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The effect of the chemical and physical environment upon the fermentative activity of zymin

Homer E. Stavely
Iowa State College

NOTE TO USERS

THE EFFECT OF THE CHEMICAL AND PHYSICAL ENVIRONMENT UPON THE FERMENTATIVE ACTIVITY OF ZYMIN

By

Homer E. Stavely

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Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

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TABLE OF CONTENTS

THE EFFECT OF THE PHYSICAL AND CHEMICAL ENVIRONMENT UPON THE FERMENTATIVE ACTIVITY OF ZYMIN

INTRODUCTION

Historical

The problem of alcoholic fermentation is one of the oldest in the realm of natural science. In the earliest chemical literature reference to it may be found, and many of the ideas of the alchemists were based upon the mysterious changes taking place in a fermenting liquid.

The first definite theory concerning the nature of the process was that of Willis (1659), later elaborated by Stahl (1697). They supposed that the particles of a fermenting substance were in violent motion, induced by an "aqueous liquid", whereby the essential constituents of the substance were loosened, and new particles (alcohol and carbon dioxide) formed.

The modern aspect of the fermentation problem dates from the classical researches of Lavoisier (1784-1789), who first established the fact that organic compounds consisted of carbon, hydrogen and oxygen. He made systematic analyses of the substances concerned in alcoholic fermentation and drew up a

balance sheet between the reactants and the products. His data are remarkably accurate when one takes into consideration the status of science at that early period.

Although yeast cells had been observed in fermenting materials as early as 1680 by Leeuwenhoek, they were regarded merely as a chemical compound. Later three observers, Cagniard-Latour (1838), Schwann (1837), and Kutzing (1837) discovered almost simultaneously that yeast is a living organism, and these workers had the audacity to announce that fermentation could no longer be regarded as a purely chemical process, but involved the action of living forms. This pronouncement was received with scorn by practically all reputable chemists. Berzelius (1839) went so far as to say that yeast was no more to be regarded as an organism than was a precipitate of alumina. The stage was now set for the famous controversy between Liebig and Pasteur.

Liebig (1839) contended that fermentation was brought about by a purely chemical body which he called the ferment, formed as the result of a change caused by the access of air to plant juices containing sugar. The ferment was supposed to contain all of the nitrogen of the nitrogenous constituents of the juice, and was remarkably susceptible to change. The fermentation of sugar was produced as a consequence of the transformation the ferment was itself undergoing. Liebig's conception of fermentation was based upon a number of chemical analogies. For example, platinum is itself incapable of dissolving

in nitric acid, but silver possesses this power. When platinum is alloyed with silver the whole mass dissolved, the ability to become dissolved possessed by the silver being transferred to the platinum.

In 1857 Pasteur began his classical investigations on the nature of alcoholic fermentation, and by 1860 he was ready to conclude:

> "The chemical act of fermentation is essentially a phenomenon correlative with a vital act, commencing and ceasing with the latter. I am of the opinion that alcoholic fermentation never occurs without simultaneous organization, development, multiplication of cells, or the continued life of cells already formed. The results.....seem to me to be completely opposed to the opinions of Liebig and Berzelius. If I am asked in what consists the chemical act whereby the sugar is decomposed and what its real cause, I reply that I am completely ignorant of it..... And the facts tell me simply that all true fermentations are correlative with physiological phenomena."

Pasteur's doctrine of "no fermentation without life" seemed firmly established. Liebig had little to say in rebuttal. Subsequent work has shown, however, that he was not completely wrong. It is now known that fermentation is a chemical process brought about by an agent similar to Liebig's ferment, but this agent is produced by a living organism, as contended by Pasteur.

Little further progress was made until 1897, when Buchner extracted the active principle (or enzymes) from yeast cells by grinding them with sand and kieselguhr and squeezing out the

juice by means of a hydraulic press. This yeast juice was found to have the ability to ferment sugar. Buchner concluded (1897,1) that fermentation could take place "without so complicated an apparatus as a yeast cell", and that the fermentation was essentially a chemical change brought about by the presence of a substance which he called zymase. Since the discovery of Buchner's yeast juice other enzyme preparations have been made from yeast and widely used in studies on the nature of alcoholic fermentation.

It is now known that the zymase of Buchner is not a single substance, but a highly complex system of many enzymes. Harden and Young (1906) demonstrated that yeast juice could be divided into a residue and a filtrate, each of which was impotent alone to ferment sugar. These fractions were called the enzyme, present in the residue when yeast juice was filtered, and the co-enzyme, present in the filtrate. In the later terminology of Neuberg and Euler the complete enzyme system necessary for fermentation is called holozymase, the co-enzyme of Harden is called co-zymase, and the holozymase freed from all co-zymase is called apozymase. But still further divisions have been made. The exact number of substances present in co-zymase is still undetermined, but the work of Myrbach (1933), Lohmann (1931), Kluyver and Struyk (1928,2), Stheeman (1929,1930) and others have clearly established its multiple nature. Co-zymase is now considered to be composed of adenylic pyrophosphate,

hexose phosphate, a hydrogen acceptor, an anti-protease, potassium and magnesium ions, and perhaps an activator similar to Euler's "Z" factors (1930).

Apozymase also is thought to be a complex substance. It is thought to be made up of the enzymes phosphatase, carboxylase, a dehydrogenase, and possibly a special enzyme to bring about primary changes in the sugar molecules, and another to bring about the esterification of phosphate and sugar. Obviously, Büchner's zymase is highly complex.

The Role of Phosphate in Alcoholic Fermentation

The indispensability of phosphates for fermentation is now almost universally accepted. Harden and Young (1905,1,2) presented the first evidence for this fact, and practically all subsequent work on fermentation by yeast preparations has verified this conclusion. These investigators observed a temporary acceleration of fermentation by yeast juice upon the addition of inorganic phosphate. They then attempted to demonstrate the necessity for phosphate by several procedures, the principle of which was to remove co-zymase and phosphate from the yeast preparation and to compare the carbon dioxide yield when co-zymase was added alone and when it was added along with inorganic phosphate. Complete removal of phosphate, which occurs to some extent naturally in the preparation, was never attained,

but the experiments showed that mixtures of apozymase and cozymase could be prepared having a very slight fermentative activity. When inorganic phosphate, or a source of inorganic phosphate such as hexosediphosphate was added, the total fermentation produced by the mixture could be increased twenty to eighty times. Moreover, it was demonstrated that the added inorganic phosphate rapidly disappeared from the substrate during the period of accelerated fermentation. All this evidence was interpreted to mean that phosphate is absolutely necessary for fermentation of suger by yeast enzymes.

A number of carbohydrate esters of phosphoric acid have been isolated from fermenting mixtures of yeast enzyme preparations. Among these are hexosediphosphate (Harden and Young, 1908), hexosemonophosphate (Harden and Robison, 1914), trehalose monophosphate (Robison and Morgan, 1928), and the "difficultly hydrolyzable" ester of Lohmann (1930). The latter compound was first isolated from a mixture of minced muscle tissue and glycogen, but was later isolated from a fermenting yeast preparation by Meyerhof and Kiessling (1933). It consists of an equimolecular mixture of phosphoglyceric acid, $CH_2(PO_4H_2)CHOHCOOH$, and glycerophosphoric acid, $CH_2(PO_4H_2)CHOHCH_2OH$.

Hexosediphosphate and hexosemonophosphate are almost invariably formed together, and the ratio of the amounts of these substances varies considerably, depending on the enzyme preparation used. The respective roles of the two have long

been a source of controversy. But in spite of the variation, the ratio of carbon dioxide evolved to phosphate esterified is always very close to unity, according to Harden. Kluyver and Struyk (1928,1) sometimes found lower ratios. The equivalence between CO_2 and phosphate led Harden to formulate his equation for alcoholic fermentation,

$2\ C_6H_{12}O_6 + 2\ R_2HPO_4 \longrightarrow 2\ CO_2 + 2\ C_2H_5OH + C_6H_{10}O_4(PO_4H_2)_2 + 2\ H_2O$. The essential feature of this equation is the decomposition of one molecule of hexose while another molecule is simultaneously esterified. To account for the formation of hexosemonophosphate Harden postulated a second equation,

$3\ C_6H_{12}O_6 + 2\ R_2HPO_4 \longrightarrow 2\ CO_2 + 2\ C_2H_5OH + 2\ C_6H_{11}O_5(PO_4H_2) + 2\ H_2O$. When all available phosphate was esterified Harden considered that the rate of fermentation was conditioned by the rate of hydrolysis of the hexose phosphates brought about by the enzyme phosphatase, as shown in the following equations,

$2\ H_2O + C_6H_{10}O_4(PO_4H_2)_2 \longrightarrow C_6H_{12}O_6 + 2\ R_2HPO_4$

$H_2O + C_6H_{11}O_5(PO_4H_2) \longrightarrow C_6H_{12}O_6 + R_2HPO_4$.

Much later Meyerhof (1926) demonstrated that hexosediphosphate could be fermented directly, so Harden supplemented his equations with the following relation:

$C_6H_{10}O_2(PO_4H_2)_2 + 2\ H_2O \longrightarrow 2\ CO_2 + 2\ C_2H_5OH + 2\ R_2HPO_4$.

Harden thought that this reaction was abnormal.

Many other investigators in the field who have proposed mechanisms for alcoholic fermentations have assumed that the hexose phosphate esters were intermediate compounds in the

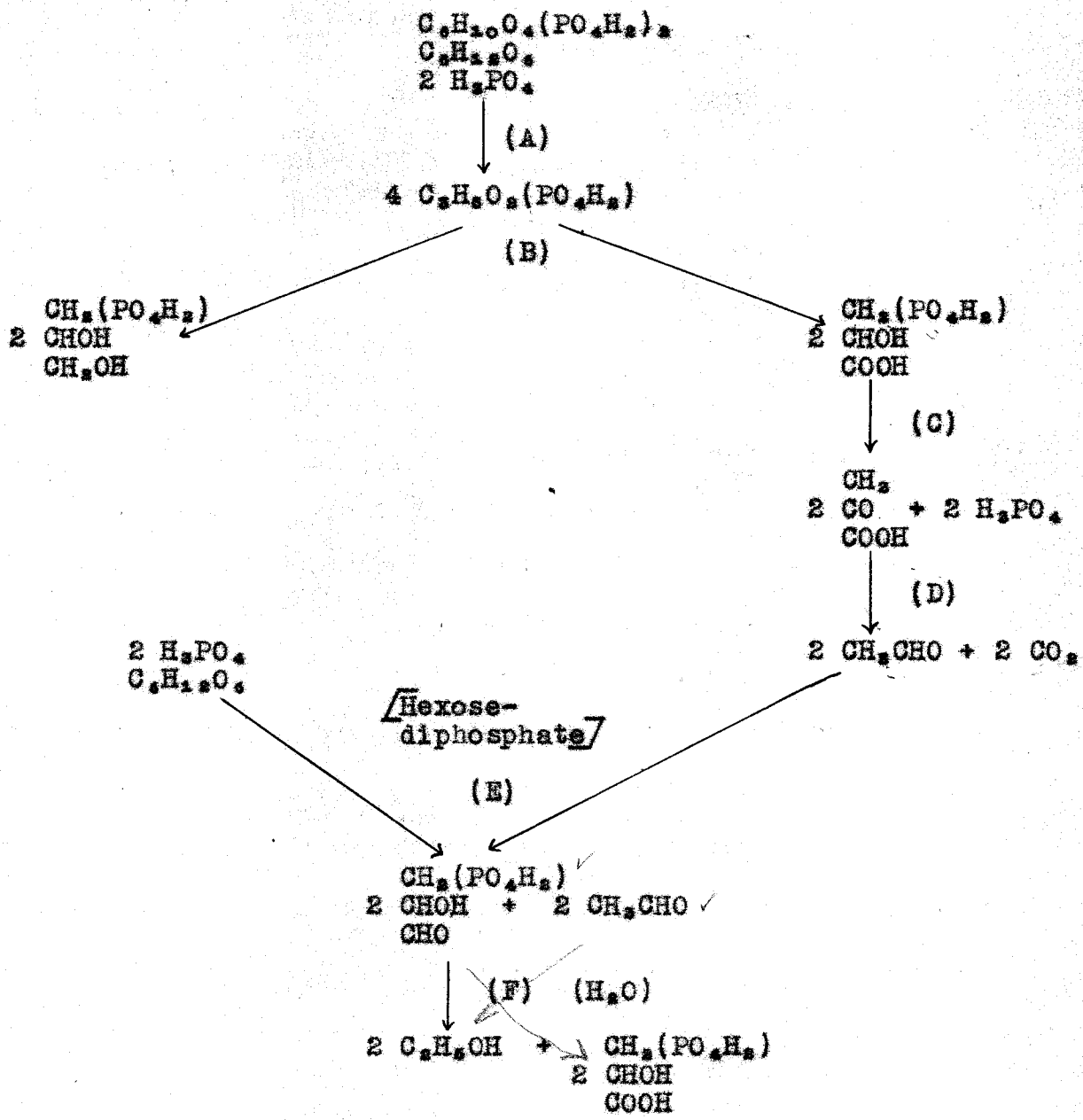
decomposition of the sugar. No attempt will be made to review all of the mechanisms which have been proposed. These have been summarized by Harden (1932,1). Only one will be considered: that recently proposed by Meyerhof (1933). This mechanism is supported by rather convincing experimental evidence.

Theories of Meyerhof

It was shown by Lohmann (1930) that an ester accumulates when muscle tissue is minced in the presence of glycogen and Na_2F_2, which is identical in composition with the Harden and Young hexosediphosphate ester but which has a much greater resistance to hydrolysis by HCl. Embden (1933) and his coworkers identified phosphoglyceric acid in the Lohmann ester. Later Meyerhof and McEachern (1933) identified another constituent in the ester, l-α glycerophosphoric acid. It was thus shown that the Lohmann ester consists of equimolecular proportions of these two triose esters. Meyerhof and Kiessling (1933) isolated the Lohmann ester from a yeast maceration juice fermentation, and Nilsson (1930) isolated phosphoglyceric acid from a dried yeast fermentation. This work furnished the clue to the importance of triose phosphoric esters in the alcoholic fermentation, and also in lactic acid production by muscle.

Further researches of Embden, Meyerhof, Neuberg and others

have furnished evidence for the Meyerhof scheme for the breakdown of carbohydrates by yeast. This may be represented as follows:



The experimental basis for this scheme has been summarized by Meyerhof (1933) and Harden (1933). Most of the data have been obtained by partial inhibition of the fermentation

with Na_2F_2 and CH_2ICOOH. The assumption is made that the poisons are specific for certain phases of the fermentation and do not affect the other phases. Thus Na_2F_2 inhibits only reactions C and D in the Meyerhof scheme, and allows the products of A and B to accumulate. But if CH_3CHO is also added the reactions E and F will take place. On the other hand, CH_2ICOOH inhibits the reactions A, B, E and F, but not C and D. If phosphoglyceric acid is added along with CH_2ICOOH it will be converted into H_3PO_4, CO_2 and CH_3CHO, which accumulate. During the initial stage in a normal fermentation phosphoglyceric acid, and hence CH_3CHO, is produced directly from hexosediphosphate with the sequence of reactions A, B, C, D. When sufficient CH_3CHO has accumulated the decomposition of hexosediphosphate ceases--it then assumes a purely catalytic role--and fermentation proceeds by the path E, F, C and D.

Conclusions based on such experiments are subject to the usual criticism that the course of the reactions is abnormal in the presence of poisoning agents. This objection may be valid, but the Meyerhof scheme agrees well with practically all experimental facts previously obtained. For example, it accounts for the importance of phosphates, the equivalence of the amount of phosphate esterified to the CO_2 evolved, the induction period sometimes observed with yeast preparations, and correlates the lactic acid (in muscle) and alcoholic fermentations, which differ as to the fate of pyruvic acid.

The Meyerhof scheme cannot be regarded as final and complete. Some parts of it need further elucidation.

Statement of the Problem

Much of the enormous amount of research on the alcoholic fermentation problem has been concerned with its mechanism, the isolation of intermediate compounds, and the establishing of the identity of individual enzymes. Less attention has been paid to environmental factors and their influence upon the activity of the zymase enzyme complex. The investigations here reported were undertaken with the idea of examining the effect of some of these factors upon yeast enzyme preparations.

The work may be subdivided as follows:

(1) The effect of inorganic salts, hydrogen ion concentration, glucose concentration, and ethanol on carbon dioxide production.

(2) The effect of the above factors on the inorganic phosphate content during the course of fermentation.

(3) The interpretation of the influence of these factors on the enzymes involved in the fermentation.

Literature Relevant to the Problem

An induction period, defined by Harden (1932,2) as "the time which elapses before the normal rate of fermentation is attained" occurs when the yeast preparations zymin, dried

yeast, or maceration extract are treated with a large volume of sugar solution, the length of the period increasing as the volume of added sugar solution increases. The induction period may be eliminated, or greatly shortened, by the addition of hexosediphosphate, CH_3CHO, and a variety of organic and inorganic salts [Harden (1925), Harden and McFarlane (1928), Katagiri and Yamagishi (1929)]. On the other hand, arsenates and cyanides greatly prolong it [Patterson (1931)]. Katagiri and Yamagishi (1929) investigated the effect of salts on the induction period of dried yeast, and concluded that the potency of the chlorides and sulfates in shortening the length of the induction period decreased in the following order:

$$NH_4^+ > 1/2Mg^{++} > K^+ > Na^+ > 1/2\ Ca^{++}.$$

These authors made the observation that the salts had no effect on the rate of carbon dioxide production after its evolution had commenced, except an inhibitory one in some cases.

Patterson (1931) investigated the changes in both the organic and inorganic "acid-soluble" phosphate (that fraction soluble in 3 per cent CCl_3COOH), during the induction period of zymin. In the control flask the inorganic phosphate gradually increased until almost the end of the period, when it began to be esterified and rapidly decreased. When either 0.13 or 0.2 M. potassium acetate was added at the start (although the initial inorganic phosphate content was the same as the control), the inorganic phosphate began to disappear much more

quickly. In every case CO_2 evolution started when the inorganic phosphate content had fallen to approximately the same low value and when the total organic phosphate content had reached about the same high value. According to McFarlane's data, the initial acid soluble organic phosphate is greater when potassium acetate is present, increasing from 33 mg. in the control to 46 mg. in the presence of 0.2 M. salt. Explanation of these results is difficult. McFarlane concludes that the salt effect in decreasing the period of induction depends upon changes brought about in the zymin rendering the phosphate more readily available. It is suggested that salts bring this about by changing the permeability of the cell wall. Against this conclusion may be cited the work of Harden and McFarlane (1928), who observed no decrease in the length of the induction period after zymin had been ground with glass to destroy the cell walls. Moreover, Harden (1925) reported that co-zymase in zymin is extracted no more rapidly with a salt solution than it is with pure water.

Whatever be the explanation, it is clear from the work of McFarlane that the effect of potassium acetate during the induction period with zymin is to decrease the time which elapses before the inorganic phosphate content reaches a minimum value.

When an excess of inorganic phosphate is added to a fermenting mixture of sugar and a yeast enzyme preparation the

time required to attain the maximum velocity of CO_2 evolution is increased and the maximum rate itself is decreased, according to Harden and Young (1908). Meyerhof (1918) examined this phenomenon and found that the addition of either 0.11 or 0.2 M. NaCl or $NaNO_3$ had the same effect as an increase in phosphate concentration. Harden and Henley (1921), using zymin as the enzyme preparation, confirmed these results with NaCl, KCl, Na_2SO_4 and K_2SO_4, but using the salts only in the concentrations 0.25 and 0.4 M. With the latter concentration the salts also decreased the final steady rate of carbon dioxide evolution. From these data Harden draws the general conclusion that salts have a depressing effect on the enzymes concerned with esterification of phosphate, and also on those concerned with the liberation of esterified phosphate. Excess of phosphate shares in this supposed depressing effect of salts.

Erdtman (1928) and Hommerberg (1933) found that magnesium ion in as small a concentration as 4×10^{-6} molar will activate the enzyme phosphatase, but the enzyme was not obtained from yeast. Lohmann (1931) demonstrated this ion to be a necessary part of the co-zymase of yeast.

Rahn (1929) studied the effect of ethanol on the rate of CO_2 production by living yeast. Analysis of his data shows a linear relationship between the relative rate of CO_2 production and the concentration of ethanol up to about 10 per cent ethanol. For this linear part of the curve the equation is:

Relative rate = 100 - 2.1 x per cent ethanol.

From the concentrations 10 to 14 per cent ethanol there is a slow but practically constant rate of CO_2 evolution. Rahn has developed the differential equation

$$\frac{-dx}{dt} = K(2L-x)$$

for the normal yeast fermentation, in which L is the limiting concentration of ethanol which just prevents fermentation, and x is the amount of sugar decomposed (approximately equal to the amount of CO_2 evolved). When ethanol is added at the beginning the equation must be corrected as follows:

$$\frac{-dx}{dt} = K(2L-x-2a)$$

in which a is the concentration of ethanol in the initial mixture.

Harden (1932,3) in his summary of the effects of antiseptics on yeast juice, reports a 0-20 per cent diminution in fermentative activity in the presence of 6 per cent ethanol and a 75 per cent decrease with 14 per cent ethanol. Comparing these data with those of Rahn, it is evident that yeast juice is less susceptible to ethanol than is living yeast.

The optimum pH range for the zymase activity of living yeast and dried yeast is 4.0 to 8.5, according to Hagglund, Soderblom and Troberg (1926). Hagglund and Rosenquist (1926) reported the values 5.5 to 8.0 for yeast juice. It is significant that none of the optimum pH ranges above is narrow. The

pH value 6.4 is recorded by Euler and Nordlund (1921) as the optimum pH for hexosediphosphate synthesis by living yeast and dried yeast. These authors supposed that the esterification was brought about by a special enzyme which they called phosphatese. Mahdihasson (1930) obtained a value of 5.9 to 6.0 for the pH of the yeast cell interior. If this is correct it might be expected that the enzymes in yeast preparations would be most active at the above values of pH.

Observations of Herzog (1902, 1904), made with zymin and yeast juice, on the effect of varying the concentration of sugar indicate that the initial velocity of fermentation is almost independent of the sugar concentration, but the rate decreases slowly as the sugar concentration increases. Experiments made by Harden (1932,4) agreed with those of Herzog.

Virtually the same conclusion was reached by Slator (1906) for fermentation by living yeast. He found that the fermentation rate with an 11 per cent sugar solution was about 10 per cent less than the rate with 4 per cent sugar.

EXPERIMENTAL

Methods of Analysis

CO_2 Evolution

For the determination of the amount of CO_2 evolved and the velocity of its evolution the apparatus of Harden, Thompson and Young (1910) was used. Several modifications were made. Fermentation flasks were placed in a constant temperature water bath at 25°C., and were connected to a Schiff azotometer filled with mercury. The level of mercury in the reservoir was kept constant by a siphon overflow, thus insuring that no change in pressure occurred in the fermentation flask. In order to prevent super-saturation of the fermenting liquid the flasks were shaken continuously during the experiment by a motor driven mechanical shaker.

The volume of CO_2 evolved was observed at intervals of 10 minutes, measured with an interval timer. When a reading was to be taken the flask was shut off by means of a stop cock and the Hg reservoir raised to the level of Hg inside the azotometer. All volumes were converted to standard conditions and milligrams of CO_2 calculated. This was deemed advisable since variations in room pressure and temperature amounted to

$40_{mm.}$ and 15°C., or a variation of 7 or 8 per cent in the volume of a definite weight of CO_2.

Readings could be duplicated to within 1-2 cc. in 100, or about 1 or 2 per cent. All CO_2 values given in the tables are the average of several determinations.

Inorganic phosphate

The inorganic phosphate was determined colorimetrically by the procedure of Kuttner and Lichtenstein (1930). Sodium molybdate is reduced by $SnCl_2$ in the presence of inorganic phosphate; within certain limits the blue color produced is proportional to the concentration of phosphate. Levene and Raymond (1928), and Raymond (1928) have used this method on zymin fermentation mixtures and agree with the above authors that the procedure is specific for inorganic phosphate. The above reaction is the basis for several colorimetric methods [see Peters and Van Slyke (1932)], the chief variation being in the reductant employed.

The exact procedure employed was as follows: A 1.0 cc. sample was withdrawn from the fermentation flask and diluted with 9.0 cc. of water in a graduate. This was immediately filtered by suction through ashless filter paper. The graduate was washed out with exactly 10 cc. of water and this was poured through the filter paper. The filtrate corresponds to a 1 to 20 dilution of the original liquid. This was further diluted

as much as necessary to bring the phosphate content within the proper limits, which are 0.002 to 0.01 mg. of phosphorus per cc. Ordinarily this necessitated an overall dilution of 1 to 100; hence 1 cc. of filtrate was diluted with 4 cc. H_2O for analysis. There is a slight but perceptible increase in phosphate content in the filtrate on standing. For this reason the reagents were added and the analysis made within 15 minutes after the removal of the sample. The reproducibility of values was about 2 per cent, which is the accuracy claimed by the originators of the method. A standard solution containing 0.1 mg. of phosphorus was made up at the beginning of the series of analysis, and every standard used for comparison with the unknown was prepared by dilution of this original standard. This insured comparability of values. It was found that arsenate ion will also produce a blue color when added to the reaction mixture. This fact is not mentioned by Kuttner and Lichtenstein (1930). Levene and Raymond (1928) or Raymond (1928) employed this procedure even though arsenate was present in their samples. Therefore, if arsenate has been added significance can only be attached to changes in the apparent phosphate content.

Hydrogen ion concentration

The pH of fermentation mixtures was determined electrometrically by the use of a quinhydrone electrode. Hopkins (1928) has used this method on zymin fermentation mixtures.

Other workers, Katagiri and Yamagishi (1929) and Harden and McFarlane (1928), have used either colorimetric or capillator methods for pH determination.

Preparation of Zymin and Dried Yeast

Since the discovery of yeast juice by Buchner (1897,1,2), quite a number of active enzyme preparations have been made which are almost entirely free from living yeast cells, but capable of bringing about the alcoholic fermentation of sugars. The most widely used of these preparations are yeast juice, zymin, the "maceration extract" of Lebedev (1911), and dried yeast. These all differ from living yeast in varying degrees but in the same general way. All of them ferment sugar more slowly than living yeast, and all respond to the presence of inorganic phosphate by fermenting sugar more rapidly than in its absence.

The activity of zymin is about 1/8 that of living yeast, whereas the ratio for yeast juice is only about 1/40. They also differ in relative stability, the dry zymin preparation retaining its activity for months, while yeast juice autolyzes rapidly with resulting loss in fermentative power. For these reasons it was decided to use zymin in the present research. Certain phases of the work were repeated with dried yeast.

An attempt was made to prepare zymin from baker's yeast (Fleischmann) grown in the laboratory in a wort medium. This

proved unsatisfactory because the zymin from this yeast had very little activity, and in some cases none at all. Zymin prepared from brewer's[1] yeast was very active, and was used throughout the investigation. Five hundred grams of yeast and 3 liters of acetone were stirred together for 10 minutes and filtered on a Buchner funnel. The mass was then mixed with 1 liter of acetone for 2 minutes and again filtered. The residue was coarsely powdered, stirred with 250 cc. of ether for three minutes, filtered and spread out on paper in a thin layer. After standing for an hour at room temperature it was dried at 40-45° for 24 hours. The average yield was about 25 per cent of the original weight of yeast. This is somewhat less than the figure given by Harden (1932,5). There was some variation in the fermentative activity of different zymin preparations, even though identical procedures were followed; in some instances this variation in evolution of carbon dioxide amounted to 50 per cent. Ordinarily from 70 to 90 mg. of carbon dioxide were evolved in 90 minutes. A clue to this variation in activity is found in the data given in Table 1. Zymin A was made on the day of arrival of a shipment of yeast by express; zymin B three days later and preparation C seven days later. The fresh yeast had been stored in the refrigerator. It is evident that the evolution of carbon dioxide increases with the time elapsed from receipt of the

[1] Furnished through the courtesy of Anheuser-Busch, St. Louis.

yeast. This fact is correlated with the increase in the amount of available inorganic phosphate. All of these experiments were run under comparable conditions.

Table 1

CO_2 Production and Inorganic Phosphate Content of Three Zymin Preparations

Minutes	Inorganic phosphate in aqueous system without glucose mg. P per cc.			Inorganic phosphate in presence of glucose mg. P per cc.		Mg. CO_2 evolved		
	A	B	C	B	C	A	B	C
5	0.69	0.79	1.13	0.72	0.97	-	-	-
10	-	-	-	-	-	6	10	29
30	0.94	1.05	1.31	0.19	0.16	29	42	73
60	1.04	1.11	1.40	0.22	0.16	55	72	110
90	-	-	1.50	0.26	0.18	71	94	143

Zymin C is richer in esterifying ability than is zymin B as shown by the inorganic phosphate content in the presence of glucose. At the end of 30 minutes, the phosphate content of C is only 12.2 per cent of what it would have been in the absence of glucose, whereas the percentage for B is 18.1. The three zymins were also progressively lighter in color.

It is, then, clearly indicated that in order to prepare an active zymin the yeast should first be allowed to age. The zymin was stable over a long period of time. No significant

change in activity was noted after four months.

Dried yeast was prepared by drying fresh yeast at 35° for 48 hours. It was always more active than a corresponding amount of zymin.

Influence of Some Environmental Factors on CO_2 Production

Hydrogen ion concentration

Duplicate flasks were prepared, one for determining the carbon dioxide evolution, and the other for obtaining pH values. Tenth normal HCl or 0.1 N NaOH was added to obtain variations in pH. The fermentation mixture consisted of 6 grams zymin, 3 grams of glucose, 1.0 cc. of toluene, and varying amounts of either acid or base and H_2O, so that the total volume of liquid added was 25 cc. Toluene was always added as an antiseptic. Samples were withdrawn at 10, 30, 60, and 90 minutes for determining pH values of the mixture.

The data are summarized in Table 2. The average rates given in the table and in all the others are the milligrams CO_2 evolved per minute over the time interval concerned.

Except in the case of extreme inhibition, the rate of carbon dioxide evolution had reached a fairly steady state after 30 minutes. It will be seen from Table 2 that this steady rate is not as sensitive to pH as is the initial rate.

Table 2

Effect of pH on CO_2 Production by Zymin

Initial pH	After 30 minutes: pH	After 30 minutes: Mg. CO_2	After 90 minutes: pH	After 90 minutes: Mg. CO_2	Average rates: 0-30 Mins.	Average rates: 30-90 Mins.
4.33	4.41	0.0	4.43	9	0	0.15
5.14	5.34	19	5.68	46	0.63	0.48
5.63	5.78	34	5.92	71	1.13	0.62
6.09	6.14	32	6.08	69	1.07	0.62
6.44*	6.28*	21*	6.30*	58*	0.70*	0.62*
7.50	7.02	10	6.65	38	0.33	0.47
7.90	7.10	2	6.85	26	0.07	0.40

* Values for flask to which only water was added.

The pH values for the flask to which only water was added varied from 6.44 to 6.30. In general the pH of other batches of zymin under the same conditions was found to be in the same range, but sometimes had decreased to 6.20 after 90 minutes.

From Table 2, the optimum pH values for zymin fermentation are 5.6 to 6.1 for the initial rate of CO_2 production (0-30 minutes), and 5.8-6.3 for the steady rate (30-90 minutes). These values are close to those given by Mahdihasson (1930), 5.9-6.0, for the yeast cell interior.

Salts

The concentration of zymin used was such that no induction period occurred. Six grams of zymin, 3 grams of glucose, 1.0 cc. toluene, and 25 cc. H_2O were added to each flask, and comprised the basic reaction mixture. To this was added the amount of salt necessary to produce the salt normality indicated in Tables 3-7.

Table 3

Effect of NH_4Cl on CO_2 Production by Zymin

Minutes	Control		.032N.		.05N.		.075N.		.093N		.112N.		.131N.		.224N.		.60N.	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
10	15	15	13	13	14	14	15	15	17	17	17	17	18	18	17	17	5	5
20	26	11	25	12	26	12	28	13	31	14	31	14	32	14	28	11	13	8
30	37	11	37	12	38	12	39	11	43	12	43	12	43	11	38	10	18	5
40	47	10	48	11	49	11	49	10	54	11	54	11	55	12	49	11	24	6
50	57	10	58	10	60	11	-	-	66	12	-	-	66	11	58	9	29	5
60	66	9	68	10	70	10	69	-	77	11	76	-	-	-	68	10	34	5
70	75	9	78	10	80	10	80	11	87	10	-	-	86	-	76	8	39	5
80	83	8	88	10	90	10	90	10	97	10	96	-	-	-	85	9	44	5
90	91	8	98	10	100	10	100	10	107	10	106	10	106	-	94	9	50	6
Average Rate 0-30 Minutes	1.22		1.22		1.27		1.30		1.44*		1.44*		1.44*		1.27		.60	
Increase	-		0%		4%		6.5%		18%		18%*		18%*		4%		-50%	
Average Rate 30-90 Minutes	.9		1.02		1.03		1.02		1.07*		1.05		1.05		.93		.54	
Increase	-		13%		14.5%		13%		19%*		16.5%		16.5%		4%		-40%	

* Optimum
I Total mg. CO_2 evolved
II Mg. CO_2 per 10 minutes

Table 4

Effect of $MgSO_4$ on CO_2 Production by Zymin

Minutes	Control		.02N.		.027N.		.034N.		.067N.	
	I	II	I	II	I	II	I	II	I	II
10	7	7	10	10	10	10	12	12	10	10
20	15	8	19	9	19	9	21	9	19	9
30	22	7	27	8	27	8	29	8	28	9
40	29	7	35	8	34	7	38	9	34	6
50	35	6	42	7	43	9	45	7	40	6
60	41	6	49	7	50	7	53	8	46	6
70	48	7	56	7	57	7	61	8	53	7
80	54	6	62	6	65	8	67	6	-	-
90	60	6	70	8	71	6	72	5	64	-
Average Rate 0-30 Minutes	0.73		0.90		0.90		0.97*		0.93	
Increase	-		23%		23%		33%		27%	
Average Rate 30-90 Minutes	0.63		0.72*		0.73*		0.72*		0.60	
Increase	-		14.5%		15.9%		14.5%		-4.7%	

* Optimum
I Total mg. CO_2 evolved
II Mg. CO_2 evolved per 10 minutes

Table 5

Effect of $CaCl_2$ on CO_2 Production by Zymin

Minutes	Control		.014N.		.022N.		.036N.		.072N.	
	I	II	I	II	I	II	I	II	I	II
10	9	9	8	8	8	8	9	9	4	4
20	17	8	16	8	15	7	17	8	11	7
30	24	7	23	7	24	9	25	8	18	7
40	31	7	30	7	32	8	32	7	24	6
50	38	7	38	8	39	7	38	6	28	4
60	44	6	44	6	46	7	45	7	35	7
70	50	6	51	7	-	-	52	7	40	5
80	56	6	58	7	60	-	-	-	45	5
90	62	6	65	7	67	7	66	-	50	5
Average Rate 0-30 Minutes	0.80		0.77		0.80		0.83*		0.60	
Increase	-		-4%		0		+4%		-25%	
Average Rate 30-90 Minutes	0.63		0.70		0.72*		0.68		0.53	
Increase	-		11%		14%		8%		-16%	

* Optimum
I Total mg. CO_2 evolved
II Mg. CO_2 evolved per 10 minutes

Table 6

Effect of NaCl on CO_2 Production by Zymin

Minutes	Control I	Control II	.034N. I	.034N. II	.068N. I	.068N. II	.102N. I	.102N. II	.137N. I	.137N. II	.205N. I	.205N. II
10	4	4	3	3	3	3	4	4	1	1	1	1
20	11	7	13	10	13	10	15	11	11	10	10	9
30	21	10	25	12	26	13	28	13	25	14	22	12
40	31	10	37	12	39	13	42	14	38	13	35	13
50	42	11	47	10	49	10	51	9	48	10	45	10
60	50	8	55	8	57	8	59	8	55	7	52	7
70	57	7	62	7	65	8	67	8	63	8	58	6
80	63	6	68	6	72	7	73	6	70	7	65	7
90	70	7	75	7	79	7	81	8	77	7	72	7
Average Rate 0-30 Minutes	0.70		0.83		0.87		0.93*		0.83		0.73	
Increase	-		19%		24%		33%*		19%		4%	
Average Rate 30-90 Minutes	0.82		0.84		0.88*		0.88*		0.87		0.84	
Increase	-		2.4%		7.3%*		7.3%*		6%		2.4%	

* Optimum
I Total mg. CO_2 evolved
II Mg. CO_2 evolved per 10 minutes

Table 7

Effect of KCl on CO_2 Production by Zymin

Minutes	Control I	Control II	.027N. I	.027N. II	.054N. I	.054N. II	.08N. I	.08N. II	.107N. I	.107N. II	.16N. I	.16N. II
10	4	4	4	4	4	4	2	2	2		2	2
20	11	7	11	7	12	8	11	9	11		11	9
30	21	10	21	10	22	10	23	12	23		22	11
40	31	10	31	10	35	13	34	11	34		33	11
50	42	11	42	11	44	9	44	10	44		42	9
60	49	7	50	8	51	7	51	7	51		50	8
70	57	8	57	7	58	7	59	8	58		56	6
80	64	7	63	6	65	7	65	6	64		63	7
90	70	6	70	7	72	7	72	7	72		69	6
Average Rate 0-30 Minutes	0.70		0.70		0.73		0.77*		0.77*		0.73	
Increase	-		0		14%		10%		10%		4%	
Average Rate 30-90 Minutes	0.82		0.82		0.84		0.82		0.82		0.78	
Increase	-		0		2.4%*		0		0		-4.8%	

* Optimum
I Total mg. CO_2 evolved
II Mg. CO_2 evolved per 10 minutes

In practically every case the maximum rate of carbon dioxide evolution was attained during the first 30 minutes, and most often in the first 10 minutes. Carbon dioxide began to collect in the mercury filled azotometer within 2 or 3 minutes after mixing. This indicates that the time necessary for saturation of the fermentation flasks is negligible. Experiments carried out with water previously saturated with CO_2 showed only a slight increase in the amount of CO_2 evolved in the first 10 minutes, and no difference whatsoever after that. Therefore it was thought unnecessary to use CO_2 saturated water in the experiments.

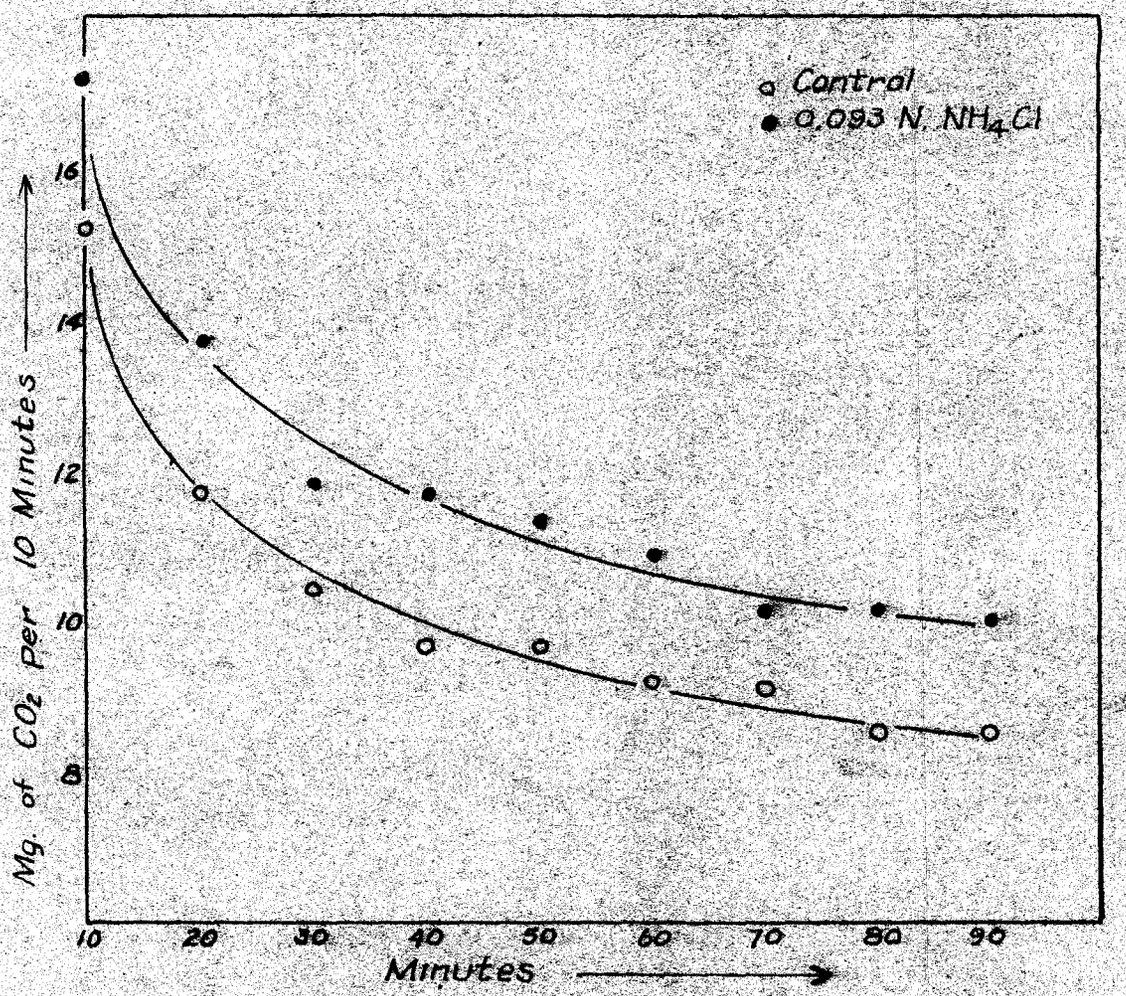


Fig 1.
Effect of NH_4Cl on Rate of CO_2 Production by Zymin.

The data show that the salts used are activators for the zymase enzyme complex, if added in the proper concentration. The first 30 minutes is a period of more rapid fermentation, and during this period most of the free phosphate in the zymin is esterified. At the end of this period the rate of CO_2 evolution had fallen to an almost constant, though gradually decreasing, value. For this reason the percentage increase in rate in the presence of the salts has been calculated for both periods. It is likely that a different part of the enzyme complex is involved in the initial period than in the second. This is suggested by the fact that the order of activation by the salts at optimum concentrations differs for the two periods. For the initial period the order is:

$$MgSO_4 = NaCl > NH_4Cl > KCl > CaCl_2,$$

whereas for the period of constant rate,

$$NH_4Cl > MgSO_4 > CaCl_2 > NaCl > KCl.$$

The above order may not be exact, since the same zymin preparation was not used in each series. However, the order is approximately substantiated by data presented later on the effect of the salts upon phosphorylation reactions.

Harden and Henley (1921) consider the rate of fermentation to be a measure of the activity of the enzyme phosphatase after a steady state is reached, since during this phase the rate of fermentation is conditioned by the amount of free phosphate available for esterification. Adopting this criterion, we may conclude that the salts NH_4Cl, $MgSO_4$, $CaCl_2$,

and NaCl are activators for the mechanism which liberates organic phosphate, while KCl has little effect.

An experiment was carried out to determine whether activation by the two most potent salts, NH_4Cl and $MgSO_4$, was additive. Optimum amounts of each salt was added to the fermentation flask containing the basic reaction mixture. Results are summarized in Table 8. Apparently the activation by NH_4Cl and $MgSO_4$ is not additive.

Table 8

Effect of NH_4Cl and $MgSO_4$ Together on CO_2 Production* by Zymin

Minutes	Control	.034N. $MgSO_4$	.075N. NH_4Cl	.034N. $MgSO_4$ .075N. NH_4Cl
10	7	12	13	12
20	15	20	23	22
30	22	29	30	31
40	29	37	39	38
50	35	44	47	46
60	41	52	54	54
70	48	59	61	62
80	54	-	69	69
90	61	71	76	77
Average Rate 0-30 Minutes	.73	.97	1.00	1.03
Increase	-	33%	37%	41%
Average Rate 30-90 Minutes	.65	.70	.77	.77
Increase	-	8%	18.5%	18.5%

* Mg. CO_2 evolved

In order to discover whether activation by NH_4Cl lasted longer than 90 minutes, a fermentation was carried out lasting 240 minutes. Data of Table 9 show that this is the case. The same basic reaction mixture was used as in former experiments.

Table 9

Effect of NH_4Cl on CO_2 Production

Over 240 Minute Period

Minutes	Mg. CO_2	
	Control	.094N. NH_4Cl
30	22	29
90	69	84
240	156	196
Average Rate 0-30 Minutes	0.73	0.97
Increase	-	33%
Average Rate 30-90 Minutes	0.77	0.92
Increase	-	19.5%
Average Rate 90-240 Minutes	0.58	0.75
Increase	-	29%

The question naturally arises as to whether activation by NH_4Cl and $MgSO_4$ is due merely to changing the reaction medium to a more favorable pH because of hydrolysis of these two salts. In Table 10 are given pH values of reaction mixtures containing

Table 10

Effect of NH_4Cl on pH of

Zymin Fermentation Mixtures

Minutes	Control	.075N.	.093N.	.224N.	.30N.
5	6.47	6.44	6.39	6.34	6.37
15	6.36	-	-	-	-
30	6.31	6.20	6.15	6.11	6.03
45	6.23	6.22	-	-	5.97
60	6.24	6.20	6.14	6.05	6.03
90	6.22	6.20	6.14	6.05	5.98

varying concentrations of NH_4Cl. As shown in Table 3, 0.075 N NH_4Cl definitely stimulates CO_2 production during the 30-90 minute period, yet the pH of this reaction mixture is virtually the same as in the control flask. Moreover, the 30-90 minute average rate is constant and at a maximum over the pH range 5.8 to 6.30, and the concentration of NH_4Cl three times as great as the optimum decreases the pH only to a value of 6.0. With more justification it might be said that NH_4Cl shifts the pH toward the optimum value for the initial rate (0-30 minutes). However, this cannot account for all of the increase. Enough NH_4Cl to bring the initial pH into the optimum range for the initial period has a depressing effect on the CO_2 rate. The

evidence makes it necessary to reject the proposition that the stimulation is caused by a change in pH.

Salts in the presence of added phosphate

When phosphate was added to either a zymin or dried yeast reaction mixture the expected increase in CO_2 production was observed. It was desired to find out if salt activation took place in the presence of the added phosphate. Tables 11 and 12 contain the data for NH_4Cl and $MgSO_4$.

Table 11

Effect of NH_4Cl on CO_2 Production by Zymin in Presence of Added Phosphate

Minutes	Control		.06 M. $(PO_4^{\equiv})$		.06 M. $(PO_4^{\equiv})$ .112N. NH_4Cl	
	I	II	I	II	I	II
10	15	15	39	39	41	41
20	26	11	82	43	88	47
30	37	11	102	20	111	23
40	47	10	121	19	129	18
50	57	10	137	16	148	19
60	66	9	154	17	165	17
70	75	9	169	15	182	15
80	83	8	181	12	-	(16)
90	91	8	193	12	214	(16)
Average Rate 0-30 Minutes	1.22		3.40		3.70	
Increase	-		0		9%	
Average Rate 30-90 Minutes	0.90		1.52		1.72	
Increase	-		0		13.2%	

I Total mg. CO_2 evolved
II Mg. CO_2 evolved per 10 minutes

Table 12

Effect of $MgSO_4$ on CO_2 Production by Zymin in Presence of Phosphate

Minutes	0.06M. ($PO_4^{\equiv}$) I	0.06M. ($PO_4^{\equiv}$) II	0.06M. ($PO_4^{\equiv}$) 0.034N. $MgSO_4$ I	0.06M. ($PO_4^{\equiv}$) 0.034N. $MgSO_4$ II
10	21	21	24	24
20	51	30	59	35
30	72	21	79	20
40	86	14	94	15
50	98	12	106	12
60	109	11	118	12
70	120	11	-	-
80	130	10	141	-
90	140	10	152	11
Average Rate 0-30 Minutes	2.4		2.64	
Increase	-		10%	
Average Rate 30-90 Minutes	1.13		1.22	
Increase	-		8%	

I Total mg. CO_2 evolved
II Mg. CO_2 evolved per 10 minutes

Concentrations of salts optimum without phosphate were added. The conclusion seems justified that stimulation by the salts is additive with that due to phosphate. It may be tentatively postulated that the salts in proper concentration increase the rate of esterification of phosphate, and since stimulation continues after the initial period the salts are also activators for the reaction which sets free the esterified phosphate, thereby speeding up the rate of CO_2 evolution.

Glucose concentration

The effect of varying the concentration of glucose in the basic reaction medium was studied, with the results summarized in Table 13.

Table 13

Effect of Concentration of Glucose

on CO_2 Production by Zymin

Minutes	12% Glucose I	12% Glucose II	4% Glucose I	4% Glucose II	2% Glucose I	2% Glucose II	1% Glucose I	1% Glucose II
10	8	8	9	9	10	10	5	-
30	34	26	44	35	45	35	41	36
50	57	23	73	29	75	30	69	28
70	77	20	99	26	103	28	94	25
Average Rate 0-30 Minutes	1.13		1.47		1.50		1.37	
Average Rate 30-70 Minutes	1.08		1.38		1.45		1.33	
Increase	0		28%		34%		23%	

I Total mg. CO_2 evolved
II Mg. CO_2 evolved per 20 minutes

The statement of Herzog (1902, 1904) and Euler (1905) that the initial velocity of fermentation is almost independent of the concentration of sugar seems unjustified for fermentation by zymin. As is to be expected, this enzymatic reaction does not follow the mass law, since the reaction velocity does not increase with increasing glucose concentration. But the

decrease of approximately 35 per cent between the steady rate in the two relatively dilute 2 per cent and 12 per cent sugar solutions was unexpected.

Salts and the induction period

The salts which have been shown to exert an influence on the rate of carbon dioxide evolution are among those which greatly shorten the induction period exhibited by zymin and dried yeast, as shown by Harden and McFarlane (1928) and Katagiri and Yamagishi (1929). It was decided to determine the effect of the salts on shortening of the induction period which occurred with the zymin used in this investigation. The basic reaction mixture for these experiments was 2.5 gms. zymin, 1.0 gm. glucose, 1.0 cc. toluene, and 25 cc. of CO_2-saturated water. To this was added enough salt to make the solution 0.056 N. The induction period was taken as the time which elapsed before 1.0 cc. of CO_2 collected in the azotometer. From Table 14, the order of potency of the cations in abolishing the induction period is:

$$NH_4^+ > 1/2\ Mg^{++} > Na^+ > K^+ > 1/2\ Ca^{++}.$$

With the exception of Na^+, this order is in agreement with that given by Katagiri and Yamagishi for shortening the induction period of dried yeast. Moreover, after evolution of CO_2

Table 14

Effect of Salts on Induction Period of Zymin

	Length of Period in Minutes, P	Mg. CO_2 Evolved after P + 30 Minutes
Control	32	7.4
.056N. NH_4Cl	16	12.0
.056N. $MgSO_4$	18	14.0
.056N. NaCl	19	10.2
.056N. KCl	22	8.8
.056N. $CaCl_2$	26	7.7

had started the salts increased the amount of CO_2 evolved in 30 minutes in every case except $CaCl_2$. The order of potency in increasing the amount of CO_2 evolved,

$$1/2Mg^{++} > NH_4^+ > Na^+ > K^+ > 1/2\ Ca^{++},$$

agrees well with the order of potency of the cations in increasing the initial rate of CO_2 evolution when no induction period occurred.

Ethanol

During the course of the investigation it became evident that zymin was very sensitive to relatively low concentrations of ethanol. A preliminary report of this phenomenon was made by Stavely, Christensen and Fulmer (1934). The reaction rates

of a series of zymin fermentations with increasing amounts of ethanol were determined. Twenty-five cc. of an ethanol water solution was added in place of 25 cc. of pure water, in order not to change the total volume of reaction mixture. In Table 15 the data are given for seven concentrations of ethanol. There is a marked diminution in the rate of CO_2 evolution up to a concentration of about 1.8 per cent ethanol, and from this point up to 4.7 per cent there is evidently a slow but almost constant rate.

Table 15

Effect of Ethanol on CO_2 Production by Zymin

Minutes	Control		Ethanol Concentration (Per cent by volume)													
			.38%		.94%		1.31%		1.86%		2.59%		3.65%		4.7%	
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
10	15	15	7	7	5	5	4	4	2	2	0	0	0	0	0	0
20	26	11	17	10	10	5	9	5	7	5	4.6	4.6	4.3	4.3	.7	.7
30	37	11	26	9	17	7	14	5	11	4	8.6	4.0	5.3	1.0	2.8	2.1
40	47	10	35	9	23	6	20	6	16	5	12.4	3.8	7.	1.7	5.6	2.8
50	57	10	43	8	30	7	26	6	20	4	-	-	9.6	2.6	8.	2.4
60	66	9	51	8	37	7	32	6	25	5	20.	-	12.	2.4	10.	2.
70	75	9	59	8	44	7	37	5	30	5	24.	4.	15.	3.0	13.	3.
Average Rate 30-70 Minutes	0.95		0.825		0.675		0.575		0.475		0.386		0.243		0.254	
Decrease	-		13%		29%		39%		50%		59%		74%		73%	

I Total mg. CO_2 evolved
II Mg. CO_2 evolved per 10 minutes

In Table 16 the data are given for the CO_2 production by dried yeast in the presence of 12 different concentrations of ethanol. The basic reaction mixture for dried yeast was the same as that for zymin except that 5 grams of dried yeast were used in place of 6 grams of zymin.

Table 16

Effect of Ethanol on CO_2 Production* by Dried Yeast

Minutes	Con-trol	Ethanol Concentration (Per cent by volume)											
		1.86	3.65	5.38	7.05	8.66	10.2	11.7	13.1	14.5	15.9	17.2	18.4
10	24	10	5	2	3	3	2	3	2	0	0	0	0
30	62	52	43	38	35	31	26	26	22	21	13	9	6
70	130	117	106	95	89	76	67	64	58	51	40	34	28
Average Rate 30-70 Minutes	1.70	1.63	1.58	1.42	1.35	1.13	1.03	.95	.90	.75	.70	.63	.55
Decrease	-	4%	7%	16%	20%	33%	39%	44%	47%	56%	59%	63%	68%

* Mg. CO_2 evolved

It is apparent that dried yeast is not nearly as susceptible to inactivation by ethanol as is zymin. This fact is clearly shown by inspection of Table 17, in which is summarized the effect of ethanol on living yeast, yeast juice, zymin and dried yeast.

Table 17

Relative Rates of CO_2 Production

in Presence of Ethanol

% Ethanol+	Yeast*	% Ethanol+	Zymin	% Ethanol+	Dried Yeast	% Ethanol+	Yeast Juice**
0	100	0	100	0	100	0	100
4.77	70.4	.38	87	1.86	96	6	80
4.93	60	.94	71	3.65	93	14	25
7.40	38.3	1.31	61	5.38	84	-	-
9.55	24.3	-	-	-	-	-	-
9.70	29.4	1.86	50	7.05	80	-	-
9.86	27.2	2.59	41	8.66	67	-	-
11.92	15.7	3.65	26	10.2	61	-	-
12.15	13.4	4.70	27	11.7	56	-	-
12.5	7.1	-	-	13.1	53	-	-
13.12	14.3	-	-	14.5	44	-	-
13.35	15.0	-	-	15.9	41	-	-
14.31	12.1	-	-	17.2	37	-	-
-	-	-	-	18.4	32	-	-

* Data of Rahn (1929)

** Data given by Harden (1932)

\+ Per cent by volume

Ethanol has about the same effect on dried yeast as it has on yeast juice. The relative rates given in the table for dried yeast and zymin are based upon the average rates in mg. CO_2 evolved per minute, over the 30 to 70 minute period of the reaction. As shown by Table 15, the rate is practically constant during this interval in a normal fermentation. These relative rates are strictly comparable to those given for living yeast by Rahn (1929), who based his figures upon the increase in pressure caused through the production of carbon dioxide by yeast in sugar solutions, and recorded as the average number of millimeters of mercury per minute during the first hour. The data for yeast juice given by Harden are the per cent decrease in the total fermentation (length of time not given), but the figures given in Table 17 should be very close to those based upon the rate after the steady state is reached.

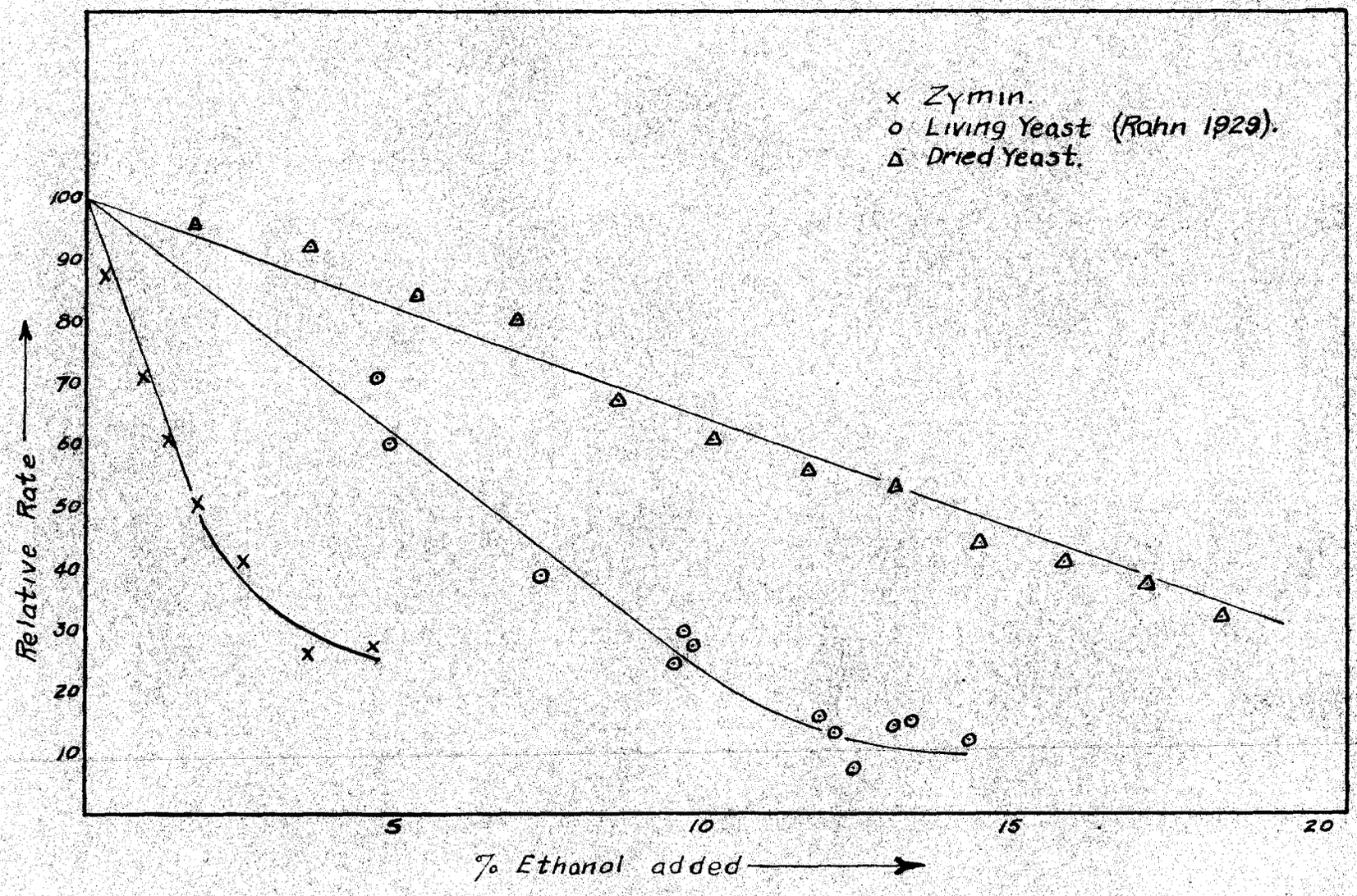


Fig 2.

Effect of Ethanol on Relative Rate of CO_2 Production.

Since dried yeast is affected by ethanol even less than living yeast, whereas zymin is very sensitive to ethanol, it appeared likely that some factor which protects the enzymes had been extracted in the preparation of the zymin. The acetone with which the yeast had been treated was distilled and evaporated on a steam bath, yielding a gummy brown residue, A. A portion of the residue A was extracted with ether in a Soxhlet extractor for 8 hours, thus separating it into two fractions, a fine brown powder, B, and a fraction present in the ether extract. Evaporation of a part of this extract resulted in an ill-smelling oily residue, C. Neither A, B, nor C had any protective action against ethanol when added to a fermentation flask.

Zymin was placed in a portion of the ether extract, and the mixture evaporated to dryness with constant stirring at room temperature, thus coating the zymin with the fatty substances. This "fatty" zymin, however, was as susceptible to ethanol as untreated zymin. In some cases a slight decrease in sensitivity was noted, but too little to be significant.

The acetone residue was shown to contain bios, as it increased the yeast count several hundred per cent when added to freshly inoculated yeast culture growing in a synthetic medium.

Table 18 gives data on the effect of ethanol on CO_2 evolution when phosphate was added at the beginning.

Table 18

Effect of Ethanol on CO_2 Production

by Zymin in Presence of Phosphate

Minutes	Control		.06 M. Phosphate		.06 M. Phosphate .38% Ethanol		.06 M. Phosphate .75% Ethanol	
	I	II	I	II	I	II	I	II
10	15	15	39	39	22	22	17	17
20	26	11	82	43	64	42	54	37
30	37	11	102	20	86	22	79	25
40	47	10	121	19	102	16	94	15
50	57	10	137	16	117	15	107	13
60	66	9	154	17	131	14	120	13
70	75	9	169	15	142	11	131	11
80	83	8	181	12	154	12	143	12
90	91	8	193	12	165	11	154	11
Average Rate 0-30 Minutes	1.23		3.40		2.87		2.63	
Decrease	-		0%		15.6%		22.6%	
Average Rate 30-90 Minutes	0.90		1.52		1.32		1.25	
Decrease	-		0%		13.1%		17.8%	

I Total mg. CO_2 evolved
II Mg. CO_2 evolved per 10 minutes

The same diminution of enzymatic activity is apparent as in the case of the normal reaction. Addition of phosphate does not overcome the ethanol inactivation. In the light of these results, the explanation presents itself that ethanol interferes with the enzyme mechanism which brings about esterification of inorganic phosphate, and hence decreases the amount of CO_2 evolved. Further evidence will be presented later which supports this conclusion.

Ethanol in presence of salts

Table 19 shows the effect of adding NH_4Cl to fermentations inactivated by ethanol.

Table 19

Effect of NH_4Cl on CO_2 Production by Zymin in Presence of Ethanol

Minutes	1.86% Ethanol								2.59% Ethanol			
	Control		.112N. NH_4Cl		.15N. NH_4Cl		.224N. NH_4Cl		Control		.224N. NH_4Cl	
	I	II	I	II	I	II	I	II	I	II	I	II
10	2	2	7	7	8	8	5	5	0	-	2.5	2.5
20	7	5	14	7	15	7	10	5	4.6	4.6	6.0	3.5
30	11	4	21	7	22	7	16	6	-	(4)	11.	5.
40	16	5	27	6	29	7	-	(6)	12.4	(4)	15.	4.
50	20	4	34	7	36	7	-	(6)	-	(4)	21.	6.
60	25	5	41	7	42	6	34	(6)	20.	(4)	26.	5.
70	30	5	48	7	49	7	40	6	24.	4.	31.	5.
Average Rate 30-70 Minutes	0.475		0.675		0.675		0.600		0.386		0.500	
Increase	-		42%		42%		26%		-		23%	
Relative Rate	50		71		71		63		41		53	

I Total mg. CO_2 evolved
II Mg. CO_2 evolved per 10 minutes

This salt causes a stimulation of 42 per cent in the constant rate of CO_2 evolution, more than twice as much activation as it produces in the normal fermentation. NH_4Cl seems to play a special role in overcoming inactivation by ethanol.

In Table 20 similar data are given for NH_4Cl and $MgSO_4$ with dried yeast.

Table 20

Effect of Salts on CO_2 Production by Dried Yeast in Presence of Ethanol

Minutes	5.38% Ethanol			10.2% Ethanol		
	Control	.135N. NH_4Cl	.034N. $MgSO_4$	Control	.135N. NH_4Cl	.034N. $MgSO_4$
10	4	16	6	3	9	4
30	40	50	44	30	38	35
50	68	81	73	50	62	60
70	95	112	102	71	87	84
Average Rate 0-30 Minutes	1.33	1.67	1.47	1.0	1.27	1.16
Increase	0%	18%	7.4%	0%	27%	16%
Average Rate 30-70 Minutes	1.37	1.55	1.45	1.02	1.23	1.23
Increase	0%	13.2%	5.8%	0%	20.6%	20.6%
Relative Rate	81	91	85	61	72.5	72.5

The influence of the salts on a dried yeast fermentation is similar to the effect on zymin.

Influence of Some Environmental Factors on the Phosphate Content

Autofermentation

When zymin is mixed with water, a slight amount of CO_2 is evolved as a result of fermentation of the carbohydrates present in the zymin. In Table 1 data are given which show that during autofermentation the inorganic phosphate content increases. There is evidently insufficient sugar present to bring about its esterification. The increase may be due to hydrolysis of organic phosphate by the enzyme phosphatase, to direct fermentation of organic phosphate, or simply to the passage of inorganic phosphate into solution through the walls of the dead yeast cells present in the zymin.

It was thought advisable to investigate the effect of several of the salts, which stimulated CO_2 production, on the phosphate content during autofermentation. In Table 21 are given the data for NH_4Cl, $MgSO_4$ and Na_2AsO_4. The latter salt was investigated since it has a profound activating action on zymase, and was thought by Harden and Young (1911,1) to stimulate phosphatase. The basic reaction mixture, without glucose, was used to obtain these data.

Table 21

Influence of Salts on Phosphate Content* During Autofermentation

Minutes	Control	.075N. NH_4Cl	.034N. $MgSO_4$	.007N. Na_3AsO_4**
5	1.13	1.12	1.20	1.49
30	1.31	1.28	1.31	1.91
60	1.41	1.38	1.51	2.00
90	1.50	1.46	-	2.04
120	1.59	1.50	1.75	2.16
150	1.73	1.60	1.82	2.06

* Data are mg. inorganic phosphorus per cc.
** Larger because $AsO_4^{\equiv}$ causes a blue coloration

The effect of ethanol is summarized in Table 22. Concentrations of ethanol up to 8.65 per cent had the same influence, causing about the same decrease in the phosphate content.

Table 22

Influence of Ethanol on Phosphate Content* During Autofermentation

Minutes	Control	% Ethanol		
		1.85	4.5	8.65
5	1.13	1.13	1.16	1.14
30	1.31	1.30	1.31	1.30
60	1.41	1.37	1.35	1.35
90	1.50	1.46	1.40	1.41
120	1.59	1.46	1.46	1.45
150	1.73	1.50	1.56	1.50

* Data are mg. inorganic phosphorus per cc.

The data of Tables 21 and 22 are summarized in Table 23, which shows that ethanol and NH_4Cl reduce the liberation of inorganic phosphate slightly, while $MgSO_4$ and Na_2AsO_4 increase it.

Table 23

Summary of Tables 21 and 22

	Increase in P* 5-120 Minutes*	Variation from Control
Control	0.46	0
1.85% Ethanol	0.33	-0.13
4.5% Ethanol	0.30	-0.16
8.65% Ethanol	0.31	-0.15
.075N. NH_4Cl	0.38	-0.08
.034N. $MgSO_4$	0.55	+0.09
.007N. Na_3AsO_4	0.67	+0.21

* Mg. inorganic phosphorus per cc.

Interpretation of these results is difficult. In the presence of $MgSO_4$ the phosphate content is markedly increased, especially in the first few minutes, whereas the effect of NH_4Cl is just the opposite. This suggests that these two salts may influence zymin fermentation in different ways.

Meyerhof (1927) has shown that the effect of arsenate on alcoholic fermentation is to stimulate direct fermentation of hexose diphosphate. This would bring about an increase in phosphate content, and is the probable cause of the increase brought about by arsenate noted in Table 23.

Ammonium chloride

The data obtained thus far in the investigation indicate that salts in the proper concentration are activators for some part of the zymase enzyme system. It was desired to find out what effect salts may have upon esterification of phosphate by studying changes in the inorganic phosphate content during a normal fermentation by zymin. Six gms. zymin, 1 gm. glucose, 25 cc. H_2O and 1.0 cc. toluene were mixed and the phosphate content determined at various time intervals. With this mixture no induction period is observed. The inorganic phosphate disappeared quite rapidly and reached a minimum value within 30 minutes. For this reason such a reaction mixture is not suitable for studying the influence of salts. Very little difference could be detected in the analysis when salts were added except in the case of NH_4Cl. In Table 24 the milligrams of inorganic phosphorus per cc. for the control and .075N. NH_4Cl are given.

Table 24

Influence of NH_4Cl on Phosphate Content

Without Induction Period

	5 Minutes	30 Minutes
Control	.73	.26
.075N. NH_4Cl	.68	.15

The data indicate that NH_4Cl activates the enzymes causing esterification of phosphate by sugar.

The same experiments were repeated with a concentration of 2.5 gms. of zymin instead of 6 gms. In this case an induction period occurred, as far as CO_2 production is concerned, and phosphorus disappeared in the control more slowly. Table 25 contains the data for this experiment, giving the inorganic phosphate content and the pH change.

Table 25

Influence of NH_4Cl on Phosphate Content* of Zymin

Minutes	Control		.056N. NH_4Cl	
	Mg. P per cc.	pH	Mg. P. per cc.	pH
5	0.38	6.58	0.37	6.42
10	0.38	-	0.41	-
20	0.42	-	0.41	-
30	0.46	6.45	0.29	6.17
60	0.19	6.27	0.06	6.12
90	0.09	6.23	-	6.12

* Mg. inorganic phosphorus per cc.

With the smaller amount of zymin used the reaction mixture had a smaller buffer capacity, as the NH_4Cl decreased the pH

0.1 to 0.3 of a unit. The same rapid decrease in inorganic phosphate occurred in the presence of NH_4Cl, however, as was observed when the larger amount of zymin was used.

Hydrogen ion concentration

The question arose as to whether the more rapid esterification of inorganic phosphate was due merely to the decrease in the pH of the reaction mixture caused by the presence of NH_4Cl. A series of flasks were prepared containing varying amounts of 0.1 N. HCl and 0.1 N. NaOH in a total added volume of 25 cc. The change in phosphate content and pH values of these were determined and the data tabulated in Table 26.

Table 26

Influence of pH* on Phosphate Content

Minutes	pH	Mg.P per cc.	pH**	Mg.P per cc.	pH	Mg.P per cc.	pH	Mg.P per cc.	pH	Mg.P per cc.	pH	Mg.P per cc.
5	6.91	0.39	6.58	0.38	6.31	0.38	6.00	0.38	5.60	0.40	4.36	0.38
30	6.71	0.47	6.45	0.47	6.20	0.43	6.03	0.42	5.80	0.45	4.39	0.41
60	6.49	0.27	6.27	0.18	6.12	0.20	6.02	0.27	5.88	0.30	4.44	0.45
90	6.37	0.13	6.23	0.09	6.16	0.11	6.06	0.21	5.88	0.25	4.48	0.48

* pH varied by addition of .1N. HCl and .1N. NaOH
** Control

It will be noted that the phosphate disappeared most rapidly in the control flask. The optimum pH for phosphate disappearance

is, then, 6.2 to 6.4. This agrees with the value 6.3 given by Hagglund (1926) for the pH optimum for phosphatese action. When enough 0.1 N. HCl was added to produce pH values comparable with those when NH_4Cl is present, a slight decrease in rate of esterification is observed. It would seem, therefore, that the effect produced by NH_4Cl is not due to the change in pH that this salt brings about. If the data are compared with those obtained without NH_4Cl, but with a comparable pH, there is an even greater difference.

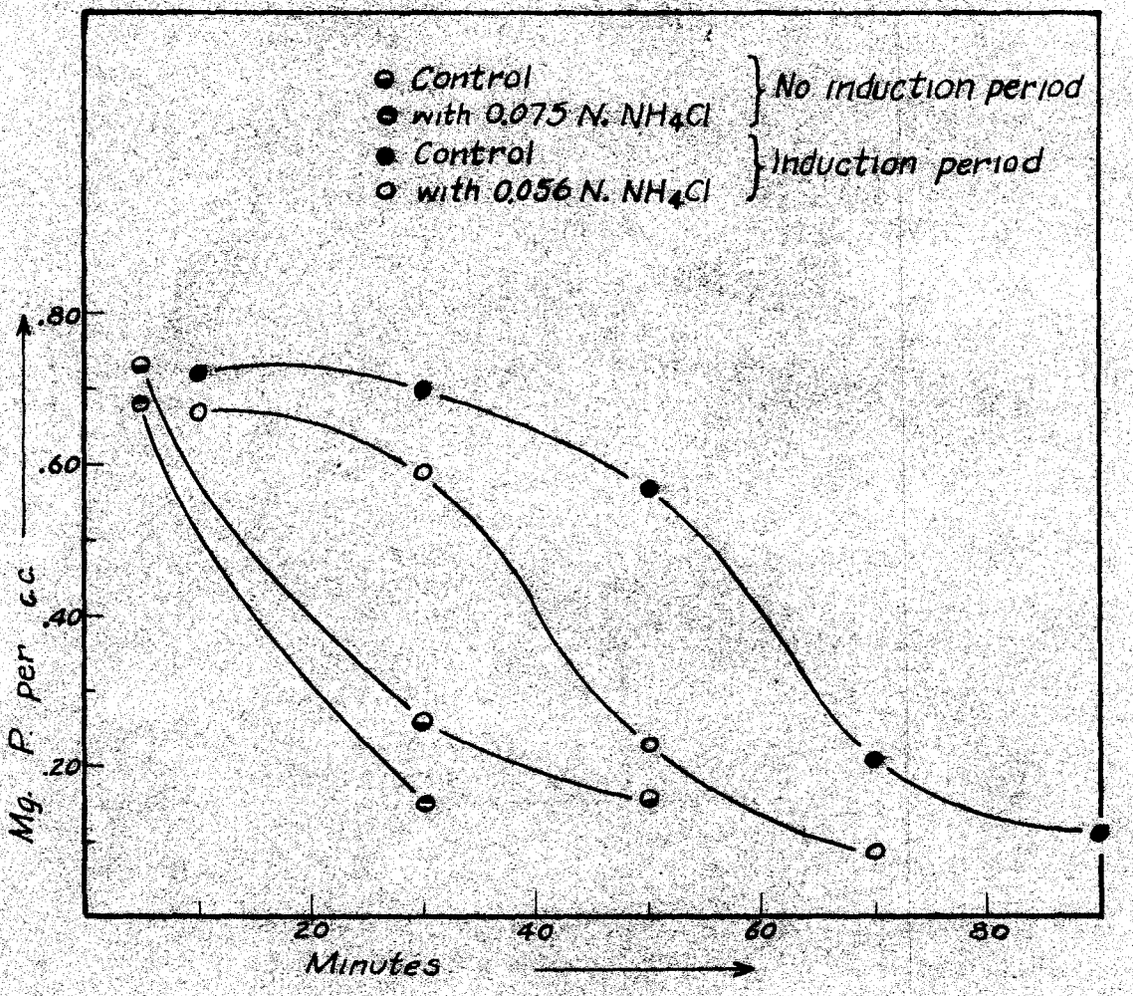


Fig 3.

Effect of NH_4Cl on Inorganic Phosphate Content of a Zymin Fermentation Mixture.

Other salts

Table 27 contains data showing the influence of other salts on the inorganic phosphate content.

Table 27

Influence of Salts* on Phosphate Content**

Minutes	Control	NH_4Cl	$CaCl_2$	$MgSO_4$	KCl	NaCl
10	0.72	0.67	0.66	0.64	0.68	0.68
30	0.70	0.59	0.68	0.56	0.64	0.66
50	0.57	0.23	0.54	0.28	0.37	0.31
70	0.21	0.09	0.23	0.10	0.12	0.10
Decrease 0-50 Minutes+	.17	.51	.20	.46	.37	.43
Decrease 0-70 Minutes+	.53	.65	.51	.64	.62	.64

* All salts .056 Normal
** Mg. inorganic phosphorus per cc.
\+ A zero value of .74 is assumed

Each flask contained 2.5 gms. zymin, 1 gm. glucose, 1 cc. toluene, 25 cc. H_2O containing about 0.3 mg. phosphorus per cc. and the amount of salt necessary to make the solution 0.056 N. During the first 50 minutes of the fermentation the potency of the salts in reducing the phosphate content is:

$$NH_4^+ > 1/2\ Mg^{++} > Na^+ > K^+ > 1/2\ Ca^{++} > \text{control}$$

The same order is obtained for phosphate decrease in the first 70 minute period, but with less variation among the salts, because KCl and NaCl exert more influence during the latter part of the period. The order of the salts given above is in agreement with that given for increase of CO_2 production on page 37, and also with that given on page 47 for the shortening of the induction period.

A series of RNH_3Cl compounds

Hixon and Johns (1927) have developed a theory on the "electron-sharing ability" of organic radicals. Experiments based upon thermodynamical measurements justify the generalization that organic radicals can be characterized and arranged in a series according to their thermodynamic "affinity", or quantity which determines the equilibrium constant for a reaction involving the radical in question. Craig (1933) has attempted to correlate "electron-sharing ability" with toxicity of a series of N-methylpyrrolidines toward insects, goldfish and tadpoles.

Another opportunity for testing the theory biologically presented itself in this investigation. NH_4Cl has a profound effect on the inorganic phosphate content of a zymin reaction mixture. If a hydrogen atom in NH_4Cl is replaced by an organic radical, R, an amine hydrochloride results, which should effect the phosphate content to a different extent than NH_4Cl.

If a series of RNH_2Cl compounds be investigated, the radicals might be arranged in the order of their potency for reducing the phosphate content, and it was desired to discover whether this order would be the same as that obtained from the "electron-sharing" series.

Three radicals were chosen (besides R = H), methyl, glucosyl, and phenyl, whose "negativity" or "electron-sharing ability" increases in the order named. These radicals are relatively far apart in the Hixon series. Putting it in another way, electron-sharing ability runs parallel to the dissociation constant of the amine, RNH_2. For the amines containing these radicals, the values for the $K_{B(H_2O)}$ are CH_3NH_2 = 5×10^{-4}, HNH_2 = 1.8×10^{-5}, $C_6H_{11}O_5NH_2$ = 10^{-7} (approximately) and $C_6H_5NH_2$ = 5×10^{-10}.

Table 28 contains the data for the effect of these amine hydrochlorides on the phosphate content. The reaction mixture contained 2.5 gms. zymin, 1.0 cc. toluene, the amine hydrochloride, and 25 cc. of phosphate solution containing about .3 mg. phosphorus per cc.

Table 28

Comparison of Influence of RNH_3Cl Compounds on Phosphate Content* of Zymin

Minutes	Control	.056 M. NH_4Cl	.056 M. CH_3NH_3Cl	Control**	.056 M. $C_6H_{11}O_5NH_3Cl$**
10	0.72	0.67	0.71	0.70	0.69
30	0.70	0.59	0.62	0.71	0.67
50	0.57	0.23	0.33	0.59	0.47
70	0.21	0.09	0.13	0.38	0.14
90	0.11	-	-	0.28	-
Decrease+ 0-70 Minutes	0.53	0.65	0.61	0.36	0.60
Deviation from Control	-	+ .12	+ .08	-	+ .24

* Mg. inorganic phosphorus per cc.
** pH = 6.0 at 70 minutes
\+ Zero value of .74 mg. is assumed

Aniline hydrochloride is missing from Table 28 because hydrolysis of the compound reduced the pH of the reaction mixture to 4.6, and an increase in phosphate was observed over the 90 minute period. The flask with $C_6H_{11}O_5NH_3Cl$ is compared with a control containing the amount of HCl added necessary to bring the pH down to a comparable value of about 6. CH_3NH_3Cl has little effect on the pH of the medium, as is to be expected

from the dissociation constant of CH_3NH_2. The NH_4Cl reduces the pH only about 0.1 of a unit (see Table 26). These two compounds are compared with the control to which pure water had been added.

When glucosyl NH_2Cl was added without glucose, the phosphate gradually increased to about the extent to be expected in autofermentation. This is taken to mean that the compound is not itself esterified by phosphate. The data show the efficacy of R in the RNH_2Cl compounds for reducing phosphate concentration to be in the order:

$$C_6H_{11}O_5 > H > CH_3$$

This is the order of increasing dissociation constants of the corresponding amines, and also the order of the radicals in the "electron-sharing" series.

Ethanol

Table 29 contains data for the influence of ethanol upon the inorganic phosphate content of fermenting zymin. Six gms. zymin, 1 gm. glucose, 1.0 cc. toluene, and 25 cc. of ethanol-water solution comprised the reaction mixture. Pure water was substituted for the ethanol solution in the control.

Table 29

Influence of Ethanol on Phosphate Content* of Zymin A

Minutes	Control	Per cent Ethanol 1.3	2.58	3.48	4.35
5	0.57	0.57	0.58	0.62	0.60
30	0.27	0.33	0.42	0.44	0.47
60	0.29	0.31	0.34	0.43	0.44
90	0.28	0.33	0.35	0.46	0.48
120	0.30	0.38	-	0.45	0.51
150	0.33	0.41	-	0.53	0.56

* Mg. inorganic phosphorus per cc.

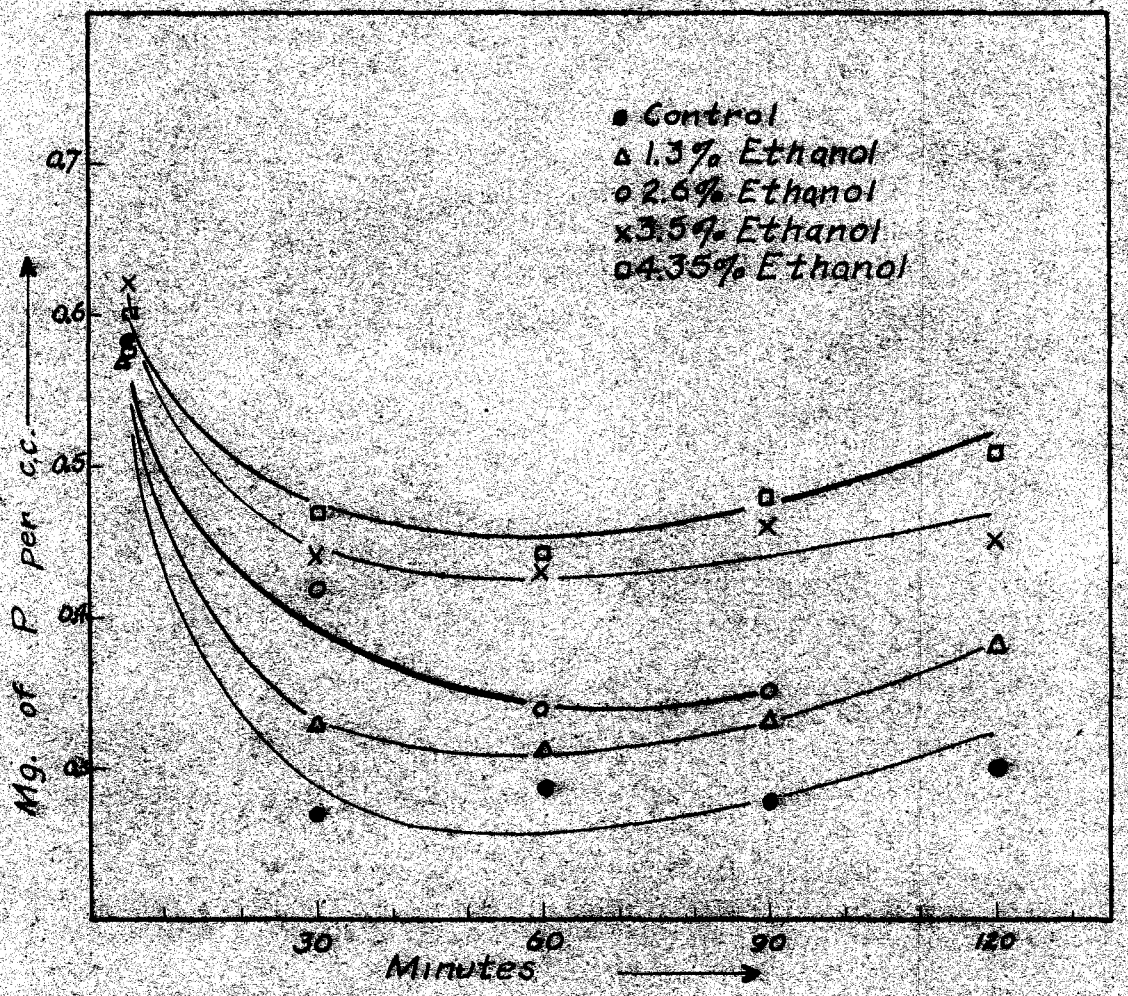


Fig. 4.

Effect of Ethanol on Inorganic Phosphate Content of a Zymin Fermentation Mixture.

In Table 30 the data are given for the influence of ethanol upon another batch of zymin, which is seen to be even more susceptible to ethanol than the zymin used in Table 29.

Table 30

Influence of Ethanol on Phosphate Content* of Zymin B

Minutes	Control	Per cent Ethanol .475	.95	2.85	4.75	7.6
5	0.73	0.84	0.80	0.79	0.81	0.80
30	0.26	0.80	0.83	0.87	0.97	1.03
50	0.16	0.32	0.59	0.80	1.01	1.02
75	0.21	0.21	0.31	0.60	0.92	1.01

* Mg. inorganic phosphorus per cc.

The conclusion seems justified that the effect of ethanol on zymin is to seriously interfere with the enzyme or enzymes which bring about the esterification of phosphate. As seen from the control in Table 29, the phosphate content reached a minimum value in about 30 minutes and stayed almost constant in the period 30 to 90 minutes. When ethanol was added less phosphate was esterified, and the minimum phosphate content became progressively greater, the more ethanol present. This explains why ethanol reduces CO_2 production by zymin to such a marked extent.

It has been shown that dried yeast is much less susceptible to ethanol than zymin, as far as CO_2 production is concerned. Ethanol should correspondingly interfere less with esterification of inorganic phosphate in a dried yeast fermentation than it does in a zymin mixture. The data of Table 31 show this to be the case. The reaction mixture consisted of 5 gms. dried yeast, 1.0 gm. glucose, 25 cc. of water or water-ethanol solution.

Table 31

Influence of Ethanol on Phosphate Content* of Dried Yeast

		Per cent Ethanol	
Minutes	Control	2.85	7.6
10	0.47	0.68	0.78
30	0.10	0.11	0.19
50	0.09	0.10	0.09
75	0.10	0.10	0.10

* Mg. inorganic phosphorus per cc.

The conclusions made on the basis of CO_2 production that zymin is more susceptible to ethanol than dried yeast seem entirely justified by these data.

Salts in presence of ethanol

It has been shown in Table 19 that NH_4Cl has a greater influence on increasing CO_2 production in the presence of ethanol than in its absence. The increase in steady rate brought about by NH_4Cl amounted to 42 per cent in the presence of ethanol, and 19 per cent when no ethanol was added. The influence of NH_4Cl and other salts on the phosphate content of fermenting zymin in the presence of ethanol is summarized in Table 32. The control flask contained 6 gms. zymin, 1.0 gm. glucose, 1.0 cc. toluene, and 25 cc. of .95 per cent (by volume) ethanol solution. The other flasks differed only as to the presence of a salt.

Table 32

Influence of Salts on Phosphate Content* of Zymin in Presence of .95 Per cent Ethanol

Minutes	Control	.075N. NH_4Cl	.034N. $MgSO_4$+	.075N. $MgSO_4$	.022N. $CaCl_2$+	.075N. $CaCl_2$	.075N. NaCl	.075N. KCl
5	0.81	0.81	0.85	0.78	0.78	0.75	0.80	0.76
30	0.87	0.75	0.88	0.85	0.80	0.59	0.80	0.80
50	0.57	0.30	0.63	0.71	0.50	0.51	0.49	0.55
75	0.27	0.19	0.30	0.33	0.27	0.27	0.20	0.24
100	0.27	0.18	0.29	0.32	0.26	0.26	0.20	0.25
130	0.30	0.22	0.34	-	0.30	0.27	0.22	0.26
Steady State Value	0.27	0.185	0.295	0.325	0.265	0.265	0.20	0.245
Deviation from Control	-	- .085	+ .025	+ .055	- .005	- .005	- .07	- .025

* Mg. inorganic phosphorus per cc.
\+ Optimum for CO_2 production without ethanol

NH_4Cl has the ability to increase the amount of phosphate esterified in the presence of ethanol, reducing the steady state value of phosphate content from 0.27 to 0.185 mg. phosphorus per cc. Other salts have the same effect to a greater or less degree. The order of potency of the cations to increase esterification is:

$$NH_4^+ > Na^+ > K^+ > 1/2\ Ca^{++} > 1/2\ Mg^{++}$$

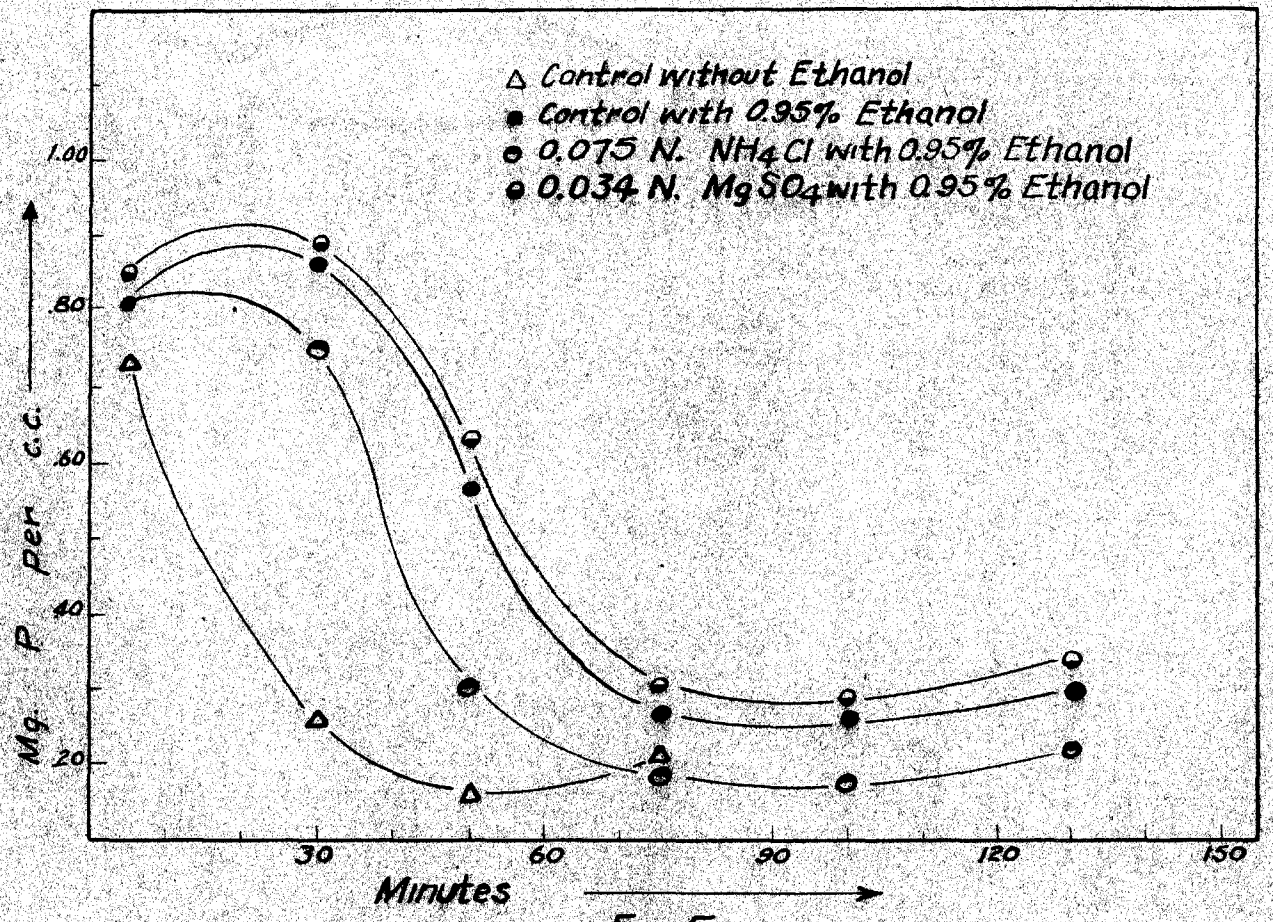


Fig. 5.

Effect of Salts on Inorganic Phosphate Content of a Zymin Fermentation Mixture in presence of .95% Ethanol

An interesting feature of these data is the difference in the effect of Mg^{++} and NH_4^+. These two salts are about equally potent in stimulating CO_2 production, and likewise they are equally potent in reducing the phosphate content without ethanol (Table 30). But as shown in Table 32, $MgSO_4$ increases the steady state phosphate content in the presence of ethanol, whereas NH_4Cl greatly decreases this value.

In view of this fact, NH_4Cl should stimulate CO_2 production by this zymin in the presence of ethanol much more than does $MgSO_4$. The data of Table 33 show that this is true.

Table 33

Influence of $MgSO_4$ and NH_4Cl on CO_2 Production* by Zymin+ in Presence of 1.9 Per cent Ethanol

Minutes	Control	.112 N. NH_4Cl	.034 N. $MgSO_4$
10	0	.7	0
30	1.4	2.1	.8
60	4.4	12.0	7.6
90	18.	38.0	21.0
Average Rate 30-90 Minutes	.28	.60	.34
Increase	0	114%	21%

* Mg. CO_2 evolved
\+ Same zymin preparation as used in obtaining data of Table 32

Thus, NH_4Cl increases the average rate of CO_2 evolution much more in the presence of ethanol than in its absence, while $MgSO_4$ increases the rate to about the same extent with or without ethanol. This fact correlates with the influence of the two salts on phosphate content, and is further evidence that NH_4Cl plays a special role in overcoming the depressing effect of ethanol.

DISCUSSION OF RESULTS

The accelerating agents for the zymase complex may be divided into three classes:

(1) Substances which have a chemical function in the reaction.

(2) Reducible substances, which probably act as hydrogen acceptors.

(3) Substances which do not enter into direct combination with the system or act as hydrogen acceptors.

Only inorganic phosphate, which is essential for fermentation by yeast preparations, has been shown to belong in class (1). In class (2) belong many reducible substances such as aldehydes, ketones, quinones, thiosulfates, colloidal sulfur, and ferric chloride. The work of Harden and Henley (1920) indicates that only the rate in the presence of phosphate is increased by reducible substances, the normal, basal rate being unaffected by them. Arsenates and arsenites have been found by Meyerhof (1927), Raymond (1928), and McFarlane (1930) to have an accelerating action upon the direct fermentation of hexosediphosphate, and belong in class (3). No other salts are given in the literature as having any influence on alcoholic fermentation other than inhibitory.

The "salt effect" as discussed by Harden and Henley has already been outlined (see page 18). He implies that, in

general, salts have a depressing action on esterification of phosphate and also on the liberation of organic phosphate, and hence decrease the basal rate of fermentation. In the light of the present investigation, this concept must be revised. If added in proper concentration, the salts NH_4Cl, $MgSO_4$, $CaCl_2$, KCl, and NaCl accelerate either the initial rate of fermentation (when inorganic phosphate is esterified and the rate attains a maximum value), or the normal steady rate (which is conditioned by the rate of liberation of bound phosphate), or both.

The conclusions of Harden and Henley (1921) were based upon results obtained with a limited range of concentration. As has been pointed out by Fulmer (1926) it is always necessary, in comparing the effect of given materials, to run each in a series. Harden and Henley based their salt theory only on experiments in which the salts were present in concentrations greater than the optimum values found in this investigation.

Of the salts studied, NH_4Cl, $MgSO_4$, and NaCl have an appreciable stimulating action upon the initial, maximum rate of fermentation, and can be considered as activators for that part of the enzyme complex which has to do with esterification of phosphate and glucose. NH_4Cl, $MgSO_4$ and $CaCl_2$ exert a marked influence on the normal, basal rate of fermentation, which depends on available phosphate, and hence are activators

for that part of the enzyme complex which has to do with liberation of organic phosphate. According to the older theories, that would involve only the enzyme phosphatase, which brings about liberation of bound phosphate by hydrolysis.

On the basis of the Meyerhof scheme (see page 13) for fermentation, the salts NH_4Cl, $MgSO_4$, NaCl and KCl, which increase the initial rate, activate reaction A. Since NH_4Cl and $MgSO_4$ influence both phases of the fermentation it may be supposed that they affect reactions A and E, both of which involve esterification of hexose. NaCl and KCl, on the other hand, can accelerate only reaction A. NH_4Cl, $MgSO_4$ and $CaCl_2$ may activate reaction C, which liberates organic phosphate, and in this manner increase the steady rate of fermentation.

However, these conclusions must be regarded as somewhat speculative. Further work is necessary to establish the conclusions as to the particular phases of fermentation influenced by the salts. By the use of the poisoning agents CH_2ICOOH and Na_2F_2, more data could be obtained on this point. But the data on hand are sufficient to justify the general conclusion that salts, in the proper concentrations, activate the fermentation of sugar by zymin.

It has been found that $MgSO_4$ and NH_4Cl both increase CO_2 production by zymin to about the same extent in the normal fermentation. Moreover, it was found that when the salts are added together there is little more increase in the rate of

CO_2 evolution than when only one is present. This could be interpreted to mean that the salts activate the enzymatic reactions similarly. However, there are three lines of evidence which indicate that the two salts play different roles:

(1) $MgSO_4$ slightly increases the available inorganic phosphate during autofermentation of zymin, while NH_4Cl slightly decreases it.

(2) $MgSO_4$ is unable to bring about a lowered minimum inorganic phosphate content in a zymin fermentation mixture inactivated by ethanol, while NH_4Cl markedly decreases this value.

(3) $MgSO_4$ increases the lowered rate of CO_2 evolution from a zymin fermentation mixture inactivated by ethanol to about the same extent that it does in the normal fermentation, while NH_4Cl increases the rate several times more in the presence of ethanol than it does in its absence.

In the normal fermentation the two salts apparently have the same effect, but in the presence of ethanol NH_4Cl has a much greater activating influence than $MgSO_4$. Therefore the two salts must affect the zymase enzyme complex in somewhat different ways. Further work is necessary to fully explain the difference between them.

It has been demonstrated that NH_4Cl decreases the time necessary for the inorganic phosphate content to reach a minimum value when the concentrations are such that no induction

period occurs, and that NH_4Cl, $MgSO_4$, KCl, and NaCl bring this about when an induction period takes place. Moreover the order of potency of the salts for decreasing the phosphate content agrees well with that for stimulating the rate of CO_2 production. Practically the same order was observed for the efficacy of salts for shortening the induction period. All this evidence points to a simple explanation of the salt effect on the induction period, namely, that salts activate the phosphate esterifying mechanism of zymase, reaction A of the Meyerhof scheme, and hence decrease the time necessary to reach reaction D, which liberates CO_2. In other words, addition of salts tends to increase hexosediphosphate concentration. Hexosediphosphate is the most effective substance known for shortening the induction period.

Salts are known to activate other enzymes. According to Haldane (1930) NaCl activates a group of amylases, and Doby and Feher (1931) found that several salts stimulate invertase. No adequate explanation for salt activation has been given. It has been assumed by the investigators that the salt increases the effective concentration or specific surface of the enzyme, or that the salt combines with either the substrate or the enzyme to form a complex which undergoes the reaction more readily, or that the salt alters the chemical potential of the substrate and resultants. These points should be studied.

Data contained in this thesis indicate that zymin is much

more sensitive to ethanol than is living yeast, dried yeast or yeast juice. Zymin is the only one of the zymase-containing materials from which any substance present in the living yeast cell has been removed. A probable explanation for the sensitivity of zymin is that some of the materials removed in its preparation normally protect the enzymes. Data on the phosphate content in the presence of ethanol indicate that the enzymes inactivated by it are those of the phosphate esterifying mechanism. Further evidence for the theory that protective substances are removed in preparing zymin arises from a comparison of the effect of ethanol on the inorganic phosphate content of zymin and dried yeast. The rate of disappearance of phosphate from the dried yeast fermentation mixture is hardly affected by ethanol in concentrations up to 7.6 per cent, whereas for zymin fermentation 2 or 3 per cent ethanol almost completely prevents esterification.

It was shown that addition of the materials extracted from yeast in the preparation of zymin to a zymin fermentation does not decrease the sensitivity toward alcohol. Superficially, this fact depreciates the validity of the theory outlined above. However, mere addition of the materials to the medium cannot insure returning the system to its original condition, especially since the added materials are partially insoluble in water. The fact that zymin is more sensitive to changes in the hydrogen ion concentration than is yeast juice is further

evidence that a fundamental change in the physico-chemical system takes place when zymin is prepared. It is possible that this sensitivity to pH is also bound up with the removal of a protective substance. In view of the evidence it must be concluded that the physico-chemical relationships of the enzyme system of zymin are different from those in living yeast, yeast juice, and dried yeast. This difference probably accounts for the sensitivity of zymin toward ethanol, hydrogen ion concentration, and glucose concentration.

SUMMARY AND CONCLUSIONS

1. Zymin is more active if prepared from yeast which has stood at refrigerator temperatures for several days.

2. The salts NH_4Cl, $MgSO_4$, NaCl and KCl, if present in appropriate concentrations, increase the initial, maximum rate of CO_2 production by zymin. For the chlorides and sulfates the cations increase the rate in the order

$$Mg^{++} = Na^{+} > NH_4^{+} > K^{+} > Ca^{++} = \text{control.}$$

3. The salts NH_4Cl, $MgSO_4$, NaCl, and $CaCl_2$ if present in appropriate concentrations increase the normal constant rate of CO_2 production by zymin in the order

$$NH_4^{+} > Mg^{++} > Ca^{++} > Na^{+} > K^{+} = \text{control.}$$

4. The increase in the rate of CO_2 production by zymin in the presence of NH_4Cl extends at least until the end of 240 minutes.

5. The salts NH_4Cl and $MgSO_4$ increase the rate of CO_2 production by zymin in the presence of added inorganic phosphate, but the effect of the salts is not additive.

6. The fermentative activity of zymin, as measured by the rate of CO_2 evolution, is 34 per cent greater in a 2 per cent glucose solution than in a 12 per cent solution. This is typical of enzyme reactions, but is a much greater variation than has been reported previously for fermentation by a yeast enzyme preparation.

7. The optimum pH for zymin activity is 5.8-6.2. Zymin is therefore more sensitive to hydrogen ion concentration than is living yeast, dried yeast, or yeast juice. This is approximately the pH value reported for the interior of a living yeast cell. The optimum pH for disappearance of inorganic phosphate from a zymin fermentation mixture is 6.2-6.4. This is the optimum value reported for the enzyme "phosphatese" in zymase, which is supposed to bring about esterification of sugar and phosphate.

8. All of the salts which increase the rate of CO_2 production shorten the induction period of zymin, or the time during which no CO_2 is evolved when the concentration of zymin is small. The potencies of the cations are in the order

$$NH_4^+ > 1/2\ Mg^{++} > Na^+ > K^+ > 1/2\ Ca^{++} > \text{control.}$$

9. Zymin is much more sensitive to inactivation by ethanol than is living yeast, dried yeast, or yeast juice. Addition of phosphate does not decrease this sensitivity. Addition of NH_4Cl causes a much greater percentage increase in the rate of CO_2 evolution in the presence of ethanol than it does in its absence. Addition of $MgSO_4$ causes about the same percentage increase in the rate of CO_2 evolution in the presence of ethanol that it does in its absence.

10. Salts markedly decrease the time which elapses before the inorganic phosphate content of a zymin fermentation reaches a minimum constant value. The potencies of the cations

are in the order

$$NH_4^+ > 1/2\ Mg^{++} > Na^+ > K^+ > \text{control} > 1/2\ Ca^{++}.$$

11. For a series of RNH_2Cl compounds the ability of the radicals R to decrease the time which elapses before the inorganic phosphate content reaches a minimum value is in the order

$$C_6H_{11}O_5 > H > CH_3.$$

This is the order of decreasing "electron-sharing ability", and also the order of increasing dissociation constants of the corresponding amines, RNH_2. Glucosyl amine hydrochloride is itself not esterified by phosphate.

12. During autofermentation of zymin the inorganic phosphate content increases with time. Any concentration of ethanol up to 8 per cent has the same effect, causing a slight decrease in the phosphate content. NH_4Cl also causes a slight decrease, and hence the stimulation of CO_2 production by this salt is not due to increasing the available phosphate. $MgSO_4$ and Na_2AsO_4 cause a slight increase in phosphate content.

13. Ethanol greatly increases the time necessary for esterification of inorganic phosphate in a zymin fermentation mixture and increases the minimum constant value of the inorganic phosphate content of a zymin fermentation mixture, but has little influence on a dried yeast fermentation.

14. In the presence of ethanol the salts NH_4Cl, NaCl, and KCl decrease the minimum, constant inorganic phosphate concentration in a zymin fermentation in the order

$NH_4^+ > Na^+ > K^+ >$ control = 1/2 $Ca^{++} >$ 1/2 Mg^{++}.

$MgSO_4$, on the other hand, slightly increases this minimum value.

15. On the basis of these observations it is suggested that the influence of salts is to activate that part of the zymase enzyme complex which has to do with the esterification of inorganic phosphate and sugar, and in some cases that part which has to do with the release of organic phosphate.

16. It is further suggested that ethanol seriously interferes with the phosphate esterifying mechanism of zymin, and hence with CO_2 production, because some protective substance is extracted in its preparation. This would also explain the greater sensitivity of zymin toward hydrogen ion concentration.

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