ON THE MECHANISM OF ENZYME ACTION.*

II. FURTHER EVIDENCE CONFIRMING THE OBSERVATIONS THAT ETHYLENE INCREASES THE PERMEABILITY OF CELLS AND ACTS AS A PROTECTOR.

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Lavoisier (1) first pointed out that during the decomposition of the sugar molecule in the course of alcoholic fermentation, the molecule not only splits in two parts to form alcohol and carbon dioxide, but he also clearly indicated that alcoholic fermentation involves the phenomena of reduction and oxidation.

Later workers have shown that the phenomenon of fermentation is not a simple process but one involving the presence of a number of intermediate products which may well differ in ease of fermentability. Indeed it seems probable that the generally accepted chemical formula for certain of these intermediate products does not express the true state of the reactive substances but that an intermediate “transportation form” (2) may well be the reactive material. One of us (Nord (3)) has presented elsewhere a critical review of the current theories. Doubts concerning the exact mechanism of enzyme action (4) and the question as to whether intermediate products are acted upon under “biological” or “unphysiological” conditions (5), as well as the question as to whether the so called activators of enzymes really increase enzyme activity or whether they simply protect and prevent


1 This paper on “Chemical Processes in Fermentations” contains a general discussion of the theories of chemical processes involved in fermentation and a more complete discussion of the literature than it is possible to include in the present paper.
enzyme destruction, induced us to undertake the experiments reported in the present paper.

An investigation (6) of the physicochemical behavior of zymase solution, such as was prepared by Lebedew (7), led to the result that (a) it is possible to prepare zymase solutions which are capable of maintaining their biological activity. Data will be presented in this paper to show that these solutions maintained their full activity even after 65 days, although it has been generally assumed (8) that even at 0° the activity is lost within 48 hours. (b) It is not only possible to maintain the full activity of such a solution for practically an indefinite period, but it was shown that the enzyme solution, which behaves as a lyophile colloid, undergoes by means of appropriate peptization, a change of its surface forces, which is indicated by an increased surface tension and by decreased viscosity (6). Hence it was considered justifiable to conclude that the surface energy of the colloids concerned was increased, and this was reflected in the measurable increase of carbon dioxide production which was observed when the enzyme acted upon glucose.

After having reached, in certain cases, a production of 6.6 cc. of carbon dioxide per minute, the observation was made that this high fermentation activity was only maintained for a short time, the activity gradually falling to the average speed usually observed in enzymatic fermentation. This decrease could not have been due to an appreciable lack of fermentable sugar. It appears to be justifiable to interpret this observation as a further confirmation of an increased surface of the enzyme concerned. The increased surface is apparently much more sensitive toward the products of yeast metabolism. It might be assumed, therefore, that there are always enzymes present in a certain concentration, which are potentially capable of acting. However, since the reactivity of the enzyme is dependent upon its surface activity, it undergoes, immediately after the initial reaction, alterations which relatively decrease the velocity of the reaction, independently of the concentration of the reactant. In the course of the reaction, the proportion of damaged enzyme to active enzyme may be greatly increased. This conclusion indicates that there is a basis for the assumption that enzymes change their colloidal behavior even when bound to cells and under conditions where they may exert their activity.
Since we have, as yet, not been able to find a means whereby we can increase the surface of the acting enzymes within the living cell, we investigated the possibility of finding some method by which the original activity of the enzymes could be maintained, or by which the decrease of original activity could be delayed. Several considerations led to the idea that, independently of its reducibility, ethylene might be electrically charged on enzyme surfaces, in this way forming an adsorbed film, and since its opposed charges

\[ \text{CH}_4^+ \quad \text{CH}_4^- \]

are neutralized (or in any event altered) in the condition of adsorption, it not only would not hinder the formation of the enzyme-substrate complex, but might act as a protector of the surface upon which it was adsorbed. Our experiments seem to have justified this viewpoint, since we not only could protect the increased surfaces which, as mentioned above, were produced by the appropriate peptization of the enzyme solution, but also found it possible to show in a striking manner that a protecting film could be formed even on enzyme surfaces which were within the cell.

The application of ethylene disclosed another very remarkable quality of this gas. It was possible to show through the action of ethylene on living single cells or cells in tissues, as for instance, tobacco leaves, that it increases the permeability when the cell systems are exposed to its influence. Since no definite data in this respect were available in the literature, it was necessary to be sure of this effect on permeability, since, for example, there has never been an agreement as to whether ether, which is also a very effective narcotic, causes an increase or a decrease of permeability, even though it inhibits cell division (9).

Through the fact that the permeability was increased, it was further possible to show that a yeast suspension, after having been exposed for a longer time to a slow current of ethylene gas, shows in the beginning a strongly increased carbon dioxide production, followed in the main course of the sugar fermentation by a decreased production as compared to the controls. If, on the
other hand, an overcharging of the surface of the enzyme was avoided, the expected magnitude of the initial increased permeability was smaller, or even not noticeable, thus enabling the film to act as a protector, even during the fermentation of the first unit of glucose.

This observation led, on the one hand, to the connecting link between the alleged mechanism of enzymatic activity, and on the other hand, to the assumption, supported by this observation, that the physiological manner of acting of so called intermediate products in the biological form is fundamentally different from that in the unphysiological condition. It was Pfeiffer (10) who felt prompted to reemphasize recently that the association between enzymes and the substrate belongs in the group of true molecular combinations. If this be true, especially so far as zymases and fermentable sugars are concerned, it would be impossible to interpret the observations noted above, as well as those which are to follow.

Accordingly it was thought necessary to go a step further. In view of the well known fact that pyruvic acid exerts, probably because of its high degree of dissociation, an injurious effect on yeast cells, suspensions of yeast cells were allowed to act upon pyruvic acid solutions of different concentrations. It was not only possible to show that a 10 per cent suspension of yeast in a 1 per cent solution of pyruvic acid was capable of evolving practically the theoretical quantity of carbon dioxide within 26 hours, but the subsequent fermentation of 1 gm. of glucose in the original mashes showed, as expected, much less damaged enzyme in the case of the presence of a protecting film of ethylene than in its absence. It would be hard to understand how, in spite of the presence of a protecting adsorption film, a true molecular combination between enzyme and substrate could be assumed in our experiments, and it is much more plausible that these reactions also belong, in accordance with Gortner (11), to that group of reactions which may be regarded as governed by purely physical forces.

Mention has already been made of the effect of ethylene on single cells, and we believe that we have demonstrated that ethylene is not only capable of being charged as a protector on sensitive biological surfaces, but that due to adsorption it is able
to increase the permeability of cells. These observations were accordingly extended to cells in tissues, where, if the above observations were correct, the effect should be demonstrated perhaps even more strikingly. For this purpose we have investigated the effect on tobacco leaves, which, as we know from the investigations of Loew (12), contain a comparatively large quantity of catalase. The selected tobacco leaves, which were exposed to ethylene and then acted upon by a 6 per cent solution of hydrogen peroxide, evolved in fact a quantity of oxygen 15 to 20 per cent greater than did the leaves which were not exposed to ethylene.

These observations indicate furthermore that there occurs in the well known effect of the artificial ripening of fruits and vegetables (13) nothing less than an increase of the permeability of the cells, thus promoting the formation of the reactant-enzyme complex, and in this way advancing the hydrolysis of starch, from which are derived the sugar and other transformation products, and simultaneously, through the building up of the adsorbed protector film, enabling the enzymes to act for a prolonged time under conditions closer to ideal cases. In accordance with our considerations, there is no escaping the conclusion that the idiom "activation" is in this connection absolutely meaningless in its present use (14), especially since the phase of zymogens—if their existence according to our present uncertain knowledge may be considered as justified—might be regarded in our experiments as undoubtedly already having been passed.

**EXPERIMENTAL.**

**Apparatus and Material.**

The apparatus used for the fermentation experiments is the same as that described in a previous paper (6). The apparatus used in the catalase experiments consisted of a 150 cc., large mouthed, extraction flask, fitted with a 2-hole rubber stopper, a dropping funnel entering through one hole, and through the other a tube leading to a 3-way stop-cock, of which the two branches entered two burettes inverted in a dish of water. By applying suction to the burette tips, they can be easily refilled, and the
use of the 3-way stop-cock permits the taking of consecutive readings without interference. The flask in which the hydrogen peroxide was acted upon by the catalase was kept in a constant temperature bath.

The ethylene used was a commercial product compressed in tanks, prepared for anesthetic purposes, and had a purity of 99.15 per cent.

The dextrose used was a chemically pure product. The yeasts were: (a) pure top yeast furnished by the Fleischmann Company, especially purified, and furnished at regular intervals, which insured its fresh condition for use in experiments; (b) bottom yeast supplied by the Minneapolis Brewing Company at regular intervals (after having been carefully washed and compressed).

Yeast juice was prepared from (b) in the following manner: The washed and carefully pressed yeast was dried under a vacuum of 34 mm. at 30° for 24 hours, in layers not exceeding 4 to 5 mm. in thickness. The dried yeast was then milled to grains of the average size of 1 to 2 mm. A 100 gm. portion of this yeast was introduced into 300 cc. of tap water, which had been previously saturated at 30° with carbon dioxide. The suspension was macerated in a mortar with a pestle until it was of a fairly uniform consistency, and was then submitted to maceration for 6 hours at 25-26°. In order to obtain a clear solution of the zymase which had partially come out of the cells, the juice was centrifuged for half an hour at approximately 4000 R.P.M. The solution was then carefully removed with a pipette from the sediment and submitted to a second centrifuging for the same length of time. The final juice had a tea color and was water-clear. Examination under the microscope indicated that it was absolutely free of cell particles, and it showed no spontaneous fermentation.

Zymin was also prepared from (b). 1000 gm. of yeast were suspended in 1000 cc. of water and most of the proteins of the plasma membrane were precipitated in an irreversible manner by adding, within 20 minutes, 6000 cc. of acetone. The product was allowed to stand 15 minutes in the acetone, then filtered, washed carefully with ether, and dried at 35° for 24 hours.

The pyruvic acid used was purified by distilling it in vacuo (at 8 mm. and 55°) in an atmosphere of carbon dioxide.
The hydrogen peroxide used was Merck's superoxol, containing about 30 per cent \( \text{H}_2\text{O}_2 \).

The tobacco leaves used were of the Blue Pryor strain.\(^2\)

**Method.**

The solution or suspension used was always made up with a carbon dioxide-saturated tap water of an average initial pH of 6.0, containing 50 parts per million of calcium. The saturation and other preparatory operations were always carried out at the thermostat temperatures in order to avoid any influences due to contracting or expanding volumes.

The parallel experiment was conducted by bubbling ethylene through an opening of a 2 mm. tube, at a speed of 70 bubbles per minute, at a pressure of a 100 mm. water column for the time noted in each experiment.

The fermentations were carried out by the addition of 1 gm. (or amounts as stated in the experiments) of glucose. This gm. would furnish, if completely fermented, 282.4 cc. of carbon dioxide at 760 mm. and 30.5°. If pyruvic acid were completely fermented, 0.1 gm. would give 28.25 cc. of carbon dioxide under the same conditions. In most cases 20 cc. of the juice or of the yeast suspension, of which the type and concentration are noted in the discussion of the experiments, were used.

In all the fermentation experiments there was introduced quantitatively a second, and in some cases, a third gm. of glucose. These additions were made after the rates of the two fermenting mashés were approaching an identical magnitude.

During the main course of the fermentation, all readings were made at 1 minute intervals. In drawing the curves, however, for technical reasons, only greater intervals were plotted.

**DISCUSSION.**

1. **Experiments on Yeast Juice.**

Ethylene was bubbled through 20 cc. of freshly prepared juice for 30 minutes, and this solution was preserved overnight at

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\(^2\) The authors are indebted to Dr. James Johnson of the University of Wisconsin, for courtesies extended to them in supplying fresh tobacco leaves as required, and to Mr. R. B. Zimmermann, superintendent of the Minneapolis Brewing Company, for bottom yeast furnished.
Fig. 1. (Experiment 1.) Fermentation of glucose with 20 cc. of yeast juice and with 20 cc. of yeast juice treated with ethylene. Curves (a) initial 1 gm. of glucose, curves (b) a second 1 gm. of glucose, curves (c) a third 1 gm. of glucose.
The control was prepared in a similar manner except for the gas treatment. 1 gm. of glucose was added to each solution just before commencing the measurements. The course of the fermentation is shown in curves (a), Fig. 1. After a period of 157 minutes a second gm. of glucose was added, and the course of the reaction, illustrated in curves (b) and (c), Fig. 1, shows the course of fermentation after the third gm. of sugar had been added. The difference in the rate of carbon dioxide produced is practically all in favor of the enzyme, treated with ethylene, especially during the course of the fermentation of the second and third gm. of glucose.

The juice used in the preceding experiment had been divided into two parts, and the results shown in Fig. 2 are those obtained 65 days after the preparation had been made.

Three different factors enter into this experiment. First, the once frozen juice was kept permanently at $-10^\circ$ in order to avoid possible influences upon the physicochemical conditions of the enzymes due to repeated thawing. Second, in view of the well known antiseptic properties of toluene (15), 1 per cent of this was added to the mash ready for fermentation. Third, ethylene was bubbled through the treated enzyme solution for only 10 minutes.

It will be noted in comparing Figs. 1 and 2, that the rate of the fermentation shows not only no decrease when viewed from the standpoint that only 15 cc. of juice and 0.75 gm. of glucose were used in this experiment, but even indicates that the absolute rate of carbon dioxide production would have been increased if the data of observations had been calculated on the magnitude of the preceding experiments (20 cc. of juice + 1 gm. of glucose). Since it is generally assumed that the loss of the activity of a zymase solution is mainly due to the action of proteases on zymase, and that the temperature and time factors are very uncertain, the results of this experiment show conclusively that it was possible to reduce the protease action so that its influence was not detectable. In spite of the fact that our solution was absolutely free of cells, there was no assurance that it was absolutely free of proteases. The remarkable maintenance of the original activity appears therefore to be due to a very significant equalization of the action of proteases by the increased surface activity of lyophilic...
FIG. 2. The fermentation of glucose by 15 cc. of yeast juice preparations identical with those used in Experiment 1 (Fig. 1) after the juice had stood for 65 days at $-10^\circ$. Curves (a) initial 0.75 gm. of glucose, curves (b) a second 0.75 gm. of glucose.
colloidal zymases. Therefore the conclusion might be drawn that in the complete absence of proteases, zymase solutions could be obtained which show not only practically indefinite keeping qualities, but are capable, in the beginning and within certain ranges, of showing a pronounced increase of their capacity to act on fermentable substrates.

The inspection of the curves indicates clearly the influence of a film adsorbed on the surface of the enzymes. This makes improbable two hypotheses; first, that in the case of fermentation, the enzyme-substrate complex may be considered as a true chemical combination, and second, that a film formed on the surface of a colloidal enzyme either by a gas, liquid, or solid compound, because of its reducibility, "activates" the processes. The main factor governing the effect is rather a physical one and depends on the capacity of the substances to be adsorbed, and in this way to interact between enzyme, substrate, or transformation products.

2. Experiments with Living Yeasts.

The preceding experiments led to the idea of applying our experiences in protecting enzyme surfaces to those in living yeast cells.

(a) Bottom Yeast.—The bottom yeast which served as a source for the zymase solutions in the above experiments was now used.

The purpose of these experiments was not only to show the possibility of the formation of a film on the surface of enzymes, bound on living cells, but to prove that it is a film, the formation of which is relatively independent of the period during which ethylene is bubbled through the preparation.

Three parallel experiments are shown in Figs. 3, 4, and 5, in each of which 20 cc. of a 10 per cent suspension acting upon each gm. of glucose were used. In the first experiment (Fig. 3) the enzyme surface was charged by bubbling ethylene for 30 minutes and kept at +5° to +8° for 12 hours. For the second (Fig. 4) the charging with ethylene was terminated after 10 minutes and the fermentation started subsequently. For the last experiment (Fig. 5) the ethylene gas was bubbled through the suspension for only 1 minute, followed by the immediate addition of sugar.
Fig. 3. The fermentation of glucose by a 10 per cent bottom yeast suspension and with a similar suspension treated for 30 minutes with ethylene. Curves (a) initial 1 gm. of glucose, curves (b) a second 1 gm. of glucose.
FIG. 4. The fermentation of glucose with a 10 per cent bottom yeast suspension and with a similar suspension treated for 10 minutes with ethylene. Curves (a) initial 1 gm. of glucose, curves (b) a second 1 gm. of glucose, curves (c) a third 1 gm. of glucose.
FIG. 5. The fermentation of glucose with a 10 per cent bottom yeast suspension and with a similar suspension treated for 1 minute with ethylene.
In all three experiments it is clearly demonstrated that the permeability of the treated yeast used was greatly increased. The decrease of the activity of the yeast enzymes, which is mainly due to the increasing concentration of the alcohol, was delayed not only during the fermentation of the first gm. of sugar, but more so during the course of the fermentation of the second and third gm. additions. In the case of the yeast treated with ethylene, this decrease is very much less than in the controls.

Comparison of the curves in Fig. 3 with those in Figs. 4 and 5 shows a lower fermentation rate, especially in the beginning, probably due to the fact that the enzyme surface was overcharged and the coating, according to Ramsden (16), passes gradually back into solution.

The fermentations represented by Fig. 5 picture the proceedings by a cumulative curve which indicates clearly the effect of the protecting surface against the transformation products, proving simultaneously that the ideal ratio of 1 between active and inactive enzymes falls much faster in those experiments where the enzyme is not protected.

(b) Top Yeast.—Similar experiments were carried out with a top yeast, whereby the general experimental conditions remained unchanged, except that the suspension used (Fig. 6) was in contact with ethylene 1 minute, and in the other case (Fig. 7) for 10 minutes.

However, in the case of fermentations represented by Fig. 7, a significant change was made in the procedure preceding the subsequent glucose additions. In order to make sure that chemical properties of the ethylene that may be present and dissolved in the mash were not responsible for the increasing delay of zymase inactivation, the liquid of the fermentation mash was removed by washing the yeast in the centrifuge after each finished fermentation, the washing being repeated three times with 70 to 80 cc. of water and the fermentation then being resumed on the addition of glucose to this washed suspension of original yeast.

The course of the reaction represented in both Figs. 6 and 7 confirms, without restrictions, the conclusions drawn from the fermentations carried out with bottom yeast.

(c) Dough Fermentations with Top Yeast.—Inasmuch as an increase of permeability in enzyme-containing cells is supposed
FIG. 7. The fermentation of glucose with a 10 per cent top yeast suspension and with a similar suspension treated for 10 minutes with ethylene. Curves (a) initial 1 gm. of glucose, curves (b) the washed yeast cells from the experiment represented by curves (a) and a second 1 gm. of glucose, curves (c) the washed yeast cells from the experiment represented by curves (b) and a third 1 gm. of glucose.
to favor the formation of all enzyme-reactant complexes, it was thought advisable to carry out experiments in which a combination of enzymatic reactions takes place. A dough fermentation was considered the most suitable, since here a starch-gluten mass is acted upon by diastase on the one hand, and the transformation products of the diastatic activity as well as added sugar are acted upon by the invertase and zymases present on the other hand. In our experiments it was not possible to demonstrate that there was an augmentation of the diastase-starch reaction in the presence of ethylene. Such a possible influence of the ethylene could only take place in so far as free ethylene remained dissolved in the water in which the yeast was suspended.

A series of baking experiments was carried out. Each batch of dough was made according to the following formula: 350 gm. of wheat flour, 10.5 gm. of sucrose, 5.25 gm. of salt, 10.5 gm. (or less) of yeast suspended in 50 cc. of water, 162.5 cc. of water, 9 gm. of compounded lard. This was fermented for 3 hours and 50 minutes. The procedure used is that which is considered as standard in baking tests.

The baking experiments carried out with 60 loaves demonstrated that there was a remarkable increase in the volume of those loaves which were prepared with the same quantity of yeast in which the cell permeability was increased by ethylene treatment. In good agreement with this fact, it was noted that the same loaf volume as the control was obtained by using 10 to 15 per cent less yeast, when the yeast had been previously treated with ethylene so that its permeability had been increased, or its enzymes were protected.

3. Experiments with Tobacco Leaf Catalase.

It was considered desirable to extend the investigations regarding the possible increase of permeability to cells in tissues. Tobacco leaves were accordingly chosen, since they contain, as already noted, a comparatively large quantity of catalase.

The experiments were carried out by shredding representative leaves of the Blue Pryor strain, and placing 5 gm. samples with 60 cc. of water in the reaction flask. The pulp, in curves (a)

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* These tests were carried out by Mr. C. C. Fifield.
The decomposition of $H_2O_2$ by catalase of tobacco leaf pulp and by tobacco leaf pulp treated with ethylene. The curves on the left show the cc. of $O_2$ evolved during the first 30 minutes of the reaction, those on the right the entire course of the reaction.
FIG. 9. The bottom curves (a) represent the fermentation of a 1 per cent pyruvic acid solution with a 10 per cent suspension of top yeast and with a similar suspension of top yeast treated with ethylene. The top curves (b) represent the fermentation of 1 gm. of glucose with the washed residual yeasts centrifuged after the completion of the above experiment.
and (b), Fig. 8, was treated by bubbling ethylene gas through it for a few minutes in the usual manner.

It is easy to see from the curves that the treated leaves have a higher production of oxygen than the controls, and also that there is an increased oxygen production due to the increased permeability from the start. This may be seen by noting the curves on the left, which picture the course of the reaction during the period of the first 30 minutes. It can be easily recognized that an increased permeability occurred from the start, as the differences in the oxygen evolution during the entire course of the reaction are overwhelmingly in favor of the treated pulp.

4. Experiments with Living Yeast on Pyruvic Acid.

The experiments with pyruvic acid were carried out from two view-points. First, in spite of the fact that the earlier contradictory opinions (17) regarding the fermentability of pyruvic acid have been cleared up, there is, judging from a search of the recent literature (18), no agreement concerning the rate of fermentation of this important compound. Second, it was regarded as necessary to demonstrate that the yeast can be protected also against the aforementioned injurious effect of the unphysiological acid; i.e., that the capacity of pyruvic acid to inactivate yeast can be decreased even during relatively long contact of the yeast with the acid.

It is believed that these view-points are met by presenting the following experiments.

In the experiment shown in Fig. 9, 10 cc. of a 20 per cent top yeast suspension were treated by bubbling ethylene through it for 20 minutes. 10 cc. of a 2 per cent pyruvic acid solution were then added, reducing in this way the yeast concentration to 10 per cent and that of the acid to 1 per cent. The bottom curves (a) show the fermentation of pyruvic acid, indicating that not only the permeability of the treated yeast was remarkably increased, but that in 26 hours about 95 per cent of the pyruvic acid had been fermented. The yeast was then washed three times with 70 to 80 cc. of tap water, separated by centrifuging, then suspended in carbon dioxide-saturated tap water, and used in the fermentation of 1 gm. of glucose (top curves (b)). The
significance of the protection obtained by the use of ethylene is evident.

In the experiment represented by Fig. 10, the conditions were somewhat altered in that 0.5 per cent pyruvic acid was fermented in a 5 per cent yeast suspension (curves (a)). The period of ethylene treatment was 10 minutes.

The pyruvic acid fermentation was followed, without washing the yeast, by three subsequent sugar fermentations, the course of which may be easily recognized by the steeper curves (b) and (c) (representing 0.5 gm. of glucose) and curves (d) (representing 1 gm. of glucose).

In order to ascertain the effect of a more unfavorable relation between yeast available for fermentation and acid to be fermented, 1 per cent pyruvic acid was fermented by a suspension containing 2.5 per cent yeast, which was previously treated with ethylene for 20 minutes. After the completion of the pyruvic acid fermentation (curves (a), Fig. 11), the yeast was carefully washed three times, each time with 70 to 80 cc. of tap water, again suspended in carbon dioxide-saturated water, and allowed to act upon 1 gm. of glucose (curves (b)). Compared with Fig. 9, the inspection of the curves conveys evidence to the effect of a smaller number of active cells during the course of the sugar fermentation, which is, moreover, more vigorous in the experiment carried out with protected enzymes.

The ability to resist the harmful effects of pyruvic acid and the possible high content of cozymase shown by the top yeast used are also evident by the inspection of the curves (a) and (b) of Fig. 12, where experiments with bottom yeast are recorded.

The experiments shown in Fig. 12 were carried out by using suspensions containing 10 per cent bottom yeast acting on 1 per cent pyruvic acid. The upper curves (a) indicate the course of

Fig. 10. Curves (a) represent the fermentation of a 0.5 per cent pyruvic acid solution with a 5 per cent suspension of top yeast and with a similar suspension of yeast treated with ethylene, curves (b) a subsequent fermentation of 0.5 gm. of glucose added following the completion of the pyruvic acid fermentation, curves (c) a second 0.5 gm. of glucose following the experiment represented by curves (b), and curves (d) a final fermentation of 1 gm. of glucose added following the experiment represented by curves (c).
the pyruvic acid fermentation and show that it yields not more than one-fourth to one-fifth of the theoretical amount of carbon dioxide. After a careful washing of the used yeast, its fermentability was tried out on glucose, but curves (b) indicate that the yeast was practically killed.

Fig. 11. Curves (a) represent the fermentation of a 1 per cent pyruvic acid solution with a 2.5 per cent suspension of top yeast and with a similar suspension of yeast which had been treated with ethylene, curves (b) the fermentation of 1 gm. of glucose with the washed yeast residues from the experiment represented by curves (a).

Fig. 12. Curves (a) represent the fermentation of a 1 per cent solution of pyruvic acid with a 10 per cent suspension of bottom yeast and with a similar suspension of bottom yeast treated with ethylene, curves (b) the fermentation of 1 gm. of glucose with the washed yeast residues from the experiment represented by curves (a).
FIG. 13. The fermentation of glucose with a zymin preparation and with a zymin preparation treated with ethylene. Curves (a) the fermentation of an initial 1 gm. of glucose, and curves (b) the fermentation of a second 1 gm. of glucose by the same zymin preparation.
5. Experiments with Zymin.

Fig. 13 represents the course of an experiment carried out with zymin, the preparation of which is indicated on p. 32. The fermenting mash consisted of 1 gm. of zymin in 20 cc. of tap water, to which was added 1 gm. of glucose, after exposure to ethylene for 10 minutes. The inspection of the curves discloses a good agreement with previous experiments; however, the extraordinarily long induction time in the first pair of curves (a) is completely overshadowed by a fermentation rate, in the course of the fermentation of the subsequent gm. of glucose, only slightly lower than that produced by a mash made up with the same, but living yeast.

In view of the fact that Harden (19) regards zymin as "quite incapable of growth or reproduction, but produces a very considerable amount of alcoholic fermentation," this observation might be regarded to be in indirect agreement with the conclusion of Giaja (20), who regards it as not yet proved that the fermentation activity of living yeast cells has to be ascribed exclusively to the zymases present.

6. Fermentation of Calcium Hexose Diphosphate by Living Yeast.

Up to date it has been considered as proved that zymophosphates are unfermentable by living yeasts (21), and even recently this observation was thought to be confirmed.

The surprising observations on our top yeast suggested that we should again take up this question, especially since the unfermentability of these products by living yeasts has given rise, during the last few years, to numerous speculations. The I. G. Farbenindustrie A. G. in Elberfeld kindly put at our disposal a purified, insoluble calcium salt, which according to a communication of Dr. Hoerlein, contained only 13.64 per cent calcium and 11.08 per cent phosphorus, instead of 18.41 per cent calcium and 14.28 per cent phosphorus. From this salt the easily soluble magnesium salt was prepared, which is mentioned for the first time by Young (22). After the fermentation for 44 hours of a 2 gm. suspension of the calcium salt in 20 cc. of a 20 per cent top yeast suspension, there was obtained a quantity of carbon dioxide which was practically equivalent to the amount of sugar which,
in accordance with the analysis, could be considered as uncombined.

The fermentation of the magnesium salt under the same conditions yielded, after 24 hours, practically no carbon dioxide.

CONCLUSIONS.

1. Zymase solutions were prepared which not only maintained their full activity for 65 days, but showed an increased activity.
2. Ethylene produces the effect of increasing the permeability of single cells and cells in tissues.
3. Enzymes outside of or bound to cells can be charged with an adsorbed film which may act as a protector against damaging transformation products.

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