant presence of the active substance in the saliva may be explained by two different theories; first, that amylase is secreted by the salivary glands; or, second, that it is due to organized ferments which are found in the mouth, and feed upon amylaceous materials. Claude Bernard, who studied this question, was a supporter of the latter theory. By heating saliva to 100°, he found the complete destruction of the active substance. This saliva, having lost the property of acting on starch, became active when left for some time at the ordinary temperature. He attributes this phenomenon to the development in the saliva of new ferments capable of furnishing to the surrounding liquid new quantities of diastase. The appearance of the diastase could, however, be explained by the action of the temperature on the ferments of the saliva. Certain cells which have not been destroyed by the heat might still retain amylase, and the enzyme, when the temperature is lowered, would diffuse into the liquid. However this may be, the appearance of amylase in the saliva may be explained otherwise than by a secretion of the glands.

To settle this question definitely, the experiment of Claude Bernard must be repeated under such conditions that the action of any organized ferments can be avoided.

Preparation of Amylase.—Amylase can be precipitated from its solutions, either by mechanical precipitation or by the action of alcohol. According to Cohnheim, the diastase

can be extracted from the saliva by the following method: quicken salivation by rinsing the mouth with ether. The saliva is then collected and a slight amount of phosphoric acid is added. The acid liquid is then neutralized with great care by the aid of very dilute lime-water. Thus calcium phosphate is formed, which carries down the diastase as well as other nitrogenous materials. This precipitate is removed by filtration, then washed on the filter with a volume of water equal to that of the saliva used. By the washing, the diastase goes into solution. It is precipitated from this solution by a suitable addition of alcohol. The preparation of amylase is accomplished much more easily by the use of an infusion of malt. Payen was the first to find that the active substances of an infusion can be precipitated from the solution by a suitable addition of alcohol. The product thus obtained is unfortunately far from pure; furthermore, it changes readily in the air, oxidizes, and becomes inactive very quickly, and assumes a very dark color.

The alteration of amylase is facilitated by foreign substances precipitated at the same time as the diastase by the alcohol. Different means have been proposed to avoid these difficulties. Payen and Persoz, for example, advise adding to the infusion of malt a quantity of alcohol insufficient to precipitate the diastase, then bringing the alcoholic solution to a temperature of 70°, which, according to them, would cause the coagulation of foreign substances, especially albuminoid matter. This operation finished, the coagulated substances are separated from the liquid which contains them and an excess of alcohol is added to cause a precipitation of the amylase. By this method there is obtained a white product which is not very changeable but which possesses only a very slight activity.

Much more satisfactory results are obtained by Lintner's method: One part of finely ground malt is mixed with four parts of 20 per cent alcohol; it is allowed to stand for 24

hours; then the liquid is poured off from the malt, and filtered, and to each volume of filtered liquid 2 volumes of absolute alcohol is added. Thus a flocculent precipitate is formed; the clear solution is decanted and the precipitate collected on a filter. After a first washing with alcohol and ether, the precipitate is ground in a small mortar with a little alcohol; it is then replaced on the filter and washed a second time with alcohol and ether, then dried *in vacuo*. By this method is obtained a product which Lintner calls raw diastase and which can be still further purified by solution in water and precipitation by alcohol. This purification leads to a product of constant composition but of slight activity.

This method gives good results if the operations are performed rapidly, in order to prevent the precipitated diastase from coming in contact with the air before it is completely dehydrated. Nevertheless, the product obtained is very rich in ash as well as foreign materials which have been precipitated from the infusion of malt by the alcohol.

To obtain purer and more active products we advise using another method.

To diminish the quantity of extractive substances in the infusion of malt which do not possess diastatic power, ferment this infusion with yeasts which have been previously limited to a scant supply of nitrogen. The alcoholic fermentation, caused by these yeasts in the infusion of malt, destroys a great part of the carbohydrates, eliminates a considerable quantity of albuminoid matter and salts, but leaves the diastase absolutely intact. This is accomplished as follows: macerate 100 grams of malt reduced to powder with 300 grams of water at a temperature of 30° for 18 hours. Stir the mixture every half hour. The mass freed from the liquid by pressure is thoroughly washed with water,—which, together with the original extract, is filtered. The filtrate is made up to 300 cubic centimetres, 10 grams of beer-yeast added and left at a temperature of 28° for 48 hours. It is then filtered, and to the clear liquid is added 700 cubic centimetres

of alcohol. The yeast used for this preparation must first of all have remained for 24 hours in a 10 per cent solution of sugar. The fermentation causes the yeast to lose a part of its nitrogen and gives it an avidity for albuminoid materials. With 100 grams of malt we have obtained from 3 to 3.5 grams of a white substance having the same activity as 80 grams of the malt used.

A new method of preparation of diastase has been recently proposed by Wroblewsky, who considers that diastase prepared by the ordinary methods is always found mixed with a pentose, arabinose. The method consists of a partial precipitation caused by the action of salts.

The author adds, drop by drop, to a solution of amylase, ammonium sulphate until a turbidity is produced in the solution. Then the liquid contains 50 per cent of ammonium sulphate; allowed to stand for some time there is produced a yellowish flaky precipitate, which is separated and washed with a 54 per cent solution of ammonium sulphate. This precipitate is very active: added to a solution of starch, it completely and almost instantaneously transforms it into sugar. According to the author this deposit would be composed of pure diastase.

To the liquid from which the precipitate has been separated is added again ammonium sulphate up to the amount of 60 per cent; a new deposit is then produced which, separated, washed, and examined, has been recognized as being a mixture of a pentose, arabinose, and diastase. Finally, in a third operation the second liquid separated from its precipitate is again taken, saturated with ammonium sulphate and a new product obtained composed wholly of the pentose. When one wishes to obtain a very active product then, one must take the first precipitate obtained in the solution containing 50 per cent of ammonium sulphate.

Amylase prepared in this manner is very soluble in water. It is not coagulated by heating, either in a neutral solution, or after acidification with acetic acid or slight amounts of

hydrochloric acid. A great addition of hydrochloric acid, however, produces, by heating, a coagulation in the form of light flakes. The solution of amylase, with a certain amount of nitric acid added, gives a light precipitate which is redissolved in an excess of the reagent. It gives Millon's reaction, also the biuret and xanthoproteic reactions. Its solution gives a light precipitate with mercuric chloride. Tannic acid added to the solution of amylase produces a voluminous precipitate soluble in dilute soda. This alkaline solution becomes somewhat discolored when left to the air at 50°, but does not entirely lose its hydrating power.

The amylase obtained by Wroblewsky gave on analysis 16.53 per cent of nitrogen.

Properties of Amylase.—Amylase is endowed with two distinct properties: it liquefies starch, and transforms starch, as well as dextrin, into maltose.

The two properties of this diastase may easily be shown by the following experiments:

To 100 cubic centimetres of water kept at the boiling point add 10 grams of potato-flour diluted in 20 cubic centimetres of tepid water. The mixture forms a thick paste which acquires still more resistance when it is kept for some time in the neighborhood of 100°. To this starch is added several cubic centimetres of an infusion of malt and the whole is left in a water-bath at a temperature of 70°–75°. The pasty mass quickly becomes fluid, and in a longer or shorter time, according to the diastatic power of the infusion, the starch is transformed into a transparent liquid passing through filter paper. This liquid, of an insipid taste, contains dextrins and traces only of sugar. With tincture of iodine it takes a deep-blue color. This solution of dextrins is cooled, a little more of the infusion added, and is left to act at a temperature of 50°–60°. If samples of this are taken from time to time and analyzed, it is found that the dextrin gradually disappears and there appears in the liquid a reducing sugar: maltose. The successive changes which oc-

cur in the mixture of dextrins, under the action of the infusion of malt, may be easily followed by the aid of tincture of iodine. The deep blue color, obtained in the solution of starch by the iodine, is gradually weakened as the saccharification proceeds. Several hues are obtained in the course of the saccharification. From the deep blue which the starch gives we pass to violet, then to red, then to yellow. Finally, when the saccharification is well advanced, the iodine no longer causes any coloration.

The action of the amylase, at the same time saccharifying and liquefying, has raised a doubt as to the individuality of this enzyme. The hypothesis has been suggested that two different enzymes are present, because the two diastatic functions of amylase appear at very different temperatures and because the saccharification and liquefaction are very differently influenced by the chemical and physical conditions of the medium. Still we must discard, at least until it is established by sure proofs, this interpretation which brings a new complication to the study of amylase. This hypothesis would only be acceptable if one could isolate completely each of the functions of amylase, that is to say obtain two products, the one having simply the liquefying power, the other simply the saccharifying power. But this separation has never been made. By keeping the malt infusion at 70° one specially favors the liquefaction, but the product obtained also contains a slight quantity of sugar. If, on the contrary, one maintains the temperature in such a way that saccharification is favored, that is at 50°–60°, a slight liquefaction is produced at the same time.

Influence of Quantities.—The study of the action of amylase leads to conclusions analogous to those furnished by the study of the mode of action of sucrase. It is found in fact if the course of saccharification is followed, that the quantity of sugar formed at the beginning of hydration is proportional to the quantity of the enzyme used. Afterwards, when the decomposition of the starch is more advanced, this pro-

portionality ceases to exist. The course of the saccharification when, for the same quantity of starch, increasing quantities of amylase are used, is shown by the following table:

Quantity of infusion of malt used.	Quantity of maltose produced.
1 cc.	0.1
3	0.31
5	0.49
10	0.82
15	1.1
20	1.1
30	1.2

By checking the action of the diastase after one hour, in a saccharification made at 50° with different amounts of amylase, it is found that with 3 cubic centimetres of an infusion of malt there is obtained fully 3 times as much maltose as with 1 cubic centimetre. If the amount of diastase is still further increased, there is observed an increase in the quantity of maltose formed, an increase which is, however, less and less regular. Beyond a certain limit the quantity of the infusion no longer influences the progress of saccharification, even though at that moment not all the dextrins contained in the solution have been transformed.

In inversion by sucrase we have observed an analogous course. Still the analogy is not complete. With amylase the proportion is maintained until nearly 40 per cent of the starch is transformed, while with sucrase the proportionality is no longer shown when 15 per cent of the sugar is inverted. Moreover, the slackening at the end of the action is much more pronounced in the case of amylase than in that of sucrase.

Influence of Time.—When one studies the effect of time on the progress of saccharification, one finds, as before, that at the beginning of the action a constant ratio exists, and

then a slackening which is more and more marked as the transformation of starch advances. To show the influence of time we cause a slight quantity of diastase to act upon 1 per cent starch; we take samples from time to time and determine the quantity of sugar formed.

The following are the results obtained at a temperature of 50°:

Numbers of the samples.	Duration of action in minutes.	Maltose produced.
1	15	0.05 gr.
2	30	0.097
3	60	0.21
4	120	0.39
5	240	0.63
6	480	0.82

In the four first samples the quantity of maltose formed is almost proportional to the duration of the action. In the others the proportionality ceases to exist and it is interesting to observe that when the slackening commences there is formed in the liquid nearly 40 per cent of maltose. It is the ratio between the quantities of transformed and non-transformed product which influences the progress of hydration. It is this ratio which determines the cessation in the proportionality.

If, instead of using, as we have just done, a very slight quantity of diastase, we repeat the experiment with double amounts of infusion, while employing the same quantity of starch, we find that the constant ratio ceases after the first hour of action. If the quantity of diastase is still further increased, the course of the transformation becomes irregular after some minutes.

Influence of Temperature.—By saccharifying starch with infusions of malt at different temperatures for 15 minutes, Kjeldahl has obtained the following results:

Temperature.	Reducing powers.
18.5	17.5
35	30.5
54	41.5
63	42
66.5	34
68	29
70	18

The action of amylase is very slow at 0°. Towards 30° saccharification begins to progress rapidly and the activity of the enzyme then increases very rapidly in intensity up to 60°. Above 60° the production of maltose diminishes, and at a temperature of 70°, which is the most favorable temperature for liquefaction, the quantity of sugar produced becomes insignificant.

Kjeldahl and Bourquelot have observed that amylase, kept for some time at temperatures higher than 60°, acts differently from a diastase that has not been heated. An infusion of malt kept for 10 minutes at different temperatures and then introduced in starch at 50° produces very different reactions.

Infusion of malt heated for 10 minutes	at 63°	furnishes	63%	maltose,	37%	dextrin.
	at 68°	"	35%	"	65%	"
	at 70°	"	17.4%	"	82.6%	"

The temperature to which the infusion has previously been brought has then produced a change in the mode of action of the amylase. The heated diastase causes a decomposition of starch according to equations which differ according to the temperatures to which the infusion has been brought.

To show the influence of temperature on the progress of saccharification, the following experiment may also be made:

Heat an infusion of malt for 12 hours at 68°, then try its fermenting power by comparing it with that of the same infusion not heated. At 50° allow 10 cubic centime-

tres of non-heated infusion and 10 cubic centimetres of the same infusion previously heated to 68° to act on 1 per cent starch.

In the first case 0.6 of maltose is obtained and in the second 0.3. The diastatic power has then diminished by half. Now the power of the infusion heated to 68° may be tried in starch of different degrees of concentration:

10 c.c. of infusion have given in a 1% starch.....0.3 maltose.
" " " 2% " .....0.6 "
" " " 3% " .....0.9 "

In 2 per cent starch the heated infusion gives a normal result, that is it furnishes the same quantity of sugar as if it had not been heated. In 3 per cent starch the infusion, kept at 68°, furnishes a still larger amount of sugar.

The variation in the energy of the infusion, according to the concentration of the starch, becomes more striking if one observes that in the three experiments cited above there are formed quantities of maltose proportional to the amounts of starch contained in the paste. There is always obtained 30 per cent of maltose.

The heated infusion then preserves all its properties when it is a question of producing limited decompositions, as long as hydration does not exceed 30 per cent, but this infusion cannot produce greater hydration.

Workers have sought to explain the difference of action of the heated diastase and that which is not heated by the hypothesis that there exist different kinds of amylase. These different diastases would possess different temperatures of destruction and coagulation and would decompose starch differently. According to this theory, by heating the infusion to 68°, an unfavorable action would be exerted on the diastases producing complete hydration, that is, giving little dextrin and much sugar, but the diastases performing the opposite work, that is, forming much dextrin and little sugar, would be left intact.

The diastases which produce a slight saccharification would, according to this hypothesis, act very favorably when

put in a heated infusion of large quantities of starch. In this particular case it would be found that the height of the temperature produced no change in the diastases.

We shall have occasion to return to this hypothesis, but let us say, at this point, that it does not at all accord with the facts which we shall set forth later.

Influence of Chemical Agents.—The conditions of the medium influence to a very great degree the action of amylase, which shows itself very sensitive towards many chemical substances.

For a long time it has been observed that the least change in the reaction of the medium has a visible influence on the progress of saccharification by the diastase. It is generally admitted that diastatic action is favored by very slight amounts of acid and that, by a greater acidity, it is possible to slacken and then arrest completely the progress of hydration.

Kjeldahl was the first to study with exactness the influence of the acidity of the medium. For this experiment he made use of dextrin solutions. In a series of samples of 100 cubic centimetres he added, to the same quantities of infusion of malt and of starch, different quantities of sulphuric acid, allowed the saccharification to continue for 20 minutes at a temperature of 59°, and then determined the sugar formed. The influence of the different amounts of acid upon the diastase is shown in the following table:

Milligrams of H_2SO_4 per 100 c.c. of solution.	Increase in sugar.
0	0.44
1	0.47
2	0.49
2.5	0.48
3	0.43
3.5	0.27
4	0.13
6	0.02
10	0.01

Thus it is seen that an amount of 1 to 2.5 milligrams of sulphuric acid produces a favorable action, while an amount of 3.5 milligrams causes a slackening which larger amounts accentuate more and more. With 10 milligrams there is obtained an almost complete arrest.

If one compares sucrase and amylase from the point of view of their sensitiveness towards the reaction of the medium, one finds a very noticeable difference between the two enzymes.

We have seen that sucrase produces its maximum effect in an acid medium. On the other hand, the favorable influence exerted by acids is extremely slight for amylase.

For the diastase which inverts cane-sugar the natural medium is acid. On the contrary the enzyme producing maltose derives little benefit from a slight acidity, and shows an extraordinary sensitiveness towards larger quantities of acid.

The figures indicated by Kjeldahl must not, however, be considered as constant, for, under conditions other than his, we have found entirely different results. We have taken an infusion of filtered malt and have added to it different quantities of sulphuric acid and hydrochloric acid, then we have determined the diastatic power of the infusion before and after acidification. We have thus obtained the following results:

Acid.	Milligrams per 100 c.c.	Diastatic power.
Sulphuric acid	0	100
	2	108
	3	104
	5	100
	10	98
Hydrochloric acid	3	107
	5	104
	10	97

Now, with 10 milligrams of sulphuric acid, Kjeldahl found an almost complete arrest in the saccharification, while in

our experiments this amount of acid showed itself almost without effect. One may then conclude that the medium by itself possesses here also an influence upon the sensitiveness of the enzyme. When a mineral acid is added to an infusion of malt, a part of this acid combines with the bases of the infusion, thus displacing organic acids which, varying in nature in different infusions, act more or less energetically on amylase.

The action of lactic acid on amylase deserves special attention because the infusion of malt, as well as the grain mashes generally, contain this acid. It is proper then, in industrial saccharification, to take account of these factors. We have studied the action of lactic acid under very varied conditions, and these experiments have led us to the following conclusions: The effect of a given amount of acid on diastase differs according to the duration of the action and also according to the temperature. Acids, furthermore, act differently upon the saccharifying power and the liquefying power.

The combined influences of time and acids may be shown by the following experiment: To an infusion of malt, filtered by the Chamberland filter, add different quantities of lactic acid, and determine the diastatic power after 1 hour and after 12 hours.

Lactic acid per 100 c.c. Centigrams.	Saccharifying power of the infusion.	
	After 1 hour.	After 12 hours.
10	48	42
100	53	24
400	57	21

By leaving the infusion of malt for an hour with 400 centigrams of acid at a temperature of 30°, a perceptible increase of the saccharifying power is observed, while the same amount of acid produces a disastrous effect if the action is allowed to be prolonged for 12 hours. The saccharifying power falls off in this case from 57 to 21.

Then the same experiment is repeated, only changing the temperature. The infusion is left at a temperature of 55° for 1 hour and the diastatic power determined.

Acid in centigrams.	Diastatic power.
10	44
100	41
400	20

The amounts of acid which produced an increase of diastatic power at 30° act quite otherwise at a temperature of 55°. At this temperature the 400 centigrams of acid have made the saccharifying power fall off from 40 to 20.

The sensitiveness of amylase becomes still more evident if we examine the changes produced in the liquefying power, after 1 and 12 hours of action, with different quantities of acid.

Lactic acid in centigrams.	Liquefying power.	
	After 1 hour.	After 12 hours.
10	100	100
100	100	50
400	51	20

The changes observed in the liquefying power show us that under the influence of acids the increase in the saccharifying power of amylase is made with a parallel reduction of the liquefying power.

After 1 hour at 30°, 400 centigrams cause the saccharifying power to rise from 48 to 57, but at the same time the liquefying power is found to be reduced almost one-half.

We shall see further on, in the chapter devoted to industrial applications, that the real value of the diastase exists in its liquefying power and that consequently the rise caused by acid is more apparent than real.

Alkaline media are unfavorable to the action of amylase. Still, the diastase can endure a certain alkalinity without

changing, for, when the alkali is neutralized, the diastase resumes its activity.

Sodium carbonate acts upon amylase in extremely small amounts. By adding 5 milligrams of sodium carbonate to 100 cubic centimetres of neutral starch, we have found the diastatic power to diminish almost 20 per cent. With .25 gr. of soda we have obtained only a quarter of the quantity of sugar which the diastase would have given without the addition of alkali. According to the experiment of Duggan, caustic soda in the amount of 2 milligrams produces a disastrous effect: under its influence amylase loses almost 75 per cent of its activity.

Salts also influence diastatic action, by either increasing or decreasing the activity of the amylase.

Mercuric chloride in the amount of one-millionth paralyzes its action.

Calcium chloride in the amount of one-hundredth diminishes the activity of amylase by half.

According to Kjeldahl, the salts of lead, zinc, and iron, as well as alum, check the action of the diastase and their more or less destructive influence may be expressed by the following figures which indicate the proportion between the normal action, expressed by 100, and the action with salts:

Potassium nitrate, 10 centigrams	20
Zinc sulphate	20
Ferrous sulphate	20
Alum	3

According to different authors, sodium chloride in the amount of one-half per cent would cause a noticeable slackening. Our experiments have not confirmed these data. With commercial salt we have often observed a paralyzing action, but the same thing has not occurred when we have used chemically pure salt. The checking action of commercial common salt must then be attributed rather to impurities.

Alcohol and most antiseptics must also be placed in the class of inhibiting agents. Salicylic acid, phenol, and formic aldehyde, used in the smallest amounts, act upon the diastase.

Still we cannot definitely place all antiseptics in the class of destructive substances. Picric acid, for example, as is shown by our experiments, does not act at all as a depressor; its action is shown rather in the opposite direction.

The study of the conditions which may influence the progress of saccharification presents a genuine interest from a practical point of view. It furnishes valuable information to distillers and brewers as well as other manufacturers who utilize the properties of amylase.

In seeking to determine conditions favoring diastatic action, we have attempted to effect the saccharification by the aid of a great number of chemical substances. These experiments have not fulfilled all our hopes. We have not succeeded in enhancing the efficiency of a malt by chemical substances, but the results of our experiments throw a special light on the mode of action of amylase and afford a firm basis for analysis of the diastase.

We have found that many chemical substances may reinforce to a very great degree the progress of saccharification by the diastase. This reinforcing action is, however, of a peculiar nature, and can only be shown under certain conditions. To the list of favorable substances belong the salts of vanadium and aluminium, the phosphates, asparagin, albuminoid substances, and picric acid. To study the action of these different substances on diastatic fermentation we have used two different methods.

The diastase has first been put in direct contact with the reagent and then introduced into the starch. After saccharification the quantity of sugar formed has been measured. In a parallel experiment, made with the same quantity of diastase not having undergone the influence of the reagent, the quantity of maltose produced has been deter-

mined and finally the results of the two experiments have been compared. In the second series of experiments the reagents have been added directly to the starch, in which is then poured the infusion of malt.

In our experiments we used an infusion of malt prepared cold with one part of malt and forty parts of water. The starch solution had a density of 1.015. For each experiment we used 1 cubic centimetre of filtered infusion and 100 cubic centimetres of starch. The saccharification took place at 50° for 1 hour.

Here are some figures which sum up the influence of the different chemical substances:

	Maltose per 100 of starch.
Without addition	8.63
With 0.7 gr. of ammonium phosphate	51.62
0.5 acid calcium phosphate	46.12
0.25 aluminium acetate	62.40
0.25 ammonium alum	56.30
0.25 potassium alum	54.32
0.05 asparagin	61.20

By the addition of 50 milligrams of asparagin the saccharification was nearly 7 times more extensive than in the proof experiments.

Aluminium acetate can produce the same effect, but it must be used in a larger quantity.

The two methods which we have used have afforded different results for calcium phosphate as well as for alum.

For the other substances we could find no difference.

The results are not changed if instead of an infusion of malt, diastase precipitated by alcohol is used. Neither do they change if the saccharification is effected at different temperatures. It is very evident that the quantities of sugar found vary according to the temperature of saccharification, but the difference between the proof experiments and the

experiments made with various substances always remains practically the same.

A series of experiments made under widely differing conditions has led us to the following conclusions:

1st. Substances which act favorably, act in proportion to their quantity up to a certain maximum amount.

Thus, by taking 0.005 of asparagin, we have obtained 25.5 maltose.

"	0.02	"	"	37	"
"	0.05	"	"	61.2	"
"	1 gr.	"	"	61.2	"

2nd. The maximum is not the same for all substances favoring diastatic action.

Thus, asparagin and aluminium acetate in maximum quantities may influence diastatic fermentation much more strongly than phosphates.

3rd. The exciting action of chemical substances is manifested only in the first phase of hydration of starch; when saccharification is very advanced it ceases to act.

It results from these facts that the same substance may possess a very different activity according to the conditions of the experiment.

If amylase is present in a very small proportion in relation to the starch to be transformed, the effect of chemical substances is easy to ascertain. In the opposite case, that is with a greater quantity of amylase, the effect of chemical substances is reduced, and, for a quantity of diastase capable of itself transforming nearly 60 per cent of starch, the accelerating substances have no longer any influence on the enzyme.

The following experiment shows the influence of asparagin with different quantities of infusion.

In each of two portions of 100 cubic centimetres of starch paste is introduced 1 cubic centimetre of malt infusion and saccharification is allowed to proceed for an hour at 50°. One of the specimens is saccharified without addition of asparagin; to the other is added, when the enzyme is introduced, 5 centigrams of that substance.

At the end of the time interval, one determines the proportion of maltose formed in the solution of starch submitted to diastatic action. These same experiments are then repeated under the same conditions with 10 cubic centimetres of infusion in place of one, and the followiing results are obtained:

		Maltose per 100.
A	1 c.c. without asparagin	18
	" with asparagin	62
B	10 c.c. without asparagin	79.25
	" with asparagin	79.25

It appears from this table that in the experiment containing 10 cubic centimetres of infusion, the asparagin no longer acts, although the saccharification must still be far from finished.

An analogous result may be obtained, even with a very small quantity of diastase. For this, instead of checking saccharification after an hour, as was just done, the diastase is left in contact with the starch for twelve hours.

		Per cent of maltose.
A	Saccharification, 1 hour at 30°.	
	1) 1 c.c. of infusion without asparagin	6.4
	2) " with asparagin	45.0
B	Saccharification, 12 hours at 30°.	
	1) 1 c.c. without asparagin	74.8
	2) " with asparagin	74.9

Another characteristic property of substances favoring diastatic fermentation is that they act exclusively on the saccharifying power, while they never influence the liquefying power of amylase.

As the liquefying power exerts its action exclusively on starch and not on dextrins, it must be assumed that the action of favoring substances is exerted only on the latter bodies.

CHAPTER IX.

CHEMICAL WORK OF AMYLASE.

Chemical work of amylase.—Theories of Payen and Musculus.—Existence of different dextrins.—Theory of Duclaux on the nature of the different dextrins.—Preservation of the diastases during saccharification.—Experiments of Effront.

When grains of starch are submitted for a short time at a low temperature to the action of amylase, they are very slightly attacked by the diastase. On the contrary, if the action is prolonged, a very complete work is effected; the grains are corroded, pass into solution and are then transformed into sugars and related bodies. The action of amylase is nevertheless more energetic and much more rapid when it is produced on starch paste. By allowing amylase to act at a suitable temperature on starch paste the solution and saccharification proceed rapidly. The chemical reactions that the diastase cause in the paste, as well as on the grains of starch, may be expressed by the following equation:

$$\underbrace{(C_{12}H_{20}O_{10})}_{\text{Starch.}} + H_2O = \underbrace{C_{12}H_{22}O_{11}}_{\text{Maltose.}}.$$

This formula shows us that starch, under the influence of amylase, is hydrated and transformed into maltose, but it does not indicate the mechanism of the transformation. In reality the phenomenon is much more complex.

In the products of the reaction there are always found dextrins whose presence shows that the reaction was complicated by the formation of intermediate products.

The saccharification of starch by malt has been the object of much research. However, in spite of the number of researches in this field, the complete solution of the problem is far from being found at present.

The simplest and also the oldest interpretation of the course of saccharification is that of Payen. According to him, diastase exercises on starch two successive actions: it first transforms it into dextrin, then into maltose. There is first produced an isomeric modification of starch, then a hydration of that isomer. According to the interpretation of Payen the transformation of starch into dextrin and into maltose would of necessity be produced not only gradually but regularly, from the beginning to the end of the action. Now, the course of saccharification presents quite a different aspect. We know, in fact, that as hydration proceeds the action of amylase becomes slower and slower. The theory of Payen is then in disagreement with the facts. Furthermore, it cannot explain to us the formation during saccharification of dextrins endowed with different properties.

According to Musculus, saccharification takes place, not by the successive transformation of starch into dextrin and then into maltose, but by hydration followed by cleavage. This author maintains that the molecule of starch is first hydrated and then decomposed into a molecule of maltose and a molecule of dextrin.

$$\underbrace{2C_{12}H_{20}O_{10}}_{\text{Starch.}} + H_2O = \underbrace{C_{12}H_{22}O_{11}}_{\text{Maltose.}} + \underbrace{C_{12}H_{20}O_{10}}_{\text{Dextrin.}}.$$

In support of this theory Musculus endeavors to establish that the dextrin and sugar formed during saccharification are in a constant ratio. He insists, moreover, that dextrin cannot be attacked by the diastase. These facts do not withstand criticism. In fact, dextrins can be transformed into sugar by amylase, and the ratio between the quantities of maltose and of dextrins does not remain at all constant dur-

ing the transformation. This ratio changes for the same temperature according to the duration of the action and the quantity of diastase used, and also depends upon the temperature of saccharification. The ratio between the products formed, the maltose and the dextrins, is then neither simple nor constant.

The theory of Musculus, based upon observations which are not very exact and reasonings which are not well founded, has however had unmerited acceptance, and at the present time it still serves as a basis for almost all the theories of saccharification. To reconcile this theory with the data on saccharification actually possessed, a repetition of the two operations, hydration and cleavage, is believed to occur. The starch is supposed to possess a very great molecular weight. This complex molecule, by hydrating, is decomposed into maltose and a primary dextrin. This dextrin, of a complicated constitution, then furnishes in its turn a second molecule of maltose and a new dextrin of a molecular weight less than that of the first, and so on.

The saccharification is then brought about by giving rise successively to dextrins of smaller and smaller molecular weight.

The ideas of Musculus as to the course of saccharification, explaining it as a progressive degradation of the dextrins, were adopted by Brown and Morris as well as by Lintner. They are, however, far from sharing the opinion of Musculus as to the formation of the intermediate products of the reaction, and as to the molecular weight of the starch and the dextrins.

According to other chemists, among the products of saccharification there are found not only dextrins and maltose, but also substances formed by the combination of these two bodies.

Existence of Different Dextrins.—It does not enter into the scope of the present work to discuss all the theories evolved to explain the saccharification of starch. Let us

confine ourselves to the essential facts underlying these theories, especially the formation, during saccharification, of dextrins which act differently towards reagents.

To show the difference between the dextrins, one may proceed in the following manner:

Treat with alcohol a paste of saccharified starch containing from 10 to 20 parts of sugar for 100 parts of starch. Dissolve the precipitate obtained, precipitate again with alcohol and repeat this operation a number of times. Thus a product is secured which contains only traces of sugar.

On the other hand, precipitate in the same manner the dextrins contained in a paste of starch in an advanced stage of saccharification and containing nearly 80 parts of maltose for 100 parts of starch. With the two kinds of dextrins thus obtained and which we will call dextrins A and B, prepare two solutions of the same concentration; add to them the same quantity of infusion of malt and allow saccharification to proceed at 50° for 2 hours.

A measurement of the maltose in the two solutions shows the difference existing between the two dextrins.

The dextrin A, extracted from slightly saccharified starch, is hydrated very easily, while dextrin B furnishes very little maltose. The two dextrins then differ in their sensitiveness towards amylase.

The difference in the nature of the two dextrins can be still further shown by the action of acids. Large amounts of a mineral acid, acting while warm on the two dextrins, give with both equal quantities of dextrose, but different results are obtained if very slight amounts of acid are used. In this case, very marked differences are observed in the progress of hydration: the dextrins coming from slightly saccharified mashes are transformed under the influence of acids, much more easily than others.

Another difference between the two dextrins is shown by the clearly different action exerted upon them by diastases with accelerating substances. Take two specimens of the

solution of dextrin A, to which add equal quantities of malt infusion and to one add also a slight amount of asparagin.

By letting the diastase act for some time and then measuring the sugar formed, it is found that the saccharification is produced in a very different manner in the two cases.

The specimen containing the asparagin shows itself much richer in sugar than the specimen lacking the accelerating substance.

If the same experiments are now repeated with the dextrin B, it is ascertained that the progress of saccharification is entirely different. The two experiments, that without asparagin as well as that which contains it, have, after saccharification, the same quantity of sugar. This proves that dextrin B is not susceptible to the combined actions of amylase and asparagin, while the transformation of dextrin A is influenced by the united action of these two substances.

The difference in sensitiveness of the dextrins A and B explains to us the irregularity in the progress of saccharification. It gives us at the same time the reason of the lack of ratio between the quantities of diastase used and of maltose formed.

It is the formation, at the end of the reaction, of dextrins of a special nature which produces the slackening in the progress of hydration and which destroys the proportionality between the quantities of active substance and product formed, which exists at the beginning of hydration before the final dextrins are formed.

The existence of different dextrins is an argument in favor of the theory which regards saccharification as hydration followed by cleavage. The authors of this hypothesis are, however, wrong in bringing forward, to demonstrate the existence of different dextrins, arguments of little value, and to attribute to these different dextrins properties which they do not have.

Thus, according to many authors, dextrins would be distinguished from each other by the difference in their rotatory

and reducing powers. Now, in reality, these differences do not exist at all. The differences found between the reducing powers and the rotatory powers of different dextrins come solely from the impurities which these dextrins contain. They arise specially from the admixture of sugar which is removed with difficulty, even by repeated precipitations with alcohol.

To obtain dextrins free from sugar, we submit impure dextrins to an alcoholic fermentation as well as to lactic fermentation. The different dextrins obtained by this method possess neither rotatory nor reducing power. Moreover, the characteristic properties of the dextrins A and B prove sufficiently the existence of different bodies of this class and is is not at all necessary to attribute to them still other characteristics which they do not possess.

The theory of Musculus in its modern form assumes that the dextrins differ in their molecular weight. By employing Raoult's method of freezing it has not been possible to establish with certainty that dextrins obtained after more or less extensive saccharification really possess different molecular weight. Lintner and Dull have found for erythrodextrin a molecular weight of 6000, and for the other dextrins a molecular weight of 2000. These figures must, however, be taken with a certain reserve, for Lintner and Dull ascertained at the same time, for various dextrins, the existence of rotatory and reducing powers. It is, therefore, to be presumed that their determinations were made with impure products, and that under those conditions they have only a relative value.

Theory of Duclaux on the Origin of the Different Dextrins.—The existence of different dextrins being demonstrated, one may ask from whence they arise and by what mechanism they are produced during the course of saccharification.

According to Duclaux it is in the structure of the molecules of starch that one must look for the origin of the dif-

ferences found between the various products of its transformation.

According to this author dextrins differ from each other, not by their chemical structure but by their physical constitution. These differences have for cause the structure of the grains of starch, which are composed of non-homogeneous superposed layers, unevenly compact, and offering a different resistance to physical and chemical agents. This hypothesis, very attractive because of its simplicity, is supported by very weighty arguments. It has been known for a long time that starch acts differently, according to its origin, in the presence of amylase in the cold. Potato-starch is very difficultly attacked, while the starches of barley and wheat saccharify with great ease.

This difference, which evidently comes from the more or less compact state of the layers forming the grain of starch, is again met with in the action of this diastase at relatively high temperatures.

Lintner, by saccharifying raw starches of different origins, has found that the attack varies considerably in energy according to the origin of the starch.

The proportions of starch dissolved at different temperatures are here tabulated:

	Temperature of Action.				Temperature of Gelatinization.
	50°	55°	60°	65°	
Potato	0	5	52	90	65
Barley	12	53	92	96	80
Fresh malt	29	58	92	96	
Brewed "	13	56	91	93	
Wheat	.....	62	91	94	75–80
Rice	6	9	19	31	80
Maize	2	.....	18	54	75
Rye	25	.....	40	94	

At a temperature of 50°, 12 per cent of barley-starch is dissolved, 2 per cent of corn-starch, and 25 per cent of rye-starch. At a temperature of 60°, 92 per cent of barley-starch is dissolved and only 18 per cent of corn-starch. The

quantity of starch which can be dissolved at a given temperature, therefore, depends distinctly upon the origin of the starch. Noticeable differences are also found between the temperatures necessary for the gelatinization of starches of different origin. Potato-starch gelatinizes at 65°, and the diastase at this temperature dissolves 90 per cent, while the barley-starch, which gelatinizes at much higher temperatures, yields at 65°, 96 per cent of dissolved substances.

These figures prove that gelatinization does not change the properties of starch which result from the variable degree of compactness of the different layers of grains.

Really, a granule of starch is irregularly attacked by diastase: corrosion occurs in very different directions and places. This manner of corrosion arises from the inequality of resistance of the surface of the grains, so that the difference existing in the compactness of the various parts of the grains is, on the whole, the initial cause of the variations in resistance to diastatic action.

Potato-starch and barley-starch are both composed of non-homogeneous granules differing in the degree of compactness of the layers which compose them.

In the granules of potato-starch more resisting layers are found than in the granules of barley-starch. Now, we have observed that with different kinds of starch, pastes are obtained which saccharify with more or less difficulty. We must then suppose that the difference of compactness between parts of the same granule does not disappear when the starch gelatinizes and that consequently the starch cannot offer an equal resistance in all its parts.

The most coherent parts of the granules will form a paste more difficult to liquefy and will then give, even when entering into solution, a dextrin which will offer more resistance.

From this point of view, the dextrins do not exist as chemically distinct bodies, but the different constituents of the starch-grains of varying coherence yield more or less refractory dextrins.

According to Duclaux, the phenomena occur in the following order:

By the action of amylase on the paste there is first produced an almost instantaneous liquefaction. There is destruction of a coagulum, analogous to that observed when a few drops of acid or of ammonium citrate are added to a gelatinous mass of calcium phosphate. Saccharification then commences in the least resistant portion of the paste. This portion is first transformed into dextrin, then into maltose, but at the same time other portions of starch are attacked and increase the quantities of dextrin and maltose in the solution.

When iodine no longer gives color, the starch is completely transformed, but there are still left some dextrins arising from the portions of starch which were least easily attacked. Some of the dextrins are so slow to disappear that they still remain at the end of the operation. Still, these dextrins disappear in their turn and are transformed into maltose if the diastatic action is sufficiently prolonged.

When precautions are taken to avoid alteration of the diastase, the course of saccharification conforms to the theory of Payen. The starch is first transformed into dextrin and then into maltose.

The hypothesis of the varying compactness of the constituents of the starch-grains has then brought a new support to Payen's theory, which, at first sight, appeared to be in direct contradiction to all the data on saccharification.

Deterioration of Diastases by Work.—In the rapid glance which we have just given at the manner of action of amylase, we have been exclusively occupied with a single factor, the progressive transformation of starch, without troubling ourselves about the fate of the acting bodies. The question which arises first of all in considering the active substance is this:

When the diastase has carried out a considerable chem-

ical work, is it still in the same state as at the beginning of its action, and has it preserved its activity?

This question has been discussed by different authors.

According to some, the diastase undergoes a weakening during the work; others claim that it possesses at the last the same fermenting power as at first.

Unfortunately, the two opinions are founded altogether on very questionable general considerations, while the experimental method alone can furnish the true solution of this question, so interesting from a theoretical point of view and so rich in practical consequences.

The following experiment gives the solution of the problem:

In 200 cubic centimetres of starch paste put 3 cubic centimetres of a malt infusion and let saccharification proceed for 4 hours at a temperature of 30°.

The volume of liquid thus saccharified is brought up to 300 cubic centimetres, so that 100 cubic centimetres of the liquid contain exactly 1 cubic centimetre of infusion of malt which has already produced a work of saccharification.

To see if the work done by the infusion has really caused weakening of the active substances, we compare the fermenting power of 100 cubic centimetres of this liquid with the fermenting power of 1 cubic centimetre of the original infusion. For this, 100 cubic centimetres of saccharified paste are mixed with 200 cubic centimetres of starch. The mixture is placed in a water-bath at 50° for an hour. This we will call specimen A.

We also take a second specimen of 100 cubic centimetres of saccharified paste; bring it very rapidly up to a temperature of 100° to destroy the diastase, after which we pour it into 200 cubic centimetres of starch with 1 cubic centimetre of fresh infusion added and place it in the water-bath. This will be specimen B.

The two saccharifications are then made during the same time, with the same quantities of infusion, but with this dif-

ference, that the infusion of specimen A has already been used once, while the infusion of specimen B has not.

The quantities of maltose obtained in a series of parallel experiments are here shown:

Specimens.	1	2	3
A	1.48	1.31	1.92
B	1.46	1.32	1.92

Thus the quantity of maltose obtained is the same in the specimens A and B. Weakening does not, therefore, occur, and all the theoretical considerations from which other conclusions are drawn must be rejected.

It is true that in changing the conditions of the experiment, exactly opposite results may easily be obtained, but in such cases there is alteration and not weakening of the diastase.

Thus, by repeating the same experiments, with the same infusion and the same starch and letting the action be prolonged, not 4 hours at 30°, but only half an hour at 60° or 65°, we reach very different results.

	Temperature 60°.	Temperature 68°.
A	2.19 maltose	2.00 maltose
B	3.25 "	3.15 "

The differences found between specimens A and B arise here from the action of heat on the diastase and not from weakening. The aqueous solution of amylase, left to a temperature of 60°, loses in fact, as is known, a great part of its diastatic power.

CHAPTER X.

AMYLASES OF DIFFERENT SOURCES.

Different amylases.—Ptyalin.—Diastase of raw grains and diastase of sprouted grains.—Action of translocation diastase on starch.—Reichler's diastase.—Mode of action of diastase brought up to a temperature of 70°.—Conditions of secretion of amylase.—Quantitative analysis of amylase.—Comparative value.—Absolute value.—Methods of Effront.

When we study amylases of different sources from the point of view of their action on starch, we are struck with certain characteristic peculiarities, which tend to confirm the existence of different kinds of amylase. The authors who have studied this question closely, first of all distinguish the salivary diastase called ptyalin and the diastase of grains. They then discriminate between the amylase of raw grains and that of sprouted grains.

The characteristic of the salivary diastase is, according to certain authors, its resistance to the action of alkaline and acid media.

This assertion is erroneous. Ptyalin really behaves toward the reactions of the medium in exactly the same manner as malt diastase.

Saliva, in reality, often possesses a very pronounced alkaline reaction which corresponds to 97 milligrams of bicarbonate of sodium per 100 c.c.

Now, Chittenden and Smith have shown that this alkalinity weakens the fermenting power of the diastase, and its power increases in a certain proportion when the saliva is neutralized.

The resistance to acids which has been claimed for ptyalin is especially based on the part played by the salivary diastase in digestion, but here also the observations made have not been exact.

In fact ptyalin acts only in the first phase of digestion when the gastric contents are not yet acid. The reaction of this enzyme is checked when acidity is developed.

The distinction between amylase of raw grains and amylase of malt appears at first sight to be based on more reliable data. Lintner and Eckhard have ascertained a perceptible difference between the action of amylase of sprouted barley and that of the diastase of barley which has not sprouted. At low temperature the diastase of raw grains accomplishes a more complete work than the diastase of malt. At the optimum temperature, on the contrary, which is practically the same for the two diastases, it is the amylase of the sprouted grain which forms the greatest amount of sugar.

A still more appreciable difference results from the study of the liquefying power of amylases of different origin.

Malt diastase liquefies starch very quickly, while the amylase of raw grains, though possessing an energetic saccharifying power, shows itself almost inactive as regards liquefaction. Lintner and Eckhard have compiled a comparative table of the action of temperatures on the two diastases and have brought forward many distinctive properties, which, they say, characterize the two enzymes.

But here, as in the case of ptyalin, the conclusions which the experimenters have drawn from their observations are not well founded.

The difference found between an infusion of raw grain and an infusion of malt comes really not from the existence of two distinct diastases, but from the presence of different foreign bodies in the two liquids. In the infusion of raw grain there is very little amylase, but the liquid is very rich in substances favoring diastatic action.

When we studied the influence of substances which favor diastatic action, we showed that this action is manifested especially in the first phase of hydration and that it ceases in the presence of residuary dextrins. It is for this reason that the low temperature is shown to be favorable to the action of amylase of raw grain and unfavorable to that of malt diastase, which under these conditions produces an incomplete hydration.

At the optimum temperature the conditions are entirely different. The small quantity of diastase contained in raw grain can by itself cause a considerable saccharification which proceeds up to the point where the foreign substances have no more influence on the course of hydration.

By using diastase of raw grain one rarely observes such a degree of saccharification that the iodine test for starch is no longer obtained. The same phenomenon is observed when the diastase is used with accelerating substances.

To obtain a decomposition of starch corresponding to 40 or 50 per cent of maltose, a very small amount of infusion is sufficient if there is present a body accelerating its action. To reach 70 per cent of maltose it needs 10 to 20 times as much malt infusion, even if the work is done in the presence of asparagin.

We shall find later that there are really in raw grains substances which accelerate the diastase. These are the cause of all the differences observed by Lintner between the diastase of malt and that of grains which have not sprouted.

Brown and Morris also make a distinction between malt amylase and the enzyme of raw grains. They call the first "secretion diastase" and the second "translocation diastase." According to these authors, the two diastases act in an entirely different manner on raw starch. Secretion diastase corrodes the granules of starch, channels them irregularly and disintegrates them. Translocation diastase, on the contrary, produces neither corrosion nor disintegration.

The solution of starch takes place layer by layer; the

grains retain their original shape, but diminish gradually in size as long as they are visible.

This singular difference in the mode of digestion appears at first sight completely to confirm the hypothesis of the existence of different amylases. But this new argument appears much less conclusive if we study with more care the different modes of attack of starch by diastase. According to Krabbe, the attack on the starch is produced in a very different manner in different plants. In potato-starch and in that of grains the digestion is accomplished in successive layers; the corrosion is centripetal and uniform. In the case of leguminous plants the amylase produces at the surface of the grain fissures which extend towards the center of the grain where they unite, forming a cavity which constantly increases. Corrosion then takes place here in two directions; it is first centripetal, then it becomes centrifugal.

With grains, on the contrary, the starch is unequally attacked; channels and grooves are formed which extend towards the center.

These facts show us that the manner of digestion of starch varies in different plants. And, in reality, the manner of action of amylase is very complex, even when it is a question of the digestion of grains of starch of the same origin. Here also is found a very variable manner of working and it is observed that the grains are not all attacked in the same manner. When cold starch is treated with an infusion of malt, digestion occurs without any regularity. In certain cells the corrosion is accompanied by clefts and holes, in others the attack is made in a regular manner. These differences evidently come from the compactness and the non-homogeneity of the granules of starch. Moreover, the manner of digestion may also be influenced by the reaction of the medium as well as by the presence of foreign bodies.

Starch, difficultly dissolved in the cold, easily digests in slightly acid media. As the acid reaction favors but slightly the saccharification of starch, the action observed on whole

grains can only be explained by the change which the acid produces in the physical state of the grains; it is probable that this acid reaction favors the contact of the diastase with the starch.

The facts announced by Brown and Morris have not, moreover, received sufficient confirmation.

It has not yet been demonstrated that two diastases of different origin always act differently on raw starch and, even if this had been proved, one ought not to conclude from it that different amylases exist. The difference in action of various diastases might in fact come from the foreign substances which accompany them.

The artificial diastase of Reichler is also cited as constituting another variety.

This worker, on digesting gluten in a certain quantity of slightly acidified water, found that the saccharifying power of the liquid gradually increased.

The enzyme obtained in this way shows all the properties of diastases of raw grain, and it is admitted that it is formed by the action of the acid on the gluten.

According to Lintner the formation of that enzyme is due to a hypothetical substance contained in the gluten, practically a zymogen, which under the action of acids is transformed into amylase.

Malt amylase is, in fact, present in a very small quantity, and the increase of diastatic power results simply from the change in the medium produced by the action of the acid.

Changes Produced in the Activity of Diastases at a Temperature of 70°.—By saccharifying starch with an infusion of malt, very different quantities of maltose are obtained according to the temperature at which one works.

Analogous results are obtained by merely heating the infusion to different temperatures.

According to O'Sullivan, there is for each temperature a certain degree of hydration of starch which is easily attained but cannot be exceeded.

The infusion heated to

64°	would cause a decomposition	1 maltose and 1 dextrin.
68°	of starch corresponding to	1 " " 2 "
70°		1 " " 5 "

By keeping the diastase successively at temperatures of 64°, 68°, and 70°, a total change is each time produced in the manner of work, and it may be concluded that either real transformations of the active substance have taken place, or that there is an artificial formation of different types of amylase.

Our knowledge of the effect of chemical conditions upon diastases leads us to quite another interpretation.

The temperature has no other effect than to reduce the diastatic power. The nearer the temperature approaches to 70°, the greater is this reduction. Only, while the diastase is losing its real activity, there is still an apparent activity owing to foreign substances contained in the infusion, which act with a still greater energy as the diastase becomes weaker.

To sum up, we have here a phenomenon which we have already observed concerning the diastase of raw grains, only the action is more complicated in the present case.

The diastase, maintained at a temperature of 68° to 70°, has not the same properties as the amylase of raw grains: the saccharifying power has largely disappeared but the liquefying power has not been affected.

From this it results that the heated infusion, though acting like the diastase of raw grains, differs from the latter in the ease with which it liquefies starch.

Condition of the Secretion of Amylase.—After having studied the action of physical and chemical agents on amylase, we will briefly discuss the method of secretion of this enzyme, as well as the conditions which favor its production.

In grain in germination, it is the embryo alone which plays an active part; the rôle of the endosperm is entirely secondary.

The embryo of grains of barley, detached with care, may be transformed, if it is put in a damp place and under suitable conditions of temperature, into a little plant. The vegetation produced under these conditions is very delicate and short-lived, but the germ nevertheless consumes its reserve food and secretes amylase. If the germ is placed on its own endosperm reduced to pulp, the vegetation becomes normal and the course of diastatic secretion may be followed by the chemical transformation which is produced in the amylaceous matter. By cultivating the germ in different nutritive media and under different conditions, very interesting data may be obtained upon the conditions which regulate the secretion of diastase.

Brown and Morris, by adopting this method, have made some interesting discoveries upon the influence of different carbohydrates and of the acidity of the medium upon the production of the diastase.

By cultivating the same number of embryos, in simple gelatine on the one hand, in gelatine with six thousandths of a part of formic acid added on the other hand, they have found a noteworthy difference in the quantities of diastase secreted.

Fifty embryos cultivated in simple neutral gelatine have furnished a quantity of diastase corresponding to 0.118 gr. of oxide of copper. The diastase was found distributed in the following manner: In the germs 0.0708 gr., in the gelatine 0.0478 gr. The 50 germs cultivated in the acidulated gelatine produced a quantity of diastase corresponding to 0.145 gr. of oxide of copper. It was distributed as follows: In the germs 0.0904 gr., in the gelatine 0.0546 gr. The acidity of the medium, therefore, clearly favors the secretion of diastase. By adding to gelatine different assimilable carbohydrates other than starch, they have found that these substances act very unfavorably on the secretion.

The property of secreting diastase is, therefore, not a fundamental property of cells.

The appearance of the diastase depends upon the method of nutrition, but let us note, however, that this appearance does not always correspond to the real needs of the cells, and that it must not be considered as an indication of intelligence of the cells which, by the aid of a diastatic secretion, would adapt themselves to different media. A barley germ cultivated in gelatine in which it cannot obtain nutritive matters secretes the same quantity of amylase as if it were cultivated in starch. The secretion is always abundant when the germ is found in poor nutritive conditions and it is checked as soon as an assimilable substance appears.

Here, as in the case of sucrase which we studied above, the secretion of diastase is a consequence of mal-nutrition, and the primary cause of all the variations observed in the secretion is nothing else than the reaction of the medium.

The secretion of amylase, as we have just seen, is favored by acidity of the medium. The degree of acidity of the cellular substances, therefore, influences considerably the intensity of the secretions.

Starting with this statement, we can explain why secretion is favored where nutriment is lacking. The cells, when they find non-assimilable substances present, consume their reserves, and this consumption produces in their interior a change of composition which favors osmosis. The saline substances of the surrounding medium then penetrate more easily into the cells and, as a result of dissociation, there is produced an accumulation of acids which favor secretion.

Analysis of Amylase.—The method used to determine the diastatic power of a solution is based on the following observation of Kjeldahl:

As long as the diastase is in the presence of a large amount of non-transformed starch, the quantity of malt produced is proportional to the quantity of diastase contained in the solution: in other words, there is a constant ratio between the quantities of maltose formed and diastase em-

ployed, as long as the latter acts in the presence of a great quantity of non-transformed starch.

This observation has been verified and confirmed by various experimenters, and it is incontestable that by submitting various specimens of the same starch, at the same temperature, to the action of increasing amounts of diastase, quantities of maltose are obtained proportional to the quantities of diastase used. The condition essential to the success of this determination is that in all the specimens a minimum amount of diastase is used, an amount capable of transforming, at the most, 40 to 50 per cent of the starch into sugar.

Starting with this principle, it is easy to determine the fermenting power of a liquid. It is sufficient to have a standard diastatic solution of known value and to make comparative experiments with starch. A 2 per cent solution of soluble starch is generally used.

To 100 cubic centimetres of solution containing 2 grams of starch add 2 cubic centimetres of a solution of standard amylase. In another dish also containing 100 cubic centimetres of a solution of soluble starch add 2 cubic centimetres of the solution to be tested. Place the two specimens in a water-bath at 50°, and, after an hour of saccharification, measure the maltose in the two solutions.

The diastatic power of the solution is expressed by the ratio between the quantities of sugar formed with equal quantities of the experimental diastatic solution and the standard solution.

If there is found, for example, 0.4 of maltose in the product saccharified with standard amylase and 0.2 of maltose in the second specimen, we should say that the activity of the solution is 50 per cent, meaning that the solution examined is half as active as the standard liquid.

This method of analysis permits a comparison of the value of two products, but it does not permit the absolute expression of the fermenting power of a diastase, because it is very difficult to maintain in a solution of amylase a constant

diastatic energy. The results are, therefore, often uncertain.

To determine absolute diastatic values, we use a method in which we take for unity the quantity of diastase which, acting for an hour at 60° on 1 gram of soluble starch, gives 50 centigrams of maltose.

The following is the regular procedure:

Ten grams of anhydrous, neutral starch are dissolved in 700 cubic centimetres of boiling water. It is cooled and the volume of the solution brought up to 750 c.c. From this solution are taken a series of specimens of 75 cubic centimetres each. To these specimens are added different quantities of the active liquid to be examined and they are left for an hour in a water-bath at 60°. The saccharification finished, all the specimens are rapidly brought up to the boiling point, cooled, brought up to 100 cubic centimetres and in each of them the quantity of sugar produced is determined. The specimen in which 50 centigrams of maltose is formed is regarded as the standard unit. If these 50 centigrams are formed in the specimen to which was added 1 cubic centimetre of the solution experimented with, we say that the diastatic power of that solution is 100. If these 50 centigrams are found in the tube to which 2 cubic centimetres of the solution were added, we say that the diastatic power is 50, and so on.

It is often difficult with a single series of experiments to succeed in producing exactly ½ gram of maltose. So it is of advantage to make first an approximate experiment with 1, 2, 4, 6, 8, 10 cubic centimetres of active substance. If the unit of diastatic activity is approximated, for example, in the experiment made with 4 cubic centimetres of infusion, we repeat the experiments with 2.5, 2.75, 3, 3.25, 3.50, 3.75 c.c. of liquid.

One must also take into account in these experiments the quantity of reducing substances which may be found in the active solution. One must of course subtract from the

total quantity of maltose found after saccharification the quantity of sugar which was introduced with the infusion.

This method may also be applied to an analysis of malt.

To estimate the diastatic power of malt we must first extract the active substances. For that we reduce the malt to fine powder, add 20 parts of water and leave it for 6 hours at a temperature of 30°, shaking the solution every quarter of an hour. With the filtered infusion, saccharification is accomplished as has just been indicated. A malt of excellent quality affords under these conditions an infusion producing 50 centigrams of maltose per cubic centimetre of infusion. Still, this method does not furnish precise data on the value of a malt from a practical point of view. In the chapter treating of the industrial applications of amylase, we shall treat particularly of such analyses.

The determination of the saccharifying power of liquids containing slight amounts of amylase often presents great difficulties. To obtain an appreciable quantity of maltose, it is necessary to use a great quantity of solution which often contains reducing materials.

In such cases it is better first to precipitate the diastase by alcohol, but this method is applicable only when one has a quite considerable volume of solution at his disposal, for when this precipitation is practiced on a small quantity of infusion, a very fine precipitate is obtained which passes through the filter and consequently gives rise to perceptible losses. To remedy this difficulty, we have sought to produce in the active solution precipitates which are more voluminous and more easily separated. We have found that tannin leads to this result. In fact our experiments have shown us that this substance completely precipitates the diastase and that the inactive precipitate becomes active again when it is carefully treated with a dilute solution of sodium carbonate.

The method of procedure is here given:

To 10 cubic centimetres of active liquid add 4 centigrams

of tannin dissolved in a few cubic centimetres of water; stir it and leave it for a half-hour. The solution is then filtered and the precipitate, well washed with water and alcohol, is placed, without being separated from the filter, in a glass capsule containing 5 cubic centimetres of sodium carbonate (1 : 10,000). The filter is washed in the solution for one or two minutes; as soon as the precipitate is redissolved add a few drops of a solution of lactic acid (1 : 1000) to neutralize it, and filter.

All these manipulations must be made as rapidly as possible because the tannin precipitate changes by a prolonged exposure to the air and becomes insoluble in the alkaline solution. The contact of the precipitate with the carbonate of sodium must also be of very short duration. When the precipitate does not redissolve except after 4 or 5 minutes of contact, the experiment must be repeated because the diastase is already changed. The solution can be greatly facilitated by triturating the filter in a mortar with the alkaline solution. By working rapidly it is possible to redissolve all the active substances precipitated and avoid all loss. The precipitate obtained by the tannin, washed in water, alcohol, and ether, and then dried, gave on analysis the following figures, a deduction being made of 32.2% of tannin:

Water	5.53%
Nitrogen	8.83
Ash	1.32

This method is of special use when one wishes to determine amylase in vegetable cells.

In analyses of this kind the substances are reduced to powder. They are left to soak in 1 to 2 parts of water for 6 hours; the liquid is expressed from the substances not dissolved. The residue is again soaked with a volume or two of water and expressed a second time. The combined liquids are filtered and the diastase is precipitated from the mixture by tannin, in the same way as with the malt infusion.

The activity of the precipitate dissolved in the water gives an idea of the diastatic value of the substances submitted to examination.

Here, for example, is an analysis of bean-leaves:

Ten grams of bean-leaves are reduced to a paste in a mortar. Ten cubic centimetres of water is added to the mass and a few drops of chloroform, then it is allowed to stand for 6 hours. The leaves are then pressed and filtered in a cloth. To the residue is then added 10 cubic centimetres of water and a small drop of chloroform, after which the whole is left quiet for three hours. Then the liquid is separated and the residue washed again with water; the liquids of the two macerations and of the washing are combined, and the total volume made up to 50 cubic centimetres. It is again filtered and precipitated with 16 cubic centimetres of tannin. The precipitate is redissolved in alkaline water and the solution is made up to 10 cubic centimetres. It is found necessary to use 2 cubic centimetres of this solution to produce 50 milligrams of maltose. Therefore, the solution has a diastatic power of 50. If we compare the diastatic power of bean-leaves with that of a good quality of malt we shall obtain the following results: Ten grams of malt furnish 200 cubic centimetres of infusion, one centimetre of which produces 50 milligrams of maltose. Ten grams of bean-leaves furnish 10 cubic centimetres of liquid of which 2 cubic centimetres are needed to furnish 50 milligrams of maltose. Since the malt has a diastatic power of 100, the bean-leaves have one of 2.5. The malt contains consequently 40 times as much active substance as the bean-leaves.

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CHAPTER XI.

INDUSTRIAL APPLICATIONS OF AMYLASE.

Malting.—Chemical transformations which accompany germination.—Methods of malting, sorting, steeping, germination, brewing.

AMYLASE is formed in considerable quantities in the grains of cereals during germination. It is for this reason that the industries which utilize diastase as an agent of hydration make use of sprouted grain, called malt, a product which at the present time is the only agent of that kind capable of being manufactured economically. All cereals produce amylase during germination, but barley furnishes the greatest yields in active substance. When one heaps up barley previously soaked, one observes a series of phenomena which, all together, characterize germination. First is found an increase in temperature, an absorption of oxygen, and a liberation of carbonic anhydride, which is augmented as the temperature of the mass increases. Along with this phenomenon of respiration are observed considerable changes in the various constituents of the grain. The reserve materials, cellulose, starch, protein matters and fatty substances as well as sugars are partially transformed by hydration.

These transformations are due to a secretion of enzymes acting on the albumen and transforming it into assimilable substances, which are in part absorbed by the embryos in the course of their development.

After 24 or 48 hours of germination, there are seen to appear, on the outside of the grains, little roots which then grow quite rapidly. The development of the plumule is much

slower. After 8 or 10 days of germination, the length of the plumule reaches half or three quarters that of the grain. It is at this phase of development that germination is generally considered as ended.

The development of the germ is largely at the expense of the starch. Under normal conditions the expenditure in amylaceous materials is 8 to 10 per cent of the starch contained in the grains, but this proportion is considerably exceeded when germination is accomplished at a temperature higher than 20°.

During germination the grain secretes, besides amylase, other active substances, among them peptase, which transforms albuminoid substances into amides, and cytase which acts on certain kinds of cellulose.

The rôle of cytase is very important from the point of view of malting.

The starch in the grains is in the form of granules enclosed within resistant cell-walls. These cell-walls protect the starch against the action of the amylase, and the attack on the carbohydrate would not be very strong without the intervention of the cytase which disintegrates the envelope of the granules.

The action on the starch, during germination, is accomplished in two successive phases. In the first phase the cellulose envelopes of the starch cells are liquefied by cytase, and it is then only that the amylase begins to act on the starch. To the action of cytase must also be attributed the differences found between the starch of raw grains and the starch of malt.

On account of the destruction of the membrane of the cell the malt-starch is liquefied at a lower temperature than the starch of raw grains.

When germination is produced at a temperature of 15–17°, the secretion of amylase commences after 35 or 40 hours and the diastatic power then gradually increases for 8 or 10 days.

In the practice of malting the grains are submitted to a succession of operations.

The first part of the work consists in sorting and cleansing the grains. Then they are soaked, then allowed to germinate. The germinated grains are utilized in a fresh state in distilleries as well as in the manufacture of maltose; for the purposes of the brewery the sprouted grains are malted.

Without entering into all the details of these different manipulations, let us consider the principal points.

The sorting of the grains is done in special apparatus which eliminates foreign substances as well as broken grains. Moreover, this apparatus separates the grains according to their dimensions.

The grain intended for germination must not be too fresh. Grain taken immediately after the harvest has a small germinating power. It is only after some time that it becomes good for germination. Grain coming from different harvests must not be mixed, nor grain having different densities. To obtain a good germination it is really indispensable that the grains should be as far as possible of a uniform weight.

In sorting, the grains are separated according to their sizes. Grains of different sizes could not be put together in germination because they would soak unequally.

Grains of a different weight are not suitable for the same use. Heavy grains are preferable for use in the brewery, while light grains, containing less starch and furnishing a much greater yield in diastase, are suitable rather for the work of the distillery.

The sorted barley is then steeped. This operation is generally carried on in special vats which easily permit of changing the water. The aim of the soaking is to make the grains absorb the quantity of water necessary for a good germination. The grains, in contact with water, swell, absorb a certain amount of oxygen, and undergo different modifications.

They also lose a part of their soluble substances, especially salts and carbohydrates other than starch. The loss in extractive substances varies from 0.8 to 1 per cent.

The elimination of sugar by the soaking of the grains is very favorable to the secretion of the enzymes during germination.

In ordinary water, there is not sufficient oxygen for normal germination, so it is advisable to pass a current of air into the mass during the soaking.

The soaking water must be frequently renewed so that the dissolved substances shall not enter into fermentation.

Generally, the grains are washed in water before soaking them to free them from germs and ferments which may adhere to their surface.

The grains are left in water for 3 to 5 days, and the water is carefully renewed every 12 or 24 hours. The duration of the soaking depends upon many factors; it depends on the temperature as well as the quality of the water, but it depends especially on the quality of the grains. Thick-glumed barley absorbs water more slowly than thin-glumed grain.

The operation of soaking the grains may be considered as ended when they have absorbed nearly 50 per cent of water. By prolonging the soaking the grains would absorb a still greater quantity of water, but in that case germination would be less regular and there would be danger of getting mouldy malts. It is very difficult to stop the soaking at exactly the right point. This difficulty comes from the differences in the grains employed. It is, therefore, advisable to stop the process before the grains are sufficiently soaked. The danger of soaking too long is especially great when rye is used. The grains having had too long a soaking become sticky, acquire a pasty aspect, and the malt they furnish is of a doubtful quality.

The grains, after having been soaked, are carried to the malt-house, where they are spread in layers of 30 to 80 centimetres in depth, according to the kind of malt-house and

the manner of aeration. Malt-houses must conform to the two following conditions:

They must be (1) well ventilated, and (2) capable of being kept at a constant temperature.

The heaped-up grains heat quite rapidly. The oxidation of starch and fatty materials frees a quantity of heat sufficient to bring the entire mass to a temperature of 100°. It is then necessary to avoid raising the temperature. This is accomplished, either by frequently changing the position of the grains, or by spreading them in thinner and thinner layers as the action becomes more energetic. In the system called "pneumatic" the layers are cooled by a current of moist air.

Germination lasts from 8 to 10 days. It is desirable to work always at the lowest possible temperature. Generally the germination is begun at a temperature of 10–11° and is continued up to 17–18°, which limit is not passed.

When the malt is spread on a cement floor, the layers are made at the beginning of 40 to 50 centimetres in thickness, and then are progressively made thinner. The fourth day a thickness of 10 to 12 centimetres is reached. In the pneumatic system the depth of the layers remains constant, but the grain is often turned over to prevent the little roots from tangling.

During germination the moisture of the grains constantly diminishes and at the end of the operation they have lost from 50 to 60 per cent of the water which they have absorbed during the soaking. It often happens that the water absorbed during soaking is insufficient to ensure germination. In this case the layers must be sprinkled from the third or fourth day. The sprinkling is done systematically in small quantities and at frequent intervals. Generally the germination is checked when the length of the plumules reaches half or three quarters that of the grains.

It is generally assumed that at this time the grains contain the greatest quantity of active substances. In reality it is not so. The researches which we have made in this sub-

ject show that one cannot trust to the length of the plumules to determine the time when the quantity of diastase contained in the grains reaches its maximum, and that it is only analysis which can show when germination should be checked.

The following table traces the course of germination, at 12°–17°, of four different malts conducted under the same conditions.

Malt.	A	B	C	D	
	Diastatic Power.				
At the beginning.....	41	60	52	35	
1 day.....	50	70	70	40	
2 days.....	60	95	80	57	
3 "	60	95	81	62	
4 "	70	97	85	80	
5 "	81	95	87	85	
6 "	85	98	88	97	
7 "	95	100	86	100	
8 "	100	100	89	94	
10 "	96	100	85	80	

These experiments, as well as a very great number of observations made in different manufactories, have led us to the following conclusion: It is when the malt possesses plumules twice as long as the grains that the diastatic power reaches its maximum; however, in some cases the maximum is not reached at this time.

The quantity of diastase contained in the grain increases gradually in the course of germination; but often reaches its maximum before the plumules have reached the length indicated above.

The quantity of diastase developed in the malt often remains stationary for a certain time.

In other cases, on the contrary, a very rapid diminution of the quantity of diastase is observed. This diminution may, in fact, be observed in the table reproduced above. We have sought the cause of this decrease and have found that it

comes from the energetic oxidation which occurs when germination is very far advanced. It is really in the pneumatic malt-houses that diminution of diastase is most frequently found, while in common malt-houses the alteration of the diastase is much rarer. It may be that in addition to the oxygen of the air other factors also come into play to produce the diminution of the diastatic power of the malt.

When a very active malt is desired, it is indispensable that it shall be analyzed after the eighth or ninth day, and the variations of its diastatic power observed twice a day. It is only in this way that losses of diastase can be avoided.

In the brewery, fresh malt cannot be employed. To make it suitable for the manufacture of beer it must pass through the malt-kiln where, under the influence of a high temperature, certain principles contained in the grains undergo transformations which give to the malt a characteristic flavor, as well as a more or less dark color.

The drying is done by the aid of hot air and, according to the kind of malt which it is proposed to make, the drying is accomplished at higher or lower temperatures.

The fundamental principle of malting consists in raising the temperature gradually, especially at the beginning of drying.

Although the grain contains from 10 to 12 per cent of water, it is extremely dangerous to go above a temperature of 50°. In fact the malt diastase changes under the action of heat and this change is the more rapid as the grain contains a greater quantity of water.

The grains dehydrated below 50° may then be brought up to a temperature of 100° without completely destroying the diastase.

The highest temperature reached during the malting is from 103° to 104° for malt of the Munich type and only from 62° to 63° for malt of the Pilsen type.

Drying always destroys a part of the diastase, even when all possible precautions are taken. In drying the malt at the

maximum temperature of 50° and avoiding raising the temperature at the beginning, we have found that nearly 20 per cent of active substances are destroyed during the process. The loss is hence seen to be considerable.

Finally, there exists a great difference between the distillery malt and brewery malt.

As we have said above, it is well to choose for brewing-malt, grain which is very heavy and very rich in starch. For the distillery, on the other hand, light grain which furnishes more diastase is to be preferred. The germination of brewing-malt should be arrested when the plumules have acquired half or three quarters the length of the grains. When, however, it is a question of distillery malt one should allow the plumule to grow as long as possible.

Brewery malt may be aerated up to the last moment, while for distillery malt, aeration should cease during the last two or three days.

Finally, there is a great difference in the drying of malt according as it is destined for the brewery or the distillery. For the distillery the temperature must be the lowest possible, while for the brewery it must be quite high.

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CHAPTER XII.

RÔLE OF AMYLASE IN THE BREWERY.

THE brewing industry was at first carried on by following empirical methods, and it is only within thirty years that the manufacture of beer has drawn the attention of investigators. The works of Pasteur, Dubrunfaut, and Hansen have brought to this domain valuable data which form at the present time the scientific basis of this industry. The researches of these workers have brought about noticeable improvements in the methods of manufacturing beer.

It must be recognized that at the present time empirical methods have not entirely disappeared from the business of the brewery, and that science cannot yet explain all the phenomena observed in the manufacture of beer. To carry out this manufacture successfully, it still requires more practice than science.

The brewery uses as raw materials malt, hops, water, and yeast. With these simple materials an almost infinite variety of fermented beverages is made. The variations in beers come, in the first place, from differences in the quality of the raw materials. Brewing-malt is far from being a substance of constant composition. It varies according to the origin and quality of the barley, and also according to the method of malting employed. The same is true of the other factors which enter into the manufacture of beer. In fact, different yeasts act very differently in the same mash and give very different results.

The difference in the character of the beers may also be influenced by the quality of the water or that of the hops.

The taste and the appearance of the fermented product may also change on account of the intervention of bacteria and of foreign yeasts. All these causes undoubtedly influence the manufacture, but variation in raw materials does not explain all the differences observed among fermented beverages.

The character of a beer depends in reality on a number of factors: on the method of work, the manner in which the malting and brewing are conducted, the methods of extraction and saccharification, as well as the mode of fermentation.

As may be seen, brewing is an exceedingly complicated industry. To understand the process, a very complete scientific knowledge is necessary, and even then one often finds problems which have not been scientifically solved. Fortunately, the brewer solves the difficulty by observation as well as by the routine he has acquired. Malt is generally very rich in enzymes and the amylase it contains can hydrate 10 to 20 times as much amylaceous matter as the malt contains. Liquefaction and saccharification take place without difficulty when there is a great deal of diastase. If complete saccharification were the sole object sought, the problem would be easily solved. But, in reality, the brewer has in view, not merely a complete transformation of starch into sugar; indeed, he often wishes to prevent complete saccharification. In fact, it is especially important to him to succeed in a particular decomposition of starch and to obtain certain dextrins which resist the action of yeasts. He often desires the production of difficultly fermentable sugars which remain intact during the principal fermentation and come into play only in the after fermentation.

The method of decomposition of the starch influences to a great degree the character of the beer, and, according to the

type of beer that the brewer proposes to produce, more or less dextrins and easily fermentable sugars must be formed.

Under these conditions, the presence of a large quantity of diastase is undesirable rather than useful. For this reason the brewer, even before he could have known the scientific reasons, always sought conditions which hinder saccharification and the action of an excess of amylase. Thus in drying, the formation of dextrins is favored, and by saccharification at a high temperature the excess of enzymes is destroyed.

The influence of the temperature of saccharification on the quantities of maltose and dextrins formed is shown in the following table from Petit, which shows the quantities of maltose and of dextrins formed at different temperatures as well as the relation between these quantities.

Temperature of Saccharification.	Maltose. %	Dextrins. %	Ratio.
60–61°	72	30	1 : 0.4
65–66	71.4	31.8	1 : 0.44
68–69	44.7	57	1 : 1.27
72–73	24.7	76.3	1 : 3

We have said above that the methods of saccharification and of drying influence, not only the quantity and nature of the dextrins, but also the nature of the sugar.

In fact, by saccharifying starch under certain conditions, combinations of maltose and dextrins are procured which act differently from maltose and dextrins alone.

Thus, when a beer-wort is left to the action of yeast, it is found that the liquid still contains, when fermentation is finished, a certain quantity of maltose. The non-fermentation of the remaining sugar is not at all due to the exhaustion of the yeasts, as one might think at first. Hence it is that the addition of pure maltose to the fermented wort induces a new fermenation which exhausts the added sugar, while the sugar remaining in the wort is hardly attacked by the yeasts during the new fermentation.

To explain this fact, it is supposed that maltose can form combinations with dextrins, which are called malto-dextrins. These bodies have not been isolated in a pure state and their chemical individuality is far from being demonstrated. Yet it is beyond doubt that a considerable difference exists in the fermentability of the various sugars obtained by saccharifying starch by malt under different conditions.

This difference may be attributed either to the actual existence of different maltoses having different geometrical structures, or to the formation of more or less stable combinations of maltose and dextrins.

The authors who have studied especially the decomposition of starch by malt generally assume the existence of different types of malto-dextrins which are characterized by the relative quantities of maltose and dextrins which they contain.

Malto-dextrins containing a great quantity of maltose are called malto-dextrins of a low type; while malto-dextrins containing dextrins in large quantity and little maltose are of a high type.

The dextrin contained in malto-dextrins is transformed by diastase at temperatures higher than 55°, while above 63° malto-dextrins remain unattacked. Beer yeast decomposes these combinations into fermentable materials and dextrins. This decomposition is always produced more or less slowly according as the yeast acts on a low or high type of malto-dextrin.

The formation of combinations of maltose and dextrins depends on the temperature of saccharification. By the action of the diastase below 50° maltose and free dextrins are formed without malto-dextrins. By allowing the diastase to act between 55° and 62°, the appearance of maltose combined with dextrins is observed and the malto-dextrins increase considerably when this temperature is exceeded. The composition of wort, from the point of view of its amount of maltose combined with dextrins, may consequently be regulated by the choice of the temperature of saccharification.

According to Petit, there is obtained, with the same malt successively saccharified at 60°, 65°, and 69°, the following respective quantities of malto-dextrins:

Temperature.....	60°	65°	69°
Malto-dextrins.....	2.4%	6.6%	16.2%

Temperature, while influencing the formation of malto-dextrins, does not greatly influence the kind of malto-dextrins transformed.

Thus the temperatures comprised between 60° and 65° all produce the same type, and it is only at a temperature of 69° that one succeeds in appreciably increasing the amount of dextrin in the malto-dextrins formed.

The temperature of malting also has a manifest influence on the course of the hydration of the starch.

Brown and Morris, by analyzing worts obtained with four malts prepared at different temperatures in ascending series, have found the following figures.

	Experiments.			
	1	2	3	4
Diastatic power.....	47	45	34	17
Per cent of malto-dextrins.....	4.25	7.9	14.9	22.4
Type of malto-dextrins obtained	1 : 0.5	1 : 1.5	1 : 2	1 : 2

As is seen, the temperature of drying acts both on the quantity and the nature of the malto-dextrins. The malt containing the least diastase furnishes both the maximum of combined maltose and the highest type of malto-dextrin. The qualities and the properties of the beer are influenced to a great degree by the quantity and the type of malto-dextrins formed during manufacture. These substances exert an influence on the attenuation and the taste, as well as the preservation of the beer.

We cannot in the present volume describe the different methods of brewing and we prefer to refer the reader to special works. Let us only remark that by modifying the manner of hydration of the starch, beers of different kinds

are produced. In fact, the method of conducting the brewing influences to a great degree the composition of the wort which in its turn acts on the quality and type of the beer.

Even before the decomposition of starch had been explained theoretically brewers understood the conditions necessary to procure a wort having the qualities required in each case. When the brewer proposed to make beers of great attenuation and rich in alcohol, he found it necessary to effect the brewing in such a way as to avoid the formation of great quantities of malto-dextrins. When it was a question, on the contrary, of a beer of low fermentation followed by a prolonged secondary fermentation, he sought to obtain a great quantity of malto-dextrins of a very high type.

For top-fermentation beers the manner of conducting saccharification also depends upon the degree of density of the wort. Wort intended for the manufacture of light beers is generally completely saccharified, while for strong beers, on the contrary, it is sought to produce dextrins in much larger proportion.

It is then by drying at a suitable temperature and by the duration of saccharification that one succeeds in producing worts of very different compositions, while using the same primary materials.

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CHAPTER XIII.

MANUFACTURE OF MALTOSE.

By the action of malt on starch one can obtain according to the duration of the process and the temperature which is employed, a series of products differing in the degree of hydration. By saccharifying a paste containing from 5 to 7 per cent of starch with an infusion of malt at a temperature of 40°–45°, an almost complete transformation of starch into maltose is obtained after 12 to 15 hours. The saccharin liquid, evaporated to the consistency of 40°–42° Baumé, forms a white crystalline mass containing only 1 to 2 parts of dextrin to 100 parts of sugar. A product of an entirely different nature is obtained by saccharification at 60°–62°. If the duration of saccharification is limited to 30 or 60 minutes and the work is performed with an excess of diastase, a strongly saccharified syrup is obtained containing from 20 to 25 parts of dextrin for 100 of sugar. By saccharification at 68° products are obtained which possess only 60 per cent of maltose.

These different products owe their industrial applications to the work of Dubrunfaut and Cuisenier.

These workers made a thorough study of saccharification by malt, and they projected an industrial process which appears to have a very great future in store for it.

Dubrunfaut, in promoting the manufacture of maltose, hoped that the different products of saccharification would find many applications in different industries. He believed that pure maltose could replace with advantage cane-sugar

in wine-making and the manufacture of liqueurs. The saccharified syrup was to have a place in all the industries which made use of glucose, for example in pastry, in preparation of preserves, bon-bons, etc. Products containing a large proportion of dextrin would be especially useful in the brewery where they would replace a great part of the malt.

The expectations of Dubrunfaut have not been completely realized.

The maltose industry had a great development at a certain period. Manufactories were started in France, Belgium, Holland, and England, and the production of this sugar reached very large proportions. Of late years, however, this industry, for various reasons, has undergone a considerable falling off.

Nevertheless, the state of the maltose industry does not warrant the prediction that it is destined to disappear.

The advantages which saccharification by malt afford over saccharification by acids are unquestionable, and we are absolutely certain that eventually this industry will replace glucose manufacture.

As the industrial preparation of maltose is very little known, we will give here some information concerning its technique.

Potato-flour, rice-flour or maize-flour is used as the source of the product. From an economic point of view maize is the raw material offering the most advantages. Unfortunately the handling of this cereal presents great difficulties as to filtering and decoloration of the syrup. To procure products of a good appearance and to obtain satisfactory yields it is necessary to have strictly constant conditions.

The successive operations are as follows:

1st. Grinding.

2nd. Drying.

3rd. Saccharification.

4th. Filtration.

5th. Clarification.

6th. Second filtration.
7th. Evaporation.
8th. Second clarification.
9th. Evaporation at 40°.

The maize, coarsely ground, is introduced into a horizontal receptacle furnished on the inside with a paddle. Each cooker receives 750 kilograms of meal and enough water to give after cooking 45 hectolitres of liquid. The pressure is quickly raised while the mass is agitated, and remains 40 minutes at 3 atmospheres. As it takes about 40 minutes to arrive at this pressure, the cooking is ended after about 80 minutes.

The cooked maize is sent into a second horizontal receptacle furnished with a double wall, a Bohm crusher, and a paddle. A small amount of malt is added at a temperature of 70°–75°, and in 5 or 10 minutes liquefaction of the mass takes place. Then it is cooled by the water jacket, the rest of the malt is added at a temperature of 65°, it is left about 20 minutes at this temperature, heated again to 70°, and the mass filter-pressed. For the manufacture of syrup containing dextrin saccharification is prolonged for 1 hour at 68°.

Malt-sugar syrup requires for its manufacture 25 per cent of fresh malt. For dextrin syrup the quantity of malt is reduced to 15 per cent.

Great importance is attached to the filtering, and this operation influences to a great degree the quality of the product as well as the yield.

The passage through the filter-press should be made very rapidly, and the filtrate should be perfectly clear. An incomplete filtration causes a change in the juices and shows at the same time a poor extraction.

The slight turbidity found in badly filtered solutions reveals the presence of a certain quantity of starch capable of producing trouble during the concentration of the juices.

To get a good filtration it is essential to use a malt whose

plumule is very long, and to reheat the saccharified wort at a temperature of 70°.

In maltose manufactories filter-presses of 70 square centimetres are generally used, furnished with 12 frames covered with linen. A battery of 7 filters furnishes in 15 minutes 45 hectolitres of juice of 2.5° to 3° Baumé.

The filtered juices are placed in copper reservoirs furnished with a double wall for the entrance of the steam. They are reheated rapidly to 75° and left for about half an hour at this temperature for clarification.

An abundant precipitate is formed which is separated by a second passage through the filter. This second filtration presents no difficulties. It is carried on in a filter-press of small dimensions.

The clear juices are evaporated in a triple-effect apparatus where they are concentrated to 22° Baumé.

The syrups are then submitted to purification and to a bone-black treatment. The syrups are placed in special reservoirs in which are added 10 kilograms of powdered bone-black and 500 grams of dried blood per 25 hectolitres of syrup.

It is kept boiling for 10 minutes, filtered and concentrated in a vacuum up to 40°–42° Baumé. For the manufacture of products which are highly clarified the syrups, after purification, are placed in the battery of bone-black filters where they remain from 5 to 8 hours.

The yields usually obtained in the manufactories are from 92 to 94 kilograms of syrup at 40° per 100 kilograms of maize, but to secure this result a very well-conducted operation and much attention are needed.

To give an idea of the influence of the method of work on the output we may state that in the first years of the manufacture of maltose the yield was only from 60 to 65 kilograms of syrup per 100 kilograms of maize, and that it was only later, owing to successive improvements, that the results mentioned above were reached.

Properly prepared syrup generally keeps well, but better in the open air than in closed reservoirs. In large reservoirs exposed to the air, change is never found, while syrup placed in barrels often ferments. The analysis of the industrial products is given below:

LUMP MALTOSE.

Water	18.9
Maltose	80.6
Dextrin	0.2

WHITE SYRUP (FECULA).

Dry substances	77.1
Maltose	59.2
Dextrin	17.4

SACCHARIFIED MAIZE.

Water	20.2
Maltose	45.
Dextrin	33.
Nitrogenous matter	2.2
Mineral substances	0.91

DEXTRINATED SYRUPS.

Water	20.
Maltose	30.2
Dextrin	48.
Nitrogenous matter	2.1
Mineral substances	0.91

SYRUPS OF RICE.

Water	18.8
Maltose	71.
Dextrin	2.4
Foreign substances	8.2

The maltose syrup affords very great advantages over that of glucose in its purity and economy.

Maltose is a nutritive substance of great value. In the living organism it is transformed into assimilable sugar more rapidly than is saccharose. It is very easy to digest and, having not so sweet a taste as cane-sugar, it can be taken in much greater quantities than the latter.

By the action of acids on starch industrial glucoses are obtained which contain, besides dextrins, foreign bodies formed under the influence of the acids at high temperature. These bodies give a disagreeable taste to glucoses and often possess poisonous properties.

The dextrins formed under the influence of acids have a scant nutritive value. The pancreatic juice acts very slowly on these dextrins and its action is always incomplete.

As shown by the experiments made by Soxhlet and Stutzer, dextrins formed by malt act quite differently; they are much more easily transformed by diastases.

Saccharification by malt affords another great advantage: that of being able to utilize amylaceous materials directly without going through the manufacture of starch.

By treating maize with acid great modifications are caused in the nitrogenous matters as well as in the fatty matters. The products obtained are black, of a disagreeable taste, and not suitable for the manufacture of beer.

To obtain the purest products it is necessary first to extract the starch, which entails great losses. Out of 60 kilograms of starch, contained in 100 kilograms of maize, only 50 to 52 kilograms are recovered in practice. One loses, therefore, 8 to 10 kilograms of starch, as well as other nutritive substances, organic and mineral, which enter into the composition of the grain and which are utilized in the manufacture of maltose.

The maltose industry also furnishes a more wholesome and more nutritive malt than that furnished by the glucose industry. It is, therefore, indisputable from a hygienic as

well as economic point of view that maltose is preferable to glucose.

The crisis through which the maltose industry is at present passing is not likely to lead to its abandonment. This manufacture presents certain advantages and the efforts made by Dubrunfaut and Cuisenier will not have been in vain.

The patents which protected this industry have fallen into disuse and this circumstance will certainly not fail to give it a new impetus.

CHAPTER XIV.

PANARY FERMENTATION.

Dumas' theory of panary fermentation.—Cerealin of Mège-Mouries.—The part played by bacteria in panary fermentation.—The origin of the sugar in flour.

The work of bread-making is done in three successive stages: kneading, fermentation (raising), and cooking.

The first of these operations has for its aim to make with the flour an elastic and homogeneous dough.

To this end a little yeast is diluted in warm water, flour is added little by little, then the mixture is stirred and the mass is kneaded. Thus a dough is formed into which a certain quantity of salty water is uniformly worked. The kneading finished, the mass is left for some time.

The incorporated yeast then causes a fermentation which modifies the structure and the chemical composition of the dough.

This fermentation constitutes the second period, which is called "raising." It takes place in the kneading-trough and generally lasts from 20 to 30 minutes.

The dough is then divided into parts of a certain size to which is given the form of a loaf of bread. They are sprinkled with flour and again left quietly for 30 to 40 minutes, after which they are baked in ovens brought up to 250° or 300°.

The leaven used in the preparation of bread comes from a previous operation. After kneading the baker takes away a small quantity of the dough and uses it as leaven in the

next operation. The same ferment is used in this way for an indefinite number of times.

The principal agent in panary fermentation is a *Saccharomyces*. But this is not the only factor; others come in play, and here, too, are found diastatic actions.

Corn, rye, and all other cereals, contain considerable quantities of amylase and substances which accelerate diastatic action. By grinding, it is true, a great part of the diastase is eliminated with the bran, but the flour is not completely deprived of active substances. These remaining enzymes play successively an important part in the various stages in the making of bread. The action of the diastases of the grains begins during the milling. This action is continued during panary fermentation, and may even be evident during baking. The part played by the yeast, as well as the physical and chemical phenomena which are manifested during bread-making, have given rise to different theories.

Dumas regards panary fermentation as an alcoholic fermentation. According to him, the starch and gluten of the flour have been already partially hydrated as a result of mixing with water. This hydration would also be favored by the kneading which scatters the yeast uniformly throughout the mass and brings it into contact with the air, a condition which favors fermentation.

During the raising the carbonic acid formed in the mass is imprisoned in the cavities of the dough, to which the gluten has given coherence. During baking the sudden elevation of temperature expands the gases enclosed in the dough, and produces a swelling of the mass as well as a closer adherence among the hydrated materials, the starch, gluten, and albumen.

According to Dumas, the carbonic acid produced by panary fermentation remains almost entirely in the bread, of which it occupies about half the volume, at a temperature of 100°.

The yeast then, according to him, would act by the car-

bonic acid it produces and the fermentation would occur at the expense of the sugar already existing in the flour.

The theory of Dumas likening panary fermentation to an alcoholic fermentation has met with various objections; certain authors have objected that, in panary fermentation, there is neither production of alcohol nor multiplication of yeasts.

According to Mège-Mouries, the bran contains an active substance which he calls cerealin and which has the property of transforming starch successively into dextrin, glucose, and lactic acid. This substance is not met with in the flour, but, according to Mège-Mouries, the gluten itself can saccharify the starch and make it ferment.

The presence of alcohol in the dough after raising escaped detection for a very long time. Moreover, different experimenters have arrived at the conclusion that the yeasts introduced with the leaven do not multiply during the raising.

Relying on these data, and on the almost invariable presence of bacteria in the leaven, some bacteriologists have evolved the hypothesis that it is the bacteria and not the yeasts which produce the fermentation.

In 1883, Chicandard described the *Bacillus glutinis* which he considers is the agent of panary fermentation.

Laurent, in his later works, has described the *Bacillus panificans.*

Popoff has isolated from baker's dough an anaerobic bacillus which, in the presence of sugar, produces carbonic acid and lactic acid.

The bacteriological analyses of leavens made by Peters and Boutroux have demonstrated the constant presence in the leaven of bacteria secreting diastase and acting on starch and albuminoid matters. Furthermore, the presence of bacteria of the same nature in corn-meal has been ascertained.

The constant intervention of ferments in bread-making may then be considered as demonstrated.

According to some, the bacteria alone cause fermentation; according to others, the bacteria act in symbiosis with the yeast: the former, by the aid of their diastase, would furnish sugar to the yeasts.

Wolffin succeeded in producing normal bread by replacing the leaven by a culture of *Bacillus levans*. Analogous experiments have been made by Popoff with the same success.

Boutroux, who has taken up these experiments again, and carefully studied bakery yeast, has reached the following conclusions:

1st. Alcoholic yeast is always present in the leaven of bread.

2nd. This yeast is cultivated from dough to dough in such a way that by sowing a first dough with imponderable traces of yeast, there will be found, at the end of several operations, a uniform distribution of yeast in the dough.

3rd. The other micro-organism found in the dough, and to which may be hypothetically attributed the power of making it rise, acts quite differently: transferred from dough to dough, it ceases to produce fermentation after the second or third operation.

The presence in the leaven of bacteria favoring bread-making is on the whole an exceptional phenomenon.

It appears from the studies of Boutroux that generally the presence of bacteria is unfavorable: they attack the gluten and prevent the bread from rising. In practical baking the destructive action of these bacteria is checked by the presence of the yeast which, in a normally constituted dough, finds an excellent field for development, antagonizes the foreign organisms, and is alone of importance in panary fermentation.

The opinion of Dumas is also confirmed by the experiments of Moussette and Aimé Girard, who have succeeded in ascertaining the presence of alcohol in the products of panary fermentation.

Moussette, by condensing the steam of bread-ovens dur-

ing baking, has obtained an alcoholic solution containing 1.6 per cent of alcohol.

According to Girard, the same weight of alcohol as of carbonic acid is formed during fermentation. He finds nearly 2.5 grams of each of these substances per kilogram of bread.

According to some authors, the sugar consumed in panary fermentation, and which equals nearly 1 per cent of the weight of the flour, comes directly from the grain.

To support this opinion we may mention barley, which always contains appreciable quantities of fermentable sugars. But the amount of sugar in corn is really very variable, though it is observed that cereals which contain quantities of fermentable materials insufficient for panary fermentation nevertheless ferment as energetically as cereals which are rich in sugar. On the other hand, flour which is deprived of certain constituent parts of the grains is found, as a result, poorer in sugars.

According to Aimé Girard, Boutroux, and Morris, there is produced during the growth of gramineous plants an accumulation of sugar in the stem; this sugar, at the time of the formation of starch, passes into the embryo of the grains and is there transformed into starch as the grain ripens.

As a result, there will be found only traces of sugar in the ripe corn, and the flour will be free from natural sugars since during grinding the greater part of the germ is carried away.

In view of this fact, it is pertinent to ask whence comes the sugar which serves for fermentation. According to Poehl, the fermentable sugar found in flour is produced during the grinding of the grains, as the result of a diastatic action on the starch. This diastatic action is manifested only with the grains containing a certain quantity of water, while the dry grains do not furnish any.

Thus, when one treats a grist of corn containing 11 to 13 per cent of water with 90° alcohol, one finds in the liquid

the presence of reducing sugar. The same grain, previously dried and then submitted to the same treatment by alcohol, furnishes no sugar.

There is really then a transformation of starch into sugar and the action of amylase is consequently shown at the time of grinding. It is quite reasonable to suppose that hydration once begun continues during kneading and raising, although the amount of sugar does not perceptibly increase during these stages of the work.

The intervention of diastase is shown with more clearness during baking. The dough, once introduced into the oven, heats very unequally. At the surface the temperature rises abruptly and causes the formation of a crust which prevents the volatilization of the gases and the water vapor formed. Inside the temperature rises very slowly, a circumstance which favors alcoholic fermentation as well as diastatic action, then the diastases continue to act up to a temperature of 80°. Under the action of the water vapor and the heat, the grains of starch are transformed into soluble starch and amylo-dextrins.

The small quantity of diastase contained in the flour is in excellent condition to cause hydration of the starch paste, which cannot be formed except in very small quantity, owing to the lack of water. It is especially during baking that maltose and dextrins are formed in the bread and give to it a characteristic taste and consistency. Flour of superior quality generally contains small quantities of diastase, while flours containing a certain quantity of bran are richer in active materials which influence to a great degree the character of the bread. Thus the soft crumb of brown bread is due exclusively to the diastase of the bran.

White bread, soaked in warm water, furnishes a half-solid mass and only about 6 per cent of the materials dissolve. Brown bread, treated in the same way, gives to the water a milky aspect and 45 to 50 per cent of the dry matter is dissolved. This difference in solubility comes from the differ-

ence between the modes of action of the diastase in the two kinds of bread.

In bran, in the germs of corn, and consequently in the flour too, there are still other enzymes which take part in the bread-making.

The transformation which the gluten undergoes during the raising and baking appears to us to be due to a diastatic action, but this question is not yet very clearly demonstrated.

The intervention of enzymes is much more evident in the coloring of flour.

In flour, there are found oxidizing enzymes to which we shall have occasion to return in studying oxidases.

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CHAPTER XV.

RÔLE OF AMYLASE IN THE DISTILLERY.

Treatment of grains by acid and by malt.—Influence of heating on saccharification.—Choice of temperature of saccharification.—Principal and secondary saccharification.—Experiments of Effront on change in diastases during saccharification.—The infusion process.—Change in diastases during the successive stages of the work.—Control of the work in the distillery.

AMYLACEOUS materials do not ferment directly by the action of yeast. To make them readily subject to the alcoholic fermentation, it is necessary to submit them to a previous saccharification.

To produce this transformation, the distiller has for a long time used mineral acids, and it is only in recent years that these agents have almost wholly disappeared from manufactories, where they have been replaced by malt.

The use of acids as saccharifying agents presents, as a matter of fact, some notable disadvantages. To obtain a complete saccharification without considerable loss of the sugar formed, it is necessary to have very dilute mashes, to keep them for a very long time at a temperature in the neighborhood of 100°, and to use considerable quantities of acid which must necessarily be neutralized before the addition of the yeast. Saccharification by acids is, therefore, not very economical, moreover it is never complete, and the greatest yields which can be obtained are never above 50 or 53 litres of alcohol per 100 kilograms of starch used.

Working with acid presents still another disadvantage: it gives a residue which cannot be utilized for food for cattle, a disadvantage sufficient to condemn the method.

By using malt all the disadvantages of the acid process disappear, and saccharification takes place comparatively rapidly. The malts obtained in this way are of good quality and the yield in alcohol exceeds 65 litres for each 100 kilograms of starch used. Nevertheless, the malt process has its difficulties, also. It is not always easy to prepare a malt corresponding to the needs of the distillery, and it is often very difficult to use it to the best advantage.

Of all the industries which use diastase as a saccharifying agent, the distillery undoubtedly has the most difficulties to contend with in the use of amylase. It is, in fact, the amylase which plays the principal part in this industry, for it regulates the course of fermentation, and influences all the stages of the work.

A thorough knowledge of the method and conditions of the action of this diastase is, therefore, indispensable in order to direct the work suitably.

For this reason, in studying the process of distillation solely from the point of view of the part played by the malt, we may review the successive operations in that industry.

Cooking.—Starch removed from the cells is not easily attacked by amylase but, when it is not freed from the grains which enclose it, its transformation by diastase is still more difficult. The intercellular substances and the cellulose membrane of starch-containing cells prevents contact of the enzyme with the granules of starch.

To render efficacious the action of the diastase on amylaceous materials, it is necessary to submit them to a cooking which dissolves the intercellular substances and frees the grains of starch.

By working with finely ground amylaceous materials, the combined actions of heat and water favor to a great degree the action on the starch, and cooking in contact with the air is sufficient to obtain a paste which is easily saccharified by amylase. However, in working with whole grains, it is necessary to work under pressure.

In practice, the steaming is done in closed vessels, where the grains are submitted for about 2 hours to a pressure of 3 to 4 atmospheres.

Increase in temperature is very favorable to the solution of the starch, but it presents great disadvantages from other points of view.

The principal part of the grains, the starch, resists high temperatures without decomposing, but this is not true of the other substances constituting the grain, of the sugars, for example, which are destroyed at high temperature. By cooking a mash containing sugar at different temperatures, it is found that the destruction of the sugar increases in a great degree in proportion as the pressure increases.

Thus a mash containing 15 per cent of maltose kept for

½	hour	at 2	atmospheres	loses 0.85	of sugar.
"	"	3	"	1.7	"
"	"	4	"	3.4	"

Grains, and especially potatoes, contain quite considerable quantities of fermentable sugars, and the destruction of these must necessarily bring about a perceptible loss in alcohol.

High pressure also has the effect of dissolving different substances which enter into the composition of the grains. The increase, in the mash, of the quantity of extractive substances under the influence of high pressures is considered by different authors as a proof of the efficiency of steaming. It is on this basis that we are sometimes advised to exceed the pressure of 3 atmospheres during cooking. It is unquestionable that high pressure increases the density of the mash, and that it favors increase in the quantity of reducing substances, but this fact does not necessarily mean an increase in the alcoholic output. On the contrary, numerous experiments made to this end have shown that a mash of much cooked grains, while giving a good saccharification with amylase, furnishes a yield in alcohol inferior to that of

a mash prepared at a moderate pressure. Thus three grain mashes, prepared at different pressures, but with other conditions constant, give the following results:

	Density Balling.	Alcohol.	Diastatic Power.
2 atmospheres.....	17	10.5	40
3 "	18.1	10.3	28
4 "	18.6	9.8	13

It is seen that the mash cooked at 4 atmospheres possesses a density of 18.6, while the mash cooked at two atmospheres shows a density of only 17. We may observe at the same time that the maximum amount of sugar does not correspond to the greatest yield of alcohol. Indeed the must prepared at 2 atmospheres furnishes 10.5% of alcohol, while the must prepared at 4 atmospheres gives only 9.8%. Under the heading " Diastatic Power " we find an explanation of this anomaly.

The grains cooked at 2 atmospheres and then saccharified under the same conditions show a fermenting power of 40; the diastatic power diminishes with the increase of pressure and at 4 atmospheres a fermenting power of only 13 is found. The cooking of the must gives rise to certain substances which weaken the enzymes during saccharification. The cooking under high pressure therefore brings about, as immediate consequence, an incomplete fermentation. The nature of the harmful substances is not exactly known, neither can it be determined what are the bodies which give rise to them; nevertheless the formation of substances impeding diastatic action cannot be doubted. It is well to take note of this fact, especially when it is proposed to work with a limited quantity of diastase.

The most rational method of work consists in making a very fine meal of the grains, and in cooking this meal for 1½ to 2 hours with water at 1½ or 2 atmospheres at the most.

Under these conditions, mashes are obtained which do not weaken the diastase. This mode of work also offers the

great advantage of furnishing a much more wholesome malt than that obtained by cooking at high pressure.

It is very difficult to show in a conclusive way the unfavorable influence which cooking at high pressure has upon the quality of the malts. Chemical analysis gives us data on the amount of nitrogen, phosphates, and organic materials in the malts, but it does not give us any information as to their nutritive value, and the comparative value of the malts cannot be determined except by experiments on animals.

Experiments of this kind would have to be made in an agricultural station having a distillery at its disposal. We do not think that experiments of this kind have been attempted, and at any rate we do not know the results which they have given. Still, our opinion on the comparative value of different malts, according to the temperatures at which the cooking has been done, results from an inquiry we have made. Information we have gained from different distillers and agriculturists proves that cattle eat more readily malts obtained by cooking the grains at slight pressure. These malts can be consumed by them in greater quantities than the malts cooked under high pressure. These same malts have not, like those prepared at high pressure, an unfavorable effect on the quantity and the quality of the milk.

The influence of cooking on the nutritive value of the malts may be particularly observed in towns possessing several distilleries. The farmer who buys liquid malts always ends, after longer or shorter trials, by giving preference to one of the distilleries, and this preference is always in favor of the manufactory using low pressure.

It appears to us probable that the same substances which influence saccharification unfavorably hinder digestion of the malts obtained by cooking at high pressure.

Saccharification of Amylaceous Materials.—By cooking, the substances which in the grains are interposed between the starch-bearing cells are partially dissolved, and

the starch-cells are liberated from the tissues where they were enclosed.

Inside the cells the grains of starch first swell and then liquefy. To remove this starch from the cells, it is necessary to have recourse to a mechanical action which bursts the cellulose membrane, which resists very strongly the action of heat. This bursting is necessary because, if the liquid starch remains enclosed in the cells, it will undergo an incomplete saccharification only.

To this end the cooked mass is vigorously stirred, the mash is expelled from the macerater by strong pressure, and the action is completed by a crushing which breaks up the mass and bursts the most resistant cells. The mash thus prepared is suitably cooled, malt is added, and it is left to saccharify.

The determination of the temperature at which saccharification should be carried on has been made both by manufacturers and by scientists. Still, in spite of all the efforts of this kind, the question is yet unsettled on account of the divergence of opinion on the subject. To understand the difficulties which are met with in the choice of the temperature for saccharification, one must first of all realize the many and varied results which it is desired to obtain by this operation, namely: the liquefaction of the starch of the raw grains and the proper utilization of the starch of the malt.

Finally one must take account of the presence of germs and bacteria in the malt, as well as the acidity of the medium and the change in the diastase.

Theoretically, by a very prolonged action of the malt on the starch, a complete saccharification is obtained, but in practice it is absolutely impossible to procure a complete transformation and the saccharification, under the best conditions, only furnishes 80 parts of maltose per 100 of starch.

The hydration of the starch is accomplished in the work of the distillery in two different stages: saccharification

proper, then the secondary saccharification, which is prolonged throughout the duration of fermentation.

Of these two saccharifications, the last is the most difficult to regulate, and it is generally believed that it is best to produce the greatest part of the sugar in the first stage of hydration, so as to leave the fewest possible dextrins for the secondary saccharification. To this end the most favorable conditions must be employed during the principal saccharification and a temperature must be adopted which furnishes the greatest effect in the least time. It is the determining of this temperature that offers the first difficulties. The optimum temperature of diastases is far from being constant. In fact, if we compare the quantities of maltose formed during the same period of time with a given quantity of malt, at different temperatures, we shall find that the maximum of sugar formed takes place at very different temperatures according to the duration of saccharification.

Thus, when different specimens of the same starch are saccharified for 1 hour with the same quantity of malt, working at temperatures increasing from 30° to 70°, an optimum temperature of 60° to 63° is found.

When the same experiment is repeated by prolonging the duration of saccharification for three hours, the optimum temperature is reduced to 50°, and it descends to 30° if saccharification is made to last for 12 hours.

From this it results that the selection of the temperatures of saccharification depends on the duration of the latter, and that the longer the duration of the action, the lower should be the temperature of saccharification.

In practice the duration of saccharification is very varied. This operation lasts, in different manufactories, from 20 minutes to 2 hours. Its duration is determined by the kind of plant, and by the general conditions of the work.

If a complete hydration of the starch is desired, it is always well to choose, when saccharification lasts a half-hour, a temperature of 62° to 63°, while one must lower the

temperature to 57°–58° for saccharifications lasting from 1 to 2 hours.

We now know the conditions necessary, in the principal saccharification, for a complete hydration.

But in reality the quantity of sugar formed during the first stage of saccharification has very little influence on the final result of the operation. A dextrinated mash affords as much alcohol as one strongly saccharified. Moreover, the quantity of diastase indispensable for the secondary saccharification is no greater for the dextrinated mash than for the mash which already contains a large amount of sugar.

A very long series of experiments performed in this way have shown us that the intensity of the first saccharification is of little importance, and that the final result depends especially on the more or less complete preservation of the diastase during fermentation.

Still, the principal saccharification cannot be completely suppressed. It has a reason for existing, especially from the point of view of liquefaction. In fact, it is this first operation which gives to the cooked mass the necessary fluidity. Moreover, it succeeds in attacking the cells which have escaped the action of steam, and it liquefies the particles of starch which adhere to the spent malts.

By raising the temperature of saccharification above 60°, excellent conditions are obtained for liquefaction.

We may now consider the change in the diastase under the action of heat, for the active substance which is to serve for the secondary saccharification must, after the principal saccharification, be absolutely unchanged. The temperature of saccharification must, therefore, necessarily be lower than that at which the diastase commences to weaken. All the authors who have investigated saccharification are completely in accord on this point, but their opinions are very different when it is a question of saying at what temperature the change begins. According to some, the active sub-

stance of the malt can withstand temperatures of 62° without changing. According to others, the degree of resistance of the malt to high temperature depends on the duration of the action as well as upon the concentration and the composition of the mash. According to some chemists, a temperature of 60°-62° would bring about in diluted mashes a pronounced change of the diastase, while in concentrated mashes the amylase would resist much better. Others finally, make a notable difference, from the point of view of the preservation of the enzyme, between a dextrinated must and a saccharified must.

It is assumed that the presence of great quantities of maltose in the solution protects the diastase against the disastrous effect of high temperatures and, on that basis, it would be advisable to conduct saccharification in two stages, during the first 30 minutes of the action of the malt at a temperature of 58°–60°, then raising it to 64°–67°.

For the support of this theory numerous experiments are cited which, however, do not lead to very clear conclusions. The various determinations, made by several chemists with different raw materials, under conditions necessarily varied, and by diverse methods, cannot furnish data of sufficient accuracy to solve this question.

The greater or less change in the diastase at different temperatures may be demonstrated by a very simple method.

To a starch paste is added a quantity of malt just sufficient to produce, under favorable conditions, a complete saccharification. After this addition, two samples are taken; one is left for 12 hours at a temperature of 30°, the other is first kept for an hour at a high temperature, then for 11 hours at 30°.

If under these conditions a difference is found in the amount of maltose, this proves the influence of high temperatures.

Here are three experiments made at different temperatures:

		Maltose formed:	
		After 1 hour.	After 12 hours.
A	12 hours at 30° C.	2.4	9.6
	1 hour at 50° C. and 11 hours at 30° C.	8.3	10.2
B	12 hours at 30° C.	2.2	9.8
	1 hour at 55° C. and 11 hours at 30° C.	9.1	11.6
C	12 hours at 30° C.	2.2	9.9
	1 hour at 59° C. and 11 hours at 30° C.	9.5	9.7

The maltose, in all these experiments, was measured after 1 hour and after 12 hours of saccharification.

Mashes kept at 45°, 50°, and 59° furnished, after the first hour of saccharification, a quantity of sugar much greater than that produced in the sample specimen left at 30°. After saccharification at 59° for 1 hour, 9.5% of maltose is obtained, in place of 2.2% obtained in the same length of time at 30°.

If the diastase had not changed during the first hour of saccharification at 59°, at the end of the 11 subsequent hours a much larger quantity of sugar would be obtained than in the sample specimens, since in the first hour of saccharification it was already much more advanced than in the sample specimens. But such was not the case. After 12 hours of saccharification, there was found in the sample specimen 9.9% of maltose, while in the experiment where the diastase was carried for an hour at 59° there was found only 9.7% of sugar. The temperature of 57° is, therefore, the limit to which amylase can be carried for 1 hour without producing a noticeable change.

The influence of high temperatures of saccharification may be equally well shown by the following experiments:

In different experiments, at different temperatures, digest a litre of paste containing 10 grams of starch and 5 cubic centimetres of malt infusion.

Experiment.	Duration of Saccharification.	Starch transformed.
1	12 hours at 30°	85%
2	1 hour at 45° and 11 hours at 30°	97
3	" 50°	96
4	" 64°	68

By repeating the same experiments with mashes of different concentrations and containing different proportions of dextrins and maltose, we have been able to ascertain definitely the concentration and the amount of maltose exercising a protective action on the diastase, but that this action is slight and that it may be entirely neglected when 58° is exceeded.

At temperatures higher than 58°, even when the mashes are very concentrated, a great destruction of diastase occurs.

By working, as is the case in most distilleries, with a great quantity of malt, the lack of diastase in the secondary fermentation is not perceived, but the result is quite different when there are rational conditions of work and when one seeks to reduce the quantity of malt to what is strictly necessary.

Those who recommend high temperatures of saccharification bring other arguments to the support of their opinion. According to them, one must employ a temperature of 60° or even a higher one, because otherwise the starch of the malt will not be utilized to the best advantage, and because only a high temperature can weaken the bacterial ferments which are always present in the malt.

The utilization of the starch in malt involves great difficulties, because its complete saccharification is secured only at a temperature of 70°.

At	65°	one finds	4%	of starch still undissolved.	
"	60°	"	8%	"	"
"	55°	"	42%	"	"
"	50°	"	73%	"	"

Hence in the choice of a temperature of saccharification

one must take into account the starch of the malt. By choosing a temperature of 55° one runs the risk of losing 42 per cent of the starch contained in the malt, while at a temperature of 60° the loss is considerably less. At this temperature there remains only 8 per cent of amylaceous material not attacked. When using from 12 to 16 per cent of malt one is obliged to choose a high temperature of saccharification, but when working with a very much reduced quantity, one may choose a lower temperature, because the loss of starch is reduced in this case to a minimum. Furthermore, the losses in amylaceous materials which may come as the result of a poor extraction of starch are never as detrimental to the yield as the weakening in the diastase under the influence of temperature. It is always preferable, therefore, to give up the attempt to secure a complete extraction of the starch and to seek to control the diastase, especially as the undissolved starch is not completely lost.

The starch of malt, which escapes solution during saccharification, is partially dissolved during fermentation. High temperatures must, therefore, be avoided, and saccharification carried out between 55° and 60°.

In certain cases, and especially when one is dealing with raw materials of doubtful quality giving mashes which contain 0.25 to 0.35 per cent of lactic acid, it is necessary to keep the temperature of saccharification still lower (not above 55°), because in an acid medium the diastase becomes more sensitive to the action of heat. In practice, unfortunately, these principles are entirely set aside. With a mouldy malt of poor quality much higher temperatures are adopted than in ordinary work, because it is supposed that by raising the temperature the micro-organisms which prevent the fermentation are killed. The results obtained by thus raising the temperature are not very satisfactory, but the distiller is consoled by the thought that if he had not employed high temperature the final result would have been still worse. In

reality, an increase of several degrees in temperature has not much influence on the purity of the fermentation and does not kill the germs at all, but destroys the diastase and hinders normal fermentation. When working with materials of poor quality, one must have recourse to antiseptics or employ only very active yeasts which can protect the mash from the invasion of foreign ferments without hindering the secondary saccharification.

The Infusion Process.—As we have just seen, the choice of temperature of saccharification presents great difficulties.

The starch paste formed during steaming must be liquefied at a temperature higher than 65°.

The starch of the malt, to be completely dissolved, requires a temperature of 70°, while the diastase cannot be brought to a temperature of 60° without undergoing a perceptible weakening.

Under these circumstances, it is always necessary to sacrifice either the enzyme or the starch, and the temperature of saccharification must necessarily vary according to the conditions and the quality of the raw materials.

An ideal process requires the separation of the active substances from the starch of the malt and their separate treatment at different temperatures.

By leaving the malt in contact with water under suitable conditions, the diastase passes into solution, is separated from the starch and can serve afterwards for saccharification.

As to the residue, it is still impregnated with sufficient quantities of the enzyme to produce liquefaction. The method, thus put in practice, leaves nothing to be desired.

The mashes cooked under pressure are liquefied at a temperature of 70° with the soaked malt and then cooled to 45°–50°. At that temperature the solution of the enzyme is added; the mixture is kept for some minutes at 45°–50°; then cooled to the temperature of fermentation; the yeast is added and it is left to ferment.

This mode of operation cannot fail to give good results

provided that the extraction of the diastase has been as complete as possible.

Let us now see how one must proceed to extract the diastase from the malt.

It is wrongly assumed that malt amylase dissolves easily in water. In reality the extraction is difficult; it depends on the temperature of the water and on the thoroughness with which the malt is ground.

We may emphasize this fact by the two following experiments: make two mixtures of malt and water and submit them to a temperature of 30°. Specimen A is not disturbed, while specimen B is constantly stirred. From time to time a few cubic centimetres of each liquid is taken and the diastatic power determined, which enables us to follow the course of the solution of the diastase.

	Diastatic Power.				
Experiments.	After 8 hours.	17 hours.	26 hours.	47 hours.	52 hours.
Liquid A	33	45	48	60	55
" B	39	58	52	50	42

The quantity of diastase dissolved in the infusion at first increases with the duration, reaches a maximum, and then decreases. In the liquid A it is after 47 hours that the diastatic power attains its maximum. Shaking renders the extraction more rapid in the liquid B, where the diastatic power reaches its maximum in 17 hours.

Numerous experiments made with different malts have shown that this maximum is reached earlier as the temperature of the infusion is higher. Our observations are summed up in the following table:

An infusion prepared at 45° reaches its maximum of diastase dissolved after	7 or 8	hours.		
" " " from 55° to 59°	"	"	"	3 hours.
" " " " 60° to 65°	"	"	"	½ hour.

The time necessary for a good extraction therefore depends on the temperature. There is, moreover, a critical time which must be borne in mind, since from this time onward the diastase begins to disappear.

The maximum quantity of active substance which can be dissolved in the infusion at the critical time is not at all constant. It varies considerably for the same malt according to the temperature, as may be seen in the following table:

	Diastatic Power of the Infusion.				
Temperature of Infusion.	After ½ hour.	3 hours.	8 hours.	17 hours.	25 hours.
30	..	..	31	60	49
45	..	44	56	51	
55	46	55			
65	36	20			

It is at a temperature of 30° that the most active solutions are obtained; from 45° to 55° the quantity of diastase which can be extracted remains almost the same, while at 65° the destruction of the ferment occurs as it passes into solution, and even at the maximum, a very weak infusion is obtained. The preparation of a cold infusion during 17 hours offers certain practical difficulties. To utilize the malt to advantage, it is well to make the solution at 55° for 3 hours.

The infusion process is especially recommended in the case of malts of maize. These malts generally give from 8 to 20 per cent of ungerminated grains, and their diastatic power is only from one fifth to one third that of barley-malt. To employ this malt it is necessary to use large quantities, and the loss in starch is greater, because the starch of maize-malt is much more slowly attacked by the enzyme than the barley-starch. An infusion of this malt must be made in the following way:

Reduce the malt to powder; dilute it in 4–5 volumes of water at a temperature of 55°. Then place it in a conical vessel and stir it during the first hour, then leave it for an hour or an hour and a half. Deposition takes place very readily and the liquid can be removed without carrying along the malt.

A filter-press may be used for the same purpose. The infusion of malted maize yields a liquid which filters easily.

The infusion of barley-malt is made in most manufactories with crushed malt, and at a temperature of 10°–15°. It is prepared in a crushing apparatus. The malt is bruised for 15–30 minutes, then put in water for one or two hours, after which the liquid which is to be used for saccharification is decanted.

The diastatic power of an infusion prepared in this way is very variable. It depends more upon the special nature of the malt than upon its richness in amylase. The quantity of diastase extracted is between 10 and 50 per cent of that contained in the malt. This method of preparing an infusion of malt is not one to be recommended.

Much more satisfactory results are obtained by preparing the infusion at a temperature of 45°–50°, and allowing the solution to proceed for 2 or 3 hours.

By this method, from 70 to 80 per cent of the enzymes contained in the malt are dissolved. The infusion process is as yet scarcely in practice, but it is unquestionably the method of the future.

The invention of a contrivance for separating barley-malt from its infusion is to be desired, because the principal difficulty always lies in this operation.

Concerning the Change which the Diastases Undergo during the Successive Stages of the Work.—From the study we have just made of the conditions of the action of amylase, it appears that a part of the enzymes of malt are destroyed during saccharification and that the varying resistance of the diastase to temperatures from 60°–62° depends upon the degree of acidity of the medium. The acidity of the musts is not the only factor which produces a change in the diastase; other conditions must be taken into account and these are not always easy to recognize.

Two malts possessing the same saccharifying power, used in the same quantity and producing in identical musts the same quantity of sugar may nevertheless yield infusions containing different quantities of diastase.

Besides richness in active materials, other factors must be taken into consideration in estimating the value of a malt.

The origin of differences in resistance is perhaps to be found in the degree of natural acidity of the grains, perhaps also in the kind of acid or in the nature of other foreign substances contained in the malt. We have made a series of experiments for the purpose of finding the cause of the differing resistance of malts and we can furnish some information on this subject, though unfortunately it is very incomplete. The resistance of malts depends on the temperature at which germination is conducted. Thus, by malting two portions of the same barley at different temperatures, one for 8 days at 19°–22°, the other for 9 days at 12°–15°, we have obtained malts which differed in their resistance at a temperature of 60°.

Malted barley, worked in the cold and for 9 hours, gave better results than barley malted at higher temperatures. On the other hand we found that barley, giving from 7 to 10 per cent of non-germinated grains after malting, possessed not only a saccharifying power less than that of completely germinated barley, but also a widely different resistance to the reaction of the medium. Incompletely germinated barley offers less resistance.

The richness of the mashes in enzymes after the principal saccharification consequently depends upon the quantity of diastase which is found in the malt, the temperature of saccharification, and finally the degree of resistance of the diastase.

The loss of enzymes which occurs during saccharification at high temperatures may, under favorable conditions, be limited to 20 per cent, but generally this limit is exceeded and the destruction reaches 30 per cent.

The secondary saccharification is made with the diastase which escapes destruction during the first stage of the operation. This saccharification is very slow and must be prolonged at least three days.

The diastase is generally preserved much better in mashes in fermentation than in sweet mashes. The diastatic power of the latter weakens considerably, even in the presence of antiseptics. The diastatic power of a mash which has fermented under favorable conditions remains almost constant for more than 70 hours. The favorable progress of fermentation depends principally on the preservation of the diastase. This preservation can be insured only in musts free from foreign ferments and for this reason the use of antiseptics is necessary in the distillery. In fact it is absolutely impossible to avoid infection by any other means.

Control of the Process in the Distillery.—The normal progress of a fermentation depends upon various factors and, besides those which have to do with the cooking and the temperature of fermentation (which is always easy to control), we must take note of the quality of the malt used, and of the nature of the yeasts as well as of the degree of infection of the must by foreign ferments.

Each of these three factors gives rise to a problem which is complicated by the interaction of the others. And it is often very hard, when there is some difficulty with the work, to recognize its cause and point out its origin.

A poor fermentation usually coincides with an infection by foreign ferments, but this is not always the first cause of the trouble observed; on the contrary, it is more often only the consequence either of lack of diastase or of weakness of the yeast. Nor should the lack of enzymes in a fermenting must always be attributed to a poor quality of malt; the destruction of enzymes may have been caused by the invasion of foreign ferments. So, too, if in a bad fermentation a degeneracy or a weakening of the yeast is found, this must not be considered as the direct cause of the trouble; the lack of diastase, among its pernicious effects, may have brought the yeast into this state.

To go back to the beginning and discover the real cause

of the trouble, it is necessary to follow the course of the weakening of the enzyme in all the stages of the work.

A quantitative determination of the diastase contained in the malt gives an idea of the quantity of germinated grain necessary for a normal operation. Then by determining the diastatic power of the saccharified mash produced with the malt under examination, one may find the extent of the change produced during saccharification and be in a position to judge if the quantity of amylase remaining is sufficient for the secondary saccharification. The determination of the diastatic power of the mash at different stages of fermentation furnishes data on the weakening of the diastase; it makes it possible to ascertain the point at which activity begins to diminish and to recognize clearly the cause of this diminution. A perceptible weakening of the enzyme in the first period of fermentation must lead to an irregular progress. The cause of the phenomenon is generally the initial acidity of the must and it is well, in such cases, to choose a saccharification temperature much below 60°.

The weakening of the diastase during fermentation may be due to other causes. It may come from the quality of the grain and in this case high pressure during cooking must be avoided, because it is generally during this process that substances which weaken the diastase are formed.

During the secondary fermentation, the acidity must be determined at the same time with the weakening of the diastase, for a perceptible increase in acidity is always followed by partial destruction of the diastase. The weakening of the diastase may be averted in this case by an addition of antiseptics.

An entirely opposite state of affairs is sometimes observed: the diastase first weakens and acidification is produced only from 6 to 10 hours later.

The appearance in the musts of foreign ferments here results from weakening of the diastase. In this case the

addition of more of the infusion to the fermenting must may prevent acidity and keep up the yield of alcohol.

Finally, if an incomplete fermentation is met with in the musts which are not abnormally acid and are rich in diastase, the cause may lie in the yeasts.

This case often arises when antiseptics are used which leave the diastase intact, but act very unfavorably on certain kinds of yeasts.

From the foregoing, it is evident that determinations of the fermenting power of malt and of musts may be of great service to distillers. In the following chapter will be found the methods to be followed in such analyses.

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CHAPTER XVI.

QUANTITATIVE STUDY OF MALT.

Determination of the diastatic power of malt and mashes by the methods of Effront.—Determination of saccharifying and liquefying powers.—Determination of the diastatic power of sweet and fermented mashes.

THE methods generally used for the quantitative study of malt take account only of its saccharifying power and entirely neglect its liquefying power as well as the resistance of the enzymes. Our researches have shown that it is indispensable to take account of these two properties. The saccharifying power of malt is subject to the influence of foreign substances contained in the grains. The intensity of the saccharifying power of a malt does not, therefore, afford an exact measure of the quantity of amylase it contains. The effect obtained in a saccharification by diastase is often the result of the combined action of the enzyme and of accelerating substances which accompany it.

The following experiment gives a clear idea of the influence of these extractive materials of the grain on the saccharifying power:

Prepare an infusion, using one part of malt and twelve parts of water, and at the same time an infusion of non-malted barley with one part of the grain and four parts of water. Filter these two infusions: from each take a certain number of cubic centimetres, which are introduced into a starch paste. Saccharify for one hour at 50°. The quantity of maltose obtained under these conditions furnishes a basis of comparison between the diastatic value of the two infusions.

In a second series of experiments, add to the starch, at the same time with the infusions of malt and of fresh barley, a certain quantity of infusion previously boiled.

The saccharification is made at the same temperature in all the experiments.

	Number of the Experiment.	Fresh Infusion.	Boiled Infusion.	Maltose formed.
Infusion of barley	1	1 c.c.	.....	0.37 g.
	2	2 "	.....	0.65 "
	3	6 "	.....	0.85 "
	4	0 "	6 c.c.	0 "
	5	1 "	1 "	0.6 "
	6	1 "	2 "	0.72 "
Infusion of malt	7	0.5 c.c.	.....	0.07 g.
	8	0.5 "	2 c.c.	0 "
	9	0 "	0.5 "	0.095 "
	10	0.5 "	1 "	0.11 "

Six cubic centimetres of the barley infusion, not boiled, give in the starch 0.85 g. of maltose (Experiment No. 3), The same quantity of infusion, previously boiled, used without fresh infusion, remains absolutely without action on the starch (Experiment No. 4). But this liquid, inactive by itself, influences saccharification to a great degree if it is with active diastase. Thus, a cubic centimetre of infusion of barley gives 0.37 g. of maltose and this same quantity of infusion produces 0.72 g. of maltose when it has 2 cubic centimetres of boiled infusion added.

The same thing is true with an infusion of malt heated to 100°; 0.5 cubic centimetres of this infusion furnish 0.07 g. of maltose and the same quantity of infusion gives 0.11 g. of maltose when saccharification occurs with a cubic centimetre of boiled infusion.

Thus it is seen that the extractive materials of raw grain have a considerable action on the amylase of malt and that with them saccharification can produce ten times as much sugar as by the action of the ferment alone.

Analogous experiments with barley of different origin

have shown us that no constant ratio exists between the real saccharifying power (due to amylase) and the accelerating power latent in raw grain. It follows that the active constituents of grains of different origin influence the saccharifying power to varying degrees.

It must be admitted that in ascertaining the value of a malt it makes no difference whether the saccharifying power comes from the amylase or some other substance, but amylase must not be confounded with the substances which accelerate saccharification; the mode of action of the latter is wholly different from that of the diastase. The substances which excite or accelerate hydration do not always increase the quantity of sugar formed and they have absolutely no influence on the distillery mash. In practice,—and that is the important point for us,—it is amylase alone which comes into play.

This results from the fact that the exciting substances act only in mashes containing little maltose, and because the effect they produce becomes weaker and weaker as saccharification advances. In distillery and brewery mashes a large proportion of maltose is always present and the effect of exciting substances is negligible.

The determination of the amylase of malt, looking solely to its saccharifying power, will consequently always give unreliable results. To reach more trustworthy results, we have sought a method which will allow of measuring quantitatively the liquefying power. The liquefying power of malt is not influenced by foreign substances. For this reason we express the value of a malt by both its powers: its saccharifying power and its liquefying power.

In the preceding chapter we have seen that malts differ much in their resistance to a temperature of 60°. This circumstance forces us, in determinations, to take account of the degree of resistance of the amylase. These factors become especially important in the case of malts destined for the distillery.

A malt of high diastatic power, but of little resistance to high temperatures, gives a less satisfactory result in the distillery than a malt less rich in amylase but which endures without change a high temperature of saccharification.

To form an idea of the greater or less resistance of malt at a high temperature, we keep the malt for an hour at 60° and determine the saccharifying power in mashes where the diastase has previously been destroyed.

We have thus established a method of determination which, we believe, answers to the needs of the industry. The determination is made in three stages:

1st. Preparation of an infusion.

2nd. Determination of the saccharifying power.

3rd. Determination of the liquefying power.

Preparation of the Infusion.—To prepare the infusion, weigh out 6 grams of crushed malt, put them in a flask containing 100 cubic centimetres of water at 60°; keep the flask in a water-bath for an hour at a temperature of 60°. During saccharification, shake the flask from time to time; the saccharification ended, cool to 30° and filter; to 50 cubic centimetres of filtered liquid then add 50 cubic centimetres of distilled water and determine the saccharifying power of this diluted infusion. The remainder of the non-diluted infusion is used to determine the liquefying power.

Determination of the Saccharifying Power.—The saccharifying power is determined by the aid of a standard solution of starch.

Dissolve 2 grams of starch in water and make the solution up to 100 cubic centimetres. To 100 cubic centimetres of this 2 per cent solution, add 55 cubic centimetres of distilled water and 5 cubic centimetres of an infusion of malt diluted as described above. The whole is then placed in a water-bath at 60° for an hour.

After saccharification, it is rapidly cooled and the content of sugar immediately determined.

To determine the maltose in the saccharine liquid, 2 cubic

centimetres of a solution of cupro-potassic tartrate is used, which corresponds to 0.01498 grams of maltose. The 2 cubic centimetres of cupro-potassic solution is put in a test-tube to which is added 3 cubic centimetres of water and a few fragments of pumice-stone. The number of cubic centimetres of saccharine solution necessary for the reduction of the copper salt varies, according to the malts, from 3 to 20; comparative experiments have shown that where 3 to 5 cubic centimetres of the saccharified solution reduce 2 cubic centimetres of cupro-potassic tartrate under the conditions indicated, the malt may be considered as having a maximum saccharifying power; 6 to 8 cubic centimetres correspond to a good malt, 9 to 12 to a malt of medium value, and if the quantity of saccharified solution necessary for the reduction is from 14 to 20 cubic centimetres, the malt may be considered poor.

The small quantity of maltose introduced with the infusion does not have much influence on the results, which, moreover, do not serve as a basis for accurate calculations, but merely as supplementary data for the estimation.

The standard starch solution which we use for the determination of the saccharifying power is prepared in the following manner: Let potato-flour soak at a temperature of 40° in a solution of 7 per cent hydrochloric acid, shaking the liquid every 6 hours.

After 3 days decant the liquid, wash the mass with water to a neutral reaction, and dry at the ordinary temperature.

The product obtained contains from 17½ to 18 per cent of water and is completely dissolved in warm water.

By submitting to this operation different potato-flours of the same origin, the same product is constantly obtained, but the result differs much when starches from different sources are used, as the diastase, in this case, shows itself more active in some samples than in others.

It is necessary that the standard starch, before being used for a determination, should be tried with a malt of known

saccharifying power. If the same power is found in the starch thus tested, this can be considered as a standard mixture. Otherwise, one must, by repeated trials, determine the composition of a new starch solution which must be taken to replace 2 grams of the standard.

Given, for example, a malt which possesses a saccharifying power of 4.5, measured by the standard starch, we will suppose that the same malt with another starch mixture would have a saccharifying power of 4.1. The question is to determine what quantity of the new starch must be taken to give the same result as 2 grams of the standard. To this end solutions are prepared which contain, instead of 2 grams per 100 of starch, 1.9, 1.8, 1.7 grams per 100, and the saccharifying power of the malt is tried with these solutions. If it is found that in the solution containing 1.7 g. of starch the saccharifying power is 4.5, 1.7 g. of the new starch will be constantly used instead of 2 grams, and under these conditions alone can it be used in place of the standard. One must always use a fresh starch solution, for we have found that this solution, although it keeps fairly well, acts differently with the same malt, according as it is fresh, or has been prepared for some time.

This peculiarity is the more unexpected since we find no difference in acidity in the two solutions of starch.

Determination of the Liquefying Power.—Weigh out 40 grams of standard rice-starch; dilute with a little water in a capsule; introduce the mixture into a 100 cubic centimetre calibrated flask; rinse the capsule with a fresh portion of water which is poured into the flask and make the volume up to 100 cubic centimetres. From the mixture of starch and water briskly stirred, take with a pipette 8 specimens of 5 cubic centimetres each, and introduce them into numbered test-tubes of 10 cubic centimetres capacity; add to the contents of each tube the same quantity of infusion of malt prepared in the manner indicated above. For each of the numbered tubes, containing 2 grams of starch and the infusion,

prepare a second larger tube having a diameter of 19 millimetres, a height of 19 centimetres and similarly numbered. In each of the large tubes place 14 cubic centimetres of distilled water, and put them in a water-bath at 80°; then bring them one after another rapidly to the boiling point, and pour into the boiling liquid the starch paste and infusion contained in the smaller tube having the same number. Stir rapidly with a glass rod, rinse the tube which contained the starch with a cubic centimetre of water and add it to the contents of the large tube. Stir again with the rod, mark the hour exactly and leave in the water-bath at 80° for 10 minutes. Take them one by one, stir the contents once more with a glass rod and plunge them into a water-bath at 100°, where they stay exactly 10 minutes. After this operation all the tubes are rapidly cooled. A thermometer placed in one of them indicates the moment when the temperature reaches 15°, and it is at this point that the degree of liquefaction is ascertained. The tubes, thus cooled to 15°, are inverted one after the other. If the contents of the tube runs out instantly and without difficulty, the specimen is considered liquefied; a tube which empties entirely, but whose contents presents the consistency of a thick syrup, shows a three-quarters liquefaction; a tube which does not become entirely empty shows a partial liquefaction.

If the tube whose contents are entirely liquid has, for example, received 2 cubic centimetres of the non-diluted infusion, the liquefying power is expressed by 2.

Comparative experiments with different malts have shown that a liquefying power of 1.5 to 2 shows a malt of excellent quality. A liquefying power of 2.5 to 3 corresponds to a malt of good quality, while a liquefying power of 3.5 to 4 shows a malt of doubtful quality whose value will depend on its saccharifying power. A malt with a liquefying power of 4 and a saccharifying power of 4 to 5 does passable work in the distillery, while a malt having the same liquefying power as the preceding and a saccharifying power of 7 to 9 must be

considered poor. The difference between the saccharifying and liquefying powers is especially marked in dry malt. By drying, the saccharifying power is considerably weakened, while the liquefying power is much less changed. In the dry malt a liquefying power of 2 to 3 does not necessarily prove an excellent product: all depends on the saccharifying power. With a moderate saccharifying power the malt may have a great value, but with a saccharifying power of 12 the malt, even when it has a great liquefying power, cannot be used in the distillery.

To determine the liquefying power, rice-starch, chosen with great care, is used. The rice-starches of different origin act differently with diastase at 80°; so rice-starches may be grouped in two classes. In the first are placed the products which, at the moment of liquefaction, become completely colorless and transparent; in the second, those which preserve a whitish tint and give an opaque liquefaction. The first liquefy with much more difficulty than the second and the liquefying power of an infusion may vary much according as one or the other type of starch is used.

At the beginning of our investigation we worked with a starch of the second type; we afterwards abandoned it because we found that the starches which give a transparent liquefaction are preferable, the exact time of liquefaction being more easy to observe.

If one wishes to obtain constant results the same starch must always be used in testing amylase.

As a standard we use Hoffman starch and whenever we make a new mixture we verify the starch with the standard specimen. The verification is made with infusion of malt. Two grams of standard starch and 2 grams of the starch to be tested are liquefied with the same quantity of infusion at a temperature of 80° for ten minutes. If the number of cubic centimetres of infusion necessary to liquefy completely the standard starch and the starch to be tested is the same, the two starches may be considered identical. Otherwise, in-

crease or diminish the quantity of starch tested in such a way as to obtain liquefaction with the same quantity of infusion.

If it happens, for example, that for the liquefaction of 2 grams of standard starch it takes 2.5 c.c. of an infusion of malt and that for the liquefaction of the same quantity of starch under examination it takes 3 cubic centimetres of the same infusion, one must weigh out 1.9 gr., 1.8 gr., 1.7 gr., of the starch to be tested and see which of these amounts is liquefied by 2.5 c.c. of infusion. If complete liquefaction is produced by 1.9 gr., it must be concluded that instead of 2 grams of standard starch one must take only 1.9 gr. of the starch which is being tested.

Another method for transforming a certain starch into standard starch consists in acidifying it or making it alkaline.

This method, which is preferable to the other, has for its basis the following observation:

The standard starch is slightly alkaline, and if rice-starch is brought to the same degree of alkalinity it acquires all the properties of the standard starch.

The liquefying power is so sensitive to the alkalinity of the liquid that the quantity of soda to be added cannot be determined by a single alkalimetric measurement. If the difference in alkalinity of the two starches corresponds to 2 cubic centimetres of decinormal solution of soda, one must add only half of the alkaline solution for 50 grams of starch, and then keep adding tenths of cubic centimetres until the two starch pastes liquefy with the same quantity of infusion of malt.

The standard starch for saccharification, as well as the standard for liquefaction * keeps without change in bottles with ground stoppers and can be used for determinations, for at least two years.

We have also observed that dry malt, kept in the same

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* Standard starches which we use are for sale at Drosten's, rue du Marais, Brussels, and at H. Koenig's, manufacturer of chemicals, Leipzig.

way, preserves for years its saccharifying and liquefying powers.

We give here two determinations of malt made according to the method indicated above.

MALT A.—Russian barley, soaked 2½ days with aeration, malted in revolving drums.

Minimum temperature 18°, maximum 21°.

Duration of germination, 4 days.

Water 48.04%.

General aspect and odor, normal.

100 grains include
- 3 non-germinated grains.
- 34 grains whose plumule was shorter than the grain.
- 30 " having a plumule of the length of the grain.
- 21 " having a plumule 1½ times as long as the grain.
- 12 " having a plumule more than twice as long as the grain.

The liquefying power and the saccharifying power of this malt were determined in three different specimens:

1st. In unsorted grains.

2nd. In grains in which the plumules are twice as long as the grains.

3rd. In grains in which the length of the plumules does not exceed that of the grain.

	Unsorted Malt.	Malt with Plumule twice the Length of the Grains.	Malt with Plumule not longer than Grains.
Maltose.....	0.6	0.585.	0.53
Saccharifying power	17. c.c.	9.5 c.c.	20.7 c.c.
Liquefying power.....	2.5 c.c. not liq. 3 " " " 3.5 " ¼ " 4 " liquid	2.5 c.c. not liq. 3 " " " 3.5 " liquid	3.5 c.c. not liq. 4 " ¼ "

The maltose indicated for each infusion shows the sugar content of the dilute infusion which was used for determining the saccharifying power.

In the grains not classified, when coming out of the apparatus, there is found a saccharifying power of 17 and a liquefying power of 4. Therefore the malt is decidedly mediocre; the trial of fermentation with different quantities of malt has shown us that it takes 18 parts of this malt per 100 of rice to insure a complete transformation.

The comparative analysis of the three specimens confirms for us the facts established by the Institute of Berlin, namely, that the development of the plumules coincides with an increase in the quantity of the enzymes.

MALT B.—Saladin pneumatic system malt-house. Malt-house of Buir, near Cologne.

Small Russian barley, soaked 2½ days without aeration.

Duration of malting, 9 days.

Temperature, minimum, 18°; maximum, 23°.

The sprouting is uniform and the plumules do not exceed the grain.

Non-germinated grains, 3.

Water, 47%.

Maltose of infusion, 0.74.

Saccharifying power, 4.65.

Liquefying power { 25 c.c. = liquid.
2 c.c. = ¼ liquid.

The liquefying power as well as the saccharifying power shows a malt of excellent quality. The quantity of malt necessary for fermenting 100 kilograms of rice is 8 kilograms.

The method we have just indicated is applicable to the investigation of malts of barley and rye.

For the analysis of malt of maize other conditions are necessary because this malt always contains relatively small quantities of diastase. For the preparation of the infusion 12 grams of ground malt are taken instead of 6, and, to de-

termine the saccharifying power, only 1 cubic centimetre instead of 2 of the cupro-potassic solution is used.

A maize malt of fine quality always contains 4 to 8 per cent of non-germinated grains. It possesses, under the conditions indicated, a saccharifying power of 4 to 6 and a liquefying power of 2.5 to 3.

By comparing the malt of maize with barley malt of first quality, it is found that it is only one fourth as active and in practice it is necessary to use 4 to 5 times as much of this as of barley-malt to secure the same result.

Method of Analysis of Sweet and Fermented Mashes.—The saccharifying power of the mash may be determined by aid of the coloration which the mash gives with iodine. This is done in the following manner: take 6 specimens of 20 cubic centimetres each of a fresh 2% solution of soluble starch and place each sample in a numbered test-tube; add with a pipette divided into tenths of a cubic centimetre .25 c.c., .50 c.c., .75 c.c., 1 c.c., 1.25 c.c., and 1.5 c.c., of the saccharine or fermented must to be tested; place the tubes in a water-bath at 60° for an hour; cool and add to the contents of each tube half a cubic centimetre of a very dilute solution of iodine and observe the coloration at the moment the iodine is added to the liquid.

A saccharification made with .25 c.c. of sweet mash, not colored by iodine, corresponds to the maximum of saccharifying power, and if this result is reached, one may be sure that a greater quantity of malt than necessary has been used for the fermentation. The absence of coloration in a tube which has received .75 c.c. of solution shows that the sweet mash possesses a saccharifying power sufficient for fermentation, at least if the liquefying power of this must is normal.

If a coloration appears in the liquid containing 1.25 c.c. of the saccharified mash, it is certain that this mash does not contain the necessary quantity of malt and it is useless to pay any attention to the liquefying power. Failure to give color with iodine of solutions which have received 1 cubic

centimetre of mash shows a sufficient quantity of diastase if the liquefying power is very great. Otherwise, the saccharified mash is not rich enough in active materials.

This method is of great service for the control of mashes during fermentation; the saccharifying power is determined by coloration with the solution of iodine at the beginning of fermentation, and this operation is repeated after 30 and 60 hours. The saccharifying power determined by this method at the beginning of fermentation should not change much up to the end of the operation. If it is found that, at a certain time, twice as much liquid is needed as at the beinning to have no coloration with iodine, one may be certain that there is a change in the diastase and it is important to add more infusion.

A mash fermented for 86 hours should have a saccharifying power of between 0.75 and 1, that is, with 0.75 to 1 cubic centimetre of mash no coloration by iodine should be obtained. A saccharifying power of 1.5 at the end of fermentation shows a lack of diastase.

These data are applicable to mashes of rice of a density of 17 to 19 Balling. Mashes of grains and potatoes act differently. In these, the weakening of the diastases during saccharification and the fermentation occur much more rapidly. One must seek to have mashes of a saccharifying power of 1 to 1.25 at the beginning of the operation and of 2 at the end of fermentation.

Our method of determination of malt and mash is now introduced in the central stations of the Association of Distillers of Bavaria and Austria-Hungary. The directors of these stations, Professors Kruis and Bücheler have expressed to us their entire satisfaction.

With a little experience, one can succeed in having a complete control of the work by means of this method.

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CHAPTER XVII.

MALTASE.

Glucase of Cusenier.—Maltase of yeast.—Properties.—Differences between the optimum temperatures of different glucases.—Maltase of moulds.—Manner of action upon starch.—Processes of secretion.—Influence of nitrogenous food.—Influence of carbohydrates.—The different amylomaltases of Laborde.

MALTASE or glucase is an enzyme which acts upon starch, dextrins, and maltose.

The existence of an enzyme acting on maltose was doubted for a long time. It is, however, evident that maltose, to be assimilated by living cells, must be hydrated and transformed into glucose.

In 1865, Béchamp found in the urine the presence of an enzyme acting on maltose which he called nefrozymase. Brown and Heron discovered an analogous active principle in the pancreatic juice and the small intestine of the pig. Later Emile Bourquelot confirmed the observation of Brown and Heron, demonstrating the presence of the same principle in the pancreas and small intestine of the rabbit.

The diastatic liquids obtained by these workers presented very varied properties. They evidently contained diastases of very different natures and it was very difficult to establish definitely the presence, in the liquids studied, of a special ferment acting solely upon maltose.

The discovery of the active principle which decomposes maltose into two molecules of glucose dates from 1886. It belongs to Léon Cusenier, who named this enzyme glucase.

Cusenier, by soaking ground maize in water at a tem-

perature of 50°, found that a great part of the amylaceous matter passed into solution, and that the rotatory power of the saccharine liquid decreased as the soaking was prolonged. This observation led to researches in regard to the nature of the sugar formed, as well as that of the agent producing this transformation.

A series of experiments undertaken with this aim resulted in finding that the maize contains a special ferment which acts on starch, giving glucose and dextrins, which in the end are themselves transformed into dextrose.

The optimum temperature of this enzyme is 60°; its temperature of destruction, about 70°.

This enzyme acts likewise on maltose and transforms it into glucose.

Its presence has been observed in almost all the cereals, but in a much smaller quantity than in maize. There exists in the latter an amount of glucase which is more than enough to transform into glucose all the starch it contains.

According to Gedulde, it is possible to isolate the glucase of maize by soaking a grist with water and then precipitating the filtered liquid with alcohol. The product obtained, and dried *in vacuo*, is a brownish mass which is friable and has the following properties:

It contains about 8 to 12 per cent of nitrogen. It gives the reaction of guaiacum and hydrogen peroxide. Precipitated by alcohol, it is redissolved in water with difficulty.

It possesses a relatively weak activity: with one part of precipitated active substance only 100 parts of maltose are transformed into glucose.

Its optimum temperature is from 56° to 60°. Above 60° there is found a perceptible slackening in the hydration it produces. Above 70° glucase is without action.

This enzyme acts more energetically on the products of decomposition of starch than on the starch itself.

According to Beijerinck, glucase can easily be prepared from maize which is hulled and deprived of its oil. The fol-

lowing method is employed: Three kilograms and a half of maize thus prepared are treated with 5 litres of water, with the addition of 500 cubic centimetres of 96% alcohol and of 2 grams of tartaric acid. This mixture is kept for 30 hours at 15° or 20°, then filtered. Thus 4½ litres of a clear liquid are obtained in which is produced a partial precipitation by adding to it an equal volume of 96% alcohol. The deposit thus procured is treated with acidulated water in the proportion of 0.4 grams of tartaric acid per litre, then a little alcohol is added. The precipitate is partially redissolved in the liquid and the insoluble part is collected on a filter. This insoluble product is very rich in glucase according to Beijerinck. It contains 1.11% of nitrogen.

Other products may be obtained by adding alcohol to the filtered liquid. The precipitates which still contain a certain quantity of diastases in solution are collected. But the precipitates obtained by these treatments, while showing a nitrogen content of 4.78 and 2.20 per cent, are less active than the insoluble part spoken of before. The glucase obtained by Beijerinck is not, however, an absolutely pure product, as he himself found. Its impurities must be due to mucilages.

According to Beijerinck, glucase acts on maltose, on starch, and on dextrins, but more actively on maltose than on dextrins; it causes the transformation of starch with much difficulty.

According to Gonnerman, glucase or an analogous ferment exists in beets frozen or in germination. Dubourg and Rhomann have discovered its presence in the blood. It is also found in the urine and in yeasts, as well as in a great number of moulds.

The secretion of maltase by yeast is of particular interest.

Maltose has been considered for a long time as a directly fermentable sugar and, in reality, during fermentation it is impossible to tell when the maltose is transformed into glucose. This is why the fermentation of maltose has been

regarded as an intracellular transformation in which the soluble ferment has no part.

Bourquelot, Lintner, and Emil Fischer have studied the question very closely and established the fact that yeast always contains a certain quantity of maltase which is retained in the cells and which is diffused with difficulty into the surrounding liquid.

To extract the enzymes of yeasts, the cells must be crushed with pumice-stone or pounded glass and the mass then soaked in water. One may also have recourse to another means which seems more expeditious: fresh yeast is spread in a very thin layer; it is slowly dried at 40° and then soaked in water. Under these conditions, the maltase of the yeast becomes soluble. The enzyme extracted from the yeasts by this method differs in many respects from the active substance of the maize which hydrates the maltose.

According to Gedulde, the glucase of maize can be precipitated from its solution in the active state by alcohol; the maltase of yeast, on the contrary, is almost completely destroyed by this reagent.

Maltases of different origin also possess a very different sensitiveness towards heat. Lintner, by exposing to different temperatures 3 specimens of a solution of maltose, to which had been added the same quantity of glucase extracted from yeasts, obtained quantities of glucose varying according to the temperature at which the action took place.

Temperature.	Duration of the Action.	Glucose formed.
35°	2 hours	2.90 gr.
40	"	3.09
45	"	2.08

The optimum temperature, according to these experiments, would be 40°, while Cusenier's glucase possesses an optimum temperature of 56° to 60°.

By trying yeast maltase at temperatures of 40° and 50°

Lintner found the formation after two hours of action of the following quantities of glucose:

Temperature.	Glucose formed.
40°	1.8
50°	0.3

Therefore the temperature of 50° almost entirely destroys the maltase of yeast, while if glucase of maize is used, the maximum result is not reached at that temperature.

A great number of moulds possess the property of transforming starch into sugar.

By studying the action of *Aspergillus orizæ*, Atkinson was the first to find that the diastase of this mould brings about the transformation of amylaceous materials in a different way from that of the diastase of malt. The final product of this reaction of *Aspergillus orizæ* is dextrose and not maltose.

Since then the same fact has been ascertained by Bodin and Rolants for *Amylomyces Rouxii*, then by Bourquelot and Laborde for *Aspergillus niger*, *Penicillium glaucum* and *Eurotiopsis Gayoni*. There is every reason for believing that it is a general fact and that many other moulds render starch assimilable by the aid of the glucases they secrete. Diastatic liquids obtained by soaking moulds act on starch, dextrins, and maltose, forming glucose.

According to Atkinson, the transformation of starch by *Aspergillus orizæ* is done by successive hydrations of the molecules, with formation of maltose as an intermediate product, but the analyses cited for the support of this opinion are not at all conclusive. Laborde, on the contrary, found that there is a direct transformation without the formation of maltose. He has found the same facts in the case of *Aspergillus niger*, *Penicillium glaucum* and *Eurotiopsis Gayoni*.

To demonstrate the presence of glucase in an active liquid, a 2 per cent solution of maltose is added to a certain

quantity of the liquid under examination. A trace of chloroform or thymol is added and the solution is left at a temperature of 45° for 24 hours. By examining the rotation of the liquid before and after the experiment, one can easily follow the course of the hydration.

The rotatory power of the maltose is $(\alpha)d + 138.4$ and that of glucose 52.4

The glucase of moulds acts more strongly on starch than on maltose.

The formation as well as the diffusion of glucases of moulds take place under the same conditions as secretion of sucrase by *Aspergillus niger*. During the development of the plant, the quantity of glucase increases as the nutritive substances diminish and the maximum amount of enzyme appears in the plant at the moment when it begins to utilize the reserve substances. Thus the glucase produced in the moulds is retained inside the cells and diffuses with great difficulty until the nutritive medium begins to be exhausted.

According to Pfeffer and Katz, the addition of sugar to the culture medium generally diminishes the production of glucase, but it is observed that the different kinds of moulds are more or less sensitive to this action. Thus *Penicillium glaucum* does not secrete glucase when there is 10 per cent of saccharose, while in an amount of 30 per cent this sugar does not entirely stop its secretion by *Aspergillus niger*. Glucose acts in the same way as saccharose.

The presence of maltose in the nutritive medium influences the secretion in a less degree. *Penicillium glaucum* still produces glucase in a medium containing 10 per cent of sugar.

Nitrogenous food also has a great influence on the production of glucase. The well-nourished cells yield the enzyme in largest quantity.

Moulds may take nitrogen from very different sources. Thus alkaline nitrates, peptone, casein, and urea are equally favorable to the cultivation of *Eurotiopsis* and furnish practi-

cally the same quantity of glucase. Sulphate and chloride of ammonium, however, furnish decidedly less, and these substances act very unfavorably from the point of view of the formation of diastase.

According to Pfeffer and Katz, the secretion of glucase by *Penicillium glaucum* and *Aspergillus niger* is limited by the quantity of enzyme already present in the nutritive medium. By removing the glucase from the medium with tannin, they have found a more abundant secretion of diastase. It is, however, not very probable that taking away the active substance can cause a further formation of diastase. It is more plausible to think that in precipitating the diastase by tannin, one favors the diffusion of the active substances already formed but retained by the cells.

Of all the moulds studied from the point of view of their action on amylaceous materials, *Aspergillus orizæ* is the most active, and it is this which really secretes the greatest quantity of maltase.

Mucor alternens and *Amylomyces Rouxii* both belong to the class of moulds which is rich in maltose. *Aspergillus niger* and *Penicillium glaucum* possess a diastatic power which is much weaker, and *Eurotiopsis* occupies the last place as regards the secretion of glucase

According to Laborde, the saccharifying diastases of *Aspergillus niger*, *Penicillium glaucum* and *Eurotiopsis Gayoni*, and which he designates under the name of amylo-maltases, have different characteristics. Starting with this observation, Laborde conceives the existence of 3 different diastases having common characteristics but distinguished by their sensitiveness towards physical and chemical agents, as well as by the intensity of their action.

By allowing enzymes of the three moulds to act, under the same conditions on 2 per cent starch paste, Laborde found perceptible differences for the 3 diastases, which are given in the following table:

Origin of the Diastatic Liquids.	Duration in Hours of the Action.	Polariscopic Rotation of the Liquids.	Glucose. %	Dextrins. %
			gr.	gr.
Aspergillus niger	12	17.5	1.31	0.56
	48	14.0	1.61	0.31
	96	14.0	1.66	0.30
Penicillium glaucum	12	12.5	1.31	0.31
	48	12.0	1.61	0.21
	96	12.0	1.72	0.18
Eurotiopsis Gayoni	12	7.0	0.80	0.16
	48	9.0	1.61	0.06
	96	9.3	1.92	0.00

The difference in the course of hydration is especially shown by the rotatory powers of the liquids as well as by the relation of the quantity of maltose and that of dextrins.

For 1.60 of glucose formed with the three active liquids, there are found noticeably different quantities of dextrins as well as different rotations. These differences probably come from the fact that the starch is not liquefied with the same facility by diastases of different origin.

Maltases of different moulds are further differentiated by their optimum temperatures as well as by their temperatures of destruction:

	Temperature Optimum.	Temperature of Destruction.
Aspergillus niger	60°	80°
Penicillium glaucum	45	70
Eurotiopsis Gayoni	50	75

As regards the action of heat, the maltase of *Aspergillus niger* approaches the maltase of maize, while the active substance of *Eurotiopsis Gayoni* resembles rather the diastase of *Penicillium glaucum* and the yeasts.

There is also found another difference between the maltase of cereals and the ferments of moulds. While the first acts with more difficulty on starch than on maltose, the second acts more vigorously on starch than on the products of hydration.

Moreover, these differences between the manner of action of diastases are apparent rather than real. An extract of maize acts energetically in the cold on starch and on maltose. But if, on the other hand, one precipitates the active substance of an infusion of maize with alcohol, one obtains a product which hardly acts at all on starch, but which, on the contrary, acts very strongly on maltose.

This difference evidently comes from the change of medium. In the first case the action is due to the diastase accompanied by foreign substances which influence its action. In the second, it is the effect of the diastase alone, or of this diastase accompanied only by substances exercising a very slight influence on the transformation.

It must then be admitted that all the differences which we have found in maltases of different origin comes exclusively from foreign substances which accompany them and influence their sensitiveness towards reagents.

Most moulds secreting maltases develop very easily in the mashes of the brewery and the distillery, as well as in yeast-water to which carbohydrates have been added.

Sanguinetti made a comparative study of *Aspergillus orizæ*, *Mucor alternans* and *Amylomyces Rouxii* as regards saccharifying and oxidizing powers. He cultivated them in mashes containing starch, dextrin or other carbohydrates, and observed the progress of diastatic secretions as well as the influence of nutrition on these secretions.

Here are some of his experiments:

In a flask of a capacity of 1,500 cubic centimetres he placed 500 cubic centimetres of yeast-water and added 15 grams of starch or dextrin.

He sterilized the liquid, and after cooling it, sowed different flasks at a temperature of 30° with the spores of different moulds.

He allowed the plants to develop for 10 days, and shook the flasks twice a day to prevent the formation of

spores. He then determined the weight of the plants formed and determined the sugar and alcohol in the liquid.

The results of experiments made with yeast-water and starch are here given:

	Control.	Aspergillus orizæ.	Mucor alternans.	Amylo-myces.
	gr.	gr.	gr.	gr.
Weight of plant	.....	2.081	0.667	2.080
Dry extract at 100°	19.00	5.20	6.27	4.50
Total acidity, H_2SO_4	0.127	0.670	0.980	0.660
Weight of alcohol	.....	2.77	1.58	3.96
Reducing sugar (as glucose)	.....	1.30	Traces	Traces
Total reducing sugar after saccharification by hydrochloric acid	16.67	2.25	2.99	3.75
Loss of alcohol per 100 of starch	.....	40%	46%	25.7%

When the quantity of carbohydrate which has disappeared is compared with the quantity of alcohol formed, it is found that there is obtained:

With *Aspergillus orizæ*	for	14.42	of sugar	transformed,	2.77	alcohol.
" *Mucor alternans*	"	13.68	" "	"	1.58	"
" *Amylomyces Rouxii*	"	12.92	" "	"	3.96	"

Aspergillus orizæ shows itself the most active and leaves in the 15 per cent solution of starch .85 gr. of untransformed dextrin, while the *Mucor alternans* and *Amylomyces* furnish twice as much unattacked dextrin.

When in these experiments starch is replaced by dextrins a still less complete saccharification is observed.

After 10 days of development there is still found 3.80 of dextrins.

If the time of the action and the weight of the plant are taken into consideration, it is seen that the quantity of active substance secreted by the moulds is relatively very slight, even with *Aspergillus orizæ;* 2.081 gr. of plant do not suffice, as we have just seen, to transform 15 grams of starch, while .5 gr. of malt produce, under similar conditions, a complete transformation.

The maltase of fungi is very sensitive to the medium. Bodin and Rolants have studied the action of oxygen and of

the acidity of the medium. The following experiment affords some data on this subject:

Bring a distillery residue to different degrees of acidity and, in the sterilized liquid, cultivate *Amylomyces* under different conditions.

In one culture let the plant develop at the surface of the liquid (culture S); in another let the development take place deep down (culture P); in a third (culture A) let a current of air pass for 48 hours.

The following results were obtained after a fermentation of 4 days at a temperature of 26°:

	Neutral Residue.			Residue with Acidity equivalent to 3.4 gr. of Sulphuric Acid.		
	S	P	A	S	P	A
Alcohol, per litre	3.4c.c.	5.5c.c.	3 c.c.	3 c.c.	1.8c.c.	1.7c.c.
Acidity	0.36	0.83	0.4	2.69	3.13	2.69
Reducing sugar in glucose	4.83	2.33	1.64	4.31	3.5	3.4
Total sugar	10.23	7.3	5.57	17.71	17.71	13.28
Weight of amylomyces obtained in a pressed state	10.23	4.6 gr.	8.15 gr	17.71	0.25 gr	2.30 gr

It is clear that aeration is very favorable to the development of the plant, since there are 8.15 gr. of plants in the aerated liquid, while a culture from which the air was excluded furnishes only 4.6 gr.

The quantity of acid formed during the development is in direct proportion with the initial acidity and is smaller according as the acidity of the medium at the beginning is stronger.

Maltase acts very well in a slightly acid medium, but its action is stopped by an amount of organic acid corresponding to 2 grams per litre of sulphuric acid.

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CHAPTER XVIII.

INDUSTRIAL APPLICATIONS OF MALTASE.

CEREALOSE.

Industrial Manufacture of Glucose by Enzymes.—Cusenier, having found glucase in maize, sought to apply this discovery to the glucose industry. By replacing, in this manufacture, acid by enzymes contained in the grains, he succeeded in making a product of great value which is found in commerce under the name of cerealose.

Cerealose is obtained in the form of a crystalline mass containing maltose and glucose.

The manufacture of cerealose is as yet little developed, for the process of Cusenier leaves much to be desired from the point of view of yield and cost of production, which is greater than that of the glucose obtained with acids.

The difficulties encountered in this industry are various.

The maize contains hydrating ferments in a quantity more than sufficient to transform all the starch into sugar, but practically it is very difficult to have conditions favorable to the action of these ferments. It results that the transformation is far from complete.

When ground maize is soaked with 4 volumes of water at a temperature of 60° for 24 hours, 60 to 65 per cent of amylaceous materials is extracted in the form of sugar. A more prolonged soaking does not bring about a perceptibly better result. The enzyme acts only on a part of the starch and unattacked grains of starch always remain, although the saccharine solution obtained is very rich in active substances. It is this peculiarity in particular which makes the manufac-

ture of glucose by glucase difficult. The operation can really never be carried to perfection.

One is obliged to have recourse to a continuous method of work.

The method of procedure is here outlined:

Five hundred kilograms of coarsely ground maize are poured into an apparatus furnished with a double wall and a stirrer. Twenty hectolitres of water at 65° are added. The temperature is kept at 58°–60° for 6 to 8 hours, the stirrer being kept in motion.

The transformation of amylaceous materials into glucose is followed by the observation of the density and rotatory power of the mash. During all the course of the operation, the density of the liquids increases, while the rotatory power diminishes. The operation is considered as finished when the mash containing 10 per cent of dry materials marks 40° to 45° on the Soleil polarimeter. Then the saccharine solution is separated by filtration from the extracted grain, which still contains quite considerable quantities of unattacked starch. The juice is decolorized with bone-black and evaporated in a vacuum to a concentration of 40° to 42° Baumé, after which the syrup is placed in vessels, where it solidifies at once after it has been primed with crystallized glucose.

The starch not attacked in the first operation is subjected to further treatment. The grist of maize remaining on the filter is submitted to cooking under slight pressure. The starch obtained by this operation is saccharified at a temperature of 63°, by the aid of a small quantity of malt (1 to 2 per cent), and the dextrinated mashes replace water in the following operation. With the malts coming from 500 kilograms of maize, nearly 20 hectolitres of dextrinated mash is obtained. Into this mash are introduced 400 kilograms of ground maize and saccharification is allowed to proceed for 6 to 8 hours at a temperature of 58°–60°.

The maltase contained in the barley malt can also be utilized for the manufacture of glucose.

The infusion of malt has very little action on the maltose, but the crushed malt acts vigorously on the maltose syrups, which are transformed into dextrose syrups. For this kind of work it is well to put the malt in contact with the syrups at 20°–25° Baumé and not with diluted juices.

In saccharified and very concentrated mashes, the extraction of the diastases of the malt is made more easily than in syrups of low concentration.

Cerealose has the following average composition:

Maltose	2.5%
Glucose	72 "
Dextrin	2.5 "
Water	20 "

CHAPTER XIX

INDUSTRIAL APPLICATIONS OF MALTASE.—(*Continued.*)

JAPANESE AND CHINESE YEASTS.

Manufacture of Japanese yeast.—Preparation of *koji*.—Changes produced in the rice.—Composition of *koji*.—Action of salts.—Manufacture of "*moto*" leaven.—Manufacture of "*saké*" beer.—Composition of *moto*.—Composition of *saké*.—Manufacture of Chinese yeast.—Properties of Chinese yeast.—Influence of temperature and chemical agents.—Oriental methods of distillation.—Utilization of Oriental processes in the distilleries of Western countries.—Works of Takamine, Collette, and Boidin.

Japanese Yeast.—In certain countries of the far East alcoholic beverages are manufactured with amylaceous materials. In Japan a kind of beer is made which is called *saké*. In China and Cochin-China a brandy is prepared from rice, "choum-choum," which contains from 34 to 42% of alcohol.

The methods employed by the Orientals differ radically from European methods. The saccharification of the amylaceous materials and the fermentation are brought about by special ferments which are cultivated as an industry.

The active agent which the Japanese use is called *koji*. The ferment which serves for the manufacture of Cochin-China brandy is called *migen* or *men*.

Chinese and Japanese yeasts owe their activity to moulds which secrete maltase and probably zymase.

In the Chinese yeast, the predominating organism is *Amylomyces Rouxii*. *Koji* owes its activity to *Eurotium orizæ*.

Korschelt and Atkinson published the first data on the preparation and utilization of Japanese yeasts.

Preparation of Koji.—The grains of rice destined for the manufacture of *koji* are first cleansed and beaten so as to free them of their covering, then they are submitted to a soaking of a dozen hours. The grains are then cooked in a current of steam until they have reached a certain consistency, then are spread on mats, which are vigorously shaken to prevent the grains from uniting in lumps. The rice is then sown with the spores of a mould, *Eurotium orizæ*. The spores of this mould, which form a commercial product in Japan, are mixed with the rice in the proportion of 1 part of spores to 40,000 parts of rice. They are distributed throughout the mass by a vigorous shaking of the mat and the whole is taken to the malt-house.

Atkinson, professor at the University of Tokio, to whom we owe the description of this industry, describes the special construction of these malt-houses. They are long subterranean passages, joining each other, 4 to 10 metres long, from 2.10 m. to 2.40 m. wide and 1.20 m. high. These malt-houses are never heated except at the beginning of the cold season. The rice, mixed with spores, is heaped up in the malt-house, covered with mats and left in this state over night.

On the second day it is sprinkled with a certain quantity of water, if it is not to be used for the manufacture of the beer called *saké*.

The *koji* is then spread in a very thin layer and left. The third day the rice is again heaped up for about 4 hours. At the end of this time the grains are covered with a light fleece coming from the mycelium of the mould. The rice is then cooled by shaking, and disposed in thin layers on mats, to which is given a lateral motion to prevent the formation of lumps.

Under these conditions, the vegetation develops; the mycelium winds about among the grains and the fourth day the *koji* forms a kind of cake which is all ready to be used.

Koji is used in different branches of Japanese industry; in

the making of bread, in the preparation of the "*soy*" sauce; but especially in the brewery for the preparation of *saké*.

Temperature of Germination.—In Atkinson's account are found some data on the variations of temperature in the course of this manufacture. When after soaking, the grains of rice have been dried by shaking the mats, they have a temperature of 28°–30°. The second day, after being scattered, the temperature falls to 23°–26°, to rise again afterwards in the malt-house to 30°. The next day, the third, Atkinson observed it to reach 40°–41°. The rice is then cooled, but it heats again to 37°. These figures are, however, only approximate; they change according to the time of year. In the month of May the temperature of the malt-house has been observed to be from 24°–26°; the temperature of the *koji* was then from 25°–26°. In the month of December the thermometer marked 27° in the malt-house and the rice showed a temperature of 39°. This increase in temperature is explained by very vigorous oxidation produced by the moulds: often a difference of 10 degrees is found between the temperature of the *koji* and the external temperature. At a temperature of 40° considerable losses of starch must occur, as well as a perceptible change in the diastase secreted by the moulds.

According to more recent data on the preparation of *koji*, the Japanese manufacturers take pains not to exceed a temperature of 25°. The whole duration of the manufacture from the moment of sowing to that of the complete development of the plants is only three days.

Changes Produced in the Rice.—The transformation of the rice into *koji* appears then like a true phenomenon of oxidation. In fact, the rice used in this process loses as much as 11 per cent of its starch; this carbohydrate is oxidized with liberation of carbonic acid and formation of water.

The *koji* presents the aspect of a cake formed of grains of rice bound together by fungus threads. The grains extracted from this cake are covered with a sort of down, and

an incision made on one of them shows that the exterior cells are penetrated by the threads of the mycelium, while the interior remains unattacked and even acquires a certain hardness.

The mould in the course of development attacks the albuminoid materials which are found in the rice; these substances become soluble and the fermenting power of the *koji* increases with their solution.

The composition of *koji* dried at 100°, according to Atkinson, is here given:

Part soluble in water: 37.76%	Dextrose	25.02
	Dextrin (by difference)	3.88
	Soluble ash	0.52
	Soluble albuminoids	8.34
Part insoluble in water: 62.24%	Insoluble albuminoids	1.50
	Insoluble ash	0.09
	Fatty bodies	0.45
	Cellulose	4.20
	Starch (by difference)	56.00

The fresh *koji* contains 25.82 per cent of water.

The growth of *Eurotium orizæ* on the grain causes the proportion of soluble nitrogen in the latter to increase. In dried *koji* there is found a total of 9.84 per cent of albuminoid materials, the soluble albuminoid materials being represented by 8.34 per cent. In the rice not transformed into *koji* the quantity of soluble albuminoid materials is only 1.38 per cent. There is also a difference of solubility between rice and *koji*. When the *koji* has not been heated to 100°, it for the most part dissolves. After a short contact with cold water 12 or 15 per cent of its total weight dissolves. If the contact with water is prolonged the diastase continues to act and at the end of a longer or shorter time 30 to 60 per cent of the *koji* enters into solution.

Action of Salts.—The enzyme of *koji* is influenced by the acidity of the medium. Lactic acid, in an amount of 0.05 per cent, is favorable; the amount of 0.1 per cent possesses a retarding action.

The diastase is equally sensitive to the action of sodium chloride. The influence of this salt was determined by

Watanabe. To 5 grams of dry starch, gelatinized and cooled, he added different amounts of sodium chloride and then added to each of these specimens the same quantity of extract of *koji*. He then left it at the ordinary temperature for 1 hour, then brought the volume up to 250 cubic centimetres and filtered. The action of the salt is determined by the aid of the reducing power and the rotatory power of the solution.

Common salt per 100 of starch.	Reducing power (on copper oxide).	Specific rotatory power.
0	30.8	173.8°
10	28.6	179.3
30	25.1	182.6
50	23.8	187.6
75	20.9	190.3
100	20.1	189.1
150	19.1	190.2
200	18.0	192.2
300	16.9	194.1
500	14.4	197.5

The increase in rotatory power is easily seen and the diminution in reducing power as the amount of sodium chloride increases.

Manufacture of Moto.—*Koji* is used in Japanese industry as agent of saccharification, fermentation, and the manufacture of the beer called "*saké*." The operations necessary for this manufacture are divided into two classes: first the preparation of a strong ferment, called *moto*, then the manufacture of the mash and its fermentation.

For the manufacture of *moto*, there are used as raw materials rice cooked by steam, *koji*, and water mixed in the following proportions:

Rice	68 parts.
Koji	21 "
Water	72 "

The manufacture of *moto* is carried on in two stages. In the first stage, which lasts from 5 to 6 days, the mixture of grains and *koji* is distributed in different vessels. Under the influence of the *koji*, the rice-starch becomes liquefied and saccharified. The temperature does not exceed a few degrees, and the fermentation is extremely slow. In the second stage, the combined liquids are heated to about 25°; fermentation commences, and the temperature rises, but never exceeds 30°. The manufacture of *moto* lasts from 16 to 18 days, and the mature leavens show as much as 10 per cent of alcohol.

The following table shows the composition of *moto* in the first stage of the manufacture:

	After 3 days. Per cent.	After 5 days. Per cent.
Dextrose	7.35	12.25
Dextrin	5.12	5.69
Glycerin, Ash, Albumen	Trace	0.48
Fixed acids	0.017	0.019
Volatile acids	....	0.008
Water by difference	87.513	81.553
Undissolved starch	20.43	15.46

Its composition, during the second stage, is shown by the following table:

	7 days.	10 days.	12 days.	14 days.
Alcohol	5.2	8.61	9.41	0.62
Dextrose	5.4	0.99	0.49	0.50
Dextrin	7.0	2.81	2.72	2.57
Glycerin	1.14	2.82	2.35	1.93
Fixed acids	0.31	0.24	0.31	0.30
Volatile acids	0.15	0.11	0.05	0.03
Water by difference	80.80	84.42	84.67	85.47
Undissolved starch.	10.68	12.46	11.55	12.05

Manufacture of Saké.—For the manufacture of *saké* the same primary materials are used as for the preparation of *moto*.

The rice is saccharified by means of *koji*, and *moto*-yeast is added. Saccharification and fermentation are produced simultaneously. After a few days of slow fermentation, the mash becomes heated and begins to ferment very vigorously.

The mashes of *saké* have a very strong concentration, reaching 35° Balling. The fermentation of these mashes lasts from 15 to 17 days. Generally the amount of alcohol produced is from 12 to 13 per cent, and in some manufactories from 14 to 15 per cent.

The table shows the composition of the must after 28 days of fermentation:

Alcohol	13.23
Dextrose	——
Dextrin	0.41
Glycerin	1.99
Fixed acids	0.107
Volatile acids	0.061
Water	84.202
Undissolved starch	4.18

The fermented mashes are filtered, and not further used; in certain manufactories, however, the mash is kept, and made to undergo a secondary fermentation.

The starch contained in the residues is used again after cooking.

To preserve the *saké*, the fermented liquids are reheated to 50°–66°. The Japanese, therefore, adopted a method of sterilization before Europe had any knowledge of the process of Pasteurization. As, after sterilization, they put the liquid back into an unsterile receptacle, they lose a part of the good of this operation.

Saké differs from beer by the small quantity of dextrin and dextrose which it contains.

In the manufacture of *moto*, a very strong alcoholic fermentation is developed. The explanations as to the cause of this fermentation are very contradictory. According to certain authors, it is due to a *Saccharomyces* which is developed spontaneously in the mash; others claim that it is due to a mucor which, under certain culture conditions, is transformed into an alcoholic ferment.

It is incontestable that *Aspergillus orizæ* as well as numerous other moulds can acquire a fermenting power when cultivated under certain conditions; but these moulds generally give little alcohol and ferment very slowly. Moreover, as *Saccharomyces* have always been found in *koji*, there is every reason for believing that the fermentation comes principally from the yeast.

Chinese Yeast.—Chinese yeast has been particularly studied by Calmette, who published detailed data on the work in the distilleries of the far East. It is from these works that we obtain the following information:

Chinese yeast possesses the double property of saccharifying and causing to ferment the amylaceous materials with which it comes in contact. It is found in commerce in the form of little loaves of rice which give out a musty odor. These loaves are filled with bacteria, yeasts, and different kinds of moulds.

The active agent contained in these loaves is a mould which penetrates all through the mass by its mycelial ramifications, a mould called by Calmette *Amylomyces Rouxii.*

The *Amylomyces* is very abundant in the loaves which constitute the Chinese yeast. When this mould is cultivated in glucose agar-agar, it develops very rapidly and forms at the end of 48 hours a sort of veil extending over the whole surface of the culture. Potato and sweet potato, sown with the spores of this mould, become covered with a light floury coating which at last becomes transparent and invisible. The *Amylomyces* develops normally in gelatine, peptone, and

beef broth which is peptonized and alkaline, although a slight acidity is more favorable for it.

The mould coagulates milk in 24 hours and reddens it when it has been previously colored blue by litmus.

As a general rule saccharine mashes containing potassium phosphate are suitable for the development of the plant; however, the media where it grows the best are beer-worts, liquid or gelatinized, and amylaceous substances which have been cooked by steam.

When the mould is developed away from the air, it takes on a fleecy aspect and produces small quantities of alcohol. If, on the contrary, it lives at the surface of the mash or wort, it consumes the sugar and produces oxalic acid. Cultivated in the air in a medium containing dextrins or starch, it transforms cane-sugar into fermentable sugar.

Amylomyces is, according to Calmette, the ferment which transforms starch into sugar with most energy.

Calmette, by following the growth of this plant, has found that in contact with the air the mycelium forms conidia; when the air is excluded they extend their hyphæ in every direction and are reproduced by direct budding.

This mould differs, from botanical and physiological points of view, from all other known species: it seems to approach the trichophytes, while by its mode of reproduction as well as by its physiological properties it recalls the branched *Saccharomyces*.

Diastase of Chinese Yeast.—The diastase contained in the cells of *Amylomyces* presents, according to Calmette, all the characteristics of the amylase of malt. This diastase is secreted by the hyphæ.

Calmette also attributes to the *Amylomyces* the property of secreting sucrase. In reality the diastase secreted is glucase, and this enzyme has nothing in common with either amylase or sucrase.

To obtain a diastatic solution of this ferment, recourse is

had to a method similar to that recommended by Fernbach for the preparation of the sucrase of yeasts.

First the mould is cultivated in sterilized Raulin's medium, or better still in beer-wort, and when the plant has reached its normal growth, the liquid is replaced by sterilized water. After a stay of nearly 60 hours in a thermostat at 38°, the diastases contained in the cells are diffused into the surrounding liquid; then the water is taken away, and shows active diastatic properties.

The following experiment has been made by Calmette to determine the activity of the enzyme of *Amylomyces*. The diastatic solution is divided into several portions of 30 cubic centimetres each, which are added to a 1 per cent solution of starch, sterilized, and weighing 120 grams. Each portion receives a drop of oil of garlic, which plays the part of antiseptic. The whole is taken to the thermostat, where saccharification proceeds. The quantities of sugar obtained are here shown:

After 1 hour.....	0.12 gr.
" 6 "	0.28
" 12 "	0.33
" 24 "	0.35

It is seen that the proportion between the duration of the action and the quantity of product formed ceases after 12 hours; it appears, therefore, that the diastase is altered after that length of time.

To estimate the fermenting power of the plant cultivated on rice, Calmette suggests the following method:

One hundred grams of steamed rice are sown with hyphæ from a pure culture of *Amylomyces*; it is left 3 days at a temperature suitable for the development of the mould. Then the mass is ground with 500 grams of water and the whole poured on the membrane of a dialyser floating on distilled and sterilized water.

The starch paste does not dialyse and the membrane al-

lows only the glucose and the diastases in solution to be transfused. There is then formed under the dialyser a new diastatic solution in which the sugar is measured; then certain quantities of these solutions are stirred in a 1 per cent solution of starch paste. After saccharification the sugar formed is measured and the sugar introduced with the infusion of *Amylomyces* is deducted.

The diastase extracted from fresh cultures produces a more intense hydration than the diastase extracted from old cultures.

The filtration of diastatic solutions in Chamberland bougies takes away all their fermenting power.

The method adopted by Calmette for determining the diastase leaves much to be desired. It is evident that by this process only a small part of the diastase contained in the plants is obtained. To take account of the diastatic power of the amylaceous materials on which moulds have developed, it is necessary to crush them, reduce them to powder or paste, and use the substance prepared in this way. For example, 1 gram of this substance may be taken, mixed with 10 grams of gelatinized starch and saccharification allowed to proceed for an hour at 40°. From the quantity of sugar found must then be deducted the sugar formed under the influence of the active materials alone, for it is particularly a question of determining the quantity of sugar which the gram of active matter can give by itself under the conditions of the experiment.

Influence of Temperature and Chemical Agents.—The temperature most favorable for the development of *Amylomyces* is from 35°–38°. At this temperature the plant produces the strongest hydration. Above 38°, or lower than 23°, growth weakens; at 72° the diastase is destroyed. The plant itself is destroyed by a stay of a half-hour at 75° or 15 minutes at 80°.

The presence of salts appears to be of little disadvantage to the diastase. Calmette has determined the amounts of

different substances which do not influence the diastase; he has found:

1.10% of phenol.
0.05 " of silver nitrate.
0.10 " of copper sulphate.
0.10 " of iron sulphate.
0.10 " of zinc sulphate.

Oil of mustard, used in small quantities, has no influence on the development of the plant. Five per cent of glycerin produces a favorable effect. Oil of garlic in very small quantity and mercuric chloride in 0.005 per cent, on the contrary, check the growth of the mould.

Manufacture of Chinese Yeast.—Chinese yeast, the preparation of which demands quite complicated operations, is in the far East the object of a very interesting industry. The apparatus needed for this preparation is quite simple; being composed of mats, shelves, sieves, a granite mortar, and a circular trough. The raw materials are hulled rice and various kinds of aromatic plants which give a special perfume to the alcohol formed and which, furthermore, undoubtedly act as antiseptics.

These plants are exceedingly numerous; the best known are the *Sinapis alba*, *Caryophyllus aromaticus*, cinnamon, *Juper nigrum*, cloves, etc. The aromatic plants and the rice are separately ground and after pulverization mixed and ground with water to form a soft paste. This paste is formed into little disks a centimetre thick, which are placed on a mat after having been sown with mould by the aid of balls of rice which are introduced into the paste. The mats are then put on shelves, covered with straw matting, and the mould allowed to develop at a temperature of 28° or 30°. After two days the moulds have covered the disks with a fine down; the yeast is then dried in the sun and prepared for sale.

The rice used for the manufacture of the yeast is not of the very first quality; grains which are broken may even be used.

In Cochin-China the manufacture of Chinese yeast is carried on everywhere in the same way. In Cambodia and China the rice is sometimes replaced by the flour of beans or maize.

Native Distilleries.—The native distilleries do not demand a complicated outfit any more than do the yeast manufactories. The plant is composed of a shed covered with a tiled roof. Under this roof are ranged furnaces in parallel lines separated by spaces containing basins full of water, in which the receivers serving to condense the alcoholic vapors are plunged. The furnaces measure 60 centimetres in height, 1.2 m. in width and 4 metres in length. They are used for the heating of two stills and a boiler devoted to cooking the rice. The furnaces are heated by a fire of mangle-wood.

The rice used for making the mash is in part hulled and mixed with a certain quantity of warm water. It is placed in the boilers, which are covered with a mat and a sheet-iron cover. In each boiler are placed 18 kilograms of grains and 22 kilograms of water, and they are cooked for 2 hours. The rice is at this time completely steeped. It is then spread on mats where it receives the Chinese yeast in a fine powdery state, after which it is placed in pots of about 20 litres capacity, which are half filled. The pots are closed and saccharification allowed to occur. When the starch is transformed, that is, at the end of about 3 days, the vessels are filled with water; fermentation at once begins and at the end of 48 hours the action is finished. The contents are then distilled.

These stills are formed of a sheet-iron vat, a wooden dome, and a terra cotta head. A bamboo tube, 2.5 m. long and inclined at 45°, joins the still to the condenser, into which it conducts the alcoholic vapors. The stills are placed directly over the fire.

The residues of the distillation are used as food for cattle.

With 100 kilograms of rice and 1.5 kilograms of Chinese yeast, there is usually obtained 60 litres of 36 per cent alcohol, or 18 litres at 100 per cent. The richness of the first

distillate varies according to the distillery; it is never less than 34° or over 42°.

Use of Moulds in Fermentation Industries in Non-Asiatic Countries.—From the point of view of the utilization of primary materials, Chinese yeast, as well as Japanese yeast, furnish very meagre results in the countries of their origin.

According to Atkinson, the yield in alcohol in the manufacture of *saké* reaches only 50 to 56 per cent of the theoretical yield.

Chinese yeast does a still less satisfactory work. According to Calmette, 100 kilograms of hulled rice, having from 81 to 84 per cent of starch, furnishes about 18 litres of alcohol in the distilleries of Cochin-China.

This unsatisfactory result must be attributed in great part to the inefficiency of the plants as well as to slovenliness in the labor.

At first sight, it must be admitted that the work by the aid of moulds is capable of being improved and of giving industrial results similar, and perhaps superior, to those of the ordinary method.

Moreover, the use of moulds presents great advantages. The work appears to be much more simple; the yeast and the malt are done away with and replaced by a mould which is very easily cultivated and less sensitive than malt and yeast to the action of heat and of the medium. But to render practicable the use of moulds it is first necessary to give up Oriental methods, adapt oneself to the conditions of European distilleries, and try to develop a practical process.

This question has been studied by the Japanese chemist Takamine, and also by Collette and Boidin.

Takamine has been engaged in the application of moulds to the fermentation industry for ten years. At first he particularly sought a medium suitable for the development of *Aspergillus orizæ*, which, in Japan, is cultivated exclusively on steamed hulled rice.

To furnish the mineral element to the plant, a certain

quantity of the ash of *Camelia Japonica* is generally added. Takamine replaced the ash by an addition of 1 to 4 per cent of the weight of the grains of a mixture of salts in which are ammonium tartrate and phosphate, potassium sulphate, and magnesium sulphate.

According to the author, this addition of salts considerably increases the yield and has the further advantage of permitting the rice to be replaced by other cereals.

To prepare, industrially, cultures of *Aspergillus orizæ*, Takamine proposes the following process:

Steam the grains until the starch-cells are swollen, cool, sprinkle with the solution of salts; mix the grains well and sow with *Aspergillus orizæ*. The cereals thus sown are left at a temperature of 30° for 24 to 36 hours.

The lumps formed are broken up and the grains placed on plates, which are left in a damp atmosphere until the complete maturity of the moulds. The mouldy mass then dried at a low temperature is sifted. Thus the spores are separated, which, again dried at a moderate temperature and then mixed with inactive materials, serve as agents of fermentation.

Takamine also makes a kind of malt which he calls *taka-koji*. For the preparation of this substance he prefers to use bran or brewery or distillery malts, and proceeds as follows:

The raw materials are sterilized by steam and sown with the spores of *Aspergillus orizæ* at a temperature of 30°. One gram of spores is used for 50 kilograms of raw material. The development of the mould takes place in a very damp malt-house at a temperature of 20°–30°. After 24 hours' stay in the malt-house, the mass is spread in thin layers and the plant is allowed to grow. Generally its development is sufficient after 4 or 5 days. Then the material is dried at a temperature not exceeding 50°.

Takamine also recommends for *taka-koji* to separate the spores from the material by sifting in a silk sieve. These spores act, according to him, as agents of alcoholic fermenta-

tion, while the *taka-koji* itself acts as agent of saccharification.

Takamine also proposes, with much reason, to use for saccharification a clear infusion prepared with *taka-koji*. For this purpose he makes a cold extraction of the active matter and decants the liquid, which is used as saccharifying agent, while the solid part is submitted to a cooking which allows of utilizing the starch.

Taka-koji serves in the manufacture of a ferment useful in the distillery and the bakery. To prepare it, bran of corn or other cereals is mixed with 3 to 10 per cent of *taka-koji* and 4 to 8 volumes of water. The mass is kept at 65° for 15 to 30 minutes and then brought to a boil. Then it is cooled to 60°; a new portion (3 to 10 per cent) of *taka-koji* is added and a second saccharification allowed to go on. This ended, the liquid is separated from the solid matter by filtration or decantation, the mash is sterilized and sown with the spores of the moulds. A fermentation is produced which lasts from 12 to 16 hours. When it is ended, the ferment is deposited at the bottom of the vats in the form of a pasty material which is pressed and used in various industries.

In distillery work, according to Takamine's system, the following is the manner of procedure:

The raw materials, grains, potatoes, etc., are cooked under pressure. The starch is then saccharified by means of *taka-koji*. Saccharification goes on for an hour at a temperature of 65°–70° and the quantity of *taka-koji* used is from 3 to 20 per cent of the quantity of grain used, according to the amount of diastase the *koji* contains.

Saccharification accomplished, the mash is cooled to 19° and leaven is added.

For the preparation of the leaven, a mash of cereals cooked under pressure and saccharified by *taka-koji* is used. The saccharification of this mash is accomplished in two stages. First it is saccharified at 60° for an hour and then slowly cooled to 19°. A new portion of *taka-koji* is added,

as well as a little leaven from a previous operation, and it is allowed to ferment.

Greatly attenuated mashes as well as yeast-mother are used for leaven in succeeding operations.

Generally 2 to 10 litres of leaven are used for 100 litres of mash submitted to fermentation.

Under a patent taken out in 1894, Takamine proposes to use industrially the active substances of moulds by precipitating them in solid state from their solutions.

To cultivate the moulds, malts, bran, or other amylaceous substances are used. The culture made, these substances are reduced to powder and soaked in cold water to extract the maltase. The liquid, separated from insoluble substances, is filtered and precipitated with 1 to 3 volumes of alcohol. The product thus obtained is placed on a filter, washed with alcohol, then with ether, and dried at a moderate temperature. According to Takamine, the active substance obtained by this process can advantageously replace malt in the distillery and the brewery.

Takamine also advises (and this he holds to be important) the addition to the active infusion, before the addition of alcohol, of an infusion of raw materials, as bran, malts, raw grains, etc. According to him, the activity of the precipitated enzymes is considerably increased by this operation.

From the description of the process, it appears attractive, and we have hastened to repeat the experiment of Takamine; but the results we have obtained are not very encouraging.

Aspergillus orizæ contains a peptonizing ferment which acts strongly on albuminoid materials. The infusion obtained is very viscid, refuses to filter, and the precipitate resulting from treatment with alcohol does not show very active properties.

The addition of infusions of bran, of malts or of raw grains increases the saccharifying power of the diastases of *Asper-*

gillus orizæ, as we demonstrated before Takamine, but it does not heighten the liquefying power. In other words, the increase is apparent rather than real.* Under a more recent patent, Takamine proposes a system for the cultivation of moulds and the preparation of active liquid which deserves mention.

To secure a great surface for the culture, without wasting the nutritive materials, he places porous objects (fragments of pumice-stone, etc.) in a nutritive solution and allows the moulds to develop on these foundations. This very ingenious idea has since been taken up by Collette and Boidin. To produce industrially a growth of *Amylomyces Rouxii* straws are saturated with the nutritive liquid, sterilized, and then sown with the mould. To favor its development he keeps up a strong current of air through the mass.

By this means and with relatively little nutritive substance, an abundant vegetation is obtained.

The fermenting of grain by *Amylomyces* is accomplished, according to the method of Collette and Boidin, in the following manner: The amylaceous materials, added to twice their weight of water, are cooked for 3 hours under a pressure of $3\frac{1}{2}$ to 4 atmospheres. The cooked mass is placed in contact with fresh crushed malt, at a temperature not exceeding 70°.

The weight of the malt, estimated in barley, is from $1\frac{1}{2}$ to 2 per cent of the total weight of the amylaceous materials used in the work. Liquefaction by the malt continues for about an hour. The mash is then sterilized in a great digester where a pressure of 2 atmospheres is maintained, after which it is inoculated and allowed to ferment.

This fermentation goes on in special vats furnished with agitators and injectors of air and steam. The boiling mash, coming from the sterilizer, is introduced into vats, constructed in such a way that all infection can be avoided. The cooling of the mash takes place in the fermentation vats,

* See Comptes Rendus de l'Academie des Sciences, 1892, vol. CIX, page 1324.

where it falls to a temperature of 38° and is neutralized. Neutrality of medium is really indispensable to the normal development of *Amylomyces*. The vats are then sown with cultures of *Amylomyces*, grown on a small quantity of amylaceous materials, after which sterilized air is injected and the agitator is used.

This agitation is to prevent the mould from developing on the surface, because if it grows in that way it will consume the sugar of the mash.

After 20 hours, the development of the mould is at a maximum. Then it is cooled to 38°–33° and sowed a second time with a pure yeast culture. This yeast produces the alcoholic fermentation. The mould, to yield the same result, would take much longer. At the end of three days the fermentation is sufficiently advanced, and the mash ready for distillation.

Critical Examination of Oriental Processes.—The attempts made by Takamine, Collette, and Boidin to introduce the use of moulds into the fermentation industries has called forth many critical articles which have appeared in different reviews of distilling and brewing. The technical journals seem, on the whole, very reserved as to the value of the new method. In general, when we consider an industrial process, the only criterion which we can admit is the practical results to which it leads. Now, as regards the use of moulds in distilling, results of this kind are lacking, at present. With the exception of a few establishments where the inventors have been experimenting with their process, not a distillery is known which is run entirely by the new method. Under these circumstances, it would be premature to pronounce definitely on the value of this method of manufacture. We have nevertheless sought to compare the different patents of Takamine with those of Collette and Boidin, since they have been considered to stand for different processes. This comparison has not led us to any definite conclusions.

Takamine's method, although seven years earlier than that of Collette and Boidin, has certain points of resemblance to the latter which may easily lead to confusion of the two.

A reading of the patents relative to the use of moulds reveals in a striking way the extravagant hopes of the inventors and the illusions which possessed them as to the scope of their discovery.

Takamine, in his patents of 1891, claims, as his exclusive right, the use in fermentation industries of any mould capable of producing saccharification and fermentation of amylaceous materials or even one of these transformations alone.

Seven years later, Collette and Boidin also aspired to this exclusive right. They claim, as the result of their researches, the use of all moulds which are both saccharifying and fermenting.

One might, possibly, condone the simplicity of the Japanese chemist, who is evidently little acquainted with our literature, but the same excuse does not exist for the French chemists. The use of moulds, as well as yeasts, has for a long time been public property. It is allowable to patent a special method of working with moulds, but not the principle of their use.

In reading the patent it is very hard to see in what the invention of Collette and Boidin really consists. One might, perhaps, characterize their process by the sterilization of the mashes and the development in them of pure cultures.

Takamine, indeed, sterilizes only the leaven and then produces fermentation in the mashes saccharified at high temperature. But, by an incomprehensible misconception, Collette and Boidin return, in additional patents, to their method of work and claim that sterilization of the mashes can be omitted. So this is not the distinctive principle of their process.

One might also get the impression by studying some of the patents of the inventors, that Takamine used *Aspergillus*

orizæ exclusively, and that Collette and Boidin used only *Amylomyces*. But when all the works which they have published are reviewed, this is found not to be so.

It is to be regretted that Takamine did not limit his pretensions to *Aspergillus orizæ*, which would have rendered his process unquestionably superior to that of his competitors.

On the whole, the practical interest of moulds centers in their saccharifying powers.

By reducing the quantity of malt for the preparation of leaven, it has been possible to reduce the cost of the yeast so low that there remains little or nothing to be done in this direction. On the contrary, an economy in the malt used for saccharification of the mashes would afford a real advantage.

Aspergillus orizæ is unquestionably a more active producer of diastase than *Amylomyces Rouxii*, and from this point of view it is of much more interest to distillers. Japanese yeast affords still other advantages over *Amylomyces Rouxii*. *Aspergillus orizæ* secretes not only maltase but also sucrase. It can consequently be used in molasses and beet-sugar distilleries where *Amylomyces Rouxii* would be of no use.

The process of Collette and Boidin does not yield mashes containing more than 4 to 5 per cent of alcohol, while *Aspergillus orizæ* produces alcohol up to 12 per cent or more.

Furthermore, with *Aspergillus orizæ* there is no need of a special equipment, while the *Amylomyces Rouxii* system cannot be adopted without a complete and costly refitting of the plant.

These faults are peculiar to the Collette and Boidin process, but there are others common to the two methods.

1. Moulds are oxidizing agents and, as such, always cause great losses of carbohydrates.

2. Alcohol produced with moulds has a peculiar taste and contains many more impurities than that resulting from the use of good yeasts.

3. Moulds generally furnish very limited quantities of diastase, and to obtain a satisfactory result an abundant culture

must be allowed to develop in the mashes, which necessarily influences the yield of alcohol.

One may conclude from the preceding observations first of all that the activity of workers is not at all restrained by patents taken, and, further, that great improvements must be introduced in processes using moulds for them to become practicable. It will be necessary to study thoroughly the conditions of development of the moulds in question and it will be necessary, also, by a systematic acclimatization, to make them produce a diastatic secretion which shall be more active and less sensitive to the conditions of the medium.

Up to the present time it has been the capitalists in particular who have been occupied with the question; it is to be hoped that disinterested investigators will apply themselves to it in their turn.

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CHAPTER XX.

ENZYMES ACTING ON CARBOHYDRATES.

TREHALASE.

TREHALASE is an enzyme which acts on trehalose, an isomeric sugar corresponding to the formula

$$C_{12}H_{22}O_{11} + 2H_2O.$$

This sugar plays the part in plants of a reserve substance. Wigers and Mitscherlich have found it in spurred rye and Berthelot in the *Trehala* of Syria. It is frequently found in great quantity in fresh fungi, from whence it almost entirely disappears during drying. For example, it constitutes 10 per cent of the dry matter of *Agaricus muscarius.* Trehalose does not reduce Fehling's solution, and is transformed into glucose by the action of acids. A similar hydration may be obtained by the use of an enzyme, trehalase, discovered by Bourquelot.

This worker discovered the presence of the enzyme in *Aspergillus niger* and *Penicillium glaucum,* as well as in other fungi. This enzyme is also found in malt, and in the small intestine.

The transformation of trehalose into glucose may be expressed by the following equation:

$$\underbrace{C_{12}H_{22}O_{11}}_{\text{Trehalose.}} + H_2O = \underbrace{2C_6H_{12}O_6}_{\text{Glucose.}}.$$

The diastatic action may be followed by the change of rotatory and reducing powers of the liquid.

Trfehalose has a rotatory power of $(\alpha)_d$ 198°, while the rotatory power of glucose is only $(\alpha)_d$ 52.4°.

Experiments with trehalase may be made in a 2 per cent solution of trehalose, at a temperature of 33°–35°.

Trehalase is much more sensitive to the action of heat than maltase. At 54° its action is checked and at 64° the enzyme is completely destroyed. The reactions of the medium also have a very great influence on trehalose. An acidity corresponding to 2 to 4 milligrams of sulphuric acid seems to favor the transformation of trehalose by the enzyme, but if the amount of acid is increased, the activity diminishes, and with 0.2 grams the action of the enzyme is almost stopped.

According to Fischer, an infusion of malt may produce the decomposition of trehalose, while the salivary diastase, ptyalin, has not this property.

Amylase, precipitated and purified according to the method of Lintner, acts energetically on trehalose. By leaving, at a temperature of 35°, 10 cubic centimetres of a 10 per cent solution of trehalose with a half-gram of amylase, the formation of 0.5 grams of glucose has been observed.

Emil Fischer recognized trehalase in Frohberg yeast. This enzyme is retained in the cells of this yeast and with difficulty passes into the surrounding medium. For this reason an aqueous extract of yeast does not possess the property of transforming trehalose, while in the cells of yeast trehalose is transformed into glucose.

By adding 5 grams of yeast to 1 gram of trehalose dissolved in 10 cubic centimetres of water, Fischer was able to find, after 40 hours action at a temperature of 33°, the formation of 0.2 grams of reducing sugar.

According to this author, the existence of trehalase is to be doubted and he believes that it is amylase which produces the transformation of trehalose into glucose.

According to Fischer, therefore, amylase must have the property of acting on starch, giving maltose, and on an isomer of maltose, giving glucose.

To prove the existence of trehalase we have made the following experiment:

Equal quantities of yeast, cultivated in sterilized wort, are added, under like conditions, to a solution of dextrins and to a solution of trehalose. The 2 solutions are left for 2 days at 30° and then tested. For these experiments 2 grams of yeast are used, 25 cubic centimetres of a 1 per cent solution of soluble starch and 20 cubic centimetres of a 10 per cent solution of trehalase. The action of the yeast takes place in the presence of chloroform.

The solution of trehalase yields, under these conditions, 0.34 grams of glucose, while in the solution of soluble starch no traces of sugar are found. The enzyme secreted by the yeast is therefore not amylase, and the fact that the diastase of Lintner acts on trehalose proves only that this diastase contains other enzymes than amylase.

LACTASE.

Pasteur has demonstrated that sugar of milk treated with mineral acids is transformed into galactose and dextrose according to the equation:

$$\underbrace{C_{12}H_{22}O_{11}}_{\text{Lactose.}} + H_2O = \underbrace{C_6H_{12}O_6}_{\text{Dextrose.}} + \underbrace{C_6H_{12}O_6}_{\text{Galactose.}}.$$

In living cells, the transformation of lactose is carried on by means of an enzyme which causes the same action as the acid.

The existence of this ferment was doubted for a long time and the transformation of the sugar of milk in the organism attributed to vital activity. Beijerinck was the first to discover the presence of lactase in certain yeasts found in cheese and kephir. Since then Duclaux, De Kayser, and Adametz have found other species of yeasts which secrete the same diastase. Emil Fischer, repeating the experiment of Beijerinck, has confirmed the fact that the filtered infusion of kephir acts upon lactose.

As in kephir the *Saccharomyces* act in symbiosis with other micro-organisms, it was of interest to find out whether the enzyme is secreted by the yeast or by the accompanying bacteria. The experiments which Fischer made with this object have established the following facts:

1st. Certain alcoholic yeasts are capable of fermenting lactose; 2nd. The action of a yeast on milk-sugar depends solely on its power to secrete lactase.

The enzyme acting on the lactose is retained inside the cells and passes with great difficulty into the surrounding medium. Even when the cells of yeast are crushed with powdered glass, it is difficult to extract the active substance. The diffusion of the diastase of the cells is accelerated by chloroform. Lactase can be precipitated from its solutions by alcohol without completely losing its activity.

The action of lactase on lactose can be determined by the aid of the polariscope; by the transformation of the lactose into dextrose and galactose the rotation of the solution increases about a third.

Lactose and dextrose have a rotatory power of $(\alpha)_d + 52.5$, while that of galactose is $(\alpha)_d + 83$.

INULASE.

Certain plants contain, as reserve substance, a carbohydrate called inulin.

These plants generally contain at the same time an active principle which transforms this carbohydrate into an assimilable sugar.

This enzyme was discovered by J. R. Green, who named it "inulase."

The presence of inulase has been observed in the tubers of Jerusalem artichokes during their development, in *Aspergillus niger*, in *Penicillium glaucum*, and in the tubers of dahlias. According to Bourquelot, it is supposed that this enzyme is also found in chicory, garlic, and onions, as well as in many other vegetables.

By the action of inulase, inulin is hydrated and transformed into levulose according to the formula:

$$\underbrace{(C_6H_{10}O_5)_{18}}_{\text{Inulin.}} + 18H_2O = \underbrace{18C_6H_{12}O_6}_{\text{Levulose.}}.$$

According to Green, this transformation is accomplished by a progressive hydration of the inulin, with formation of intermediate substances. Granting that the molecule of inulin is very complex, one may assume that, during hydration, there are formed, besides levulose, different inulins having different molecular weights.

However, inulin, after having undergone a partial hydration, possesses the same rotatory power as before undergoing the action of the enzyme.

The existence of intermediate substances is somewhat problematical, as the formation of these bodies by the action of acids has not been determined. The optimum temperature of inulase is found between 50° and 60°. The action of the enzyme is influenced by the reaction of the medium. In a neutral liquid, or with 0.005 part of hydrochloric acid, hydration progresses regularly. In the presence of increasing amounts of acid, the activity of the enzyme decreases. With 0.2 of acid or 1.5 of sodium carbonate, the diastase is destroyed.

The influence of the reaction of the medium is more strongly shown at 40° than at 10°–15°.

The transformation of inulin into levulose may be followed either by observation of the rotatory power or by that of the reducing power.

Inulin has a rotatory power of $(\alpha)_d - 36$, while levulose gives a rotation to the left which is almost double.

In the distilleries which use Jerusalem artichokes as a raw material, the inulin has to be inverted when a satisfactory yield in alcohol is desired. To effect this transformation, the use of barley malt is advised.

This method is very poor indeed, for amylase is without action on inulin and malt does not contain inulase.

The transformation of inulin into levulose can be brought about very easily: it is sufficient to cook the raw materials under low pressure to effect a complete inversion.

PECTASE.

In the pulp of carrots and beets, and also in the soft parts of fruits, Frémy found a reserve substance to which he gave the name of pectose.

This substance is insoluble in water and in alcohol. It very much resembles cellulose.

Pectose undergoes a succession of transformations during the ripening of fruits: it is transformed into pectin and finally into pectates.

Pectin is a neutral substance which gives with water a viscous solution from which it is precipitable by alcohol.

The transformation of pectose into pectin is very probably produced by an enzyme which, however, has not yet been isolated.

The transformation of pectin into pectates is better known, and the intervention of an enzyme is here definitely established.

This active substance is called pectase. This name ought to belong to the substance acting on pectose and not to the enzyme transforming pectin. This latter enzyme, according to the correct nomenclature, should rather be called "pectinase."

The composition of pectin is not definitely established. According to Frémy it has the formula:

$$C_{32}H_{48}O_{32}.$$

and according to Chandnew:

$$C_{28}H_{42}O_{24}.$$

The mechanism of the reaction produced by the pectase is little known.

It is not even clearly established that the reaction is caused by hydration, and it may well be that the mechanism of the reaction consists in a molecular change of the same nature as that found in the transformation of sugar into lactic acid.

The action of pectase on a solution of pectin is shown by the gelatinization of the solution, and by the formation of a reducing substance.

Bertrand and Mallevre have shown that the reaction takes place only in the presence of certain salts. A solution of pure pectin, with the addition of pectase, free from calcium salts, never becomes gelatinous.

The solidification of the solution takes place instantly if to the mixture are added several drops of a solution of calcium chloride, a substance, which, without pectase, could not produce gelatinization.

The calcium salt may be replaced by salts of barium or strontium, which play just the same part.

To obtain a solution of pectase, carrots gathered in the process of growth are used, because it is then that these plants contain the most diastase. It is well to pare the carrots and use only the central part, as the skin contains but little pectase.

The substance is reduced to a pulp and the juice extracted by pressure; in this way 70 to 80 per cent of a turbid liquid is obtained, which is filtered after addition of a little chloroform.

This liquid is very active in a solution of pure pectin. To preserve the filtered solution of pectase, precipitate the salts of lime and magnesium by the addition of alkaline oxalate, previously determining by analyses the amount of oxalate to use. The quantity of salts contained in the juice varies slightly with the species of carrots; for three different samples Bertrand obtained the following figures:

Lime.....	0.016%	0.018%	0.013%
Magnesia	0.029		0.021

It is well, in practice, to use a third of alkaline oxalate in excess of the amount calculated for the salts present. The solution of pectase to which oxalate has been added rapidly becomes clear and, after filtration, gives a transparent solution.

This product can be kept for a long time if chloroform is added and it is put fresh into full bottles sheltered from the light. Jelly is not produced in a solution of pectin free from salts.

To prepare pectase in a pure state, clover is used. The plants are crushed in an iron mortar; the mass is then pressed, and the juice, with chloroform added, is placed away from the light. At the end of 24 hours, a coagulum is produced in the liquid which permits of filtration. In the filtered liquid the diastase is precipitated by alcohol as has been done with the other enzymes.

Measurement of Pectase.—Pectase is very widely distributed in the vegetable kingdom. It is found in stems, flowers, and leaves of different plants.

Bertrand and Mallevre propose the following method for measuring it:

To one volume of a 2 per cent pectin solution add one volume of the juice under examination and measure the diastatic power by the time required for the liquid to become gelatinized.

The results obtained with the juices of different plants are given below:

Tomatoes	48 hours.
Grape-vine	24 "
Carrots	2 "
Maize (leaves)	8 "
Clover	10 minutes.

By using this method, Bertrand and Mallevre studied the influence of the medium on pectase.

Different samples of the same solution of pectin were

acidified in different degrees and the same quantity of pectase was added:

Hydrochloric Acid. Per cent.	Coagulation at the end of
0	$\frac{3}{4}$ hour.
0.02	1 "
0.06	$3\frac{3}{4}$ hours.
0.1	20 "

Pectase is unfavorably influenced by the acid reaction of the medium; 0.06 per cent of acid in the liquid produces a delay of three hours in coagulation. However, acid does not easily destroy this active substance.

By neutralizing the acid solutions, which have become weak and slightly active, new liquids are obtained which act very rapidly. This resistance of pectase to the acid reaction explains why the action of pectase is weak in green fruits: before ripening the enzyme is in the presence of a large amount of acid and does not act, or acts only very feebly, while during ripening the acidity disappears and the action of the pectase is shown with much more intensity.

CYTASE.

Cellulose is often assimilated by vegetable cells. This assimilation is preceded by liquefaction and a more or less complete transformation. The agent which produces the change is cytase.

As celluloses exist whose properties differ considerably, it is necessary, at the outset, to assume also the existence of different cellulose-dissolving enzymes.

Sachs was the first to discover that, during the germination of the stones of dates, the cellulose of the endosperm is gradually dissolved and that the products formed are absorbed by the young plants which, with the cellulose, produce the transitory starch.

Green, by treating germinated date-seeds with glycerin,

obtained an active solution which causes swelling as well as partial solution of certain celluloses.

The destruction of vegetable tissues by moulds must also be attributed to a secretion of cytases; however, the isolation of these enzymes is accomplished with much difficulty and their existence was doubted for a long time.

The difficulty met with, when it is desired to isolate this diastase, comes probably from its decomposition. It is probable that these enzymes are destroyed as rapidly as they appear, and that it is for this reason that they are not found accumulated in the cells.

A more stable cytase was discovered by Brown and Morris in malt dried in the air. To obtain this enzyme in a solid state, an infusion of malt is precipitated with alcohol, and the precipitate dried *in vacuo*.

The product obtained contains, besides amylase, a cellulose-dissolving enzyme.

The activity of this ferment is shown by its property of dissolving the cellulose envelope of grains of starch. This may be verified by causing it to act on the endosperm of barley. For this, very thin shavings of barley endosperm are put into an infusion of malt and it is found that the cellular walls soften and then enter partially into solution.

Cytase is present from the beginning of germination of cereals, appearing even before amylase.

The dissolving action of cytase during malting is exercised upon the whole of the endosperm and, as a result, the germinated grain becomes friable and mealy.

This transformation may also be produced artificially by placing a grain of barley from which the embryo has been removed in a malt infusion. By a prolonged stay, the endosperm changes its appearance completely; it becomes mealy and friable, while if the infusion is previously heated to 60°, the same effect is not obtained. At this temperature, the amylase of the solution does not lose its power to hydrolyze starch, while the cytase is destroyed.

The transformation which cytase produces during germination has been studied from a chemical point of view. It is very probable that the cellulose is transformed into sugar, but it may also be that the action of the cytase is less complete. During germination, according to J. Gruss, the cellular walls are only partially liquefied, and the action of cytase is reduced to freeing the amylaceous cells and indirectly facilitating the action of amylase.

By cultivating the germ of cereals in different media, Brown and Morris found that the presence of an assimilable hydrocarbon influences unfavorably the secretion of cytase. They also found that a slight acidity of the medium is, on the contrary, very favorable to the secretion.

In general, all the conditions which favor the secretion of amylase are equally favorable to that of cytase.

CAROUBINASE.

Caroubinase is an enzyme acting on a carbohydrate isolated from grains of the *Ceratonia siliqua* to which we have given the name of caroubin.

This enzyme causes a liquefying and saccharifying action on the endosperm of carob-seeds and plays a very important part during the first period of the development of this plant.

The endosperm of the seeds of *Ceratonia siliqua* is found to be partially composed of a carbohydrate which occurs in the form of a homogeneous and horny mass, not colored by iodine, and possessing some properties like those of agar-agar. To prepare this carbohydrate in a pure state, the seeds are freed of their exterior envelope as well as their embryo, and the endosperm is dissolved in warm water. The solution is then precipitated by alcohol.

The operation is carried out in the following manner: The seeds are allowed to soak for five or six days, the liquid being renewed three or four times a day. The grains swell a great deal and absorb three times their weight of water. In

this state it is easy to separate the endosperm from the testa and the embryo. One hundred grams of dry seeds furnish 53 grams of albumen. The swelling of the grain during soaking is due almost entirely to the mucilaginous substance which they contain and which constitutes an elastic and resisting mass.

By submitting the endosperm to the action of warm water, in a water-bath, a transparent jelly is obtained which can be filtered through a silk filter. It is well to use enough water to obtain a thick syrup. To precipitate the caroubin, add to the cooled syrup twice its volume of 98 per cent alcohol. The carbohydrate is thrown down in long filaments which are collected on a cloth.

The first precipitate thus obtained contains 2 to 3 per cent of albuminoid materials and salts which are easily eliminated by redissolving the product in water and again precipitating it with alcohol. By treating the endosperm eight to ten times successively with warm water, there is obtained an almost complete extraction of the carbohydrate it contains.

The product, purified and dried at 100°, is a white substance, which is spongy, very friable, and having the chemical formula of celluloses. Instead of alcohol, one may just as well use barium hydrate, which precipitates the carbohydrate in a pure state. Caroubin is easily hydrated by acids as well as by a special diastase, caroubinase.

To isolate this enzyme, we used an infusion of germinated carob-beans. A hundred grams of germinated seeds reduced to a paste were put to soak in water, at a temperature of 30°, for twelve hours. To the filtered liquid 3 volumes of alcohol were added; the precipitate was washed with alcohol, then with ether, then dried *in vacuo*.

The active substance obtained by this method dissolves easily in water and gives a reaction with guaiacum and hydrogen peroxide.

Caroubinase acts energetically at 40°, and its activity increases with the temperature up to 50°, which is its optimum

temperature; at 70°, the action becomes very weak and at 80° the enzyme is destroyed.

Caroubinase acts very slightly in a neutral medium. An addition of 0.01 to 0.03 per cent by volume of formic acid favors the action of the enzyme.

To determine the diastatic power of caroubinase, the degree of fluidity produced in a jelly of caroubin is taken as a starting point.

The diastatic power may also be estimated by the greater or less facility with which the liquid may be filtered.

The solution of caroubin not transformed by the enzymes does not pass through the filter, while the solution of caroubin with a sufficient quantity of diastase added passes through very rapidly.

The process employed is as follows:

Pour into test-tubes 50 cubic centimetres of water; add 0.1 c.c. of normal formic acid and 1 gram of pulverized caroubin. Mix and add to different tubes 2, 5, 7, 10, 15 cubic centimetres of the liquid to be examined. If there is room, bring the volume up to 65 cubic centimetres and leave it for three hours at 45°.

All the samples receive the same amount of chloroform, and the experiments are conducted in duplicate: on the one hand with fresh infusion, on the other with this same infusion previously kept for a half-hour at 90°.

The tubes which have not received any infusion, or in which the substance has been destroyed by heating, may be turned over without the liquid running out, while the tubes which have received a sufficient quantity of enzyme contain a very fluid substance which easily passes through the filter.

To study the secretion of caroubinase, we allowed the embryos of *Ceratonia siliqua* to grow in varied conditions and followed the transformation of the nutritive materials as well as the quantity of diastase formed.

The embryo, separated from the endosperm and cultivated in the dark, develops very slowly and gives, after eight

to ten days, a rootlet of the same length as itself. Then placed in calcareous earth and in the light, the germ develops into a puny plant which generally dies at the end of three to four weeks.

The progress of growth is quite different when the isolated embryo is cultivated in hydrated caroubin; the germination is more rapid; a rootlet of the length of the seed is obtained, and the embryo, set out in the earth, rapidly develops into a plant of several branches.

During germination away from the light, the caroubin used swells a great deal and is partly liquefied, but the quantity of carbohydrate absorbed is inconsiderable.

The liquefaction and absorption of the caroubin progresses much more rapidly as soon as chlorophyll appears in the plantlet. The embryo, developed in the dark and transplanted in a calcareous soil, absorbs in three or four days a quantity of caroubin equal to its own weight.

By taking specimens at different stages of germination, we found that the active substance appears abundantly when the plantlets are completely developed and that the enzyme becomes more active when the chlorophyll begins to appear.

Caroubinase is both a liquefying and a saccharifying agent. When jelly of caroubin is analyzed immediately after liquefaction, the liquid is found to contain no trace of reducing-sugar. Caroubin liquefied by the enzyme is easily precipitated by alcohol, but the precipitate no longer has the properties of the caroubin. It is strongly dextro-rotatory and easily dissolves in water.

By a prolonged action of caroubinase on caroubin, a solution is obtained in which alcohol no longer produces precipitate and a reducing-sugar easily fermenting under the influence of beer-yeast is produced.

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CHAPTER XXI.

FERMENTS OF GLYCERIDES AND GLUCOSIDES.

Saponifying ferments.—Ferments of glycerides.—Serolipase and pancreatolipase.—Measurement of lipase.—Influence of temperature and alkalinity of the medium.—Differences between lipases of different origins.—Ferments of glucosides.—Myrosin, Emulsin, Rhamnase, Erythrozyme, Betulase.

FERMENTS OF GLYCERIDES.—LIPASE.

THE pancreatic juice has the property of splitting fats into fatty acids and glycerin. This property is due to the presence of a soluble ferment to which has been given the name of steapsin or lipase. The reaction which steapsin causes may be represented by the following equation:

$$\underbrace{C_3H_5(C_{18}H_{35}O_2)_3}_{\text{Stearin.}} + 3H_2O = \underbrace{C_3H_5(OH)_3}_{\text{Glycerin.}} + \underbrace{3C_{18}H_{36}O_2}_{\text{Stearic acid.}}.$$

To obtain steapsin in solution, the pancreas is macerated in a solution of sodium or potassium carbonate. It can then be extracted from the pancreas by glycerin.

The pancreatic juice acts upon fats as a saponifying and emulsifying agent. The emulsion is produced by the pancreatic juice, owing to the alkaline reaction and to the viscosity of the liquid, and not by the action of the enzyme contained therein.

The pancreatic juice as well as the products of maceration of the pancreas contain relatively little of the enzyme and the saponification of the fatty substances is always incomplete.

The enzyme of the pancreatic juice also acts upon other substances than the fats; it attacks the lecithins, decompos-

ing them into glycero-phosphoric acid, choline, glycerin, and free fatty acids. Steapsin acts also on some other ethers: on the benzoic ether of glycerin, and on phenyl succinate, as well as on salol.

It decomposes this latter body into salicylic acid and phenol.

The ferment of the glycerides is very abundant in the vegetable kingdom. Its presence has been observed in the poppy, hemp, maize, rape-seeds, as well as many other plants.

To obtain an active liquid containing lipase, Green ground the germinated seeds of the castor-oil plant in a 5 per cent solution of sodium chloride, with the addition of a small quantity of potassium cyanide. He then dialysed the liquid to separate the salts from it. This solution, mixed with an emulsion of castor-oil, soon began to decompose the oil, setting free the fatty acid.

An active substance showing all the properties of lipase is met with in *Penicillium glaucum.*

The presence of a similar substance called serolipase is also found in the blood. It plays an important part in the assimilation of fats. Hanriot, who studied this enzyme with much care, pointed out a method for measuring the active substance and determined the influence of temperature, and of the reaction of the medium upon this enzyme. According to him there is a difference between the lipase of the pancreatic juice and the lipase of the blood.

Measurement of Lipase.—To measure the lipase, Hanriot and Camus make use of a solution of monobutyrin.

They take 1 cubic centimetre of the liquid containing the lipase to be measured, add to it 10 cubic centimetres of a 1 per cent solution of monobutyrin. The solution is carefully neutralized with sodium carbonate, then heated to 25° for 20 minutes. Under the influence of lipase, the liquid becomes acid, and this acidity is estimated by again neutralizing the solution with sodium carbonate: the number of drops used serves to measure the diastatic activity.

The solution of sodium carbonate used for the saturation is prepared in such a way that each drop of the alkaline liquid neutralizes 0.000001 of a gram-molecule of acid. The diastatic power is expressed by the number of millionths of a gram-molecule of acid freed during 20 minutes at 25°; 1 cubic centimetre of serum, for example, is said to possess a diastatic force of 33 if, in 20 minutes at 25°, it frees a quantity of butyric acid, molecular weight 88, equal to $\frac{33 \times 88}{1{,}000{,}000}$.

Influence of Temperature and the Reaction of the Medium.—Heat exerts a considerable influence on the activity of lipase. Between 0° and 50° it acts with an increasing energy, but beyond that point the diastatic activity begins to diminish and the enzyme is soon destroyed.

Temperature of the reaction.	Quantities saponified. In 10 minutes.	In 1 hour.
0°	4.5	13.5
10	....	
20	6.7	29.3
25	10.1	35.
37	13.5	39.5
40	16.9	56.5
50	22.6	71.2
60	27.1	36.1
70	22.6	22.6

The temperature of 60° appears favorable at the beginning, but in the end destroys the diastase.

The influence of temperature on lipase may be shown by warming the serum to different temperatures and causing it to act then on monobutyrin at 37°.

Serum heated.	Diastatic activity.
50°–55°	41.5
60°–62°	0.7
65°–66°	Extremely slight.
70°–72°	0

The action of lipase is proportional to the quantity of enzyme used, at least at the beginning of the action. The following table shows this fact:

Duration of the Action.	Quantity of Lipase.			
	0.5 c.c.	1 c.c.	1.5 c.c.	2 c.c.
20 minutes	6	11	16	22
1 hour.	12.5	25	37	48
1 h. 20 m.	20	36	53	62
2 hours	30	54	73	66

Cessation of the proportionality is noticed, in the case of lipase as well as in that of other diastases, when the action is prolonged or takes place at high temperatures.

The glycerin and the sodium butyrate formed during the action have no influence at all on the diastatic activity; the presence of monobutyrin is also almost without effect on the saponification.

The alkalinity of the medium influences considerably the course of saponification by serolipase.

Hanriot, to show this action, made the following experiment:

To indentical mixtures of serum, monobutyrin, and water (10 cubic centimetres) were added varying amounts of sodium carbonate. After twenty minutes he determined the quantity of monobutyrin saponified by neutralizing with sodium carbonate. He obtained the following results:

Excess of carbonate of sodium in milligrams	0	2	4	6	8	10	15	20
Activity of the lipase	22	33	40	44	46	52	74	86

Difference between Lipases of Different Origin.—Hanriot, having remarked that the ablation of the pancreas in the organism does not prevent the secretion of lipase, attributed to the blood the property of secreting a lipase different from that of the pancreas. He called it serolipase, in distinction from the first, which he called pancreatolipase.

But the ablation of the pancreas is a very delicate operation and impossible to perform without leaving active fragments of the gland. Lipase, on the other hand, may be preserved in the blood. The existence of two lipases needs, therefore, to be clearly established.

Hanriot sought to differentiate the two enzymes by their mode of action and their sensitiveness to physical and chemical agents. For that, he prepared two solutions having the same activity, that is, producing the same quantity of butyric acid by acting on monobutyrin during the same time.

These two solutions should, therefore, if there exists merely a single lipase, contain the same quantity. Now, when the action of the serolipase and pancreatolipase is prolonged for 20 minutes, it is observed that the serum enzyme produces a quantity of butyric acid double that obtained by the pancreatolipase.

On the other hand, the enzyme of the pancreas acts with great difficulty in an acid medium, while serolipase produces a very energetic transformation under the same conditions.

	Pancreatic juice.	Serum.
Activity in alkaline medium (excess of sodium carbonate, per litre 0.2 gr.)	23	22
Activity in acid medium	9	16

The serolipase and the pancreatolipase act differently at the same temperatures: two solutions of these enzymes possessing the same activity at 14° gave the following figures at other temperatures:

	Serolipase.	Pancreatolipase.
At 15°	11	10
" 30°	15	10
" 42°	21	11

It is seen by this table that the action of pancreatolipase is, up to a certain limit, independent of temperature, while serolipase produces a much more energetic action at 42° than at 15°.

Finally, the two enzymes differ as regards stability. In fact, serolipase remains unchanged during whole months, while the pancreas enzyme becomes inactive at the end of a few days.

The lipases of the pancreas and the serum act differently, therefore, at the same temperatures and are influenced differently by the reaction of the medium. Furthermore, they present different characteristics of stability. These properties, however, are not enough to demonstrate that serolipase and pancreatolipase are two very distinct chemical substances. In the case of lipase, as we have seen for amylase and glucase, the conditions of the medium produce variations in the properties of the diastase. The foreign substances found in the blood, as well as the extractive materials of the pancreas, give different characteristics to the two diastatic extracts, but the enzyme is really the same in the two cases.

FERMENTS OF GLUCOSIDES.

Glucosides are combinations of sugars and organic substances containing one or more hydroxyls.

Glucosides exist in which the sugar is found combined with alcohols, phenols, aldehydes or organic acids. These ethers are frequently found in plants, especially in the bark and roots.

The manner of formation of glucosides in living cells is as yet little known. It is very probable that their formation is due to a molecular concentration followed by a dehydration which is produced by special enzymes.

According to Gautier, the formation of certain glucosides may be explained by a transformation of formic aldehyde:

$$12CH_2O + H_2 = C_{12}H_{16}O_7 + 5H_2O.$$

Formic aldehyde. Arbutin.

$$13CH_2O + 2H_2 = C_{13}H_{18}O_7 + 6H_2O.$$

Formic aldehyde. Salicin.

The part played by glucosides in the cells is also little known at the present time.

In some cases they evidently play the part of reserve materials. In other cases the assimilation of the products of cleavage of glucosides appears of little probability. In fact, these bodies contain, besides sugar, poisonous substances which must act unfavorably on the cells.

In the parts of plants where the presence of glucosides is observed, there are almost always found enzymes under the influence of which these ethers are hydrated, then split, regenerating the sugar. The enzymes of glucosides are generally enclosed in special cells which separate them from the substances on which they can act.

The glucoside-splitting enzymes have this peculiarity that they act, not on a single body, as is the case with sucrase, for example, but on a whole series of bodies.

Their action may be exerted on numerous ethers resulting from the combination of glucose with bodies belonging either to the fatty series or the aromatic series.

Emulsin.—By treating bitter almonds, reduced to powder, with water, an aromatic oil is produced which did not exist in the almonds before the treatment.

This reaction is caused by an enzyme, emulsin, on a special substance contained in the almond: amygdalin. The reaction may be represented by the following equation:

$$\underset{\text{Amygdalin.}}{C_{20}H_{27}NO_{11}} + 2H_2O = 2\underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Benzoic aldehyde.}}{C_7H_6O} + \underset{\text{Hydrocyanic acid.}}{HCN}.$$

Emulsin and also amygdalin were discovered by Robiquet and Boutron.

This diastase is found in the leaves of cherry-laurel as well as in sweet almonds. With the latter, oil of bitter almonds is not obtained on account of the absence of amygdalin.

Bourquelot discovered the presence of emulsin in fungi. Fungi parasitic on trees, especially, contain great quantities

of this substance; thus he discovered the presence of this enzyme in *Polyporus sulfureus*, in *Armillaria mellea*, and in *Polyporus fomentarius*.

Emulsin has also been met with in *Penicillium glaucum*, in *Aspergillus niger*, as well as in other moulds.

Emulsin acts on a great number of glucosides, causing the reactions expressed by the following equations:

$$\underset{\text{Arbutin.}}{C_{12}H_{16}O_7} + H_2O = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Hydroquinone.}}{C_6H_6O_2}.$$

With helicin, a product of oxidation of salicin:

$$\underset{\text{Helicin.}}{C_{13}H_{16}O_7} + H_2O = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Salicylic aldehyde.}}{C_7H_6O_2}.$$

With salicin, extracted from the bark of poplar or the flowers of *Spirea ulmaria*:

$$\underset{\text{Salicin.}}{C_{13}H_{18}O_7} + H_2O = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Saligenin.}}{C_7H_8O_2}.$$

With phloridzin, extracted from the bark of the apple-tree:

$$\underset{\text{Phloridzin.}}{C_{21}H_{24}O_{10}} + H_2O = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Phloretin.}}{C_{15}H_{14}O_3}.$$

With daphnin, extracted from the *Daphne gnidium*:

$$\underset{\text{Daphnin.}}{C_{15}H_{16}O_9} + H_2O = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Daphnetin.}}{C_9H_6O_4}.$$

With coniferin, extracted from the *Larix Europæ*:

$$\underset{\text{Coniferin.}}{C_{16}H_{22}O_8} + H_2O = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Coniferic alcohol.}}{C_{10}H_{12}O_3}.$$

With esculin of *Æsculus hippocastanum*, which certain authors consider as an isomer of daphnin, glucose and esculitin are obtained:

$$\underset{\text{Esculin.}}{C_{15}H_{16}O_9} + H_2O = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Esculitin.}}{C_9H_6O_4}.$$

Emulsin acts also on the chlorinated and brominated derivatives of the glucosides.

According to Fischer emulsin can also transform lactose into galactose and dextrose. But this assertion needs to be verified, for it is very probable that the emulsin having served for these experiments contained a certain proportion of lactase.

Emulsin, which acts on bodies very differently from a chemical point of view, acts differently on the various monosaccharids, according to their configuration. Thus, while it acts on the β-methyldextro-glucoside, it is without action on the α-methyldextro-glucoside.

In living plants, amygdalin is not transformed because it is localized in special cells, and is thus separated from the glucosides. Mechanical action is necessary to bring the two bodies into contact.

Thus the transformation of amygdalin into oil of bitter almonds and hydrocyanic acid occurs very rapidly when the plants containing the glucoside and the enzyme are macerated in water.

According to Guignard, the emulsin cells are located in the cotyledons. In the cherry-laurel the enzyme is localized in the cells of the endodermis.

Emulsin gives characteristic reactions with the solution of orcin as well as with Millon's reagent. With the latter, the vegetable cells containing emulsin are colored an orange-red. When the cells containing emulsin are carefully heated with a solution of orcin, a violet coloration is obtained. This solution is prepared by adding 2 cubic centimetres of hydrochloric acid to a 1 : 10 solution of orcin.

The physical and chemical conditions of the action of emulsin are but little known.

Chloral, up to 3.5 per cent, does not influence the course of hydration by emulsin, but the enzyme is sensitive to the action of 8 per cent alcohol.

Neutral salts do not appear to influence the course of the

hydration. Alkaline salts, on the contrary, have a retarding influence.

Emulsin plays an important part in the manufacture of the oil of bitter almonds as well as in the manufacture of laurel-water.

To manufacture the oil of bitter almonds, the almonds are reduced to powder, the oil extracted, water added, and the mass left at the ordinary temperature for the reaction to take place. Fermentation ended, they are distilled with steam.

To obtain a good yield one must avoid beginning to distill before the fermentation is finished.

For the manufacture of laurel-water, the fresh leaves of the plant are used. They are crushed, cold water is added, and finally distilled.

It is necessary to leave cold water for some time in contact with the leaves before heating.

Emulsin is used in pharmacy, where it is prepared in the following manner:

Sweet almonds are blanched, powdered, and submitted to a strong pressure which presses out the oils. The press-cakes are put to soak in three times their volume of water; the mass is again pressed and thus is obtained an oily liquid which is clarified by leaving it for some time at a temperature of 30°.

Then the upper layer of the liquid, which consists of oil, is removed and the enzyme in the clear liquid is precipitated by alcohol. The precipitate is collected on a filter, washed in 95 per cent alcohol, and dried *in vacuo*.

Thus a yellowish powder is obtained, very rich in phosphates and mineral salts. Completely dried, it can be heated to 100° without losing its activity.

Emulsin is soluble in water and in a dry state remains unchanged for a long time.

MYROSIN.

Myrosin was discovered by Bussy in mustard-seed.

The characteristic odor of black mustard-seed, when ground with water, is due to the presence and action of this enzyme.

Myrosin is very widely distributed in the vegetable kingdom; it is frequently found in plants of the family *Cruciferæ*. This diastase, like emulsin, is located in special cells scattered in the different organs of the plant, but chiefly in the root and the leaves.

It acts on sinigrin or potassium myronate, which is decomposed by hydration. This chemical reaction is generally considered as taking place according to the equation:

$$\underset{\text{Sinigrin.}}{C_{10}H_{18}NKS_2O_{10}} = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Allyl iso-thiocyanate.}}{C_3H_5NSC} + \underset{\text{Potassium bisulphate.}}{KHSO_4}.$$

According to this equation, the decomposition would be produced without hydration. But free myronic acid has not yet been studied; it is very probable that potassium myronate has the formula:

$$C_{10}H_{16}KNS_2O_9 + H_2O,$$

and it is, therefore, probable that the diastase produces a hydration and not a simple decomposition.

In the seeds of white mustard myrosin is also found, but the sinigrin is replaced by another glucoside, sinalbin. The reaction produced may be expressed by the following equation:

$$\underset{\text{Sinalbin.}}{C_{30}H_{44}N_2S_2O_{16}} =$$

$$\underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Oxy-benzyl-thiocyanate.}}{C_7H_7O\text{–}NCS} + \underset{\text{Sinapine sulphate.}}{C_{16}H_{24}NO_5} - HSO_4.$$

Myrosin may also act upon many other glucosides. To this diastase is attributed the formation of essential oils of different plants such as: water-cress, *Reseda odorata*, and *Cochlioria officinalis*.

RHAMNASE.

This enzyme is found in the fruits of the Avignon berry (*Rhamnus infectoria*). It acts on a yellow coloring matter having the characteristics of a glucoside, xanthorhamnin, and transforms it into rhamnetin and isodulcite:

$$\underset{\text{Xanthorhamnin.}}{C_{24}H_{32}O_{14}} + 3H_2O = \underset{\text{Rhamnetin.}}{C_{12}H_{10}O_3} + 2\underset{\text{Isodulcite.}}{C_6H_{14}O_6}.$$

ERYTHROZYME.

This diastase is secreted by the root of the madder. It acts on a glucoside of alizarin: rubian, which is also found in fresh madder-root. The reaction probably occurs according to the following equation:

$$\underset{\text{Rubian.}}{C_{26}H_{28}O_{14}} + 2H_2O = 2\underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Alizarin.}}{C_{14}H_8O_4}.$$

BETULASE.

Betulase is met with in the bark of *Betula lenta*. This enzyme acts on gaultherin and the reaction may be expressed by the following equation:

$$\underset{\text{Gaultherin.}}{C_{14}H_{18}O_8} + H_2O = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Methyl salicylate.}}{C_6H_4\begin{matrix}OH\\CO\end{matrix}.OCH_3}.$$

To prepare this enzyme, take the bark of the *Betula lenta* and reduce it to powder, treat it with 4 volumes of glycerin and leave it at the ordinary temperature for 30 days.

The mass is then pressed, and the enzyme precipitated from the solution with 5 volumes of alcohol. The deposit is filtered off, washed, and dried.

A kilogram of bark gives with this treatment nearly a gram of enzyme.

Betulase does not color with tincture of guaiacum and does not act on other glucosides than gaultherin.

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CHAPTER XXII.

ZYMASE.

Zymase or alcoholic diastase.—Preparation of the sap of yeasts and its properties.—Determination of the fermenting power of zymase.—Chemical and physical conditions of the action of zymase.—Experiment of Effront on intracellular fermentation.—Industrial applications of zymase.

Zymase or Alcoholic Diastase.—The phenomena observed in alcoholic fermentation have for a long time occupied the scientific world and given rise to numerous theories and hypotheses.

In 1858, Traube sought to explain the decomposition of sugar into alcohol and carbonic acid by the intervention of a diastase secreted by the yeast. This opinion was accepted by Berthelot as well as by some other scientists. None of them, however, brought experimental proofs to show that alcoholic fermentation constitutes a chemical reaction capable of being produced outside of living cells.

The first attempts in this direction were made in 1871, by Mme. Manisseim, who found that the cells of dead yeast can still produce, under certain conditions, a decomposition of sugar into alcohol and carbonic acid.

The experiments of Mme. Manassein were, however, far from convincing, and did not clearly establish the non-intervention of cells.

It was Buchner who, in 1897, clearly showed the existence, in the cells of yeast, of an enzyme causing alcoholic fermentation. By submitting yeast to a strong pressure he succeeded in obtaining a very active liquid causing alcoholic fer-

mentation in the absence of any cells. He gave to the enzyme contained in this extract the name of "zymase."

This discovery gives a definite explanation of alcoholic fermentation; it will certainly have a great influence on the study of similar phenomena, and will lead to the discovery of many other enzymes. Once established that alcoholic fermentation is caused by a chemical substance, there is reason to assume that other similar phenomena, such as butyric, viscous, and acetic fermentation, are likewise due to diastases secreted by the bacteria producing these fermentations. The isolation of these diastases seems to be only a matter of time.

Preparation and Properties of the Sap of Yeast.—Buchner advises the following method for the preparation of the extract of yeast.

Take 1 kilogram of yeast, to which add 1 kilogram of quartz sand and 250 grams of infusorial earth. Crush the mass to make it plastic and pasty. This operation requires much care. The crushing must be done with a special machine and lasts about two hours per kilogram of yeast. The crushed mass is then submitted to a pressure of 500 atmospheres. For this purpose a hydraulic press is used and the pressure should be produced slowly and gradually.

In this way about 320 cubic centimetres of liquid are obtained. The mass, from which the sap has been extracted, is ground with 140 cubic centimetres of water and then pressed again very slowly at 500 atmospheres. Thus there is obtained, after 2 hours, 180 cubic centimetres of an extract, which is added to the liquid produced by the first pressure. By this means, 1 kilogram of yeast furnishes 500 cubic centimetres of extract. The liquid is stirred with 4 grams of infusorial earth, filtered through paper, and poured into a cooled receptacle.

The extract obtained by Buchner's process is clear, light yellow, and has a characteristic odor. According to the origin of the yeast which has been used in its preparation, the liquid contains from 7 to 10 per cent of dry substance.

The analysis of the liquid shows the following figures:

Dry substance	6.7
Ash	1.15
Albuminoid substances	3.7

In this analysis the albuminoid substances are calculated according to the richness of the liquid in nitrogen.

The extract of yeast is saturated with carbonic acid and when it is brought to the boiling-point, one observes an abundant liberation of this gas and a strong coagulation which gives the liquid a semi-solid aspect.

This liquid acts differently towards different sugars; lactose and mannite remain intact in presence of the extract as in presence of yeast-cells; saccharose, dextrose, levulose, and maltose, mixed with an equal amount of the yeast extract, at the end of a quarter of an hour disengage carbonic acid, an action which sometimes lasts several days.

The fermenting power of the liquid persists after it has been through a Berkefeld filter; the activity of the liquid is not destroyed by passage through the Chamberland filter, but the fermenting power weakens, however, to a greater degree than in passing through the Berkefeld filter. The fermentation is retarded by these operations: the extract filtered in a Berkefeld filter produced fermentation only at the end of a day.

The active substance contained in the extract is capable of diffusing through dialyser paper; in fact, when a dialyser containing a certain quantity of yeast extract is placed in a 37 per cent solution of saccharose, numerous bubbles of carbonic acd are seen to appear at the surface of the sugar solution.

Yeast extract can be dried at 30°–35° without losing its activity. By drying *in vacuo*, a hard product is obtained presenting the appearance of white of egg. A filtered solution of this product possesses the same properties as yeast extract; in a dry state it keeps several months.

For the preparation of concentrated extract of yeast, one proceeds in the following manner: 500 cubic centimetres of sap are evaporated *in vacuo* at 20° or 25° to a syrupy consistency. The evaporation must be done very rapidly and lasts about half an hour. The syrup obtained is then spread in thin layers on glass plates and replaced *in vacuo*, or else left in the air at a temperature of 30° or 35° so that it can evaporate. After 24 hours the dried substance is scraped from the glass, reduced to powder and completely dried over sulphuric acid. Five hundred grams of yeast extract furnish 70 grams of a very soluble powder which shows great activity.

It is to be observed that the concentrated extract of the juice keeps much better than the diluted extract. The solution of diluted extract is rapidly destroyed in the presence of oxygen, while this same solution, brought to a syrupy consistency, keeps for a very long time, even at a temperature of 30° and in the air.

Buchner succeeded in separating the diastase from the yeast sap by adding to the latter 12 times its volume of absolute alcohol.

The precipitate thus obtained, and dried, is a white powder having the same properties as the extract but possessing a very slight fermenting power.

The zymase enclosed in the cells resists quite high temperatures. A yeast dried in the air at a temperature of 37° and then heated to 100° for 6 hours is still capable of producing alcoholic fermentation in a solution of saccharose. The cells of yeast which could not resist this temperature are killed and no longer reproduce.

If, instead of bringing it to 100°, the yeast is brought to a temperature of 140°–145°, the cells lose all fermenting power. Zymase is then more resistant to the action of heat than the cells which secrete it.

Determination of the Fermenting Power of Zymase.—The fermenting power of yeast is measured by a method

recommended by Meissel for the determination of the alcohol in fermented solutions. This method is based on the measurement of the carbonic acid formed during the fermentation.

Forty cubic centimetres of extract are introduced into a flask of 120 cubic centimetres capacity, and a quantity of powdered saccharose sufficient to obtain a 12 to 15 per cent sugar solution is added. The flask is left alone for a few minutes, after which it is shaken and closed with a rubber stopper through which two tubes are passed. One of them is furnished with a tap on the outside, and descends to the surface of the liquid. The other tube is open and communicates with a washing-bottle containing 2 cubic centimetres of sulphuric acid; the open end is provided with a rubber Bunsen valve.

At the end of the experiment the tap is opened, air is allowed to pass into the apparatus so as to drive out the carbonic acid and the apparatus is placed on the balance. The difference in weight shows the quantity of carbonic acid disengaged.

Method of Decomposition of Sugar by Zymase.—A solution of cane-sugar with arseniate of potassium added and fermented with 450 cubic centimetres of yeast extract at 12° for 40 hours furnished 6.67 gr. of carbonic acid and 7.72 gr. of alcohol, the alcohol which was in the extract at the beginning being deducted.

In fermentation produced by yeast-cells there is theoretically obtained 48.89 parts of carbonic acid and 51.11 parts of alcohol. By comparing these figures with the preceding, Buchner found that the relation between the quantities of carbonic acid and alcohol is practically the same in the two cases, and that the decomposition of sugar by yeast extract is accomplished in the same way as by the cells.

It appears from these data that the fermenting power of yeast extract can be measured by the carbonic acid liberated during its action. These experiments of Buchner show us only the general lines of the course of decomposition of sugar

by zymase. The method he adopted for the determination of alcohol and carbonic acid is far from being exact and from giving information on the degree of purity of the fermentation obtained with zymase. Pasteur demonstrated that the entire amount of sugar which disappears during fermentation is not transformed quite in accordance with the following equation:

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH.$$

There is always a little sugar which is broken up in a different way, and furnishes glycerin and succinic acid. It is very probable that zymase acts quite differently from beer-yeasts, and that the fermentation brought about by its use may give much purer products than those obtained by yeasts.

Still, the zymase isolated from the cells is relatively weak, one hundred cubic centimetres of this extract, representing 200 grams of yeast, produce, by acting on a sugar solution, less carbonic acid than a gram of yeast. Probably there exists in the yeast-cells only a small quantity of zymase which, further, undergoes a change during extraction.

Influence of Physical and Chemical Conditions.—Zymase is very sensitive to the action of the temperature. A 27 per cent solution of sugar, with yeast sap added, produces very different quantities of carbonic acid according to the temperature of fermentation.

Temperature.	Carbonic acid (in grams) formed after			
	6 hours.	21 hours.	24 hours.	40 hours.
12–14°	0.43	1.11	1.14	1.27
22°	0.76	1.01	1.02	1.00

At the beginning of the experiment the temperature of 22° is very favorable to the action of the enzyme; after 6 hours of fermentation there is found, at this temperature, a liberation of 0.76 grams of carbonic acid, while at 12° to 14° there is formed only 0.43 gr. of this gas. But if the operation is prolonged at a temperature of 22°, the action

slackens; this slackening is evidently because of a partial destruction of the diastase.

The course of fermentation is also influenced by the concentration of the sugar solutions.

Saccharose, per cent.	CO_2 (in grams) after		
	16 hours.	24 hours.	40 hours.
16	1.33	1.46	1.48
27	0.70	0.80	0.82
37	0.60	0.72	0.74

In liquids containing 16 per cent of sugar the fermentation is more active than in those which contain 27 per cent, but the enzyme still acts on solutions containing 37 per cent of sugar. In the solutions containing from 40 to 50 per cent of sugar the fermentation is almost completely stopped, but the enzyme is not at all changed, for, by diluting the solution, fermentation can be brought about anew.

The diastatic activity of zymase diminishes in proportion as its action is prolonged; if we compare the quantity of carbonic acid formed by the action of the enzymes during the first 16 hours with the quantity of gas freed during the following 16 hours, we find a rapid decrease of the fermenting power. By calculating this power per 100 cubic centimetres of extract per hour, Buchner found that zymase furnishes, on the average, the following quantities of carbonic acid:

	From 1 to 16 h.	16 to 24 h.	24 to 40 h.	40 to 60 h.
Average of 3 experiments...	0.17 gr.	0.060 gr.	0.020 gr.	0.002 gr.
" " 2 " ...	0.11 "	0.010 "	0.002 "	
" " 2 " ...	0.08 "	0.016 "	0.004 "	

Yeast extract, like all diastatic solutions, produces in hydrogen peroxide a liberation of oxygen.

When hydrocyanic acid has been added to the extract it loses this characteristic property. But if the extract with hydrocyanic acid added is then submitted to prolonged action of the air, the reaction with hydrogen peroxide reappears.

It is, therefore, probable that hydrocyanic acid combines with the diastases, forming a combination which impedes their activity, and that this combination is destroyed by contact with the oxygen which regenerates the enzyme.

An experiment of Buchner relative to the action of hydrocyanic acid and air on zymase may be given:

Four cubic centimetres of extract were mixed with 6 cubic centimetres of 2 per cent hydrocyanic acid; to one half (A) of the mixture were added 3 grams of cane-sugar; the other half (B) was submitted to the action of the air for 1 hour, then 3 grams of saccharose were added. The liquids were placed in U tubes closed at one end. In experiment A not a trace of carbonic acid was produced, while in experiment B the closed part of the tube was filled with this gas at the end of 20 hours, the liberation having begun at the end of 5 hours.

Zymase is influenced by the chemical conditions of the medium.

Neutral salts, like ammonium sulphate, calcium chloride, etc., have a retarding influence on fermentation.

A direct relation is also found to exist between the activity of the yeast extract and the presence of coagulable albumen in the extract.

Yeast extract, kept for some time at a temperature of 35° to 40°, becomes turbid, contains flocculent masses, and loses its activity. On the other hand, it has been observed that when an extract becomes inactive for any reason, it scarcely coagulates at all at a temperature of 40° to 50°. This relation between the presence of coagulable materials and the diastatic activity is explained by Buchner in the following way. According to him, zymase is an albuminoid substance which is coagulated by heat, but this coagulation does not occur when the diastatic substance is transformed.

Buchner also interprets the great instability of zymase by the presence of a peptonizing diastase in the yeast extract; this enzyme would act on zymase rendering it inactive. It

is the simultaneous presence of these two ferments, the one peptonizing and the other producing the decomposition of sugar, which explains the activity of yeast extract at relatively low temperatures.

At 22° peptase acts with more energy than zymase, while at a low temperature the peptonizing action is not complete and zymase can produce greater quantities of alcohol.

The favorable influence of sugars on the preservation of zymase also speaks in favor of the hypothesis of the digestion of zymase by the peptonizing enzyme.

It is known that in concentrated solutions of saccharose the digestion of fibrin by pepsin is retarded. Now, if one volume of yeast extract is mixed with one volume of a 75 per cent solution of saccharose, a solution is obtained which keeps for a week at the ordinary temperature and for 15 days in a refrigerator. Sugar, therefore, has a very favorable action on the preservation of zymase.

The activity of yeast extract varies noticeably according to the variety of yeast used in its preparation. Generally bottom yeasts give a very active extract, while bakery yeasts contain hardly any zymase. A great difference is also observed between extracts of fresh yeasts and those of yeasts which have remained in the air for some time. The latter give less active extracts. However, not all brewery yeasts give an extract of the same activity. The origin of the yeast plays an important part here. The differences observable according to the origin and age of the yeast bring up an argument for the hypothesis explaining the alteration of zymase by the action of another enzyme.

Buchner divided a certain quantity of yeast into two parts A and B. The extract was pressed out from A immediately, while the portion B was left to itself for 3 days at 7°–8° before the sap was expressed. The extract of A possessed a strong activity, while the liquid coming from B gave only a small quantity of carbonic acid. Buchner attributed the diminution of activity in the second case to a peptonization

produced during the 3 days' standing at 7°–8°. The action of pepsin would also, according to him, be the cause of alteration of the diastase contained in compressed yeast.

This opinion is also reinforced by an experiment of Hohn, who quite recently demonstrated the presence of the proteolytic enzyme in the sap of yeasts.

Buchner has, however, shown directly the peptonization of zymase by the enzyme by the following experiment:

He placed on ice 3 tubes containing 3 cubic centimetres of yeast extract. To two of these tubes were added 0.1 gr. of trypsin, the third serving as the control. After 12 hours each tube received 2 grams of pulverized saccharose. The experiments made with trypsin remained absolutely inactive after the addition of cane-sugar; on the other hand, in the control tube a very active fermentation was produced.

The discovery of Buchner has had numerous opponents, who have tried to show that yeast extract always contains either cells or ferments and who have attributed the decomposition of sugar to the intervention of living cells.

Buchner successfully refuted all these objections by means of conclusive experiments.

The existence of zymase is demonstrated by the following facts:

1st. An alcoholic fermentation can be obtained with the solid substance obtained by the precipitation of the sap by alcohol;

2nd. With yeast extract an almost instantaneous fermentation may be obtained, whose intensity diminishes with time. If it is a question of living cells a contrary phenomenon is observed: the fermentation increases in intensity as the cells develop;

3rd. Yeast extract produces a fermentation in the presence of amounts of antiseptics which check the activity of living cells;

4th. By passage through a porcelain filter, active liquids

are obtained without it being possible to discern the presence of organisms.

To sum up, alcoholic fermentation is produced by chemical agents, without, and in the absence of, living cells.

It is true that the material which produces this transformation is elaborated by the vital activity, and that its formation is intimately related to the growth and multiplication of the cells. The fermenting power of the cells is, therefore, reduced to their ability to produce zymase.

Intracellular Fermentation. —Zymase should be found in many other living cells. The fermenting power which can develop in certain fungi should, it seems to us, be attributed to a secretion of zymase which occurs under special conditions.

There is also reason to believe that, in the phenomena of intracellular fermentation, it is zymase again which plays an active part. Pasteur found that fruits plunged in carbon-dioxide gas enter into fermentation and transform sugar into alcohol and carbonic anhydride.

Muntz, by replacing air by nitrogen, found the same phenomenon for leafy plants. Under these conditions alcohol is formed in the leaves of the plant. This phenomenon is explained by vital activity and it is assumed that the changes in the work are due to changes in the conditions of nutrition.

It is easier, we believe, to suppose that the absence of oxygen is, under these conditions, favorable to the secretion of zymase. The fermentation observed in this case is similar to that produced by the action of alcoholic yeast; in both cases the zymase is the cause.

The action of zymase in fruits protected from the air has furnished us with the subject of interesting researches which we are at present pursuing, and which are still far from being completed; but we can even now give some information which will find its complete development in a later work.

The numerous experiments we have made have confirmed, in our opinion, the presence of zymase in fruits, especially cherries and plums, in peas, and in barley.

The first experiments were made with cherries in the following way:

The fresh fruit was washed in a dilute solution of formic aldehyde to destroy the micro-organisms, then carefully wiped and submerged in flasks containing olive oil. At the end of 3 days the cherries were covered with little bubbles of gas and there was then found, above the oil which covered the fruits, a liberation of carbonic acid which increased after the 5th day.

Fermentation continued very slowly for 20 days at a temperature of 10°. After this time the oil was poured off, the cherries, and also the stones, were crushed in a mortar and the juice pressed out by squeezing the mass in a cloth.

The residue was removed from the cloth, treated cold with ether to free it from the oil, then dried *in vacuo*, and reduced to a fine powder, which was soaked in 2 volumes of water with a small amount of ether added. It was left in a corked bottle at 5° for 12 hours, after which the mass was submitted to strong pressure. Thus a liquid was obtained which, filtered through filter-paper, was a viscous, transparent solution, of slightly acid reaction, and giving the reactions of guaiacum and hydrogen peroxide.

The presence of zymase in such a liquid may be found by the following experiments:

To 50 cubic centimetres of the liquid add 7 grams of powdered cane-sugar and leave for 6 hours at 22° in a small flask furnished with a delivery-tube. After 2 hours, the formation of carbonic acid is observed, and after 6 hours a diminution in weight of 3 decigrams.

A parallel experiment is made with the same liquid previously kept at 40° for an hour in a closed flask, then cooled to 22° and left at this temperature for 5 hours, leaving the delivery-tube open. In this second experiment neither liber-

ation of gas nor diminution of weight is found. The alcoholic diastase has therefore been destroyed by heat.

The analysis of the unfermented sugar solution shows that a change has occured in its chemical composition. We have, for example, found in one of our experiments that 3.4 grams of saccharose had been transformed into invert-sugar.

This transformation cannot be attributed to the acidity of the medium, for by heating the active liquid in a closed vessel for 10 minutes at 80° before the addition of sugar, and by then keeping the liquid with sugar added for an hour at 40°, then for 5 hours at 22°, we obtained only 0.15 gr. of invert-sugar instead of 3.4 gr. The active liquid evidently contains zymase and sucrase, and while the zymase is destroyed by the heat, the sucrase is not changed.

The existence of zymase in the juice of cherries has been confirmed by other experiments in which it has been possible to measure the alcohol produced. The following method of procedure was employed:

To 200 cubic centimetres of active juice were added sugar and 2 grams of chloroform. In a parallel experiment, a little chloroform and 2 grams of yeast were added to a 15 per cent solution of sugar. The two liquids, left 5 days at 10°, gave different results.

In the solution containing yeasts fermentation did not take place, while in the juice of the cherries 0.8 gr. of alcohol was found.

The non-existence of yeasts in the fermented solution was confirmed by microscopic analysis as well as by cultivation on plates.

Experiments made with a view to precipitating the diastase by alcohol have not furnished satisfactory results. The active liquid loses its properties in passing through porcelain bougies, and the zymase which we obtained differs in this respect from the enzyme isolated by Buchner.

In the course of our experiments we found, furthermore,

that fresh peas, as well as barley, furnish quite considerable quantities of alcohol by intracellular fermentation. Sugar-peas, left in oil, gave on analysis 2 per cent of alcohol. Barley, previously soaked, dried, and put in oil, gave 1.6 per cent of alcohol.

By treating these seeds by processes similar to those used for cherries, we were able to discover the presence of zymase.

Industrial Applications of Zymase.—Zymase, while very interesting from a theoretical point of view, will perhaps also have in the future numerous industrial applications.

The fermentation produced by the enzyme, without direct intervention of yeasts, presents theoretically a great advantage. In this way much more rapid fermentations can be obtained, and purer and better products.

At the present time distillers and brewers cultivate their own yeasts and seek to adapt them to their kind of work. However, even starting with pure yeasts, good results are not always obtained; often infection takes place and consequently a degeneration of the yeast.

It is to be hoped that in the future the cultivation of yeasts and the subsequent preparation of zymase will be done in special manufactories, where the brewers and distillers will produce preparations of great activity and producing an immediate action.

It is true that in the brewery yeast plays also an important part from the point of view of the elimination of nitrogenous materials, a work which zymase alone could not do. It would be necessary, therefore, in working with enzymes alone, to change completely the technique and invent new processes.

The discovery of zymase is too recent to have caused great industrial changes immediately; however, the first attempts at practical application have already been made by Buchner, who has invented a new process having for its aim

the preparation of a durable yeast intended to replace pressed yeast in bread-making.

This process consists in first drying the yeast at low temperature, then heating to 50°, then to 100°, and then, when the yeast is completely dried, grinding it to powder. It is then ready for the market.

This process has many advantages. The yeast prepared in this way keeps much better than compressed yeast, dead cells being less subject to change than living cells. Moreover, the greater drying of this yeast recommends it from a hygienic point of view, for the micro-organisms which yeast breads always contain, are here destroyed and can no longer affect the dough, even when it is insufficiently sterilized.

Buchner's yeast is called enduring yeast (Dauerhefe); it is used in the bakery in the same way as the ordinary compressed yeasts. According to the author of the patent, experiments have proved that the addition of 5 to 10 per cent of enduring yeast are sufficient to produce a satisfactory dough.

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CHAPTER XXIII.

OXIDASES.

Presence of oxidases in vegetable and animal cells.—General properties.—Laccase.—Tyrosinase.—Influence of the medium.—Action of oxidases on phenols insoluble in water.—The "breaking" of wines: œnoxidase.—Oxidin.—Olease.

SOLUBLE ferments have for a long time been considered as substances acting only as hydrolyzing agents, that is causing the fixation of one or more molecules of water at the same time with a molecular decomposition. Oxidation and dehydration, molecular change without fixation of water, all these chemical phenomena were attributed to the direct action of vital energy without any diastatic intervention.

This entirely erroneous theory has lately been successfully refuted by a series of discoveries accomplished in the domain of biological chemistry by Bertrand, Bourquelot, Hikorokuro Yoshida, Cazeneuve, Martinand, etc., whose works we shall have occasion to examine.

The studies of these scientists have demonstrated the existence of a series of substances having characteristics of true oxidizing agents, and causing oxygen to unite with certain bodies. These substances, secreted by living cells, have received the name of oxidases. These enzymes facilitate the oxidation of certain substances, either by dehydrating them or by enlarging their molecule by the addition of oxygen.

Certain vegetable juices, such as wine, the latex of the lac-tree, the juice of pears, plums, and other fruits, as well as

certain fungi, change when exposed for some time to the air. This phenomenon, which is generally shown by a change of color or, in case of solid bodies, by an increase in temperature, does not occur *in vacuo*. It therefore possesses all the characteristics of oxidation, because the intervention of air, and consequently of oxygen, is indispensable to its production.

The direct cause of this oxidation remained for a long time unknown. It was discovered in 1883 by a Japanese chemist, Hikorokuro Yoshida, who, by making experiments on the oxidation of the latex of the lac-tree, discovered in this phenomenon the intervention of a diastase.

This discovery gave a new impetus to studies upon enzymes; the question was taken up in different laboratories, and discoveries bearing on the most varied subjects were soon numerous and conclusive. There were successively studied vegetable tissues, muscles, organic secretions, and each investigation carried on in this field brought new proofs of the existence of oxidases.

The oxidation of the latex of the lac-tree and its transformation into a black varnish was clearly recognized as a phenomenon of the diastatic order. A transformation presenting some similar characteristics and occurring in the juices of numerous vegetables, such as mushrooms, potatoes, beets, and the rhizomes of *Canna indica*, was attributed by Bourquelot, Lindet, Bertrand, etc., to another oxidizing enzyme.

The decoloration of wine and the deposition of the coloring matter were recognized as being phenomena of the same order, and were attributed to the action of a diastase which certain authors regard as pre-existent in the must, while others consider it as the product of a mould: *Botrytis cinerea*.

In the animal kingdom the experimenters had occasion to make quite as numerous and quite as interesting discoveries.

An oxidizing ferment was found in the saliva as well

as in other secretions—nasal mucus, tears, semen—while the urine, bile, and intestinal secretions were found to be free from any ferment of the kind.

Jacquet, in 1882, made experiments on the oxidation of benzyl alcohol and salicylic aldehyde with pieces of lungs, loins, and muscles of the horse, previously treated with carbolised water, then frozen and reduced to pulp. These fragments of organs caused an oxidation which was no longer produced when they had been cooked in boiling water.

Even at that time Jacquet realized that the oxidation did not come solely from the cells, because the aqueous extract of these tissues, as well as the cells themselves, produced a fixation of oxygen on benzyl alcohol and salicylic aldehyde.

Aleloos and Brauwer confirmed these results by collecting a substance, extracted from a horse's liver, which, precipitated from its aqueous solution by alcohol, oxidized formic aldehyde and transformed it into acid with liberation of carbon dioxide. This substance lost, moreover, all oxidizing action after having been heated to 100°.

Spitzer and Rhomann found this substance in the blood and in the organs of several mammals.

Finally, the phenomena of internal destruction which we have had occasion to observe in yeasts, can be attributed to oxidizing diastatic actions.

We have found that by reducing a certain quantity of compressed yeast to minute fragments and then heaping them up, an increase in temperature is soon manifest, which may reach 40° at the end of 2 hours. This temperature may, for example, be obtained with 2 kilograms of fresh yeast ground and massed in heaps 20 centimetres in height at a temperature of 20°. The same experiment, made *in vacuo*, does not result in the least elevation of temperature. The experiment may be made in the following manner:

In a half-litre flask, provided with three tubes, dispose layers of yeast reduced to small fragments and alternating with layers of pumice-stone, which prevents the yeast from

settling. A thermometer is introduced in the center tube and a current of air established with the other two. As soon as the air enters the flask the temperature rises, and if the tap is closed to the air it is immediately found to decrease.

By allowing the experiment to continue for several hours, it can be renewed several times with the same yeast, for 3 or 4 consecutive days; and it will be observed that at each entry of the air in the bottle the temperature rises.*

When, on the contrary, air is allowed to pass into the bottle for 5 or 6 consecutive hours, the yeast liquefies and is completely exhausted.

By crushing the yeast with pumice-stone in a powerful crusher a paste is obtained which, allowed to stand with cold water and filtered, gives a liquid free from cells and yet offering, from the point of view of oxidation, the same properties as the yeast itself.

The fragments of pumice-stone, impregnated with liquid, put in a mass of glycogen in the air produce there an elevation of temperature of from 4 to 6 degrees. This extract is less active than the yeast itself, but a series of experiments have shown us that it possesses, like yeast, an oxidizing diastatic power.

In view of all these facts it is unquestionable that the phenomena of respiration and oxidation of vegetables and animals must be generally attributed to oxidases.

It is seen, after this short exposition, that the discovery of oxidases was of considerable importance, because it has thrown some light on phenomena still unexplained, or which were explained by erroneous theories.

The study of oxidizing enzymes has also much interest from a chemical point of view for they constitute very sensitive reagents for many organic substances.

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* This experiment is specially interesting from the point of view of gas analysis. We have, in fact, observed that one can in this way distinguish 1 per cent of oxygen when mixed with inactive gases.

General Properties of Oxidases.—Like all diastases, oxidases are extremely unstable bodies. They are destroyed by heat above 60°.

Antiseptics, in general, appear capable of simply retarding the oxidation produced by these agents. This retarding action of antiseptics has not, however, been generally established. We think, on the contrary, that the different diastases belonging to this class are more or less sensitive to the action of antiseptics, and that to this fact must be attributed the negative results of a number of investigations carried on with bodies which certainly contain oxidases.

Alcohol, when sufficiently dilute, does not appear to hinder the action of enzymes of this class. The diastase of latex, laccase, still produces an oxidation in a 50 per cent alcoholic solution.

Soluble oxidizing ferments give a strong blue color to tincture of guaiacum to which hydrogen peroxide is not added, guaiaconic acid being formed with the oxygen absorbed from the air.

Temperature, as well as the reaction of the medium, influences the action of oxidases.

Finally, the greater number of oxidases act especially on bodies of the aromatic series: phenols, amines, and their substitution products.

The oxidation products brought about by diastases are as yet poorly defined. The oxidation of bodies of the aromatic series is produced either by an elimination of the hydrogen or by direct addition of oxygen. This oxidation is never complete. The oxidation of fatty substances is much more energetic; it leads to a complete destruction and to the formation of carbonic acid.

The action of oxidases is not at all specific. Laccase, for example, transforms hydroquinone (diatomic phenol) just as well as pyrogallol (triatomic phenol).

The position of the groups appears, however, to play a

principal part; the para position, for example, seems to influence the reaction favorably.

Among the diastases producing hydrolysis the individuality is more strongly marked; sucrase, for example, can only decompose saccharose and is incapable of acting on very closely related bodies which differ only by their molecular configuration.

The quantity of oxygen absorbed under the action of oxidizing enzymes may serve, in most cases, to measure the intensity of oxidation.

Preparation of Oxidases.—Oxidases are extracted from bodies which contain them by the methods generally used for the extraction of soluble hydrolyzing ferments.

The bodies serving in the preparation are ground and then extracted in the presence of chloroform. The use of the latter body constitutes a danger, however, for it is not known whether this antiseptic, which leaves most hydrolyzing diastases intact, is also without action on all the oxidases. It is, therefore, to be recommended, in the preparation of oxidases, to make two triturations, one with water and chloroform, the other with water containing ether. In certain cases the oxidases will be found in the water and ether, while the chloroform infusion will not contain a trace of active substances.

The infusion is then precipitated by alcohol; the precipitate formed is redissolved and reprecipitated several times to purify it.

The method of extraction with glycerin is also applicable to the preparation of oxidases.

LACCASE.

Laccase is a soluble ferment producing the oxidation of the latex of the lac-tree and transforming it into a very beautiful varnish which the Japanese, the inhabitants of Tong-

king, and the Chinese use for varnishing their furniture. The latex is a clear liquid presenting the appearance and the consistency of honey. It is collected in eastern Asia by making incisions in the bark of certain resinous trees of the order Anacardiaceæ (*Rhus vernicifera*).

The odor of the latex is very slight and somewhat resembles that of butyric acid; it has an acid reaction.

The latex changes with extraordinary rapidity. Exposed to oxygen it turns brown and its surface becomes covered with a resistant film of a beautiful black color and absolutely insoluble in ordinary solvents. *In vacuo*, change does not occur and the latex can be kept for a very long time.

The first data on laccase were obtained by the Japanese chemist Hikorokuro Yoshida. The study of the oxidation of the latex revealed to him the presence of a body which he called uruschic acid ($C_{14}H_{19}O_2$), a body which by oxidation changes into oxyuruschic acid, as is shown by the following equation:

$$\underset{\text{Uruschic acid.}}{2C_{14}H_{19}O_2} + 3O = \underset{\text{Oxyuruschic acid.}}{2C_{14}H_{18}O_3} + H_2O.$$

Bertrand, by diluting the latex in a great quantity of alcohol, discovered in it two products, one which enters into solution, while the other is precipitated.

This precipitate, separated from the liquid, is a sort of gum. It is carefully washed with alcohol, taken up again with distilled water, then precipitated again with 10 volumes of alcohol. Then it can be collected in the form of flakes and dried *in vacuo*. The product obtained by this method resembles ordinary gums and is, like them, transformed by hydration into a mixture of galactose and arabinose.

This body possesses a diastatic power.

The alcoholic solution, after the gummy precipitate has been removed, is quickly distilled *in vacuo*. The residue is

shaken in water, then in ether; the water retains the glucose, mineral salts, etc., and the ether dissolves the resinous extract of the latex. The ether is then decanted, and evaporated in an atmosphere of hydrogen.

The product obtained by this method is laccol; it is an oily liquid, with a high density, not dissolving in water but entirely soluble in alcohol, ether, chloroform, and benzol. The manipulation of this product presents certain dangers: traces of laccol may act in an injurious way on the skin. In the air, it turns a reddish-brown color, becomes somewhat viscous, and is finally converted into resin.

Oxidation, favored by potash and soda, is produced in different stages. The liquid becomes warm, turns green, then inky black, and absorbes a great quantity of oxygen. Laccol gives with ferric chloride and lead acetate reactions much resembling the reactions which the polyatomic phenols produce with the same agents.

In the presence of laccase, the oxidation of laccol is much more pronounced, much more rapid, and finally gives a black insoluble substance which is not obtained in the absence of the enzyme.

Bertrand, at the beginning of his studies, thought that the addition of oxygen was effected by simple chemical affinity, and that laccase then acted on the oxidized bodies in the manner of a hydrating agent.

In the course of his experiments, the French chemist succeeded in determining the true mechanism of oxidation. He observed that the quantity of oxygen absorbed by the laccol in contact with the air increases with the amount of laccase used, which can only be explained by a direct oxidizing action of laccase.

Conclusive proofs were afterwards furnished by Bertrand. He caused a certain quantity of laccase to act on bodies nearly related to laccol, principally on hydroquinone and pyrogallol, and found that in the presence of laccase all the polyatomic phenols absorbed a certain quantity of oxygen,

liberating carbonic acid. In the absence of the enzyme, on the contrary, or even with a diastatic solution heated to 100°, no oxidation occurred. The oxidizing action of laccase is therefore well demonstrated.

Bertrand then discovered a very sensitive reaction for discerning the presence of oxidases in plants. He found that the alcoholic tincture of guaiacum takes, in the presence of laccase, a deep blue color by the action of air alone, while to obtain the same result with hydrolyzing diastases hydrogen peroxide must be used.

The same reaction takes place also when cuttings of organs containing an oxidizing diastase are treated with tincture of guaiacum.

The sensitiveness of this reaction allowed Bertrand to recognize the presence of laccase in a great number of vegetables, and to evolve the hypothesis, which is moreover quite justifiable, that laccase is distributed all through the vegetable kingdom. This diastase has been found in the following list of plants:

Beets, carrots, turnips (roots), dahlias (roots, tubers), potatoes (tubers), asparagus (yellow stem), lucerne, clover, ray grass (stems and leaves), Jerusalem artichokes, apples, pears, chestnuts, gardenias (flowers), lac-tree (latex).

For the extraction of laccase, secreted by the vegetables we have just named, Bertrand made use of a method slightly different from that used for the latex. The juice extracted from the parenchymatous organs of the rhizomes or tubers is precipitated immediately after its extraction. As to the liquid extracted from the green parts of the plant, chloroform is added to it and it is allowed to stand at the ordinary temperature for 24 hours; then a coagulum forms which is separated from the rest of the liquid, and in the filtered liquid the precipitation by alcohol is accomplished. This precipitation is made in the same way as for the latex of the lac-tree.

Bertrand observed that the greatest quantity of laccase is secreted by the organs in the course of development.

Emile Bourquelot and Bertrand sought the presence of laccase in mushrooms, plants which, as we know, cause energetic phenomena of oxidation.

Schönbein, in 1856, had already made the curious observation, which, moreover, remained as a simple observation, that the juice of two mushrooms, *Boletus luridus* and *Agaricus sanguineus*, colored blue tincture of guaiacum without addition of hydrogen peroxide and lost this faculty when it was heated to 100°.

The presence of oxidases was sought for by the French scientists in more than two hundred kinds of cryptogams and the reaction of guaiacum was tried in the various organs of these plants. They examined especially the Basidiomycetes, some Ascomycetes, a Myxomycete,—*Reticularia maxima*, the Polypori, and the Agaricines. *Russula fœtens*, Persoon, was studied particularly on account of the peculiarity which all its parts have of coloring blue with a solution of guaiacum. The investigators cut and crushed 125 grams of *Russula*, then soaked it in water with chloroform added. The filtered liquid took in the course of an hour pale yellow, then dirty red tints; it presented all the characteristics of a solution of laccase.

The oxidizing diastase of these different plants is soluble, at least in part, in alcohol, for when an excess of this reagent is added to the diastatic solution, even when the latter is very active, only a very weak precipitate is obtained.

Below is the table which Bourquelot and Bertrand give as a summing up of their experiments, from which it is seen that the oxidizing enzyme is found in plants destitute of chlorophyll.

In the mushrooms it is distributed throughout the whole reproductive portion; it is found localized in the lamellæ of certain hymenomycetes, or at the base of the stipe.

Genus or sub-genus.	Number of species examined.	Species.	
		With laccase.	Without laccase.
Russula	18	18	0
Lactarius	20	18	2
Psalliota	5	4	1
Boletus	18	10	8
Clitocybe	9	5	4
Marasmius	6	0	6
Hygrophorus	6	0	6
Cortinarius	12	1	11
Inocybe	6	1	5
Amanita	7	2	5

Manner of Action of Laccase.—Laccase acts on a large number of substances. Added to a solution of hydroquinone in an open vessel, it produces comparatively rapid oxidation. The solution takes a deep color and at the end of some time crystalline plates of a green color are formed.

The oxidized liquid has the characteristic odor of quinone, and the reaction may be expressed by the following equation:

$$2C_6H_4\!<\!\begin{matrix}(OH)\\(OH)\end{matrix} + O_2 = 2C_6H_4\!<\!\begin{matrix}O\\O\end{matrix}.$$

Hydroquinone. Quinone.

The diastase also acts on gallic acid, but the product of the reaction has so far been little studied.

By causing a certain quantity of laccase extracted from *Russula* to react on gallic acid, Bourquelot and Bertrand obtained the following results:

Quantities used:

Gallic acid	1 gr.
Water	100 c.c.
Laccase solution	5 c.c.

After one hour:

Oxygen absorbed	15.9 c.c.
Carbonic acid freed	13.9 c.c.

After four hours:

Oxygen absorbed..... 17.6 c.c.
Carbonic acid freed..... 11.1 c.c.

After an hour the ratio $\frac{CO_2}{O}$ equals 0.874 and after four hours 0.630. These quite high ratios show that the oxidizing power of laccase is very great.

By trying the action of laccase on three isomeric polyphenols: on hydroquinone, pyrocatechin, and resorcin, the following figures have been obtained which give an idea of the rapidity of the oxidation:

		Oxygen absorbed.	CO_2 freed.
Hydroquinone (para-diphenol).	After 4 h.....	32.0 c.c.	1.7 c.c.
Pyrocatechin (ortho-diphenol).	" 4 h.....	17.4 "	2.8 "
Resorcin (meta-diphenol)	" 15 h.....	0.6 "	0.0 "

It is seen that the quantity of oxygen absorbed is almost nought for the meta-diphenol, while the para-diphenol oxidizes very strongly.

These facts are reproduced in all Bertrand's experiments; phloroglucin, where all the hydroxyls are in the meta position, refuses, so to speak, all oxidation, while its isomer, pyrogallol, absorbs oxygen with rapidity.

The different polyphenols examined by Bertrand have shown that their oxidizability is in direct proportion to the facility with which they are transformed into quinones.

The whole or a part of the hydroxyls of polyphenols may be replaced by amido radicals (NH_2), without the progress of oxidation being modified.

The paramidophenol:

$$C_6H_4 < \begin{matrix} OH\ (1) \\ NH_2(4) \end{matrix}$$

is easily oxidized; metamidophenol, on the contrary,

$$C_6H_4 < \begin{matrix} OH\ (1) \\ NH_2(3) \end{matrix}$$

takes up only the smallest quantities of oxygen.

According to J. de Rey Pailhade, laccase exists in germinating grains. The enzyme acts on an oxidizable material,

philothion, also contained in these grains. Laccase consequently would play a part in the respiration of vegetable cells. Still, he has not shown at all that the oxidizing enzyme found in the grains is laccase. It is quite possible to believe that it is some other oxidase.

We can now define in a more general way the manner of action of laccase.

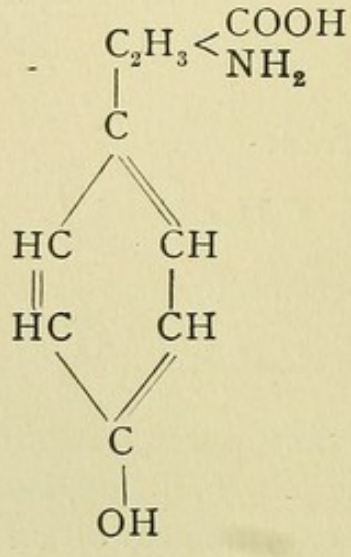
Laccase is a soluble ferment producing the oxidation of bodies of the benzene series, which possess at least two groups, OH or NH_2, when these groups occupy the para- or ortho position.

Here the observations specially applicable to laccase cease. The later works of Bourquelot and Bertrand relate to another diastase, or rather to a mixture of laccase and another enzyme, tyrosinase, whose presence investigators have recognized in a great number of vegetables.

TYROSINASE.

The juices extracted from beets and some other plants, when put in contact with the air, become red in color, then black. This phenomenon is due to the oxidation of the tyrosin which is found in these plants, and which is caused by the action of a diastase.

The rational formula of tyrosin or oxyphenyl-amidopropionic acid is:



It is seen by this formula that tyrosin does not belong wholly to the class of bodies we have recognized as being surely oxidizable by laccase, that is, to the class of polyphenols whose hydroxyls are in the para- or ortho-position.

Tyrosin, in fact, when submitted to the action of laccase does not absorb oxygen.

On the other hand, Bertrand found by various experiments that the oxidation of tyrosin did not occur when the juice extracted from the plants had been heated to 100°. This fact indicated the intervention of a diastase.

The oxidation of tyrosin may be explained by the action of laccase on a product of decomposition of tyrosin, previously elaborated by another non-oxidizing enzyme contained in the liquid.

To test this hypothesis, Bertrand placed in a flask a certain amount of an extract of *Russula* and a few grams of tyrosin. After 24 hours, the whole was heated to 100°; the addition of laccase to the liquid and exposure to the air did not then cause any oxidation.

It was therefore demonstrated by this experiment that laccase is absolutely without action on tyrosin, and that there is not produced in the liquid a previous diastatic action which permits the oxidation by laccase.

In reality, the absorption of oxygen is caused by the mediation of tyrosinase, an enzyme similar to laccase but acting on other bodies.

Tyrosinase has been isolated by Bertrand from many plants. Extracted from potatoes, dahlias, etc., it possesses only a very feeble oxidizing power; but extracted from fungi it oxidizes tyrosin very rapidly.

Bourquelot prepared tyrosinase with *Russula nigricans*, which he crushed in water to which chloroform had been added. The liquid, after filtration, constituted the diastatic solution.

To demonstrate the action of tyrosinase, 5 cubic centimetres of the diastatic solution are put in reaction tubes,

then 5 cubic centimetres of a solution of tyrosin, and the mixture is shaken from time to time to introduce the air. The liquid becomes first red, then black.

By this reaction and that of guaiacum, Bourquelot demonstrated the existence of tyrosinase in the following fungi:

Boletus, Russula, Lactarius, Parillus, Psalliota, Hebeloma, Amanita, Scleroderma.

Certain fungi give no reaction, either with guaiacum or with tyrosin, and it may be concluded from this that they contain no oxidizing enzyme.

Tyrosinase is not so widely distributed in nature as laccase; but it is very often met with, simultaneously with the latter, in the same vegetable juice. Certain diastatic solutions extracted from plants really transform tyrosin as well as polyphenols.

In a series of experiments made at 50°, 60°, and 70° Bertrand observed that the faculty which vegetable juices possess of transforming tyrosin disappear at relatively low temperatures, while the similar properties of laccase still persist in the liquid at higher temperatures. This difference in resistance of the two diastases allows the separation of one from the other in the following manner:

One thousand five hundred grams of fresh *Russula delica* are reduced to a pulp and macerated in an equal weight of cold water to which chloroform has been added. By pressing out the juice of the paste thus obtained, about 2 litres of a mucilaginous liquid is obtained, to which are added 3 litres of 95 per cent alcohol; a precipitate is formed which is separated by filtration. The alcoholic liquid, from which the precipitate has been separated, is reduced, by distillation *in vacuo* at 50°, to about half a litre. The product thus obtained oxidizes hydroquinone and pyrogallol very rapidly, leaving the tyrosin perfectly intact. The precipitation with alcohol and the heating to 50° have destroyed every trace of tyrosinase.

This latter diastase is found in the precipitate which has been separated from the alcoholic liquid. This precipitate is

purified by diluting with water containing chloroform; it is again precipitated with 2 volumes of alcohol and separated from the liquid. The product, after a second similar treatment, is dried at 35°. It reacts with difficulty on polyphenols, but causes a very rapid oxidation of tyrosin.

The individuality of the two enzymes is then well proved.

Influence of the Medium on Oxidation.—Bourquelot, in a very complete work, has shown the relation existing between the composition of the medium and the diastatic activity of the oxidizing ferment of fungi, a ferment composed, as we have seen, of at least two oxidizing enzymes: laccase and tyrosinase.

A solution of anilin, in the presence of an infusion of fungi rich in oxidase, oxidizes very slowly, for only a slight change of color is observed.

Bourquelot was then led to inquire if the alkalinity which anilin gives to the medium did not exercise an unfavorable influence on the oxidizing action of the enzyme, and he studied the oxidation of anilin in the presence of increasing amounts of acetic acid.

The fungus chosen for these experiments was the *Russula delica*, because the filtered juice obtained from its maceration gives a clear aqueous solution, which consequently makes it easy to observe the changes in color. It was soaked by taking 5 parts of water for one part of fungus, and thus was obtained by filtration a liquid but slightly colored yellow.

This extract, with the addition of glacial acetic acid in amounts varying from 1 to 50 parts per thousand, was tested with tincture of guaiacum.

Bourquelot then saw the blue coloration appear with the same intensity and the same speed in all the experiments which he made. Therefore, the reagent is not influenced by great amounts of acetic acid and, under these conditions, the influence of the acid on the action of oxidase may be studied. This action is shown, for different amounts of acid, in the following table:

	Control Test.	Exper. 1.	Exper. 2.	Exper. 3.	Exper. 4.	Exper. 5.	Exper. 6.
Solution of saturated aniline.	5 c.c.	5 c.c.	5 c.c.	5 c.c.	5 c.c.	5 c.c.	5 c.c.
Water.	8 c.c.	8 c.c.	8 c.c.	8 c.c.	8 c.c.	8 c.c.	8 c.c.
Acetic acid, %	0	0.1	0.2	0.4	1	2	5
Diastatic solution.	5 c.c.	5 c.c.	5 c.c.	5 c.c.	5 c.c.	5 c.c.	5 c.c.
Result.	Slight oxidation.	A little stronger oxidation.	Strong oxidation.	Very strong oxidation.	Strong oxidation.	Very slight oxidation.	No oxidation.

Oxidation hardly appears in the control tube, which takes a dirty yellow tint; it increases with extraordinary rapidity in experiments 1, 2, 3, 4, where the solution is immediately colored a dirty yellow, with the formation of a brownish-yellow precipitate, soluble in ether. As to experiments 5 and 6 containing, respectively, 2 and 5 per cent of acetic acid, the first furnishes a slight oxidation, while in the second there is absolutely no oxidation. Therefore, 2 per cent acetic acid is unfavorable to oxidation.

With orthotoluidin and paratoluidin, tried under the same conditions, with the same quantities of acid, the same reactions occur, although giving different colorations.

Orthotoluidin gives a transparent violet color, becoming opaque at the end of several hours.

An aqueous solution of phenol takes a brown tint in the presence of the diastatic solution. This reaction, which takes place very slowly, is wholly prevented by acetic acid and favored by amounts of 0.1 to 0.4 per cent of carbonate of sodium. In general, the oxidation of substances of basic nature is favored by the acidity of the medium, while substances of acid nature oxidize more readily in an alkaline medium. This influence of the medium on the progress of oxidation is very considerable.

Action of Oxidase on Phenols Insoluble in Water.—Bourquelot first occupied himself with the action of oxidase on phenols which are soluble in water. He then turned his attention to the action of oxidase on phenols insoluble in water but soluble in ethyl alcohol or methyl alcohol. He previously assured himself that the alcohols used as solvents and suitably diluted produced no change in the oxidase and that the phenomenon of oxidation occurred there in the same way as in the watery solutions.

Assured of this, Bourquelot made various experiments on phenols soluble in these reagents. The results of his researches is here given:

The action of oxidase was tried on three solutions of different xylenols containing 0.50 gr. of xylenol, 100 grams of absolute alcohol, and 50 cubic centimetres of water.

The orthoxylenol (1, 2, 4), a body melting at 55° to 60°, produced a white precipitate which then became salmon pink and soluble in ether.

Metaxylenol (1, 3, 4), a liquid whose alcoholic solution becomes green under the action of ferric chloride, was immediately oxidized and gave a white precipitate which then became dirty pink, and is soluble in ether.

Paraxylenol, melting at 74° or 75°, was made slightly turbid, and gave a pure rose-colored precipitate insoluble in ether.

Experiments upon the oxidation of thymol were made in a solution having the following composition:

Thymol	0.50 gr.
Water	40 c.c.
Alcohol	10 c.c.
Solution of carbonate of sodium (2%)	5 c.c.
Diastatic solution	50 c.c.

The solution absorbs 19 cubic centimetres of oxygen and a white precipitate is formed in the liquid.

Carvacrol, tried under the same conditions, gives rise

little by little to a turbidity, then to a white precipitate, absorbing 27.5 c.c. of oxygen.

"BROWNING" OF WINES.

The "browning" of wines is a disease characterized by the oxidation of the coloring matter of the wine and the precipitation of this material, while the entire liquid becomes yellow.

In 1895, Gairaud recognized that this phenomenon was due to the action of a diastase, yet without clearly attributing it to an oxidase.

P. Martinand, in a work published later, identified the diastase producing oxidation of the coloring matter of wine with the laccase recently discovered by Bertrand. This identification was entirely erroneous. Indeed, it was later recognized that the oxidase of wine transforms polyphenols, while laccase, though hastening the oxidation of browning wines, is incapable, of itself, of producing it in wines.

Cazeneuve, having added to wines a certain quantity of laccase, observed only an imperceptible alteration, although the diastatic solution used was very active and strongly colored blue the tincture of guaiacum. The diastase causing the oxidation of the coloring matter is then a well-determined enzyme. Cazeneuve gave to it the name of œnoxidase.

Preparation of Œnoxidase.—Cazeneuve observed the phenomenon of oxidation in Beaujolais wine, which was very sensitive to the action of the air; he isolated the diastase from it by the following process:

The wine is submitted to the action of an excess of alcohol, which precipitates a substance having the appearance of a gum. This precipitate is taken up again with distilled water, in which it dissolves, giving an opaline, uncolored solution. The liquid obtained is again precipitated; the new precipitate is dried *in vacuo* and then obtained under the form of a gum impregnated with oxidase.

Secretion of Œnoxidase.—The various reactions which characterize the diastase of the browning of wines are identical with those of all oxidases. Like other soluble ferments they color blue the tincture of guaiacum.

The reaction of guaiacum was tried by Martinand on ripe grapes, and it revealed the presence of oxidases. With the juice of the grape he succeeded in transforming hydroquinone and pyrogallol.

Ripe grapes secrete a greater quantity of œnoxidase than green grapes, and raisins are completely destitute of it. The fermented juices of pears, plums, and apples are richer in œnoxidase than wine.

The secretion of œnoxidase was attributed by Laborde to the presence, at the root of the vine, of the mould *Botrytis cinerea* (" sweet rot ").

Measurement and Properties of Œnoxidase.—The measurement of oxidases presents great difficulties. In fact these enzymes do not always exercise their action with liberation of carbonic acid, which is easy to measure; oxygen is sometimes combined with hydrogen to form water or is directly combined with the oxidizable materials. Under these conditions, the analysis of the products of oxidation becomes very difficult.

Laborde has based a method of measurement on the coloration which a diastatic liquid assumes in the presence of tincture of guaiacum. He takes as unit the coloration which is acquired by 20 cubic centimetres of alcoholic solution of guaiacum with the addition of 0.5 gr. of iodine, and he compares the coloration obtained in the same tincture by oxidase with this unit in a Dubosc colorimeter.

Œnoxidase oxidizes the coloring matter of French and Italian wines; Spanish and Turkish wines undergo its action with more difficulty.

Cazeneuve found that the coloring matter of wine is a phenol-like body. It is transformed by oxidation, likewise

the ethers, alcohols, essences, etc., to which is due the bouquet of the wine.

When wine is shaken with ether, it yields to this reagent a substance having the characteristics of a tannin. After oxidation of the wine, only the smallest quantities of this substance are found, often indeed not a trace is to be discovered.

Now, neutral wine after having been treated with ether undergoes no alteration under the action of oxidases.

The browning of wines, according to this experiment, therefore, appears to be due to the oxidation of a particular substance.

Œnoxidase is weakened in proportion as it acts, for the quantity of oxygen absorbed at the beginning is greater than that absorbed at the end of oxidation.

By introducing air into half a litre of wine, Laborde found that absorption occurred during the first eight days and that at the end of this time a sudden check occurred. The measurement of the gas absorbed gave the following figures for three different wines:

	Oxygen absorbed per litre.	CO_2 set free per litre.	Ratio $\frac{CO_2}{O}$.
1st experiment...	50.8 c.c.	32.4 c.c.	0.63
2nd experiment...	81.0 c.c.	38 c.c.	0.47
3rd experiment...	110.2 c.c.	63.8 c.c.	0.58

This table shows that there is not only oxidation of the coloring matter, but combustion of this matter and production of carbonic acid.

Lagati observed that by the addition of ferrous salts the wines oxidize just as under the action of a diastase. The precipitate which he thus obtained is identical with the precipitate of browned wines; it is not produced if protected from the air, nor in the presence of sulphurous anhydride.

This author attributed oxidation to the action of ferrous salts alone, but this opinion was refuted by Laborde in a con-

clusive manner. Indeed, the greatest amount of iron in the ferrous state contained in a wine can absorb only 10 cubic centimetres of oxygen, while browning wine absorbs as much as 110 cubic centimetres per litre. Therefore, besides the action of the ferrous salt, that of a diastase is also exercised.

Action of Temperature.—According to Cazeneuve, œnoxidase is but slightly sensitive to low temperatures: at 0° and even below oxidation still occurs. At 65° the diastase is not entirely destroyed; for the destruction to be complete, the temperature must be raised to 70°–72°. Martinand fixed the temperature of destruction at 72° for 4 minutes or at 35° for an hour.

Bouffard made interesting experiments on this subject. In 3 tubes, A, B, C, he put in A, an aqueous solution of the enzyme; in B, the same solution, with a certain quantity of 10° alcohol added; in C, a solution of the same diastase with 0.5 gr. of tartaric acid added. The temperature of destruction was determined for each experiment and the following results obtained:

	Temperature of destruction.
Neutral aqueous solution.....	72.5°
Solution + alcohol at 10°.....	60°
Solution + tartaric acid.....	52.5°

It is seen that the presence of alcohol and tartaric acid lower the temperature of destruction. When 20 per cent of alcohol is added, the temperature of destruction is lowered by 5° more. At 60°, according to the same author, the activity lasts for 2 minutes, then decreases and disappears completely at the end of 20 minutes.

Laborde studied the action of temperature in an acid diastatic liquid containing 5 parts of oxidase. He brought these liquids to different temperatures and, after cooling, he determined the quantity of active substance remaining. These experiments gave the following figures:

Temperature.	Oxidase.	
	Active.	Destroyed.
60°	2.30	2.70
65	1.50	3.5
70	0.90	4.1
75	0.75	4.25
80	0.45	4.55
85	0	5

The temperature of destruction of œnoxidase is, therefore, situated between 70° and 75°, but the activity of the enzyme diminishes considerably at 60°.

Action of Chemical Agents.—According to Martinand, an addition of acid retards oxidation and an addition of alkali, on the contrary, is favorable to the combination of oxygen.

However, when the wine already possesses a quite large natural acidity of itself, oxidation occurs, even without the addition of diastase.

Concentrated alcohol decomposes the diastase, but dilute alcohol and wine containing up to 9 per cent leave it absolutely intact.

Tricalcium phosphate and tartaric acid are without action, either accelerating or retarding, on oxidation. Formol (formic aldehyde) is also without action.

Gallic, pyrocatechuic, and salicylic acids hinder oxidation.

Sulphurous acid, in an amount of 0.01 to 0.08 parts per litre, checks the action of œnoxidase and causes its destruction. This fact was demonstrated by Bouffard and Cazeneuve. Cazeneuve, by adding to a certain quantity of wine 0.004 grams of sulphurous acid, precipitated the diastase of this wine by the ordinary methods, washed the precipitate in alcohol, and collected it. After some time the precipitate, redissolved in water, no longer gave coloration with tincture of guaiacum. The sulphurous acid, therefore, acted directly on the diastase.

Œnoxidase is extremely unstable. In the air it is rapidly

destroyed by absorption of oxygen. By exposing a solution of oxidase to the air, Laborde obtained the following figures:

Duration of aeration.	Oxidase	
	Remaining.	Lost.
2 days.....	3.5	2.0
4 "	2.8	2.7
6 "	2.4	3.1
12 "	0.8	4.7

It will be noticed that the destruction, which is rapid at the beginning, slackens very perceptibly after the second day.

Other Oxidations of Wine.—According to Martinand, oxidase plays an important part in the improvement of wines with age. He was able, in fact, to produce artificially, by the addition of oxidase, an ageing of a Burgundy wine.

The wine, with oxidase added, and exposed to the air for 48 hours, took on a yellower color and the perfume of an old wine. The coloration of this wine corresponds to red-violet 354 of the Salleron wine colorimeter before oxidation; after being exposed to the air, in the presence of oxidase, the tint corresponds to the third red 404.

The oxidation of the sugar and tartaric acid of the wine must, according to Martinand, be attributed to a cause of the same kind.

A special action of oxidase has been found in certain American grapes.

These grapes have a disagreeable taste which is lost by aeration; but when they are kept at a temperature of 100°, they retain the special flavor which disappears by the addition of oxidizing diastase.

OXIDIN.

Boutroux, in studying the cause of the coloration of brown bread, discovered in the bran an active substance resembling laccase which he called oxidin.

When the bran is left to soak for a half-hour with its

volume of water, there is obtained by filtration through a porcelain filter a clear light-colored liquid, which, protected from the air, keeps without changing its color.

Put in contact with the air, this liquid takes on a brown tint which deepens with age and finally becomes black. This coloration does not occur in an infusion brought up to 100°.

Boutroux succeeded in separating from the infusion the oxidizing enzyme, and the substance which undergoes oxidation. By adding alcohol to the filtered infusion, the oxidase precipitates without carrying with it the oxidizable substance.

In this way two solutions may be obtained which separately do not change color in the air and which, mixed, grow brown under the influence of oxygen.

To prepare oxidin the bran is soaked in an atmosphere of carbonic acid gas, and filtered under the same conditions. To the filtered liquid is added 3 volumes of 95 per cent alcohol and the precipitate is washed with 82 per cent alcohol on a paper filter. The filter is impregnated with an amorphous substance, which is brown and difficult to detach. The filter is cut in pieces and dried in a vacuum. This paper, impregnated with active substance, acts energetically on the sterilized infusion of bran; it also oxidizes hydroquinone like laccase.

Oxidin is also precipitated by sodium chloride. An infusion of bran saturated with this salt does not color in the air. The enzyme is evidently precipitated, but the precipitate is not active.

Oxidin plays a very important part in the coloration of brown bread, but in this phenomenon amylase also is concerned. The two enzymes contained in the bran act in different ways.

The intervention of oxidase is manifested during the preparation of the dough and in the first stages of panary fermentation.

The oxidizable material of the bran is at this point trans-

formed into coloring matter. The oxidation which oxidin produces is checked by the acidity, and when panary fermentation has become more active, oxidin ceases to act.

The color of the dough becomes still deeper by cooking. In this stage of the work, amylase intervenes. The starch, which is in suspension in the dough before cooking, is partially liquefied through the influence of the amylase of the bran. An intimate mixture is brought about between the parts not yet liquefied. The mass changes in structure and this change causes coloration. The coloration of the flour may be also influenced by a substance found in the germ of the wheat. According to a verbal communication made to me by Albiana, Jr., of Barcelona, who is very expert on questions of milling, the flour obtained with grain deprived of the germ is white and unchangeable, while the presence of the germ, even in relatively small quantity, furnishes a dough which colors very rapidly. It is probable that the germ contains an oxidase or some similar diastase.

OLEASE.

Fresh olives, when in heaps, easily undergo fermentation. One finds an increase in temperature, a liberation of carbonic anhydride with formation of acetic acid and other fatty acids. Talomei showed that this fermentation was caused by an enzyme which he called olease.

This agent is sometimes found in olive oil, in which it causes a very great change. Under its action, the oil becomes rancid, on account of the formation of fatty acids, and discolors on account of the precipitation of the coloring matter. This discoloration is favored by light.

Olease is isolated from the oil by stirring with water. Thus a watery solution of the enzyme is obtained and the oil remains unchanged.

The optimum temperature for the action of olease is below 35°. The acidity of the medium checks the diastatic ac-

tion and it is owing to this circumstance that the change in the oil is often not very extensive, the fatty acid formed hindering the action of the olease.

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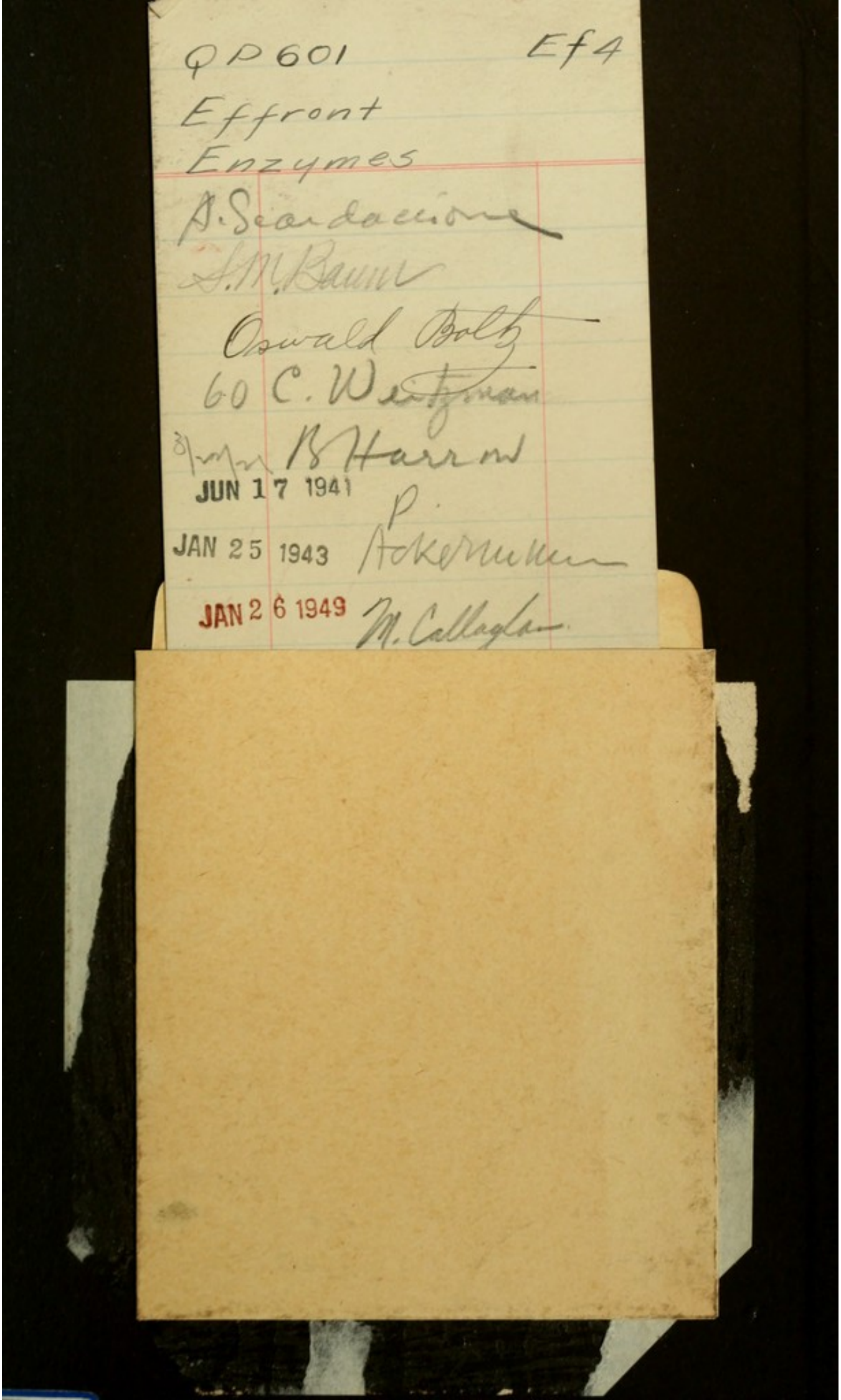
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