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ALCOHOLIC FERMENTATION

BY

ARTHUR HARDEN

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ALCOHOLIC FERMENTATION

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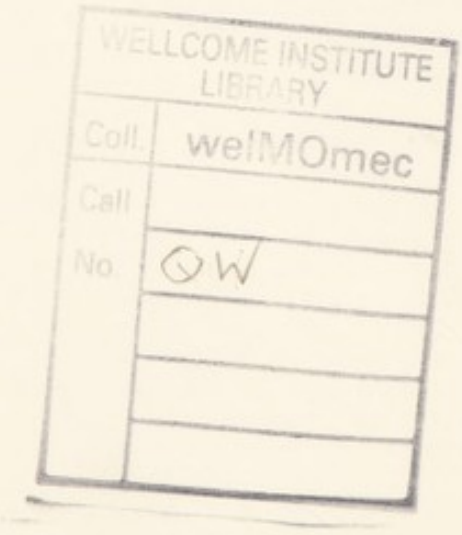

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PREFACE TO THE FOURTH EDITION.

THE amount of work on the subject of alcoholic fermentation which has appeared since the publication of the third edition of this book has been so great that it has been impossible to attempt to give a complete account of it, but it is hoped that nothing of fundamental importance has been overlooked.

I am indebted to my colleagues Dr. R. Robison, F.R.S. and Dr. M. G. Macfarlane for helpful criticism and to the latter also for invaluable assistance in preparing the manuscript for the press and in revising the proofs.

A. H.

November, 1931.

PREFACE.

THE following chapters are based on courses of lectures delivered at the London University and the Royal Institution during 1909-1910. In them an account is given of the work done on alcoholic fermentation since Buchner's epoch-making discovery of zymase, only in so far as it appears to throw light on the nature of that phenomenon. Many interesting subjects, therefore, have perforce been left untouched, among them the problem of the formation of zymase in the cell, and the vexed question of the relation of alcoholic fermentation to the metabolic processes of the higher plants and animals.

My thanks are due to the Council of the Royal Society, and to the Publishers of the "Journal of Physiology" for permission to make use of blocks which have appeared in their publications.

A. H.

CONTENTS.

CHAPTER I.

HISTORICAL INTRODUCTION.

The problem of alcoholic fermentation, of the origin and nature of that mysterious and apparently spontaneous change which converted the insipid juice of the grape into stimulating wine, seems to have exerted a fascination over the minds of natural philosophers from the very earliest times. No date can be assigned to the first observation of the phenomena of the process. History finds man in the possession of alcoholic liquors, and in the earliest chemical writings we find fermentation, as a familiar natural process, invoked to explain and illustrate the changes with which the science of those early days was concerned. Throughout the period of alchemy fermentation plays an important part; it is, in fact, scarcely too much to say that the language of the alchemists and many of their ideas were founded on the phenomena of fermentation. The subtle change in properties permeating the whole mass of material, the frothing of the fermenting liquid, rendering evident the vigour of the action, seemed to them the very emblems of the mysterious process by which the long-sought-for philosopher's stone was to convert the baser metals into gold. As chemical science emerged from the mists of alchemy, definite ideas about the nature of alcoholic fermentation and of putrefaction began to be formed. Fermentation was distinguished from other chemical changes in which gases were evolved, such as the action of acids on alkali carbonates (Sylvius de le Boë, 1659); the gas evolved was examined and termed gas vinorum, and was distinguished from the alcohol with which it had at first been confused (van Helmont, 1648); afterwards it was found that like the gas from potashes it was soluble in water (Wren, 1664). The gaseous product of fermentation and putrefaction was identified by MacBride, in 1764, with the fixed air of Black, whilst Cavendish in 1766 showed that fixed air alone was evolved in alcoholic fermentation and that a mixture of this with inflammable air was produced by putrefaction. In the meantime it had been recognised that

only sweet liquors could be fermented (" Ubi notandum, nihil fermentare quod non sit dulce," Becher, 1682), and finally Cavendish [1766] determined the proportion of fixed air obtainable from sugar by fermentation and found it to be 57 per cent. It gradually became recognised that fermentation might yield either spirituous or acid liquors, whilst putrefaction was thought to be an action of the same kind as fermentation, differing mainly in the character of the products (Becher).

As regards the nature of the process very confused ideas at first prevailed, but in the time of the phlogistic chemists a definite theory of fermentation was proposed, first by Willis (1659) and afterwards by Stahl [1697], the fundamental idea of which survived the overthrow of the phlogistic system by Lavoisier and formed the foundation of the views of Liebig. To explain the spontaneous origin of fermentation and its propagation from one liquid to another, they supposed that the process consisted in a violent internal motion of the particles of the fermenting substance, set up by an aqueous liquid, whereby the combination of the essential constituents of this material was loosened and new particles formed, some of which were thrust out of the liquid (the carbon dioxide) and others retained in it (the alcohol).

Stahl specifically states that a body in such a state of internal disquietude can very readily communicate the disturbance to another, which is itself at rest but is capable of undergoing a similar change, so that a putrefying or fermenting liquid can set another liquid in putrefaction or fermentation.

Taking account of the gradual accumulation of fact and theory we find at the time of Lavoisier, from which the modern aspect of the problem dates, that Stahl's theoretical views were generally accepted. Alcoholic fermentation was known to require the presence of sugar and was thought to lead to the production of carbon dioxide, acetic acid, and alcohol.

The composition of organic compounds was at that time not understood, and it was Lavoisier who established the fact that they consisted of carbon, hydrogen, and oxygen, and who made systematic analyses of the substances concerned in fermentation (1784-1789). Lavoisier [1789] applied the results of these analyses to the study of alcoholic fermentation, and by employing the principle which he regarded as the foundation of experimental chemistry, " that there is the same quantity of matter before and after the operation," he drew up an equation between the quantities of carbon, hydrogen, and oxygen in the original sugar and in the resulting substances, alcohol, carbon dioxide, and

acetic acid, showing that the products contained the whole matter of the sugar, and thus for the first time giving a clear view of the chemical change which occurs in fermentation. The conclusion to which he came was, we now know, very nearly accurate, but the research must be regarded as one of those remarkable instances in which the genius of the investigator triumphs over experimental deficiencies, for the analytical numbers employed contained grave errors, and it was only by a fortunate compensation of these that a result so near the truth was attained.

Lavoisier's equation or balance-sheet was as follows :—

	Carbon.	Hydrogen.	Oxygen.
95·9 pounds of sugar (cane sugar) consist of	26·8	7·7	61·4
These yield :—			
57·7 pounds of alcohol containing . .	16·7	9·6	31·4
35·3 ,, carbon dioxide containing	9·9	—	25·4
2·5 ,, acetic acid containing .	0·6	0·2	1·7
Total contained in products . . .	27·2	9·8	58·5

The true composition of the sugar used was carbon 40·4, hydrogen 6·1, oxygen 49·4.

Lavoisier expressed no view as to the agency by which fermentation was brought about, but came to a very definite and characteristic conclusion as to the chemical nature of the change. The sugar, which he regarded in harmony with his general views as an oxide, was split into two parts, one of which was oxidised at the expense of the other to form carbonic acid, whilst the other was deoxygenised in favour of the former to produce the combustible substance alcohol, " so that if it were possible to recombine these two substances, alcohol and carbonic acid, sugar would result."

From this point commences the modern study of the problem. Provided by the genius of Lavoisier with the assurance that the hitherto mysterious process of fermentation was to be ranked along with familiar chemical changes, and that it proceeded in harmony with the same quantitative laws as these simpler reactions, chemists were stimulated in their desire to penetrate further into the mysteries of the phenomenon, and the importance and interest of the problem attracted many workers.

So important indeed did the matter appear to Lavoisier's countrymen that in the year 8 of the French Republic (1800) a prize—consisting of a gold medal, the value of which, expressed in terms of the newly introduced metric system, was that of one kilogram of gold—was offered by the Institute for the best answer to the question :

"What are the characteristics by which animal and vegetable substances which act as ferments can be distinguished from those which they are capable of fermenting ? "

This valuable prize was again offered in 1802 but was never awarded, as the fund from which it was to be drawn was sequestrated from the Institute in 1804. The first response to this stimulating offer was an important memoir by citizen Thenard [1803], which provided many of the facts upon which Liebig subsequently based his views. Thenard combats the prevailing idea, first expressed by Fabroni (1787-1799), that fermentation is caused by the action of gluten derived from grain on starch and sugar, but is himself uncertain as to the actual nature of the ferment. He points out that all fermenting liquids deposit a material resembling brewer's yeast, and he shows that this contains nitrogen, much of which is evolved as ammonia on distillation. His most important result is, however, that when yeast is used to ferment pure sugar it undergoes a gradual change and is finally left as a white mass, much reduced in weight, which contains no nitrogen and is without action on sugar. Thenard, moreover, it is interesting to note, differs from Lavoisier, inasmuch as he ascribes the origin of some of the carbonic acid to the carbon of the ferment, an opinion which was still held in various degrees by many investigators (see Seguin, quoted by Thenard).

Thenard's memoir was followed by a communication of fundamental importance from Gay-Lussac [1810]. A process for preserving food had been introduced by Appert, which consisted in placing the material in bottles, closing these very carefully and exposing them to the temperature of boiling water for some time. Gay-Lussac was struck by the fact that when such a bottle was opened fermentation or putrefaction set in rapidly. Analysis of the air left in such a sealed bottle showed that all the oxygen had been absorbed, and these facts led to the view that fermentation was set up by the action of oxygen on the fermentable material. Experiment appeared to confirm this in the most striking way. A bottle of preserved grape-juice was opened over mercury and part of its contents passed through the mercury into a bell-jar containing air, the remainder into a similar vessel free from air. In the presence of air fermentation set in at once, in the absence of air no fermentation whatever occurred. This connection between fermentation and the presence of air was established by numerous experiments and appeared incontestable. Fermentation, it was found, could be checked by boiling even after the addition of oxygen, and hence food could be preserved in free

contact with the air, provided only that it was raised to the temperature of boiling water at short intervals of time. Gay-Lussac's opinion was that the ferment was formed by the action of the oxygen on the liquid, and that the product of this action was altered by heat and rendered incapable of producing fermentation, as was also brewer's yeast, which, however, he regarded, on account of its insolubility, as different from the soluble ferment which initiated the change in the limpid grape-juice. Colin, on the other hand [1825], recognised that alcoholic fermentation by whatever substance it was started, resulted in the formation of an insoluble deposit more active than the original substance, and he suggested that this deposit might possibly in every case be of the same nature.

So far no suspicion appears to have arisen in the minds of those who had occupied themselves with the study of fermentation that this change differed in any essential manner from many other reactions familiar to chemists. The origin and properties of the ferment were indeed remarkable and involved in obscurity, but the uncertainty regarding this substance was no greater than that surrounding many, if not all, compounds of animal and vegetable origin. Although, however, the purely chemical view as to the nature of yeast was generally recognised and adopted, isolated observations were not wanting which tended to show that yeast might be something more than a mere chemical reagent. As early as 1680 in letters to the Royal Society Leeuwenhoek described the microscopic appearance of yeast of various origins as that of small, round, or oval particles, but no further progress seems to have been made in this direction for nearly a century and a half, when we find that Desmazières [1826] examined the film formed on beer, figured the elongated cells of which it was composed, and described it under the name of Mycoderma Cerevisiae. He, however, regarded it rather as of animal than of vegetable origin, and does not appear to have connected the presence of these cells with the process of fermentation.

Upon this long period during which yeast was regarded merely as a chemical compound there followed, as has so frequently occurred in similar cases, a sudden outburst of discovery. No less than three observers hit almost simultaneously upon the secret of fermentation and declared that yeast was a living organism.

First among these in strict order of time was Cagniard-Latour [1838], who made a number of communications to the Academy and to the Société Philomatique in 1835-6, the contents of which were collected in a paper presented to the Academy of Sciences on 12 June,

1837, and published in 1838. The observations upon which this memoir was based were almost exclusively microscopical. Yeast was recognised as consisting of spherical particles, which were capable of reproduction by budding but incapable of motion, and it was therefore regarded as a living organism probably belonging to the vegetable kingdom. Alcoholic fermentation was observed to depend on the presence of living yeast cells, and was attributed to some effect of their vegetative life (quelque effet de leur végétation). It was also noticed that yeast was not deprived of its fermenting power by exposure to the temperature of solid carbonic acid, a sample of which was supplied to Cagniard-Latour by Thilorier, who had only recently prepared it for the first time.

Theodor Schwann [1837], whose researches were quite independent of those of Cagniard-Latour, approached the problem from an entirely different point of view. During the year 1836 Franz Schulze [1836] published a research on the subject of spontaneous generation, in which he proved that when a solution containing animal or vegetable matter was boiled, no putrefaction set in provided that all air which was allowed to have access to the liquid was previously passed through strong sulphuric acid. Schwann performed a very similar experiment by which he showed that this same result, the absence of putrefaction, was attained by heating all air which came into contact with the boiled liquid. Wishing to show that other processes in which air took part were not affected by the air being heated, he made experiments with fermenting liquids and found, contrary to his expectation, that a liquid capable of undergoing vinous fermentation and containing yeast did not undergo this change after it had been boiled, provided that, as in the case of his previous experiments, only air which had been heated was allowed to come into contact with it.

Schwann's experiments on the prevention of putrefaction were unexceptionable and quite decisive. The analogous experiments dealing with alcoholic fermentation were not quite so satisfactory. Yeast was added to a solution of cane sugar, the flask containing the mixture placed in boiling water for ten minutes, and then inverted over mercury. About one-third of the liquid was then displaced by air and the flasks corked and kept inverted at air temperature. In two flasks the air introduced was ordinary atmospheric air, and in these flasks fermentation set in after about four to six weeks. Into the other two flasks air which had been heated was led, and in these no fermentation occurred. As described, the experiment is quite satisfactory, but

Schwann found on repetition that the results were irregular. Sometimes all the flasks showed fermentation, sometimes none of them. This was correctly ascribed to the experimental difficulties, but none the less served as a point of attack for hostile and damaging criticism at the hands of Berzelius (p. 8).

The origin of putrefaction was definitely attributed by Schwann to the presence of living germs in the air, and the similarity of the result obtained with yeast suggested the idea that alcoholic fermentation was also brought about by a living organism, a conception which was at once confirmed by a microscopical examination of a fermenting liquid. The phenomena observed under the microscope were similar to those noted by Cagniard-Latour, and in accordance with these observations alcoholic fermentation was attributed to the development of a living organism, the fermentative function of which was found to be destroyed by potassium arsenite but not by extract of Nux vomica, so that the organism was regarded rather as of vegetable than of animal nature. This plant received the name of " Zuckerpilz " or sugar fungus (which has been perpetuated in the generic term Saccharomyces). Alcoholic fermentation was explained as " the decomposition brought about by this sugar fungus removing from the sugar and a nitrogenous substance the materials necessary for its growth and nourishment, whilst the remaining elements of these compounds, which were not taken up by the plant, combined chiefly to form alcohol."

Kützing's memoir, the third of the trio [1837], also dates from 1837, and his opinions, like those of Cagniard-Latour, are founded on microscopical observations. He recognises yeast as a vegetable organism and accurately describes its appearance. Alcoholic fermentation depends on the formation of yeast, which is produced when the necessary elements and the proper conditions are present and then propagates itself. The action on the liquid thus increases and the constituents not required to form the organism combine to form unorganised substances, the carbonic acid and alcohol. " It is obvious," says Kützing, in a passage which roused the sarcasm of Berzelius, " that chemists must now strike yeast off the roll of chemical compounds, since it is not a compound but an organised body, an organism."

These three papers, which were published almost simultaneously, were received at first with incredulity. Berzelius, at that time the arbiter and dictator of the chemical world, reviewed them all in his " Jahresbericht " for 1839 [1839] with impartial scorn. The microscopical evidence was denied all value, and yeast was no more to be

regarded as an organism than was a precipitate of alumina. Schwann's experiment (p. 6) was criticised on the ground that the fermenting power of the added yeast had been only partially destroyed in the flasks in which fermentation ensued, completely in those which remained unchanged, the admission of heated or unheated air being indifferent, a criticism to some extent justified by Schwann's statement, already quoted, of the uncertain result of the experiment.

Berzelius himself regarded fermentation as being brought about by the yeast by virtue of that catalytic force, which he had supposed to intervene in so many reactions, both between substances of mineral and of animal and vegetable origin [1836], and which enabled " bodies, by their mere presence, and not by their affinity, to arouse affinities ordinarily quiescent at the temperature of the experiment, so that the elements of a compound body arrange themselves in some different way, by which a greater degree of electro-chemical neutralisation is attained."

To the scorn of Berzelius was soon added the sarcasm of Wöhler and Liebig [1839]. Stimulated in part by the publications of the three authors already mentioned, and in part by the report of Turpin [1838], who at the request of the Academy of Sciences had satisfied himself by observation of the accuracy of Cagniard-Latour's conclusions, Wöhler prepared an elaborate skit on the subject, which he sent to Liebig, to whom it appealed so strongly that he added some touches of his own and published it in the " Annalen," following immediately upon a translation of Turpin's paper. Yeast was here described with a considerable degree of anatomical realism as consisting of eggs which developed into minute animals, shaped like a distilling apparatus, by which the sugar was taken in as food and digested into carbonic acid and alcohol, which were separately excreted, the whole process being easily followed under the microscope.

Close upon this pleasantry followed a serious and important communication from Liebig [1839], in which the nature of fermentation, putrefaction, and decay was exhaustively discussed. Liebig did not admit that these phenomena were caused by living organisms, nor did he attribute them like Berzelius to the catalytic action of a substance which itself survived the reaction unchanged. As regards alcoholic fermentation, Liebig's chief arguments may be briefly summarised. As the result of alcoholic fermentation, the whole of the carbon of the sugar reappears in the alcohol and carbon dioxide formed. This change is brought about by a body termed the ferment, which is formed as the result of a change set up by the access of air to plant juices containing

sugar, and which contains all the nitrogen of the nitrogenous constituents of the juice. This ferment is a substance remarkably susceptible of change, which undergoes an uninterrupted and progressive metamorphosis, of the nature of putrefaction or decay, and produces the fermentation of the sugar as a consequence of the transformation which it is itself undergoing.

The decomposition of the sugar is therefore due to a condition of instability transferred to it from the unstable and changing ferment, and only continues so long as the decomposition of the ferment proceeds. This communication of instability from one substance undergoing chemical change to another is the basis of Liebig's conception, and is illustrated by a number of chemical analogies, one of which will suffice to explain his meaning. Platinum is itself incapable of decomposing nitric acid and dissolving in it ; silver, on the other hand, possesses this power. When platinum is alloyed with silver, the whole mass dissolves in nitric acid, the power possessed by the silver being transferred to the platinum. In like manner the condition of active decomposition of the ferment is transferred to the sugar, which by itself is quite stable. The central idea is that of Stahl (p. 2) which was thus reintroduced into scientific thought.

In a pure sugar solution the decomposition of the ferment soon comes to an end, and fermentation then ceases. In beer wort or vegetable juices, on the other hand, more ferment is continually formed in the manner already described from the nitrogenous constituents of the juice, and hence the sugar is completely fermented away and unexhausted ferment left behind. Liebig's views were reiterated in his celebrated "Chemische Briefe," and became the generally accepted doctrine of chemists. There seems little doubt that both Berzelius and Liebig in their scornful rejection of the results of Cagniard-Latour, Schwann, and Kützing, were influenced, perhaps almost unconsciously, by a desire to avoid seeing an important chemical change relegated to the domain of that vital force from beneath the sway of which a large part of organic chemistry had just been rescued by Wöhler's brilliant synthetical production of urea and by the less recognised synthesis of alcohol by Hennell (see on this point Ahrens [1902]). A strong body of evidence, however, gradually accumulated in favour of the vegetable nature of yeast, so that it may be said that by 1848 a powerful minority adhered to the views of Cagniard-Latour, Schwann, and Kützing [see Schrohe, 1904, p. 218, and compare Buchner, 1904]. Among these must be included Berzelius [1848], who had so forcibly repudiated the idea only ten years before, whereas Liebig in the 1851 edition of his

letters does not mention the fact that yeast is a living organism (Letter XV).

The recognition of the vegetable nature of yeast, however, by no means disproved Liebig's view of the nature of the change by which sugar was converted into carbon dioxide and alcohol, as was carefully pointed out by Schlossberger [1844] in a research on the nature of yeast, carried out in Liebig's laboratory but without decisive results.

Mitscherlich was also convinced of the vegetable character of yeast, and showed [1841] that when yeast was placed in a glass tube closed by parchment and plunged into sugar solution, the sugar entered the glass tube and was there fermented, but was not fermented outside the tube. He regarded this as a proof that fermentation only occurred at the surface of the yeast cells, and explained the process by contact action in the sense of the catalytic action of Berzelius, rather than by Liebig's transference of molecular instability. Similar results were obtained with an animal membrane by Helmholtz [1843], who also expressed his conviction that yeast was a vegetable organism.

In 1854 Schröder and von Dusch [1854, 1859, 1861] strongly reinforced the evidence in favour of this view by succeeding in preventing the putrefaction and fermentation of many boiled organic liquids by the simple process of filtering all air which had access to them through cotton-wool. These experiments, which were continued until 1861, led to the conclusion that the spontaneous alcoholic fermentation of liquids was due to living germs carried by the air, and that when the air was passed through the cotton-wool these germs were held back.

At the middle of the nineteenth century opinions with regard to alcoholic fermentation, notwithstanding all that had been done, were still divided. On the one hand Liebig's theory of fermentation was widely held and taught. Gerhardt, for example, as late as 1856 in the article on fermentation in his treatise on organic chemistry [1856], gives entire support to Liebig's views, and his treatment of the matter affords an interesting glimpse of the arguments which were then held to be decisive. The grounds on which he rejects the conclusions of Schwann and the other investigators who shared the belief in the vegetable nature of yeast are that, although in some cases animal and vegetable matter and infusions can be preserved from change by the methods described by these authors, in others they cannot, a striking case being that of milk, which even after being boiled becomes sour even in filtered air, and this without showing any trace of living organisms. The action of heat, sulphuric acid, and filtration on the air is to remove, or destroy, not living organisms but particles of decomposing matter, that is to

say, ferments which would add their activity to that of the oxygen of the air. Moreover, many ferments, as for example diastase, act without producing any insoluble deposit whatever which can be regarded as an organism.

" Evidemment," he concludes, " la théorie de M. Liebig explique seule tous les phénomènes de la manière la plus complète et la plus logique ; c'est à elle que tous les bons esprits ne peuvent manquer de se rallier."

On the other hand, it was held by many to have been shown that Liebig's view of the origin of yeast by the action of the air on a vegetable infusion was erroneous, and that fermentation only arose when the air transferred to the liquid an active agent which could be removed from it by sulphuric acid (Schulze), by heat (Schwann), and by cotton-wool (Schröder and von Dusch). Accompanying alcoholic fermentation there was a development of a living organism, the yeast, and fermentation was believed, without any very strict proof, to be a phenomenon due to the life and vegetation of this organism. This doctrine seems indeed [Schrohe, 1904] to have been widely taught in Germany from 1840-56, and to have established itself in the practice of the fermentation industries.

In 1857 commenced the classical researches of Pasteur which finally decided the question as to the origin and functions of yeast and led him to the conclusion that " alcoholic fermentation is an act correlated with the life and organisation of the yeast cells, not with the death or putrefaction of the cells, any more than it is a phenomenon of contact, in which case the transformation of sugar would be accomplished in presence of the ferment without yielding up to it or taking from it anything " [1860]. It is impossible here to enter in detail into Pasteur's experiments on this subject, or indeed to do more than indicate the general lines of his investigation. His starting-point was the lactic acid fermentation.

The organism to which this change was due had hitherto escaped detection, and as we have seen the spontaneous lactic fermentation of milk was one of the phenomena adduced by Gerhardt (p. 10) in favour of Liebig's views. Pasteur [1857] discovered the lactic acid-producing organism and convinced himself that it was in fact a living organism and the active cause of the production of lactic acid. One of the chief buttresses of Liebig's theory was thus removed, and Pasteur next proceeded to apply the same method and reasoning to alcoholic fermentation. Liebig's theory of the origin of yeast by the action of the oxygen of the air on the nitrogenous matter of the fermentable liquid was con-

clusively and strikingly disproved by the brilliant device of producing a crop of yeast in a liquid medium containing only comparatively simple substances of known composition—sugar, ammonium tartrate and mineral phosphate. Here there was obviously present in the original medium no matter which could be put into a state of putrefaction by contact with oxygen and extend its instability to the sugar. Any such material must first be formed by the vital processes of the yeast. In the next place Pasteur showed by careful analyses and estimations that, whenever fermentation occurred, growth and multiplication of yeast accompanied the phenomenon. The sugar, he proved, was not completely decomposed into carbon dioxide and alcohol, as had been assumed by Liebig (p. 8). A balance-sheet of materials and products was constructed which showed that the alcohol and carbon dioxide formed amounted only to about 95 per cent. of the invert sugar fermented, the difference being made up by glycerol, succinic acid, cellulose, and other substances [1860, p. 347]. In every case of fermentation, even when a paste of yeast was added to a solution of pure cane sugar in water, the yeast was found by quantitative measurements to have taken something from the sugar. This "something" was indeterminate in character, but, including the whole of the extractives which had passed from the yeast cells into the surrounding liquid, it amounted to as much as 1·63 per cent. of the weight of the sugar fermented [1860, p. 344].

Pasteur was therefore led to consider fermentation as a physiological process accompanying the life of the yeast. His conclusions were couched in unmistakable words: "The chemical act of fermentation is essentially a phenomenon correlative with a vital act, commencing and ceasing with the latter. I am of opinion that alcoholic fermentation never occurs without simultaneous organisation, development, multiplication of cells, or the continued life of cells already formed. The results expressed in this memoir 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 is its real cause, I reply that I am completely ignorant of it.

"Ought we to say that the yeast feeds on sugar and excretes alcohol and carbonic acid? Or should we rather maintain that yeast in its development produces some substance of the nature of a pepsin, which acts upon the sugar and then disappears, for no such substance is found in fermented liquids? I have nothing to reply to these hypotheses. I neither admit them nor reject them, and wish only to restrain myself from going beyond the facts. And the facts tell me simply that all true fermentations are correlative with physiological phenomena."

Liebig felt to the full the weight of Pasteur's criticisms; his reply was long delayed [1870], and, according to his biographer, Volhard [1909], caused him much anxiety. In it he admits the vegetable nature of yeast, but does not regard Pasteur's conclusion as in any way a solution of the problem of the nature of alcoholic fermentation. Pasteur's "physiological act" is for Liebig the very phenomenon which requires explanation, and which he still maintains can be explained by his original theory of communicated instability. On some of Pasteur's results, notably the very important one of the cultivation of yeast in a synthetic medium, he casts grave doubt, whilst he explains the production of glycerol and succinic acid as due to independent reactions. The phenomenon of fermentation is still for him one which accompanies the decomposition of the constituents of the cell, rather than their building up by vegetative growth. "When the fungus ceases to grow, the bond which holds together the constituents of the cell contents is relaxed, and it is the motion which is thus set up in them which is the means by which the yeast cells are enabled to bring about a displacement or decomposition of the elements of sugar or rather organic molecules." Pasteur replied in a brief and unanswerable note [1872]. All his attention was concentrated on the one question of the production of yeast in a synthetic medium, which he recognised as fundamental. The validity of this experiment he emphatically reaffirmed, and finally undertook, from materials supplied by Liebig himself, to produce as much yeast as could be reasonably desired. This challenge was never taken up, and this communication formed the last word of the controversy. Pasteur had at this time firmly established his thesis, *no fermentation without life*, both for alcoholic fermentation and for those other fermentations which are produced by bacteria, and had put upon a sound and permanent basis the conclusions drawn by Schulze, Cagniard-Latour, Schwann, and Kützing from their early experiments. It became generally recognised that putrefaction and other fermentative changes were due to specific organisms, which produced them in the exercise of their vital functions.

Pasteur subsequently [1875] came to the conclusion that fermentation was the result of life without oxygen, the cells being able, in the absence of free oxygen, to avail themselves of the energy liberated by the decomposition of substances containing combined oxygen. This view, which did not involve any alteration of Pasteur's original thesis but was an attempt to explain the physiological origin and function of fermentation, gave rise to a prolonged controversy, which cannot be further discussed in these pages.

Nevertheless, Liebig's desire to penetrate more deeply into the nature of the process of fermentation remained in many minds, and numerous endeavours were made to obtain further insight into the problem. In spite of an entire lack of direct experimental proof, the conception that alcoholic fermentation was due to the chemical action of some substance elaborated by the cell and not directly to the vital processes of the cell as a whole found strenuous supporters even among those who were convinced of the vegetable character of yeast. As early as 1833 diastase, discovered still earlier by Kirchoff and Dubrunfaut, had been extracted by means of water from germinating barley and precipitated by alcohol as a white powder, the solution of which was capable of converting starch into sugar, but lost this power when heated [Payen and Persoz, 1833]. Basing his ideas in part upon the behaviour of this substance, Moritz Traube [1858] enunciated in the clearest possible manner the theory that all fermentations produced by living organisms are caused by ferments, which are definite chemical substances produced in the cells of the organism. He regarded these substances as being closely related to the proteins and considered that their function was to transfer the oxygen and hydrogen of water to different parts of the molecule of the fermentable substance and thus bring about that apparent intramolecular oxidation and reduction which is so characteristic of fermentative change and had arrested the attention of Lavoisier and, long after him, of Liebig.

Traube's main thesis, that fermentation is caused by definite ferments or enzymes, attracted much attention, and received fresh support from the separation of invertase in 1860 from an extract of yeast by Berthelot, and from the advocacy and authority of this great countryman of Pasteur, who definitely expressed his opinion that insoluble ferments existed which could not be separated from the tissues of the organism, and further, that the organism could not itself be regarded as the ferment, but only as the producer of the ferment [1857, 1860]. Hoppe-Seyler [1876] also supported the enzyme theory of fermentation, but differed in some respects from Traube as to the exact function of the ferment [see Traube, 1877; Hoppe-Seyler, 1877].

Direct experimental evidence was, however, still wanting, and Pasteur's reiterated assertion [1875] that all fermentation phenomena were manifestations of the life of the organism remained uncontroverted by experience.

Numerous and repeated direct experimental attacks had been

made from time to time upon the problem of the existence of a fermentation enzyme, but all had yielded negative or unreliable results.

As early as 1846 a bold attempt had been made by Lüdersdorff [1846] to ascertain whether fermentation was or was not bound up with the life of the yeast by grinding yeast and examining the ground mass. A single gram of yeast was thoroughly ground, the process lasting for an hour, and the product was tested with sugar solution. Not a single bubble of gas was evolved. A similar result was obtained in a repetition of the experiment by Schmidt in Liebig's laboratory [1847], the grinding being continued in this case for six hours, but the natural conclusion that living yeast was essential for fermentation was not accepted, on the ground that during the lengthy process of trituration in contact with air the yeast had become altered and now no longer possessed the power of producing alcoholic fermentation, but instead had acquired that of changing sugar into lactic acid [see Gerhardt, 1856, p. 545].

Similar experiments made in 1871 by Marie von Manasseïn [1872, 1897], in which yeast was ground for six to fifteen hours with powdered rock crystal, yielded products which fermented sugar, but they contained unbroken yeast cells, so that the results obtained could not be considered decisive [Buchner and Rapp, 1898, 1], although Frau von Manasseïn herself drew from them and from others in which sugar solution was treated with heated yeast, but not under aseptic conditions, the conclusion that living yeast cells were not necessary for fermentation.

Quite unsuccessful were also the attempts made to accomplish the separation of fermentation from the living cell by Adolf Mayer [1879, p. 66], and, as we learn from Roux, by Pasteur himself, grinding, freezing, and plasmolysing the cells having in his hands proved alike in vain. Extraction by glycerol or water, a method by which many enzymes can be obtained in solution, gave no better results [Nägeli and Loew, 1878], and the enzyme theory of alcoholic fermentation appeared quite unjustified by experiment.

Having convinced himself of this, Nägeli [1879] suggested a new explanation of the facts based on molecular-physical grounds. According to this view, which unites in itself some of the conceptions of Liebig, Pasteur, and Traube, fermentation is the transference of a state of motion from the molecules, atomic groups, and atoms of the compounds constituting the living plasma of the cell to the fermentable material, whereby the equilibrium existing in the molecules of the latter is disturbed and decomposition ensues [1879, p. 29].

This somewhat complex idea, whilst including, as did Liebig's theory, Stahl's fundamental conception of a transmission of a state of motion, satisfies Pasteur's contention that fermentation cannot occur without life, and at the same time explains the specific action of different organisms by differences in the constitution of their cell contents. The really essential part of Nägeli's theory consisted in the limitation of the power of transference of molecular motion to the living plasma, by which the failure of all attempts to separate the power of fermentation from the living cell was explained. This was the special phenomenon which required explanation ; to account for this the theory was devised, and when this was experimentally disproved, the theory lost all significance.

For nearly twenty years no further progress was made, and then in 1897 the question which had aroused so much discussion and conjecture, and had given rise to so much experimental work, was finally answered by Eduard Buchner, who succeeded in preparing from yeast a liquid which, in the complete absence of cells, was capable of effecting the resolution of sugar into carbon dioxide and alcohol [1897, 1].

In the light of this discovery the contribution to the truth made by each of the great protagonists in the prolonged discussion on the problem of alcoholic fermentation can be discerned with some degree of clearness. Liebig's main contention that fermentation was essentially a chemical act was correct, although his explanation of the nature of this act was inaccurate. Pasteur, in so far as he considered the act of fermentation as indissolubly connected with the life of the organism, was shown to be in error, but the function of the organism has only been restricted by a single stage, the active enzyme of alcoholic fermentation has so far only been observed as the product of the living cell. Nearest of all to the truth was Traube, who in 1858 enunciated the theorem, which was only proved for alcoholic fermentation in 1897, that all fermentations produced by living organisms are due to ferments secreted by the cells.

Buchner's discovery of zymase has introduced a new experimental method by means of which the problem of alcoholic fermentation can be attacked, and the result has been that since 1897 a considerable amount of information has been gained with regard to the nature and conditions of action of the enzymes of the yeast cell. It has been found that the machinery of fermentation is much more complex than had been surmised. The enzyme, now termed apozymase, which is essential for fermentation, cannot of itself bring about the alcoholic fermentation of sugar, but is dependent on the presence of a second

substance, termed, for want of a more reasonable name, the co-enzyme or co-zymase. The function of this mysterious coadjutor is still unknown. As the co-enzyme withstands the temperature of boiling water and is dialysable, it is probably more simple in constitution than the enzyme, and considerable progress has been made towards its isolation and identification. This, however, is not all; for the decomposition of sugar a phosphate is also indispensable. It appears that in yeast-juice, and therefore also most probably in the yeast cell, the phosphorus present takes an active part in fermentation and goes through a remarkable cycle of changes. The breakdown of sugar into alcohol and carbon dioxide is accompanied by the formation of complex hexosephosphates, and the phosphate is split off from these compounds and thus again rendered available for action by means of a special enzyme, termed hexosephosphatase. In addition to this complex of ferments, the cell also possesses special enzymes by which the zymase and the co-enzyme can be destroyed, and, further, at least one substance, known as an anti-enzyme, which directly checks this destructive action. The view is now generally held that the decomposition of the sugar molecule takes place in stages, although much doubt yet exists as to the nature of some of these. It appears, however, almost certain that in one of them pyruvic acid is produced and that this is subsequently decomposed by a specific enzyme, carboxylase, into carbon dioxide and acetaldehyde, the latter of which then undergoes enzymic reduction to ethyl alcohol.

This mechanism for the decomposition of the sugar molecule is not confined to the yeast cell, for it has been found that the same processes are involved in the conversion of carbohydrate into lactic acid in muscle and in the decomposition of carbohydrates by bacteria, moulds and the higher plants. It appears in fact that the fundamental biological mode of attack on carbohydrates is that revealed by the study of alcoholic fermentation.

The subject still remains one of the most interesting in the whole field of biological chemistry, the limited degree of insight which has already been gained into the marvellous complexity of the cell lending additional zest to the attempt to penetrate the darkness which shrouds the still hidden mysteries.

CHAPTER II.

ZYMASE AND ITS PROPERTIES.

Discovery of Zymase.

THE history of Buchner's discovery is of great interest [Gruber, 1908 ; Hahn, 1908]. As early as 1893 Hans and Eduard Buchner found that the cells of even the smallest micro-organism could be broken by being ground with sand [Buchner, E. and H., and Hahn, 1903, p. 20], and in 1896 the same process was applied by these two investigators to yeast, with the object of obtaining a preparation for therapeutic purposes. Difficulties arose in the separation of the cell contents from the ground-up mixture of cell membranes, unbroken cells, and sand, but these were overcome by carrying out the suggestion of Martin Hahn (at that time assistant to Hans Buchner) that kieselguhr should be added and the liquid squeezed out by means of a hydraulic press [Buchner, E. and H., and Hahn, 1903, p. 58]. The yeast-juice thus obtained was, in the first instance, employed for animal experiments, but underwent change very rapidly. The ordinary antiseptics were found to be unsuitable, and hence sugar was added as a preservative, and it was the marked action of the juice upon this added sucrose that drew Eduard Buchner's attention to the fact that fermentation was proceeding in the absence of yeast cells.

As in the case of so many discoveries, the new phenomenon was brought to light, apparently by chance, as the result of an investigation directed to quite other ends, but fortunately fell under the eye of an observer possessed of the genius which enabled him to realise its importance and give to it the true interpretation.

In his first papers [1897, 1, 2 ; 1898], Eduard Buchner established the following facts : (1) yeast-juice free from cells is capable of producing the alcoholic fermentation of glucose, fructose, sucrose, and maltose ; (2) the fermenting power of the juice is neither destroyed by the addition of chloroform, benzene, or sodium arsenite [Hans Buchner, 1897], by filtration through a Berkefeld filter, by evaporation to dryness at 30° to 35°, nor by precipitation with alcohol ; (3) the

fermenting power is completely destroyed when the liquid is heated to 50°.

From these facts Eduard Buchner drew the conclusion " that the production of alcoholic fermentation does not require so complicated an apparatus as the yeast-cell, and that the fermentative power of yeast-juice is due to the presence of a dissolved substance." To this active substance he gave the name of zymase.

Buchner's discovery was not received without some hesitation. A number of investigators prepared yeast-juice, but failed to obtain an active product [Will, 1897 ; Delbrück, 1897 ; Martin and Chapman, 1898 ; Reynolds Green, 1897 ; Lintner, 1899]. A more accurate knowledge of the necessary conditions and of the properties of yeast-juice, however, led to more successful results [Will, 1898 ; Reynolds Green, 1898 ; Lange, 1898], and it was soon established that, given suitable yeast, an active preparation could be readily procured by Buchner's method. Criticism was then directed to the effect of the admitted presence of a certain number of micro-organisms in yeast-juice [Stavenhagen, 1897], but Buchner [Buchner and Rapp, 1897] was able to show by experiments in the presence of antiseptics and with juice filtered through a Chamberland candle that the fermentation was not due to living organisms of any kind.

The most weighty criticism of Buchner's conclusion consisted in an attempt to show that the properties of yeast-juice might be due to the presence, suspended in it, of fragments of living protoplasm, which, although severed from their original surroundings in the cell, might retain for some time the power of producing alcoholic fermentation. This, it will be seen, was an endeavour to extend Nägeli's theory to include in it the newly discovered fact.

In favour of this view were adduced the similarity between the effects of many antiseptics on living yeast and on the juice, the ephemeral nature of the fermenting agent present in the juice, the effect of dilution with water, and the phenomenon of autofermentation which is exhibited by the juice in the absence of added sugar [Abeles, 1898 ; v. Kupffer, 1897 ; v. Voit, 1897 ; Wehmer, 1898 ; Neumeister, 1897 ; Macfadyen, Morris, and Rowland, 1900 ; Bokorny, 1906 ; Fischer, 1903 ; Beijerinck, 1897, 1900 ; Wroblewski, 1898, 1899, 1901].

A brief general description of the actual properties of yeast-juice and of the phenomena of fermentation by its means is sufficient to show the great improbability of this view.

The juice prepared by Buchner's method forms a somewhat viscous opalescent brownish-yellow liquid, which is usually faintly acid in

reaction [compare Ahrens, 1900] and almost optically inactive. It has a specific gravity of 1·03 to 1·06, contains 8·5 to 14 per cent. of dissolved solids, and leaves an ash amounting to 1·4 to 2 per cent. About 0·7 to 1·7 per cent. of nitrogen is present, nearly all in the form of protein, which coagulates to a thick white mass when the juice is heated.

Powerful digestive enzymes [see Dernby, 1917; Oppenheimer, 1926, p. 1120; and Haldane, 1930 *passim*] are also present, so that when the juice is preserved its protein undergoes digestion at a rate which depends on the temperature [Hahn, 1898; Geret and Hahn, 1898, 1, 2; 1900; Buchner, E. and H., and Hahn, 1903, pp. 287-340], and is converted into a mixture of bases and amino-acids. After about 6 days at 37°, or 10 to 14 days at the ordinary temperature, the digestion is so complete that no coagulation occurs when the juice is boiled. These proteoclastic enzymes, like the alcoholic enzyme, cannot be extracted from the living cells.

Fresh yeast-juice produces a slow fermentation of sugar, which lasts for forty-eight to ninety-six hours at 25° to 30°, about a week at the ordinary temperature, and then ceases, owing, not to exhaustion of the sugar, but to the disappearance of the fermenting agent. When the juice is preserved or incubated in the absence of a fermentable sugar this disappearance occurs considerably sooner, so that even after standing for a single day at room temperature, or two days at 0°, no fermentation may occur when sugar is added. The reason for this behaviour has not been definitely ascertained. As will be seen later on (p. 80) the phenomenon is a complex one, but the disappearance of the enzyme was originally ascribed by Buchner to the digestive action upon it of the proteases of the juice [1897, 2], and no better explanation has yet been found. Confirmation of this view is afforded by the fact that the addition of a tryptic enzyme of animal origin greatly hastens the disappearance of the alcoholic enzyme [Buchner, E. and H., and Hahn, 1903, p. 126], and that some substances which hinder the tryptic action favour fermentation [Harden, 1903]. The amount of fermentation produced is almost unaffected by the presence of such antiseptics as chloroform or toluene, although some others, such as arsenites and fluorides, decrease it when added in comparatively high concentrations, and it is only slightly diminished by dilution with three or four volumes of sugar solution, somewhat more considerably by dilution with water. When it is filtered through a Chamberland filter the first portions of the filtrate are capable of bringing about fermentation, but the ferment-

ing power diminishes in the succeeding portions and finally disappears. The juice can be centrifuged without being in any way altered, and no separation into more or less active layers takes place under these conditions.

The amorphous powder obtained by drying the precipitate produced when the juice is added to a mixture of alcohol and ether is also capable of producing fermentation, and the process of precipitation may be repeated without seriously diminishing the fermenting power of the product.

These facts clearly show that the various phenomena adduced by the supporters of the theory of protoplasmic fragments are quite consistent with the presence of a dissolved enzyme as the active agent of the juice, and at the same time that the properties demanded of the living fragments of protoplasm to which fermentation is ascribed are such as cannot be reconciled with our knowledge of living matter. If living protoplasm is the cause of alcoholic fermentation by yeast-juice, a new conception of life will be necessary; the properties of the postulated fragments of protoplasm must be so different from those which the protoplasm of the living cell possesses as to deprive the theory of all real value [Buchner, 1900, 2; Buchner, E. and H., and Hahn, 1903, p. 33].

Further and very convincing evidence against the protoplasm theory is afforded by the behaviour of yeast towards various desiccating agents. When yeast is dried at the ordinary temperature it retains its vitality for a considerable period. If, however, the dried yeast be heated for six hours at 100° it loses the power of growth and reproduction but still retains that of fermenting sugar, and when ground with sand, kieselguhr, and 10 per cent. glycerol solution yields an active juice [Buchner, 1897, 2; 1900, 1]. Preparations (known as zymin) obtained by treating yeast with a mixture of alcohol and ether [Albert, 1900, 1901], or with acetone and ether [Albert, Buchner, and Rapp, 1902], show precisely similar properties (p. 38). The proof in this case has been carried a step further, for the active juice obtained by grinding such acetone-yeast, when precipitated with alcohol and ether, yields an amorphous powder, still capable of fermenting sugar.

The contention of Kostytschev and his collaborators [Kostytschev and Chomitsch, 1928; Kostytschev and Faermann, 1928; Kostytschev, Medvedev and Kardo-Sysojeva, 1927], that the fermentations produced by the various yeast preparations are in reality due to the presence of living organisms (yeast cells, lactic acid bacteria) does not appear to be well founded, and has been refuted by various authors.

The Preparation of Yeast-Juice.

Buchner's process for the preparation of active yeast-juice is characterised by extreme simplicity. The yeast employed, which should be fresh brewery yeast, is washed two or three times by being suspended in a large amount of water and allowed to settle in deep vessels. It is then collected on a filter cloth, wrapped in a press cloth, and submitted to a pressure of about 50 kilos. per sq. cm. for five minutes. The resulting friable mass contains about 70 per cent. of water and is free from adhering wort. The washed yeast is then mixed with an equal weight of silver sand and 0·2 to 0·3 parts of kieselguhr, care being taken that this is free from acid. The correct amount of kieselguhr to be added can only be ascertained by experience and varies with different samples of yeast. The dry powder thus obtained is brought in portions of 300 to 400 grams into a large porcelain mortar and ground by hand by means of a porcelain pestle fastened to a long iron rod which passes through a ring fixed in the wall (Fig. 1). The mortar used by Buchner had a diameter of 40 cm. and the pestle and rod together weighed 8 kilos.

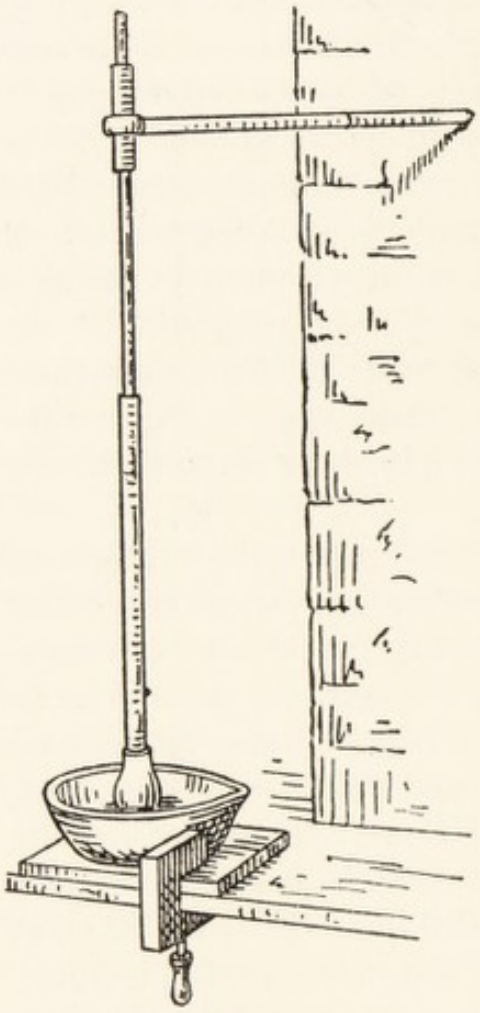


Fig 1.

As the grinding proceeds the light-coloured powder gradually darkens and becomes brown, and the mass becomes moist and adheres to the pestle, until finally, after two to three minutes' grinding, it takes the consistency of dough, at which stage the process is stopped. The mass is next enveloped in a press cloth and submitted to a pressure of 90 kilos. per sq. cm. in a hydraulic hand press, the pressure being very gradually raised in order to avoid rupture of the cloth. The cloth required for 1000 grams of yeast measures 60 by 75 cm. and is previously soaked in water and then submitted to a pressure of 50 kilos. per sq. cm., retaining about 35 to 40 c.c. of water.

The juice runs from the press on to a folded filter paper, to remove

kieselguhr and yeast cells, and passes into a vessel standing in ice water.

The yield of juice obtained by Buchner in an operation of this kind from 1 kilo. of yeast amounts to 320 to 460 c.c. It may be

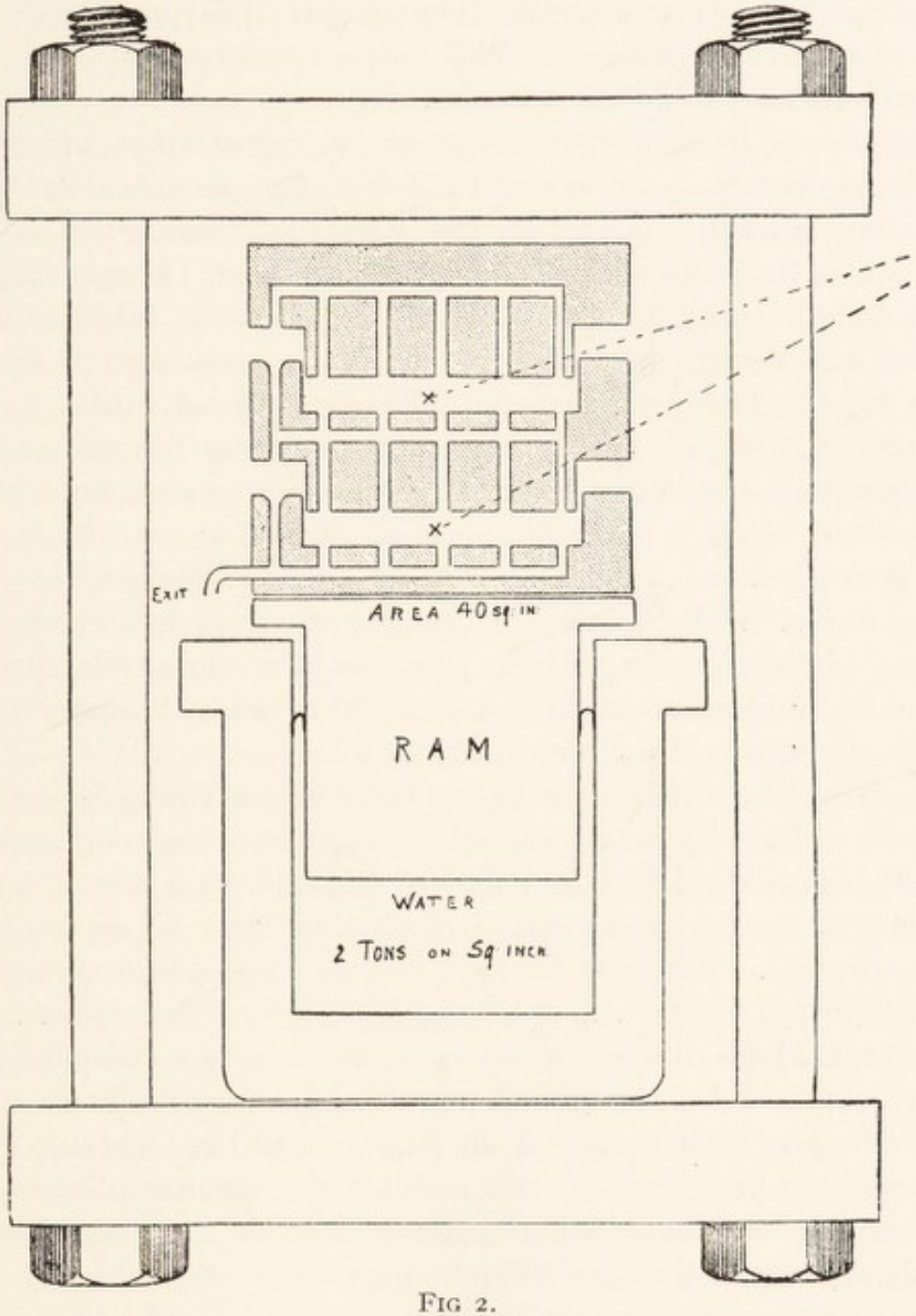


FIG 2.

increased by re-grinding the press cake and again submitting it to pressure, and then amounts on the average to 450 to 500 c.c.

Since the cell membranes constitute about 20 per cent. of the weight of the dry yeast, this yield corresponds to more than 60 per cent. of the total cell contents of the yeast. It has been computed by Will [quoted by Buchner, E. and H., and Hahn, 1903, p. 66] that

only about 20 per cent. of the cells are left unaltered by one grinding and pressing, and only 4 per cent. after a repetition of the process, at least 57 per cent. of the cells being actually ruptured by the double process, and the remainder to some extent altered. It seems probable from these figures that a certain amount of the juice may be derived from the unbroken cells, and Will expressly states that many unbroken cells have lost their vacuoles.

If the yeast be submitted to a process of regeneration, which consists in exposure to a well-aerated solution of sugar and mineral salts until fermentation is complete, the juice subsequently obtained is more active than that yielded by the original yeast [Albert, 1899, 1].

A slight modification of Buchner's process was introduced by Harden and Young, the mass being ground in a mixing mill instead of by hand. The ground mass is then well mixed with a further quantity of kieselguhr until a nearly dry powder is formed, and the mass packed between two layers of chain cloth in steel filter plates and pressed out in a hydraulic press at about 2 tons to the square inch (300 kilos. per sq. cm.). The press and plates are shown in section in Fig. 2. It has also been found convenient to remove yeast cells and kieselguhr from the freshly pressed juice by centrifugalisation instead of by filtration through paper, and to wash the yeast before grinding by means of a filter-press.

Working with English top yeasts Harden and Young have found the yield of juice extremely variable, the general rule being that the amount of juice obtainable from freshly skimmed yeast is smaller than that yielded by the same yeast after standing for a day or two after being skimmed. The yield for 1000 grams of pressed brewer's yeast varies from 150 to 375 c.c., and is on the average about 250 c.c.

As a rule a juice which gives a more vigorous reaction with phosphate (p. 43) is obtained if the ground mass be kept for two hours at air temperature before it is pressed out [Harden and Henley, 1927, 1].

Very fresh yeast occasionally presents the peculiar phenomenon that scarcely any juice can be expressed from the ground mass, although the latter does not differ in appearance or consistency from a mass which gives a good yield.

Extraction of Zymase from Unground Yeast.

1. *Maceration of Dried Yeast.*

A valuable addition to the methods of obtaining an active solution of zymase was made in 1911 by Lebedev [1911, 2; 1912, 2; see also

1911, 3, 6, and 1912, 1]. This investigator had been in the habit of grinding dried yeast with water for preparing samples of yeast-juice of uniform character, and observed that when the dried yeast was digested with sugar solution and the mixture heated, coagulation took place throughout the whole liquid, the proteins of the yeast having passed out of the cells. Further examination revealed the interesting fact that dried yeast readily yielded an active extract when macerated in water for some time. The quality of the resulting " maceration extract " depends on a considerable number of factors, the chief of which are : (1) the temperature of drying of the yeast ; (2) the temperature of maceration ; (3) the duration of maceration ; and (4) the nature of the yeast, as well as, of course, the amount of water added in maceration.

In general the yeast should be dried at 25°-30° and then macerated with 3 parts of water for two hours at 35°.

The temperature of maceration may as a rule be varied, without detriment to the product provided that the time of maceration is also suitably altered ; thus with dried Munich yeast, maceration for 4·5 hours at 25° is about as effective as 2 hours at 35°, whereas treatment for a shorter time at 25° or a longer time at 35° produces in general a less efficacious extract. Yeast dried at a lower temperature than 25° tends to yield an extract poor in co-enzyme (p. 76) and hence of low fermenting power, this being especially marked at air temperature.

The subsequent treatment of the yeast during maceration may, however, be of great influence in such cases. Thus a yeast dried at 15° gave by maceration at 25° for 4·5 hours a weak extract (yielding with excess of sugar 0·33 g. CO_2), whereas when macerated at 35° for two hours it yielded a normal extract (1·36 g. CO_2).

The nature of the yeast is of paramount importance. Thus while Munich (bottom) yeast usually gives a good result, a top yeast from a Paris brewery was found to yield extracts containing neither zymase nor its co-enzyme in whatever way the preparation was conducted. The existence of such yeasts is of great interest, and it was probably due to the unfortunate selection of such a yeast for his experiments that Pasteur was unable to prepare active fermenting extracts and therefore failed to anticipate Buchner by more than 30 years (see p. 15). The English top yeasts as a rule give poor results [see Dixon and Atkins, 1913] and sometimes yield totally inactive maceration extract. Good results can sometimes be obtained by macerating such yeasts with 0·15*M* K_2HPO_4 or Na_2HPO_4 instead of water [Hägglund and Rosenqvist, 1927]. A solution of co-enzyme has also sometimes

been substituted for the water [see Neuberg and Kobel, 1925]. It is not understood why the enzyme passes out of the cell during the process of maceration and the whole method gives rise to a number of extremely interesting problems.

Method.—A suitable yeast is washed by decantation, filtered through a cloth, lightly pressed by means of a hand-press, and then passed through a sieve of 5 mm. mesh, spread out in a layer of 1-1·5 cm. thick and left at 25°-35° for two days. Fifty grams of the dried yeast are thoroughly and carefully mixed with 150 c.c. of water in a basin or Erlenmeyer flask, and the whole digested for two hours at 35°. The mass often froths considerably. It is then filtered through ordinary folded filter paper, preferably in two portions, and collected in a vessel cooled by ice. It may also be filtered at the pump using a Buchner funnel. The separation may also be effected by centrifuging or pressing out the mass, and the maceration may be conveniently conducted in a flask immersed in the water of a thermostat. It is not advisable to macerate more than 50 grams in one operation. Under these conditions 25-30 c.c. of extract are obtained after 20 minutes' filtration, 70-80 c.c. in twelve hours.

This extract closely resembles in properties the juice obtained by grinding the same yeast, but it is usually more active, gives rise to a much smaller autofermentation or even none at all, contains more inorganic phosphate (see p. 47) and often exhibits a long induction period before fermentation of added sugar begins (see p. 166) [see Lebedev, 1912, 2; Neuberg and Rosenthal, 1913].

2. *Other Methods.*

Attempts to prepare active extracts from undried yeast in an analogous manner have so far not been very successful. Thus Rinckleben [1911] found that plasmolysis by glycerol (8 per cent.) or sodium phosphate (5 per cent.) sometimes yielded an active juice and sometimes a juice which contained enzyme but no co-enzyme, but more often an inactive juice incapable of activation [see also Kayser, 1911].

Giglioli [1911] by the addition of chloroform also obtained an active liquid. It appears in fact as though almost any method of plasmolysing the yeast cell may yield a certain proportion of zymase in the exudate.

An ingenious process has been devised by Dixon and Atkins [1913] who applied the method of freezing in liquid air which they had found efficacious for obtaining the sap from various plant organs. They thus

succeeded in obtaining from yeast, derived from Guinness' brewery in Dublin, liquids capable of fermenting sugar and of about the same efficacy as the maceration extracts prepared by Lebedev's method from the same yeast. The results were, however, in both cases very low, the maximum total production of CO_2 by 25 c.c. of liquid from excess of sugar being 32·5 c.c. (air temperature) or about 0·06 g. Munich yeast, on the other hand, yields, either by maceration or grinding, a liquid giving as much as 1·5-2 g. of CO_2 per 25 c.c., whilst English yeast-juice prepared by grinding often gives as much as 0·5-0·7 g. of CO_2.

No direct comparison with the juice prepared by grinding was made by Dixon and Atkins, but it may be concluded from their results that the best method of obtaining an active preparation from the top yeasts used in this country is that of grinding. Maceration, freezing, and plasmolysis alike yield poor results. With Munich yeast, on the other hand, the maceration process yields excellent results, whilst the liquid air process has not so far been tried.

Practical Methods for the Estimation of the Fermenting Power of Yeast-Juice.

In order to estimate the amount of carbon dioxide evolved in a given time and the total amount evolved by the action of yeast-juice on sugar, Buchner adopted an extremely simple method, which consisted in carrying out the fermentation in an Erlenmeyer flask provided with a small wash-bottle, which contained sulphuric acid and was closed by a Bunsen valve, and ascertaining the loss of weight during the experiment. Corrections are necessary for the carbon dioxide present in the original juice and retained in the liquid at the close of the experiment and for that present in the air space of the apparatus, but it was found that for most purposes these could be neglected. In cases in which greater accuracy was desired, the carbon dioxide was displaced by air before the weighings were made. A typical experiment of this kind, without displacement of carbon dioxide, is the following :—

March 22, 1899, Berlin bottom yeast V. 20 c.c. juice + 8 grams sucrose + 0·2 c.c. toluene as antiseptic at 16°. Grams of carbon dioxide after

24	48	72	96 hours.
0·40	0·64	0·99	1·11

The total weight of carbon dioxide evolved under these conditions is termed the fermenting power of the juice (Buchner).

A more accurate method [Macfadyen, Morris, and Rowland, 1900] consists in passing the carbon dioxide into caustic soda solution and estimating it by titration. The yeast-juice, sugar, and antiseptic are placed in an Erlenmeyer flask provided with a straight glass tube, through which air can be passed over the surface of the liquid, and a conducting tube leading into a second flask which contains 50 c.c. of 10 per cent. caustic soda solution and is connected with the air by a guard tube containing soda lime. The juice can be freed from carbon dioxide by agitation in a current of air before the flask is connected to that containing the caustic soda solution, and at the end of the period of incubation air is passed through the apparatus, the liquid being boiled out if great accuracy is required. The absorption flask is then disconnected and the amount of absorbed carbon dioxide estimated

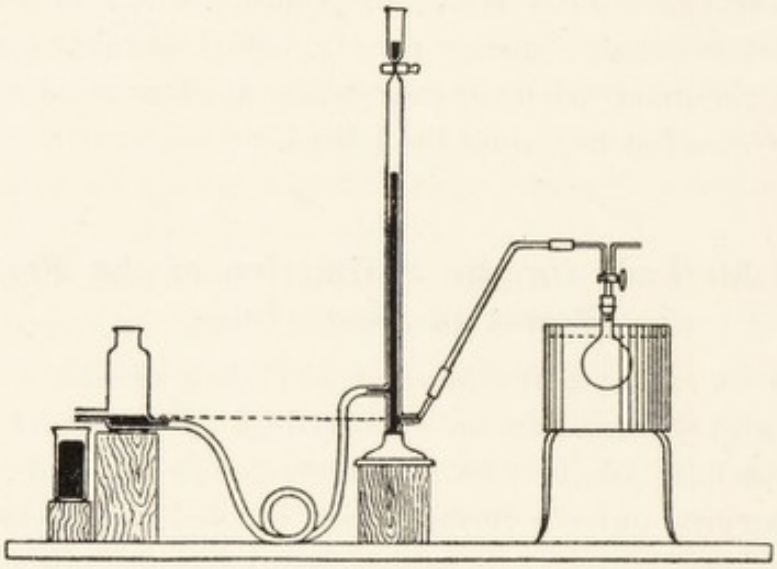


FIG. 3.

by titration. This is carried out by making up the contents of the flask to 200 c.c., taking out an aliquot portion, rendering this exactly neutral to phenophthalein by the addition first of normal and finally of decinormal acid, adding methyl orange and titrating with decinormal acid to exact neutrality. Each c.c. of decinormal acid used in this last titration represents 0·0044 gram of carbon dioxide in the quantity of solution titrated.

These methods are only suitable for observations at considerable intervals of time. For the continuous observation of the course of fermentation Harden, Thompson, and Young [1910] connect the fermentation flask with a Schiff's azotometer filled with mercury and measure the volume of gas evolved, the liquid having been previously saturated with carbon dioxide (Fig. 3). The level of the mercury in the reservoir is kept constant by a syphon overflow, or, as shown in the

figure, according to a modification introduced by S. G. Paine, by a specially constructed bottle provided with two tubulures near the bottom. This ensures that no change in the pressure in the flask occurs, and the volume of gas observed is reduced to normal pressure by means of a table. Before making a reading it is necessary to shake the fermenting mixture thoroughly, as the albuminous liquid very readily becomes greatly supersaturated with carbon dioxide, so much so in fact that very little gas is evolved in the intervals between the shakings. The exact procedure in making an observation consists in shaking the flask thoroughly, replacing in the thermostat, allowing to remain for one minute, and then reading the level of the mercury in the azotometer. After the required time, say five minutes, has elapsed from the time at which the flask was first shaken, it is again removed from the bath, shaken as before, replaced, allowed to remain for one minute and the reading then taken. In this way readings can be conveniently made at intervals of three or five minutes or even less, and much more detailed information obtained about the course of the reaction than is possible by means of observations made at intervals of several hours.

This apparatus can be constructed on a small scale so that observations can be made with a total volume of 2 c.c. of fermenting liquid. A micro-apparatus has also been employed by Euler and Myrbäck [see Oppenheimer and Pincussen, 1929, p. 1297] which consists of a short test-tube in which the fermenting mixture is placed, connected by a capillary tube with a gas burette provided with a movable reservoir. The test-tube is completely immersed in the water of the thermostat and mechanically shaken throughout the experiment. The gas is collected under slightly diminished pressure and is brought to atmospheric pressure before being read.

Another form of volumetric apparatus, designed by Walton [1904], has been used by Lebedev [1909], and a number of others have been described [see Dann and Quastel, 1928, Nord and Franke, 1928].

An apparatus on a different principle has been designed by Slator [1906] for use with living yeast, but is equally applicable to yeast-juice, and a very similar form has been more recently employed by Ivanov [1909, 2]. In this apparatus the change of pressure produced by the evolution of carbon dioxide is measured at constant volume, and comparative rates of evolution can be obtained with considerable accuracy, although the method has the disadvantage that the absolute volume of gas evolved is not measured. The apparatus consists of a bottle or flask connected with a mercury manometer. The fermenting

mixture is placed in the bottle along with glass beads to facilitate agitation, the pressure is reduced to a small amount by the water-pump, and the rise of pressure is then observed at intervals, this being proportional to the volume of gas produced. As in the preceding case, the liquid must be well shaken before a reading is made.

Dorner [1912] adapted the Haldane-Barcroft blood-gas manometer [Barcroft and Haldane, 1902] for use in this manner, and this instrument has also been used by Meyerhof [1918, 1, 2]. The manometer liquid consists of water, of which about 10,000 mm. equal one atmosphere pressure. This method is therefore very sensitive and enables accurate measurements to be made with 1-2 c.c. of liquid.

The Alcoholic Fermentation of the Sugars by Yeast-Juice.

Yeast-juice brings about a slow fermentation of those sugars which are fermented by the yeast from which it is prepared, as well as of dextrin, and of starch and glycogen, which are not fermented by living yeast.

(*a*) *Relation to Fermentation by living Yeast.*

Both in rate of fermentation and in the total fermentation produced, yeast-juice stands far behind the equivalent amount of living yeast. Taking 25 c.c. of yeast-juice to be equivalent to at least 36 grams of pressed yeast containing 70 per cent. of moisture, it is found that whereas the yeast-juice (from English top yeast) gives with glucose a maximum rate of fermentation of about 3 c.c. in five minutes, the living yeast ferments the sugar at the rate of about 126 c.c. in the same time, or about forty times as quickly. The total carbon dioxide obtainable from the yeast-juice, moreover, corresponds to the fermentation of only 2 to 3 grams of sugar, whilst the living yeast will readily ferment a much larger quantity, although the exact limit in this respect has not been accurately determined. The reasons for this great difference in behaviour will be discussed later on, after the various factors concerned in fermentation have been considered (p. 183).

(*b*) *Relation of Alcohol to Carbon Dioxide.*

In all cases of fermentation by yeast-juice and zymin, the relative amounts of carbon dioxide and alcohol produced are substantially in the ratio of the molecular weights of the compounds, that is as 44 : 46, so that for 1 part of carbon dioxide 1·04 of alcohol are formed. This

has been shown for the juice and zymin from bottom yeasts by Buchner [Buchner, E. and H., and Hahn, 1903, pp. 210, 211], who obtained the ratios 1·01, 0·98, 1·01, and 0·99 from experiments in which from 8 to 15 grams of alcohol were produced. Similar numbers, 0·90, 1·12, 0·95, 0·91, and 0·92, have been obtained for the juice from top yeasts by Harden and Young [1904], who worked with much smaller quantities. The variable results obtained with juice from top yeast by Macfadyen, Morris and Rowland [1900], have not been confirmed.

(*c*) *Relation of Carbon Dioxide and Alcohol Produced to the Amount of Sugar Fermented.*

The construction of a balance-sheet between the sugar fermented and the products formed is of special interest in the case of alcoholic fermentation by yeast-juice, because, there being no cell growth as in the case of living yeast, an opportunity appears to be afforded of ascertaining whether the whole of the sugar is converted into alcohol and carbon dioxide, or whether some fraction of the sugar passes into any of the well-known subsidiary products of alcoholic fermentation by yeast, such as glycerol, fusel oil, or succinic acid. Unfortunately the question cannot be settled in this way. When the loss of sugar during the fermentation is estimated directly, it is usually found to be considerably greater than the sum of the alcohol and carbon dioxide produced from it. This fact was first observed by Macfadyen, Morris and Rowland [1900], and was then confirmed by Buchner [Buchner, E. and H., and Hahn, 1903, p. 212] in one instance, the excess of sugar lost over products being in this case about 15 per cent. of the total sugar which had disappeared. The matter was then more thoroughly investigated by Harden and Young [1904].

The conditions under which the experiment must be carried out are not very favourable to the attainment of extreme accuracy. Yeast-juice contains glycogen and a diastatic enzyme which converts this into dextrins and finally into sugar. This process goes on throughout fermentation, tending to increase the sugar present and to make the apparent loss of sugar less than the sum of the products. In spite of this it was found that a certain amount of sugar invariably disappeared without being accounted for as alcohol or carbon dioxide, and this whether the fermentation lasted sixty or a hundred and eight hours, and independently of the dilution of the juice. This disappearing sugar amounted in some cases to 44 per cent. of the total loss of sugar, and on the average of twenty-five experiments was 38 per cent. Further

information was sought by converting all the sugar-yielding constituents of the juice into sugar by hydrolysis before and after the fermentation. This process revealed the fact that when the glucose equivalent of the juice before and after fermentation was determined after hydrolysis with three times normal acid for three hours (and a correction made for the loss of reducing power experienced by glucose itself when submitted to this treatment), the difference was almost exactly equal to the alcohol and carbon dioxide produced. In other words, accompanying fermentation, a change proceeds by which sugar is converted into a less reducing substance, reconvertible into sugar by hydrolysis with acids. Similar results were subsequently obtained by Buchner and Meisenheimer [1906], who employed 1·5 normal acid and observed a small nett loss of sugar. Still later Lebedev [1909, 1910, see also 1913,] carried out similar estimations with the same result. It is doubtful whether the experiments which have so far been made on this point are sufficiently accurate to decide with certainty whether or not the loss of sugar is exactly equal to the sum of the carbon dioxide and alcohol produced. It has been shown by Buchner and Meisenheimer [1906] that glycerol is a constant product of alcoholic fermentation by yeast-juice, and it has now been established that this is produced from the sugar (p. 177), so that it is certain that a small amount of sugar is normally converted into non-carbohydrate substances other than carbon dioxide and alcohol. In addition to this the possibility exists that a hexosephosphoric ester of a lower reducing power than glucose and resistant to hydrolysis may be formed (see p. 59).

It has been shown [Harden and Young, 1913, see also Naganishi, 1926] that the deficit of sugar is not due to the formation of hexosediphosphate (p. 48), which has a lower reduction than glucose, and that the solution from which the sugar (either glucose or fructose) has disappeared actually contains some substance of relatively high dextrorotation and of low reducing power.

It may, therefore, be considered as established that during alcoholic fermentation sugar is converted by an enzyme into some compound of less reducing power, which again yields sugar on hydrolysis with acids. The exact nature of this substance has not been ascertained, but it appears likely that the process is a synthetical one resulting in the formation of some polysaccharide, possibly intermediate between the hexoses and glycogen.

(d) Fermentation of Different Carbohydrates. Autofermentation.

Yeast-juice and zymin ferment all the sugars which are fermented by the yeast from which they are prepared, and, in addition, a number of colloidal substances which cannot pass through the membrane of the living yeast cell, but which are hydrolysed by enzymes in the juice and thus converted into simpler sugars capable of fermentation[Buchner and Rapp, 1898, 3 ; 1899, 2]. Of the simple sugars which have been examined, glucose, fructose, and mannose are freely fermented, *l*-arabinose not at all, whilst the case of galactose is doubtful. Galactose is, however, fermented by juice prepared from a yeast which has been "trained" to ferment galactose [Harden and Norris, 1910]. As regards both the rate of fermentation and the total amount of carbon dioxide evolved from glucose and fructose by the action of a definite amount of yeast-juice, Buchner and Rapp obtained practically identical numbers. Harden and Young [1909], using juice from top yeast, found that fructose was slightly more rapidly fermented and gave a somewhat larger total than glucose, whilst mannose was initially fermented at almost the same rate as glucose, but gave a decidedly lower total, the following being the average results :—

	Relative Rates.	Relative Totals.
Glucose	1	1
Fructose	1·29	1·15
Mannose	1·04	0·67

Among the disaccharides, sucrose and maltose are freely fermented, and the juice can be shown like living yeast to contain invertase and maltase. The extent of fermentation does not differ materially from that attained with glucose. Lactose is not fermented.

Of the higher sugars raffinose is fermented by juice from bottom yeast, but more slowly than sucrose or maltose. No experiments seem to have been made with juice from top yeast.

As regards the fermentation of the higher carbohydrates, very little experimental work has been carried out. Buchner and Rapp found that the fermentation of starch paste was doubtful, but that soluble starch and commercial dextrin were fermented with some freedom. Inulin and the erythrose prepared from the yeast cell-wall are slowly fermented, yeast gum hardly at all [Gottschalk, 1926]. No special study has been made of the diastatic enzymes which bring about the hydrolysis of these substances.

The fermentation of glycogen by yeast-juice is of considerable interest, since it is known that the characteristic reserve carbohydrate of the yeast cell is glycogen [see Harden and Young, 1902,

where the literature is cited], and moreover, that in living yeast the intracellular fermentation of glycogen proceeds readily, whereas glycogen added to a solution in which yeast is suspended is not affected. Yeast-juice contains a diastatic enzyme which hydrolyses glycogen to a reducing and fermentable sugar, so that in a juice poor in zymase to which glycogen has been added, the amount of sugar is found to increase, the hydrolysis of the glycogen proceeding more quickly than the fermentation of the resulting sugar [Harden and Young, 1904], but the course of this enzymic hydrolysis of glycogen by yeast-juice has not yet been studied. As a rule, it is found both with juices from top and bottom yeast that the evolution of carbon dioxide from glycogen proceeds less rapidly and reaches a lower total than from an equivalent amount of glucose.

Since nearly all samples of yeast contain glycogen, yeast-juice and also zymin usually contain this substance as well as the products of its hydrolysis. These provide a source of sugar which enters into alcoholic fermentation, so that a slow spontaneous production of carbon dioxide and alcohol proceeds when yeast-juice is preserved without any addition of sugar. The extent of this autofermentation varies considerably, as might be expected, with the nature of the yeast employed for the preparation of the material, but is generally confined within the limits of 0·06 to 0·5 gram of carbon dioxide for 25 c.c. of juice.

In juice from bottom yeast it amounts to about 5 to 10 per cent. of the total fermentation obtainable with glucose [Buchner, 1900, 2], whereas in juice from top yeasts, which gives a smaller total fermentation with glucose, it may occasionally equal, or even exceed, the glucose fermentation, and frequently amounts to 30 to 50 per cent. of it. It is therefore generally advisable in studying the effect of yeast-juice on any particular substance to ascertain the extent of autofermentation by means of a parallel experiment.

The maceration extract of Lebedev (p. 24) is usually, but not invariably [Oppenheimer, 1914, 2], free from glycogen, which is hydrolysed and fermented during the processes of drying and macerating, and therefore as a rule shows no appreciable autofermentation.

The fermentation of glycogen, like that of glucose, requires the presence of the coenzyme and is accompanied by esterification of phosphoric acid (see p. 142) [Gottschalk, 1926].

(*e*) *Effect of Concentration of Sugar on the Total Amount of Fermentation.*

The kinetics of fermentation by zymase will be considered later on (p. 180), but the effect on the total fermentation of different concentrations of sugar, this substance being present throughout in considerable excess, may be advantageously discussed at this stage. The subject has been investigated by Buchner [Buchner, E. and H., and Hahn, 1903, pp. 150-8; Buchner and Rapp, 1897] using sucrose, and he has found both for yeast-juice and for dried yeast-juice dissolved in water that (*a*) the total amount of fermentation increases with the concentration of the sugar; (*b*) the initial rate of fermentation decreases with the concentration of the sugar. The following are the results of a typical experiment, 20 c.c. of yeast-juice being employed in presence of toluene at 22° :—

Sucrose.		CO_2 in grams after		
Weight. Grm.	Per Cent.	6 hours.	24 hours.	96 hours.
2·2	10	0·17	0·50	0·55
3·52	15	0·14	0·53	0·64
5	20	0·13	0·54	0·73
6·66	25	0·13	0·52	0·80
8·56	30	0·12	0·46	0·81
10·76	35	0·12	0·40	0·82
13·33	40	0·11	0·36	0·82

The results as to the total fermentations in experiments of this kind are liable to be vitiated by the circumstance that when a low initial concentration of sugar is employed, the supply of sugar may be so greatly exhausted before the close of the experiment as to cause a marked diminution in the rate of fermentation and hence an unduly low total. Even allowing, however, for any effect of this kind, the foregoing table clearly shows the increase in total fermentation and the decrease in initial rate accompanying the increase of sugar concentration from 10 to 40 per cent. Working with a greater range of concentrations (3·3-53·3 grm. per 100 c.c.) Lebedev has obtained similar results with maceration extract [1911, 4], but has found that the total amount fermented diminishes after a certain optimum concentration (about 33·3 grm. per 100 c.c.) is reached.

A practical conclusion from these experiments is that a high concentration of sugar tends to preserve the enzyme in an active state for a longer time. Simultaneously it prevents the development of bacteria and yeast cells.

(f) *Effect of Varying Concentration of Yeast-Juice.*

This subject, which is of considerable importance with reference to the question of the protoplasmic or enzymic nature of the active agent in yeast-juice, has been examined in some detail by Buchner [Buchner, E. and H., and Hahn, 1903, pp. 158-65] and by Meisenheimer [1903] for juices from bottom yeast, by Harden and Young [1904] for those from top yeast, and by Lebedev [1911, 4] for maceration extract, the results obtained being in substantial agreement.

Dilution of yeast-juice with sugar solution, so that the concentration of the sugar remains constant, produces a small progressive diminution in the total fermentation, which only becomes marked when more than two volumes are added, and this independently of the actual concentration of the sugar. Dilution with water produces a somewhat more decided diminution, which, however, does not exceed 50 per cent. of the total for the addition of three volumes of water. The effect on maceration extract is somewhat greater but of the same kind. The autofermentation of juice from top yeast is scarcely affected by dilution with four volumes of water.

On the whole, therefore, yeast-juice may be said to be only slightly affected by dilution even with pure water, and the effect of the latter can in no way be regarded as comparable with the poisonous effect which it exerts on living protoplasm, as suggested by Macfadyen, Morris, and Rowland [1900].

(g) *The Effect of Antiseptics on the Fermentation of Sugars by Yeast-Juice.*

Buchner has paid special attention to the effect of antiseptics on the course of fermentation by yeast-juice [Buchner and Rapp, 1897 ; 1898, 2, 3 ; 1899, 1 ; Buchner and Antoni, 1905, 1 ; Buchner and Hoffmann, 1907 ; Buchner, E. and H., and Hahn, 1903, pp. 169-205 ; see also Albert, 1899, 2 ; Gromoff and Grigorieff, 1904 ; Duchaček, 1909] in order (1) to obtain evidence as to the possibility of the active agent in yeast-juice consisting of fragments of protoplasm and not of a soluble enzyme, and (2) also to provide a safe method of avoiding contamination, by the growth of bacteria or yeasts, of the liquids used, which were often kept at 25° for several days. The results of these experiments as far as they bear on these two points are briefly summarised in the following table, in which the effect of each substance on the total fermentation produced is noted :—

Substance.	Effect on Total Fermentation.
Concentrated solution of glycerol .	Slight diminution
,, ,, ,, sugar . .	,, increase
Toluene (to saturation or excess) .	Less than 10 per cent. diminution
Chloroform 0·5 per cent. . . .	Slight increase
,, 0·8 ,, (saturation)	No change
,, large excess (17 per cent.)	64 per cent. diminution
Chloral hydrate 0·7 per cent. . .	Increase up to 27 per cent.
,, 3·5-5·4 per cent. .	Completely inhibited
Phenol 0·1 per cent. . .	No change
,, 0·5 ,, . .	40 per cent. diminution
,, 1·2 ,, . .	Completely inhibited
Thymol 1 ,, . .	Slight diminution
,, 5 ,, . .	Marked ,,
Benzoic acid 0·1 ,, . .	7 per cent. diminution
,, 0·25 ,, . .	26 ,, ,,
Salicylic acid 0·1 ,, . .	10 ,, ,,
,, 0·27 ,, . .	35 ,, ,,
Formaldehyde 0·12 ,, . .	20 ,, ,,
,, 0·24 ,, . .	30-60 ,, ,,
Acetone 6 ,, . .	20 ,, ,,
,, 14 ,, . .	80 ,, ,,
Alcohol 6 ,, . .	0-20 ,, ,,
,, 14 ,, . .	75 ,, ,,
Sodium fluoride 0·5 ,, . .	99 ,, ,,
,, 2 ,, . .	Almost completely inhibited
Ammonium fluoride 0·55 per cent. .	Completely inhibited
Sodium azoimide, NaN_3 0·36 per cent.	Slight diminution
,, ,, 0·71 ,,	Marked ,,
Quinine hydrochloride 1 ,,	Slight increase
Ozone 10·4-34·8 milligrams per 20 c.c.	Marked diminution
Hydrocyanic acid 1·2 per cent. . .	Completely inhibited

The general result of these experiments is to show that quantities of antiseptics which are sufficient to inhibit the characteristic action of living cells have only a slight effect on the fermentative activity of yeast-juice. A large excess of the antiseptic in many cases produces a very decided diminution or total destruction of the fermenting power, and accompanying this a precipitation of the constituents of the juice. The decided increase of activity produced by small quantities of chloral hydrate, and to a less marked extent by chloroform and a few other substances, is of considerable interest. It is ascribed by Duchaček to a selective action on the proteoclastic enzyme, but without satisfactory evidence. The effect of some of these substances on the various reactions involved in alcoholic fermentation, such as the esterification of phosphoric acid, the action of hexosephosphatase, etc., has also been determined (see Chapter VIII.).

Hydrocyanic acid, even in dilute solution, completely suspends the fermenting power of the juice, without, however, producing any permanent change in the fermenting complex, as is shown by the fact that when the hydrocyanic acid is removed by a current of air, the juice regains its fermenting power. In this respect therefore hydrocyanic acid behaves as with certain other enzymes and with colloidal platinum [Bredig, 1901]. Sodium arsenite is a pronounced proto-

plasmic poison, which rapidly destroys the power of growth and reproduction in living cells, and was therefore applied to yeast-juice to differentiate between protoplasmic and enzymic action. It was, however, found that the action of this substance was complicated by some unknown factor and very irregular results were obtained[Buchner, E. and H., and Hahn, 1903, pp. 193 ff.]. These phenomena appear to be of the same order as those produced by the addition of arsenates to yeast-juice [Harden and Young, 1906, 3], and will be discussed along with the latter (p. 158).

Permanent Preparations Containing Active Zymase.

A considerable number of preparations have been obtained in the dry state which retain some proportion of the fermenting power of yeast or yeast-juice.

Starting with yeast-juice, it is possible to arrive at this result either by evaporation or precipitation. When the juice is very rapidly evaporated to a syrup at 20° to 25° and then further dried at 35°, either in the air or in a vacuum, and finally exposed over sulphuric acid in a vacuum desiccator, a dry brittle mass is obtained which is soluble in water and retains practically the whole of the fermenting power of the juice. The success of the preparation depends on the nature of the yeast from which the juice is derived, Berlin yeasts V and S yielding much less satisfactory results than Munich yeast. The powder when thoroughly dry is found to retain its properties almost unimpaired for at least a year, and can be heated to 85° for eight hours without undergoing any serious loss of fermenting power [Buchner and Rapp, 1898, 4 ; 1901 ; Buchner, E. and H., and Hahn, 1903, pp. 132-9].

Active powders can also be obtained by precipitating yeast-juice with alcohol, alcohol and ether, or acetone. The preparation is best effected by bringing the juice into 10 volumes of acetone, centrifuging at once and as rapidly as possible, washing, first with acetone and then with ether, and finally drying over sulphuric acid. The white powder thus obtained is not completely soluble in water but is almost entirely dissolved by aqueous glycerol (2·5 to 20 per cent.), forming a solution which has practically the same fermenting power as the original juice. The precipitation can be repeated without any serious loss of fermenting power. Prolonged contact of the precipitate with the supernatant liquid, especially when alcohol or alcohol and ether is used, causes a rapid loss of the characteristic property [Albert and Buchner, 1900, 1, 2 ; Buchner, E. and H., and Hahn, 1903, pp. 228-246 ; Buchner and Duchaček, 1909].

A preparation of this character has been obtained by Boyland [1930, 1] which is of great interest inasmuch as when it is ground with sugar and water and the liquid centrifuged the solution produces only a very slow fermentation with glucose or fructose but ferments them readily when inorganic phosphate is present. This is apparently due to lack of hexosephosphatase (see p. 70).

Dry preparations capable of fermenting sugar can also be readily obtained from yeast without any preliminary rupture of the cells. Heat alone (yielding a product known as hefanol) or treatment with dehydrating agents may be used for this purpose, and a brief allusion has already been made (p. 20) to the different varieties of permanent yeast (Dauerhefe) obtainable in these ways. The most important of these products are dried yeast, and the material known as zymin, which is now made under patent rights for medicinal purposes. Dried yeast has been very largely used in the investigation of the enzymes of yeast. It is manufactured commercially and can readily be prepared in the laboratory as already described (p. 25). Some discussion has taken place as to whether the fermentation produced by dried yeast, which is often preceded by a long induction period (see p. 165) is due to the presence of living cells [Abderhalden, 1926; Kostytschev, Medvedev and Kardo-Sysojeva, 1927; Sobotka, 1924, 1925]. Further experiments [see Euler and Barthel, 1926; Barthel, Euler and Myrbäck, 1929; Myrbäck and Euler, 1929; Harden, 1925] have shown that the proportion of living cells in many dried yeasts is far too small to account for the fermenting power, especially in the presence of toluene, and that under proper conditions of dilution (see p. 40) fermentation sets in long before the few living cells present have had time to produce fresh yeast cells. Dried yeast possessing 50 to 75 per cent. of the fermenting power of fresh yeast has been described by Myrbäck and Euler [1929] but this is unusual, 10-30 per cent. being a more general value.

Zymin has proved of value in the investigation of the production of zymase in the yeast cell [Buchner and Spitta, 1902], and of many other problems concerned with alcoholic fermentation. In order to prepare it 500 grams of finely divided pressed brewer's yeast, containing about 70 per cent. of water, are brought into 3 litres of acetone, stirred for ten minutes, and filtered and drained at the pump. The mass is then well mixed with 1 litre of acetone for two minutes and again filtered and drained. The residue is roughly powdered, well kneaded with 250 c.c. of ether for three minutes, filtered, drained, and spread on filter paper or porous plates. After standing for an hour in the air it is dried at 45° for twenty-four hours. About 150 grams of an almost

white powder containing only 5·5 to 6·5 per cent. of water are obtained. This is quite incapable of growth or reproduction but produces a very considerable amount of alcoholic fermentation, far greater indeed than a corresponding quantity of yeast-juice. Two grams of the powder corresponding to 6 grams of yeast and about 3·5 to 4 c.c. of yeast-juice, are capable of fermenting about 2 grams of sugar, whereas the 4 c.c. of yeast-juice would on the average only ferment from one-quarter to one-sixth of this amount of sugar. The rate produced by this amount of zymin is about one-eighth of that given by the corresponding amount of living yeast [Albert, 1900; Albert, Buchner, and Rapp, 1902]. The proteoclastic enzyme is still present in zymin, which undergoes autolysis in presence of water in a similar manner to yeast-juice [Albert, R. and W., 1901].

As already mentioned an active juice can be prepared by grinding acetone-yeast with water, sand, and kieselguhr, and this process presents the advantage that samples of yeast-juice of approximately constant composition can be prepared at intervals from successive portions of a uniform supply of acetone-yeast.

Preparations of acetone-yeast, made from yeast freed from glycogen by exposure in a thin layer to the air for three or four hours at 35° to 45°, or eight hours at the ordinary temperature [Buchner and Mitscherlich, 1904], show practically no autofermentation and may be used analytically for the estimation of fermentable sugars.

According to Bokorny [1916] an active preparation can be obtained by digesting yeast with 0·1 per cent sulphuric acid for several hours, washing and drying at 35°.

All the foregoing preparations exhibit the same general properties as yeast-juice, as regards their behaviour towards the various sugars, antiseptics, etc.

When zymin is mixed with sugar solution without being previously ground, it exhibits a peculiarity which is of both theoretical and practical interest. The time which elapses before the normal rate of fermentation is attained, which may be termed the induction period, and the total fermentation obtainable vary with the amount of sugar solution added, the time increasing and the total diminishing as the quantity of this increases. This phenomenon appears to have been noticed by Trommsdorff [1902], and a single experiment of Buchner shows the influence of the same conditions [Buchner, E. and H., and Hahn, 1903, p. 265, Nos. 700-1]. Harden and Young have found that when 2 grams of zymin are mixed with varying quantities of 10 per cent. sugar solution the following results are obtained :—

Volumes of Sugar Solution.	Total Gas Evolved in				
	1 hour.	2 hours.	3 hours.	4 hours.	22·5 hours.
5 c.c. . . .	15·7	31·6	44·8	56·5	233·3
10 ,, . . .	2·2	10·5	23	31·8	202·3
20 ,, . . .	0·9	2·4	13·6	23·7	125·5
40 ,, . . .	1·4	1·7	2·3	2·9	56·3

It follows that when zymin is being tested for fermenting power, a uniform method should be adopted, and all comparative tests should be made with the same volumes of added sugar solution. The nature of this induction period, which is also shown by dried yeast, is discussed later on (p. 165).

CHAPTER III.

THE FUNCTION OF PHOSPHATES IN ALCOHOLIC FERMENTATION.

In the course of some preliminary experiments (commenced by the late Allan Macfadyen, but subsequently abandoned) on the production of anti-ferments by the injection of yeast-juice into animals, the serum of the treated animals was tested for the presence of such anti-bodies both for the alcoholic and proteoclastic enzymes of yeast-juice, and it was then observed that the serum of normal and of treated animals alike greatly diminished the autolysis of yeast-juice.

As the explanation of the comparatively rapid disappearance of the fermenting power from yeast-juice had been sought, as already mentioned (p. 20), in the hydrolytic action of the tryptic enzyme which always accompanies zymase, the experiment was made of carrying out the fermentation in the presence of serum, with the result that about 60 to 80 per cent. more sugar was fermented than in the absence of the serum [Harden, 1903].

This fact was the starting-point of a series of attempts to obtain a similar effect by different means, in the course of which a boiled and filtered solution of autolysed yeast-juice was used, in the hope that the products formed by the action of the tryptic enzyme on the proteins of the juice would, in accordance with the general rule, prove to be an effective inhibitant of that enzyme. This solution was, in fact, found to produce a very marked increase in the total fermentation effected by yeast-juice, the addition of a volume of boiled juice equal to that of the yeast-juice doubling the amount of carbon dioxide evolved [Harden and Young, 1905, I]. This effect was found to be common to the filtrates from boiled fresh yeast-juice and from boiled autolysed yeast-juice, and was ultimately traced in the main, not to the anti-tryptic effect which had been surmised, but to two independent factors, either of which was capable in some degree of bringing about the observed result.

Boiled yeast-juice was indeed found to possess a decided anti-autolytic effect, as determined by a comparison of the amounts of

nitrogen rendered non-precipitable by tannic acid in yeast-juice alone and in a mixture of yeast-juice and boiled juice on preservation [Harden, 1905]. The anti-autolytic effect, however, appeared to vary independently of the effect on the fermentation, and the conclusion was drawn, as stated above, that the increase in the alcoholic fermentation was not entirely dependent on the decrease in the action of the proteoclastic enzyme but was due to some independent cause. The property possessed by boiled yeast-juice of diminishing the autolysis of yeast-juice was subsequently examined by Buchner and Haehn [1910, 1] and more recently by Haehn and Schifferdecker [1923], and ascribed by them to a soluble anti-protease (p. 82).

The two factors to which the increase in fermentation produced by the addition of boiled juice was ultimately traced were (1) the presence of phosphates in the liquid, and (2) the existence in boiled fresh yeast-juice of a co-ferment or co-enzyme, the presence of which is indispensable for fermentation [Harden and Young, 1905, 1, 2].

The former of these factors will be here discussed and the co-enzyme will form the subject of the following chapter.

The general fact that sodium phosphate increases the total fermentation produced by a given volume of yeast-juice was observed on several occasions by Wroblewski [1901] and also by Buchner [Buchner, E. and H., and Hahn, 1903, pp. 141-2], who ascribed the action of this salt to its alkalinity, comparing it in this respect with potassium carbonate and remarking that the increase in both cases took place chiefly in the first twenty hours of fermentation. The increased amount of fermentation following the addition of boiled yeast-juice was also noted by Buchner and Rapp [1899, 2, No. 265, p. 2093] in a single experiment.

Observations made at intervals of a few minutes instead of twenty hours have, however, revealed the fact that phosphates play a part of fundamental importance in alcoholic fermentation and that their presence is absolutely essential for the production of the phenomenon.

Effect of the Addition of Phosphate to a Fermenting Mixture of Yeast-Juice and Sugar.

When a suitable quantity[1] of a soluble phosphate is added to a fermenting mixture of glucose, fructose, or mannose with yeast-juice, the rate of fermentation rapidly rises, sometimes increasing as much as twenty-fold, continues at this high value for a certain period and

[1] The effect of an excess of phosphate is discussed later on, p. 150.

then falls again to a value approximately equal to, but generally somewhat higher than, that which it originally had. Careful experiments have shown that during this period of enhanced fermentation the amounts of carbon dioxide and alcohol produced exceed those which would have been formed in the absence of added phosphate by a quantity almost exactly equivalent to the phosphate added in the ratio CO_2 or C_2H_6O : R'_2HPO_4 [Harden and Young, 1906, 1].

This result is of fundamental importance, and the evidence on which it rests deserves some consideration. Quantitative experiments on this subject require certain preliminary precautions. The acid phosphates are too acid to permit of any extended fermentation and the phosphates of the formula R'_2HPO_4 absorb a considerable volume of carbon dioxide with production of a bicarbonate, according to the reaction

$$R_2HPO_4 + H_2CO_3 \rightleftharpoons RHCO_3 + RH_2PO_4.$$

The method which has been adopted, therefore, is to employ either a secondary phosphate saturated with carbon dioxide at the temperature of the experiment, or a mixture of five molecular proportions of the primary phosphate with one molecular proportion of a secondary phosphate ($p_H = 6{\cdot}2$) in which the amount of bicarbonate formed is negligible. In the former case it is necessary to ascertain how much of the carbon dioxide evolved is derived from the bicarbonate by a disturbance of the original equilibrium owing to the chemical changes which occur (p. 55). This is done by acidifying duplicate samples with hydrochloric or trichloroacetic acid before and after the fermentation and measuring the gas evolved in each case. Any necessary correction can then be made.

The calculation of the extra amount of carbon dioxide evolved from yeast-juice containing sugar when a phosphate is added involves an estimation of the amount which would have been evolved in the absence of added phosphate, and this is a matter of some uncertainty. With some yeast preparations, especially dried yeast or zymin, the final steady rate of fermentation is often greater than the initial rate (owing to the greater concentration of hexosephosphate; see p. 71) [Boyland, 1929], and the practice has been adopted of ascertaining this final rate and then calculating the total evolution corresponding to it for the whole period from the time of the addition of the phosphate to the end of the observations. This amount deducted from the observed total leaves the extra amount of carbon dioxide formed, and it is this quantity which is equivalent to the phosphate added. This

correction is probably somewhat too large so that the volume of carbon dioxide found is rather less than that actually evolved. This method of calculation is discussed later. Alcohol is simultaneously produced in the normal ratio.

An experiment is illustrated graphically in Fig. 4, in which the volume of carbon dioxide evolved is plotted against time. The determination was made by adding 25 c.c. of an aqueous solution containing 5 grams of glucose to one quantity of 25 c.c. of yeast-

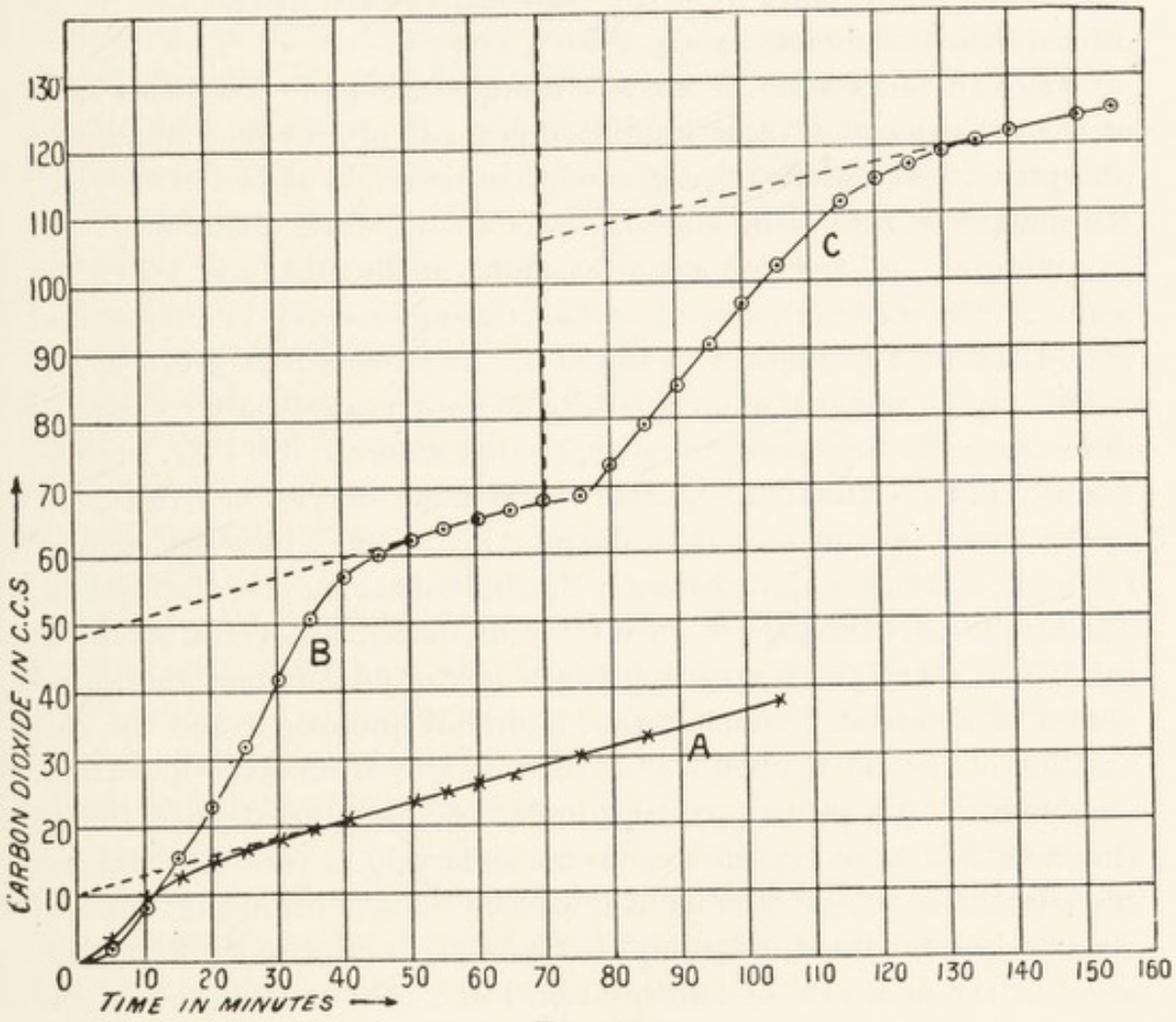


FIG. 4.

juice (curve A) and 5 c.c. of 0·3 molar solution of the mixed primary and secondary sodium phosphates, and 20 c.c. of a solution containing 5 grams of glucose to a second equal quantity of yeast-juice (curve B). Curve A shows the normal course of fermentation of yeast-juice with glucose. There is a slight preliminary acceleration during the first twenty minutes, due to free phosphate in the juice, and the rate then becomes steady at about 1·4 c.c. in five minutes. During this preliminary acceleration 10 c.c. of extra carbon dioxide are evolved, this number being obtained graphically by continuing the line of steady

rate back to the axis of zero time. Curve B shows the effect of the added phosphate. The rate rises to about 9·5 c.c. in five minutes, i.e. to more than six times the normal rate, and then gradually falls until after an hour it is again steady and almost exactly equal to 1·4 c.c. per five minutes. Continuing the line of steady rate back to the axis of zero time, it is found that the extra amount of carbon dioxide is 48 c.c. Subtracting from this the 10 c.c. shown in curve A as due to the juice alone, a difference of 38 c.c. is obtained due to the added phosphate. The amount calculated from the phosphate added in this case is, at atmospheric temperature and pressure, 38·9 c.c.

After the expiration of seventy minutes from the commencement of the experiment, a second addition is made of an equal amount of phosphate. The whole phenomenon then recurs, as shown in Curve C, the maximum rate being slightly lower than before, about 6 c.c. per five minutes, and the rate again becoming finally steady at 1·4 c.c. as before. The extra amount of carbon dioxide evolved in this second period obtained graphically as in the former case, is 107—68 = 39 c.c.

It may be noted that in this case the observations after each addition last fifty to seventy minutes, so that an error of 0·1 c.c. per five minutes in the estimated final rate would make an error of 1 to 1·4 c.c. in the extra amount of carbon dioxide, i.e. about 3 to 4 per cent. of the total, and this is approximately the limit of accuracy of the method. The results are more precise when the yeast-juice employed is an active one, since, when the fermenting power of the juice is low, the initial period of accelerated fermentation is unduly prolonged and the calculation of the extra amount of carbon dioxide is rendered uncertain.

Zymin (p. 39) yields precisely similar results to yeast-juice, but in this case the rate of fermentation is not so largely increased. This has the effect that the extra amount of carbon dioxide cannot be quite so accurately estimated for zymin, because a slight error in the determination of the final rate of fermentation has a greater influence on the result. The equivalence between the extra amount of carbon dioxide evolved and the phosphate added is, however, unmistakable, as is shown by the following results of an experiment with zymin, in which 6 grams of zymin (Schroder) + 3 grams of fructose (Schering) + 25 c.c. of water were incubated at 25° in presence of toluene until a steady rate had been attained. Five c.c. of a solution of sodium phosphate equivalent to 32·2 c.c. carbon dioxide at N.T.P. were then added.

Maximum rate attained, c.c. per five minutes	14·1
Final rate of fermentation	6·2
Total evolved by fermentation in eighty minutes after addition of phosphate	131
Correction for evolution in absence of phosphate in eighty minutes	99·2
Extra carbon dioxide at 10° and 767·1 mm.	31·8
„ „ „ „ N.T.P.	29·8

Considering the small proportional rise in rate and the long period of accelerated fermentation, the agreement between the volume observed, 29·8 c.c., and that calculated from the phosphate, 32·2, is quite satisfactory [Harden and Young, 1910, 1]. Precisely the same relations hold for maceration extract, but in this case it must be remembered that a large amount of free phosphate is present in the extract, as much as 0·3129 grm. $Mg_2P_2O_7$ being obtained from 20 c.c. in one preparation, so that the original extract had the concentration of a 0·14 molar solution of sodium phosphate. When this phosphate is removed by incubation with glucose or fructose, the subsequent addition of phosphate produces the characteristic action, and the extra carbon dioxide evolved is, as with other yeast preparations, approximately equivalent to the phosphate added. An actual estimation carried out in this way gave 35 c.c. of CO_2 for an addition of phosphate equivalent to 32·9 c.c. [Harden and Young, 1912].

The behaviour towards phosphate of yeast which has been dried by exposure to air at about 37° varies with the strain of yeast employed. The German bottom yeasts when dried as a rule react very readily, whilst many of the dried German top yeasts only react to a very small extent, in some cases owing to lack of co-enzyme [see Neuberg and Gottschalk, 1924, 2]. The dried English top yeasts as a rule react readily.

Yeast which has been treated with toluene [Harden, 1911; Euler and Johansson, 1912, 2] or with benzene, xylene, ether or ethyl butyrate [Kerr and Young, 1926] also reacts with phosphate in a similar manner to other yeast preparations.

Within the limits imposed by the experimental conditions, then, the evidence is strongly in favour of the conclusion that the addition of a soluble phosphate to a fermenting mixture of a hexose with yeast-juice, maceration extract, dried yeast or zymin causes the production of an equivalent amount of carbon dioxide and alcohol.

This fact indicates that a definite chemical reaction occurs in which sugar and phosphate are concerned, and this conclusion is confirmed when the fate of the added phosphate is investigated. If an experiment, such as one of those described above, be interrupted as soon as the rate of fermentation has again become normal, and the liquid be boiled and filtered, it is found that nearly the whole of the phosphorus present passes into the filtrate, but that only a very small proportion of this exists as mineral phosphate, whilst the remainder, including that added in the form of a soluble phosphate, is no longer precipitable by magnesium citrate mixture [Harden and Young, 1905, 2]. The

phosphate has been esterified and converted into a phosphoric ester of a sugar.

A similar observation was made at a later date by Ivanov [1907], who had previously observed [1905] that living yeast, like many other vegetable organisms, converted mineral phosphates into organic derivatives. Ivanov employed zymin and hefanol (p. 39) instead of yeast-juice, and found that phosphates were thereby rendered non-precipitable by uranium acetate solution, but did not observe the accelerated fermentation caused by their addition.

A considerable number of estimations have been carried out in which the amount of phosphorus esterified has been determined experimentally as well as the volume of carbon dioxide evolved [Euler and Johansson, 1913; Harden and Henley, 1927, 2; 1929; Boyland, 1929, 1930, 1, 2], with the result that the ratio of CO_2/P esterified has been found usually to be slightly greater than 1 for dried yeast, slightly less (0·85-0·97) for zymin, yeast-juice, and maceration extract. Experiments with preparations obtained by adding acetone and ether to yeast-juice, which (owing to lack of hexosephosphatase, see p. 70) did not ferment sugar in absence of inorganic phosphate, and for which therefore no correction was necessary, gave ratios 1·05-0·93 [Boyland, 1930, 1, 2].

On the other hand, Kluyver and Struyk [1928, 1, Struyk, 1928], using maceration extract, have obtained in some cases lower ratios (0·6-0·7) than were ever found by Harden and Henley in their experiments.

When dried yeast is employed as the fermenting agent the amount of phosphate esterified in the earlier stages is greater than would be expected, but ultimately becomes equivalent to the carbon dioxide evolved [Euler and Johansson, 1913; Boyland, 1929].

The addition of phosphate has no effect on the rate of fermentation by living yeast (see p. 183).

The Phospho-organic Compounds formed by Yeast Preparations from the Hexoses and Phosphate.

Phosphates undergo this characteristic change when the sugar undergoing fermentation is glucose, mannose, fructose, or sucrose, and it may be said at once that no definite distinction has been established between the products formed from these various sugars. Three different well-characterised compounds have so far been isolated from the products of this reaction, all in the form of salts, and it is possible

that others are present [see Robison and Morgan, 1930; Robison and King, 1931]. These are a hexosediphosphate [Harden and Young, 1908, 1; Young, 1909], a hexosemonophosphate [Harden and Robison, 1914; Robison, 1922], and a trehalosemonophosphate [Robison and Morgan, 1928, 2]. The last of these has only been found in quantity in the products formed by dried yeast. There is some evidence that the hexosemonophosphate from mannose differs from that obtained from glucose and fructose [Neuberg, I. S., and Ostendorf, 1930], but this ester has not as yet been obtained pure. In addition to these, two isomerides of hexosediphosphoric acid, which are only hydrolysed with difficulty, have been obtained [Lohmann, 1930; Lipmann and Lohmann, 1930]. A hexosemono- and hexosedi-phosphoric acid have also been isolated from muscle (p. 64), and a hexosemonophosphoric acid has been prepared by the partial hydrolysis of hexosediphosphate [Neuberg, 1918, 2]

Preparation of the Hexosephosphates.

Several methods are available for the treatment of the products of fermentation with the object of isolating the phosphoric esters. The mixture contains a large amount of yeast protein which must be removed at some stage of the process. According to Robison's earlier method [1922] this is precipitated by the addition of an equal volume of alcohol to the liquid after the addition of a weight of solid barium acetate equal to the weight of the crystalline sodium phosphate added during the fermentation and sufficient baryta to make the mixture alkaline to phenolphthalein. After standing overnight the precipitate, which consists of the protein and the barium salts of any orthophosphoric acid and of the hexosephosphoric esters, is washed with 70 per cent. alcohol, treated with boiling absolute alcohol and allowed to remain in contact with the alcohol overnight. The protein is thus denatured and rendered insoluble and the barium salts can then be extracted fractionally by treatment with successive quantities of water. Neuberg and Leibowitz on the other hand [1927, 1] precipitate the proteins by heat-coagulation after the solution has been neutralised with baryta. The protein-free filtrate, after the addition of barium acetate, is again heated to boiling in a brine-bath and filtered hot, the phosphate and nearly all the hexosediphosphate (the barium salt of which is less soluble in hot than in cold water) being thus removed as insoluble salts, from which the hexosediphosphate can be obtained by extraction with cold water. This affords a rapid method for the preparation of the monophosphate, but there is some

danger of hydrolysis of the hexosediphosphate during this process [Robison and Morgan, 1930] leading to the formation of the hexosemonophosphate of Neuberg (p. 62).

Robison and Morgan [1930] therefore recommend the precipitation of the protein by trichloroacetic acid. In a typical preparation according to this method, which is recommended as the best at present available, 30 g. of glucose or fructose are added to 300 c.c. of yeast-juice or maceration extract, or to 50 g. of dried yeast or zymin mixed with 180 c.c. of water and brought to 28° to 30°. Dried yeast or zymin should be used for the preparation of hexosediphosphate, dried yeast for trehalosemonophosphate, and yeast-juice or maceration extract for hexosemonophosphate. The dried yeast or zymin is left in contact with the sugar solution for one hour before the addition of phosphate. To the fermenting mixture at 28° to 30° about 350 c.c. of a 20 per cent. solution of $Na_2HPO_4 . 12H_2O$(0·56 *M*) containing also 20 per cent. of the sugar, or a corresponding solution of K_2HPO_4 which is more easily soluble (35 g. in 200 c.c. solution with 60 g. sugar), is either added in five or six equal portions, the rate of fermentation being allowed to fall to the normal before each addition, or is run in continuously at a controlled rate. Potassium phosphate, however, gives a smaller proportion of hexosemonophosphate. It is convenient to collect the gas evolved over brine in a large nitrometer (500 c.c. capacity) as the rate of evolution of CO_2 affords a good indication of the progress of the reaction. The fermentation can also conveniently be carried out at room temperature.

At the conclusion of the fermentation sufficient 25 per cent. solution of trichloroacetic acid is added to bring the trichloroacetic acid content to 4 per cent., and the mixture is then well shaken, allowed to stand for two hours, preferably at 0°, and filtered after the addition of a few drops of capryl alcohol. In the protein-free filtrate the barium salts are precipitated by adding an amount of barium acetate equal in weight to the crystalline sodium phosphate added, and then bringing the reaction to p_H 8·4 by the addition of baryta, the bulk of which is added in hot saturated solution, and the final adjustment made with cold saturated solution. The precipitate is filtered off, washed with small quantities of water, pressed out, washed with absolute alcohol and dried *in vacuo*. This precipitate contains the bulk of the hexosediphosphate along with barium phosphate resulting from any excess of inorganic phosphate, whilst the hexosemonophosphate and trehalosephosphate are present in the filtrate. The separation is not a sharp one because (*a*) the hexosediphosphate is slightly soluble in

water, and (*b*) in presence of much monophosphate some of this is carried down (probably as a double salt) along with the hexosediphosphate.

Preparation of Barium Hexosediphosphate.—The diphosphate can readily be obtained pure [Robison and Morgan, 1930] by extracting the precipitate obtained with barium acetate and baryta with 200 parts of water at p_H 8·6 at 0°. The clear liquid, which should be free from inorganic phosphate, is treated with alcohol until a slight cloud forms and is then heated in 200 c.c. portions to 70° in a water-bath and filtered hot. The diphosphate remaining in solution can be recovered in a somewhat less pure condition by adding more alcohol and again heating at 70°. Purification by means of precipitation by lead acetate and decomposition of the washed precipitate with H_2S, the method originally used by Young [1909], is of little advantage and often leads to hydrolysis during the decomposition by sulphuretted hydrogen. Barium hexosediphosphate should be dried at low temperatures, otherwise hydrolysis may occur. Other less satisfactory methods of preparation have been described by Lebedev [1910] and Euler and Fodor [1911]. Salts of hexosediphosphoric acid are manufactured by Messrs. Bayer, the calcium salt being known as candiolin.

Preparation of the Hexosemonophosphates.—The treatment of the filtrate from the sparingly soluble precipitate obtained in the preparation of the hexosediphosphate varies according to the nature of the yeast preparation used and the hexosemonophosphate sought for.

Trehalosemonophosphoric Ester [Robison and Morgan, 1928, 2] (Fermentation carried out with dried yeast).—One-tenth of its volume of alcohol is added to the filtrate from the sparingly soluble barium salts and any precipitate which forms is filtered off. This consists largely of hexosediphosphate. To the filtrate a slight excess of basic lead acetate is added and the precipitate washed and decomposed by H_2S. The filtrate from the PbS is freed from H_2S by aeration, brought to p_H 8·4 with baryta and poured into two volumes of alcohol, the precipitated barium salts being washed with alcohol and rapidly dried *in vacuo*. The dried precipitate is then ground with 10 parts of water, a volume of alcohol equal to one-tenth of the volume of the water is added, the residue (which may contain some hexosediphosphate, hexosemonophosphate, and trehalosemonophosphate) is filtered off, washed and dried, and the filtrate poured into two volumes of alcohol. This final soluble fraction consists of varying proportions of hexose- and trehalosemonophosphate, and may still contain a small

proportion of diphosphate and P-free impurities. It serves for the isolation of trehalosemonophosphate, for which purpose it is dissolved in water and fractionally precipitated with alcohol up to 70 per cent., the fractionation being several times repeated. In this process the trehalosephosphate accumulates in the fraction precipitated between 30 to 70 per cent. of alcohol. The barium salt thus obtained is then converted into the brucine salt, which can be readily recrystallised from hot aqueous solution. The barium salt regenerated from the pure brucine salt may be obtained in crystalline form by adding alcohol to its saturated aqueous solution until the first signs of precipitation are observed and allowing the liquid to stand at room temperature.

Preparation of Hexosemonophosphate (Fermentation carried out with yeast-juice or maceration extract).—The hexosemonophosphate originally isolated by Harden and Robison [1914] and studied by Robison [1922] is now known to contain both glucose- and fructose-monophosphate. The preparation of the mixed monophosphoric esters free from diphosphate and trehalosephosphate precedes the separation of these two compounds.

For this purpose the soluble barium salts, obtained from the salts directly prepared from the lead acetate precipitate by trituration with 10 parts of water and addition of 1 part of alcohol (as in the process for the isolation of trehalosemonophosphate), are repeatedly dissolved in 10 per cent. alcohol, and the clear filtrate is precipitated with an equal volume of alcohol. It is sometimes advantageous to supplement the first precipitation with lead acetate by a second precipitation which is preceded by the addition of mercuric acetate to the filtered solution of the barium salt in 10 per cent. alcohol.

A rapid method of isolation [Neuberg and Leibowitz, 1927, 1] is to add basic lead acetate directly to the filtrate obtained by heating the neutralised fermentation products in a water-bath and adding barium acetate and filtering hot (p. 49). The washed lead salt is decomposed by H_2S, and reprecipitated with basic lead acetate and then converted into the barium salt which is precipitated by 50 per cent. alcohol. As already pointed out, some danger is incurred of hydrolysing a portion of the diphosphate present.

Isolation of Aldosemonophosphoric Ester and Ketosemonophosphoric Ester.—The soluble barium salts, which probably contain from 55 to 66 per cent. aldosemonophosphate (estimated iodimetrically), are converted into brucine salts, which are then fractionally crystallised from 20 per cent. ethyl alcohol. In order to obtain the aldosemonophosphate the less soluble salt, which crystallises in needles, is then fractionally

crystallised from boiling 90 to 95 per cent. methyl alcohol, and the salt which first separates is finally recrystallised from boiling absolute methyl alcohol [Robison and King, 1929, 1931].

The fraction of the brucine salts which are more soluble in 20 per cent. ethyl alcohol yields platelets containing a ketosemonophosphate which is probably identical with that obtained by the partial hydrolysis of hexosediphosphate (Neuberg). This ketosemonophosphate can be isolated from the platelets by treating the corresponding barium salt with bromine and barium carbonate, which oxidises any aldose-constituent to a phosphohexonic acid, leaving the keto-constituent untouched. The phosphohexonic acid is removed by the precipitation of its acid barium salt with 4 volumes of alcohol at p_H 3-4, and from the filtrate the barium salt of ketosemonophosphoric ester is obtained by neutralising with baryta to phenolphthalein, and is purified by solution in 10 per cent. alcohol and reprecipitation.

The final mother liquors from the solution of the brucine salts in 20 per cent. ethyl alcohol after the successive removal of the needles and platelets contain one or more phosphoric esters of unknown composition.

Hydrolysis of the Hexosephosphates by Acid.

All the hexosephosphates are hydrolysed when boiled with acid, but the rate at which phosphoric acid is liberated varies greatly for the different esters. The esters hitherto obtained, except that of trehalose and possibly the aldose- and keto-esters separated from fermentation hexosemonophosphate, are probably mixtures of at least two components (see pp. 56, 61, 62), and in agreement with this it is in general found that decomposition by acids is not a simple process but the resultant of two reactions proceeding at different rates [Lohmann, 1928, 1]. Thus fermentation hexosemonophosphate and Embden's ester from muscle are at first rapidly decomposed according to the equation of a unimolecular reaction with a definite value for k, but after a short time (when the readily hydrolysable keto-ester, present to the extent of 35 to 45 per cent., has all been decomposed), the value of k falls to a much lower level, about $\frac{1}{20}$ of its first value. With the Neuberg ester (p. 62), which contains 80 to 90 per cent. of keto-ester, a similar change takes place when about 80 per cent. of the total phosphoric acid has been liberated.

Hexosediphosphate loses its first phosphoric acid group five times as quickly as its second, which is lost at the same rate as that of the

Neuberg ester. A very small residue of more resistant ester is also left in this case, which is probably an aldose-ester. The following figures are given by Lohmann for *N* HCl at 100° :—

	k
Hexosemonophosphate.	
Fermentation (Robison)	$0{\cdot}2 \times 10^{-3}$
Embden	$0{\cdot}2 \times 10^{-3}$
Neuberg	$3{\cdot}5 \times 10^{-3}$
Hexosediphosphate.	
Harden and Young, 1st group	$22{\cdot}0 \times 10^{-3}$
,, ,, 2nd ,,	$4{\cdot}0 \times 10^{-3}$
Esters resistant to hydrolysis (see p. 59).	
Ester I.	$0{\cdot}1 \times 10^{-3}$
Ester II.	$0{\cdot}1 \times 10^{-3}$

A method for the rapid estimation of mixtures of esters has been based upon these facts by Lohmann [1928].

Properties of Hexosediphosphoric Acid.

A solution of the free acid can readily be prepared by the action of sulphuretted hydrogen on the lead salt suspended in water, by decomposition of the barium salt with sulphuric acid or of the calcium salt by oxalic acid. It forms a strongly acid liquid, which decomposes when evaporated, leaving a charred mass containing free phosphoric acid. The acid is slightly optically active and has $[\alpha_D] = + 3{\cdot}4°$, that of the barium salt being about $+ 3{\cdot}2°$ ($c = 0{\cdot}67$). The acid is tetrabasic and requires two equivalents of base for each atom of phosphorus when titrated with phenolphthalein as indicator. It is a stronger acid than orthophosphoric acid (see the following Table) and, as is the

Apparent Dissociation Constants of the Hexosephosphoric Acids.

	$p_{K'_1}$.	$p_{K'_2}$.
1. Phosphoric acid [1]	1·99	6·81
Hexosemonophosphate :		
Neuberg ester [2]	0·97	6·11
Embden ester [3]	—	6·12
Robison ester [3]	0·94	6·11
Hexosediphosphate :		
Harden and Young ester [1]	1·48	6·29
,, ,, ,, 1st group [1]	—	(6·1)
,, ,, ,, 2nd ,, [1]	—	(6·5)
Lohmann ester, I [4]	—	6·3

case with phosphoric acid, the dissociation constant of one of the two replaceable hydrogen atoms in each PO_4H_2 group is considerably greater than that of the second. The hydrolysis of the ester into hexose and phosphoric acid is therefore accompanied by a rise of p_H, and the reverse change occurs during the esterification of phosphoric acid.

[1] [Meyerhof and Suranyi, 1926.]
[2] [Irving and Fischer, 1927.]
[3] [Meyerhof and Lohmann, 1926.]
[4] Lohmann [Meyerhof, 1930, p. 74].

This is the reason why some of the bicarbonate present in phosphate solution which has been saturated with CO_2 is displaced chemically during esterification (see p. 44).

A number of amorphous salts have been prepared by precipitation from a solution of the sodium salt, or by neutralising the acid with the appropriate oxide [see Neuberg and Sabetay, 1925, 1], and of these the silver, barium, calcium, and magnesium salts have been analysed with results agreeing with the general formula $C_6H_{10}O_4$ $(PO_4R_2')_2$. The *barium salt* is sparingly soluble (0·6 g. in 100 c.c. of cold water) as are also the *calcium* and *manganese salts*, whilst the *magnesium salt* is more readily soluble (30 parts in 100 of cold water). They are all partially reprecipitated when their solutions are heated, but redissolve on cooling and this property can be utilised for their purification. The calcium and barium salts can be obtained in a readily soluble modification by dissolving in lactic acid, neutralising with ammonia and precipitating with alcohol [Neuberg and Sabetay, 1925, 1]. The alkali salts have only been obtained as viscid residues. The ferrous salt is a greyish-green, tasteless and odourless powder [Schweizer, 1920].

Some difference of opinion has existed as to the molecular weight and constitution of this substance. Ivanov [1909, 1] regarded it as a triosephosphoric acid, $C_3H_5O_2(PO_4H_2)$, basing this view on the preparation of an osazone which melted at 142°, but when recrystallised from benzene gave a product melting at 127° to 128°, which had the same appearance, melting-point, and nitrogen content as the triose-osazone formed by the action of phenylhydrazine on the oxidation products of glycerol. Neither Lebedev [1909] nor Young could obtain Ivanov's osazone, and all attempts to reduce the acid with formation of glycerol either by sodium amalgam or hydriodic acid were unsuccessful (Young). There is therefore practically no serious experimental evidence in favour of Ivanov's view.

On the other hand, Harden and Young regard the acid as a diphosphoric ester of a hexose. This view is based on the fact that when the acid is boiled with water, or an acid, free phosphoric acid is produced along with a lævo-rotatory solution containing fructose and possibly a small proportion of some other sugar or sugars. (Euler and Fodor, however, did not obtain a hexose in this way [1911].) From the solution obtained by boiling the aqueous acid Neuberg and his colleagues [1917] isolated pure fructose in the crystalline state and consider that this is the only hexose produced and that the original compound is fructose-diphosphoric ester. The fact observed by Young [1909] that the ratio

of the reducing power to the rotation of the product of hydrolysis is greater than corresponds with pure fructose is explained by Neuberg as due to the formation of a reducing by-product of low rotation, a similar effect being produced when synthetic fructosemonophosphoric acid is hydrolysed [Neuberg and Kretschmer, 1911].

The hexosemonophosphate obtained by partial hydrolysis of the diphosphate with acid (Neuberg), even when prepared from a carefully purified specimen, reacts to a small extent with iodine, the amount of aldose thus indicated being about 7 per cent. [Robison and King, 1931].

The reducing power towards Bertrand's solution is about 42 per cent. [Meyerhof, 1930, p. 74], and to Hagedorn and Jensen's reagent [Robison and Morgan, 1930] 40 per cent. of that of the equivalent amount of glucose in presence of 0·5 *M* sodium hydroxide. The acid itself only reduces Fehling's solution after some hours in the cold, rapidly when boiled, whereas when its solution is first boiled, and then treated with Fehling's solution in the cold, the products of decomposition bring about reduction in a few minutes. With sodium hypoiodite (which oxidises aldoses but not ketoses) hexosediphosphate gives only a slight reaction, according to Meyerhof equal to about 4 per cent. of that of the hexose present (whilst fructose itself gives 2·5 per cent). This indicates that the hexose residue is almost exclusively a ketose. On the other hand, when the phosphate groups are removed by the hydrolytic action of bone phosphatase the sugar obtained (like that obtained by Young by acid hydrolysis) is considerably less lævo-rotatory than fructose [Martland and Robison, 1929]. The same enzyme on the other hand liberates pure trehalose from trehalosemonophosphoric ester. This affords an instance of the complicated and little understood nature of the changes which the sugar residues of these phosphoric esters undergo. (See also p. 68.)

The behaviour of the compound towards phenylhydrazine is also in complete agreement with this view. Lebedev found [1909, 1910] that the acid or its salts heated with phenylhydrazine in presence of acetic acid gave an insoluble compound, which was ultimately found to be the *phenylhydrazine salt of hexosemonophosphoric acid osazone*

$$C_6H_5NH \cdot NH_2 \cdot H_2PO_4 \cdot C_4H_5(OH)_3 \cdot C(N_2HC_6H_5)CH(N_2HC_6H_5)$$

[Lebedev, 1910; 1911, 5; Young, 1911]. After recrystallisation from alcohol this compound forms yellow needles, melting at 151° to 152°. It is decomposed by caustic soda yielding a *sodium salt*

$$Na_2PO_4 \cdot C_4H_5(OH)_3 \cdot (CN_2HC_6H_5) \cdot CH(N_2HC_6H_5)$$

and on boiling with caustic soda decomposes giving a hexosazone (free from phosphorus) which is probably glucosazone, and in addition glyoxalosazone, probably as the result of a secondary decomposition. Towards acid it is remarkably stable yielding with hydrochloric acid a *hexosonephosphoric ester* from which the original osazone can be regenerated (Lebedev). Lebedev at first [1910] argued from the formation of this osazone that the original hexosephosphate contained only one phosphoric acid group per molecule of hexose. It was, however, shown by Young [1911] and subsequently confirmed by Lebedev [1911, 5] that one molecule of phosphoric acid is split off during the formation of the osazone, even in neutral solution. Moreover, it has been found that in the cold hexosediphosphoric acid reacts with 3 molecules of phenylhydrazine forming the *di-phenylhydrazine salt of hexosediphosphoric acid phenylhydrazone*

$$(C_6H_5NH_2 \cdot NH \cdot H_2PO_4)_2 \cdot C_6H_7(OH)_3 \cdot N_2HC_6H_5.$$

This compound crystallises out when one volume of alcohol is added to a solution of three molecules of phenylhydrazine in one of the acid and forms colourless needles melting at 115°-117.° *p*-Bromophenylhydrazine yields an analogous compound melting at 127°-128°.

Precisely the same products are given with phenylhydrazine by the hexosediphosphoric acid prepared from glucose, mannose, and fructose, proving that all these sugars yield the same hexosediphosphoric acid, a point of fundamental importance.

A series of phenylosazones has been obtained by Lebedev [1924, 2] from the fermentation products of sucrose and dried yeast in presence of the primary and secondary phosphates of sodium and potassium, and of the primary phosphate with the addition of sodium sulphite, but their exact nature has not yet been made out.

Direct measurements of the molecular weight of the acid by the freezing-point method, combined with the determination of the degree of dissociation by the rate of sucrose inversion, are indecisive, but indicate that the acid has a molecular weight considerably higher than that required for a triosephosphoric acid.

A similar uncertainty attaches to the determination of the molecular weight from the freezing-point depression and conductivity of the acid potassium salt [Euler and Fodor, 1911]. Euler, however, concludes that both a hexosediphosphoric acid and a triosemonophosphoric acid are formed, but has not prepared any derivatives of the latter.

Constitution of Hexosediphosphoric Acid.

As regards the constitution of hexosediphosphoric acid Young was able to make several suggestions.

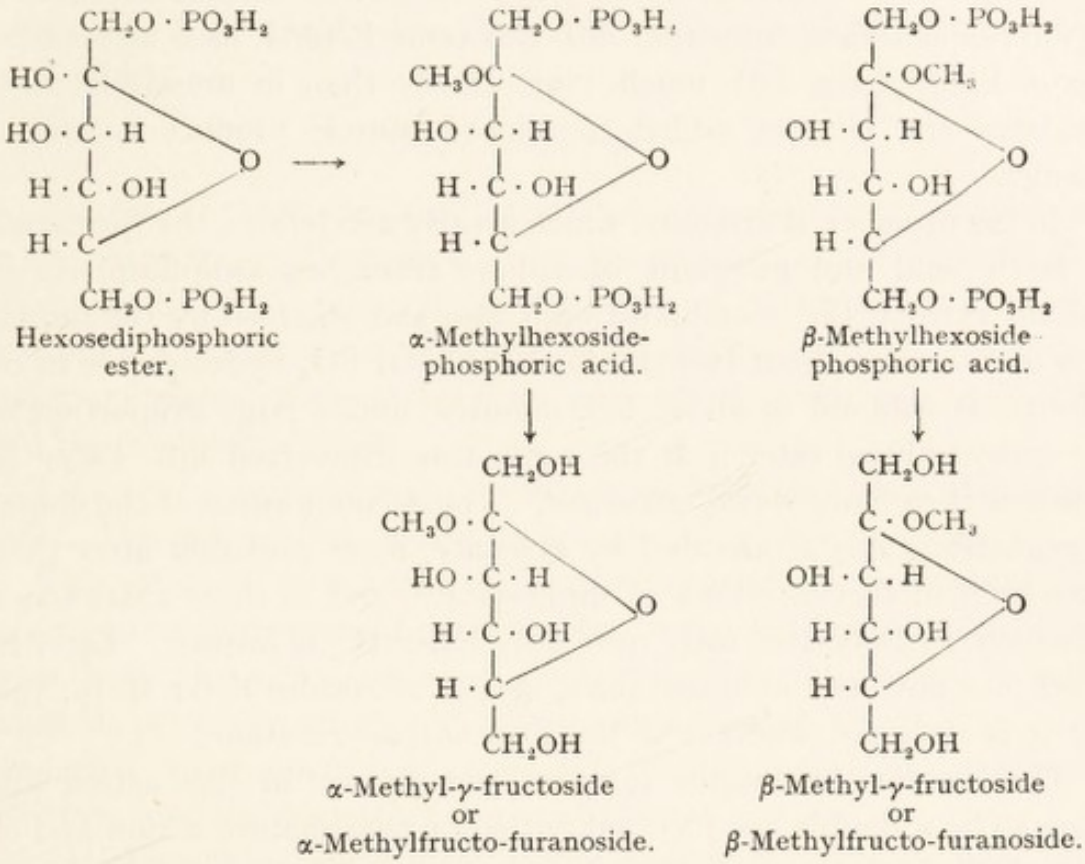
The identity of the products from glucose, mannose, and fructose may be explained by regarding the acid as a derivative of the enolic form common to these three sugars (p. 110), or by supposing that portions of two sugar molecules may be concerned in its production. The formation and composition of the hydrazone and osazone are of great importance as they indicate that in all probability one of the phosphoric acid residues is united with the carbon atom adjacent to the carbonyl group of the hexose. They moreover render it certain that the original phosphoric ester is a hexosediphosphoric ester and not a triosemonophosphoric ester.

Further information [Morgan, 1927; Robison and Morgan, 1928, 1] on this subject has been obtained by converting the diphosphoric ester into the corresponding α- and β-methylhexosediphosphoric acids by treatment with HCl in methyl alcoholic solution [Morgan, 1927] and then separating these by crystallisation of their brucine salts and removing the phosphoric acid groups by the action of the bone phosphatase of Martland and Robison [1929]. This resulted [Robison and Morgan, 1928, 1] in the production of two isomeric α- and β-methylfructosides, the specific rotation ($[\alpha]_{5461}$ = approx. $\pm$ 50°), and sensitivity to hydrolysis of which, as well as the properties of their tetramethyl derivatives, showed that they were derivatives of fructo-furanose, the γ-form of fructose. Hence it may be concluded that hexosediphosphoric acid is itself a derivative of fructo-furanose. (See also Levene and Raymond [1928], who have used the same argument.)

The criticism that treatment of the stable form of fructose (fructo-pyranose) with methyl alcohol and HCl may also yield under some conditions fructosides of the furanose form, and hence that some transformation may have been effected during the preparation of the methylhexosediphosphoric acids, has been answered by carrying out the methylation at 65° with the same result, although the methylhexoside of the fructo-furanose type is not produced from the stable fructo-pyranose at this temperature [Morgan, 1929].

If this conclusion be accepted, it may be inferred that one phosphoric acid group is in position 6, since if this group were unoccupied we should expect the pyranose form of fructose to be produced. Considering this with Young's conclusion that one phosphoric acid group is in

position 1, it follows that the constitutions of the diphosphoric acid and the methylhexosides derived from it are as follows :—



Hexosediphosphates Resistant to Hydrolysis (Esters I. and II.).

Ester II.—When hexosediphosphate is added to natural or diluted frog muscle extract at 20° a small amount of it is hydrolysed with liberation of phosphate, whilst at the same time a considerable portion of it, up to 83 per cent., is converted into a new ester (difficultly hydrolysable ester II.) which is much more resistant to boiling *N* HCl [Lipmann and Lohmann, 1930]. In the undiluted extract, lactic acid is also frequently produced from part of the original ester, and, when this is exhausted, also from the new ester, but with diluted (and in some cases with undiluted) extract, the production both of lactic acid and inorganic phosphate is negligible, whilst about 70 per cent. is converted into the new ester.

Ester I.—The glycogen of muscle-mince and glycogen or starch added either to mince or extract of muscle (frog, rabbit, crab) are converted in presence of fluoride into a difficultly hydrolysable, hexosediphosphate (Ester I.), which is also formed by muscle extract (containing fluoride) from added hexosediphosphate [Lohmann, 1930].

The same ester is formed from the hexosemonophosphates, approximately one molecule of inorganic phosphate being simultaneously taken up from the medium. In yeast maceration extract in presence of fluoride starch is converted into this same Ester I. as is also added hexosediphosphate, but much more slowly than in muscle extract. Oxalates and citrates added to minced muscle produce a similar change.

In the presence of arsenate, which greatly accelerates the formation of lactic acid and inorganic phosphate from hexosediphosphate in muscle extract, the decomposition is also accompanied by the formation of Ester I.; about two-thirds of the total PO_4 hydrolysable in 60 minutes is split off in about five minutes, and a large proportion of the unhydrolysed ester is at the same time converted into Ester I., which is then more slowly attacked. The decomposition of the monophosphates is also accelerated by arsenate, most probably after they have been further esterified to diphosphate, and in these cases too a difficultly hydrolysable ester (probably Ester II.) is formed. Ester I., which has not been obtained pure, closely resembles Ester II. (p. 59), but it is doubtful whether it has any optical rotation.

The function of fluoride (oxalate and citrate) in this action appears to be a double one; (1) inhibition of phosphatase action and of desmolysis; (2) conversion of previously formed hexosediphosphate into a difficultly hydrolysable ester.

These new esters do not appear to be formed in appreciable amount in the course of the ordinary transformation into lactic acid by frog muscle extract of starch or fructose (in presence of hexokinase), the products in these cases being hexosemonophosphate (Embden) 23-50 per cent. and hexosediphosphate (Harden and Young) 77-50 per cent.

Properties of Esters I. and II.—Neither of these esters has been obtained quite pure. Their properties are summarised below:—

	Ester I.	Ester II.
Specific rotation . . .	0	$[\alpha]_D = -2{\cdot}8°$ to $-3{\cdot}5°$
Reduction to Bertrand's solution	about 7 per cent.	about 12 per cent.
Reduction to hypoiodite . .	about 10 per cent.	about 9·5 per cent.
Hydrolysis constant (both groups)	$0{\cdot}1 \times 10^{-3}$	$0{\cdot}1 \times 10^{-3}$
Solubility of Ba salt . .	——	1·5 g. per 100 c.c.

Both esters resemble the normal hexosediphosphate in their resistance to oxidation in presence of phosphate (p. 63); Ester I. has the same dissociation constant ($p_{K'_2}$) as the Harden and Young ester (p. 54).

Properties of the Hexosemonophosphoric Acids.

Two compounds of this composition have been described, both of which have been obtained by the aid of yeast, but which differ in their properties.

The Robison Ester.

The first of these, frequently termed the Robison ester, was isolated in the manner described above from the products of the fermentation of sugars by yeast-juice in the presence of phosphate. The lead, barium, and calcium salts are all readily soluble in water. The acid and its salts are dextro-rotatory, the barium salt having $[\alpha]_D = + 12{\cdot}5°$ and the free acid $+ 25{\cdot}0°$.

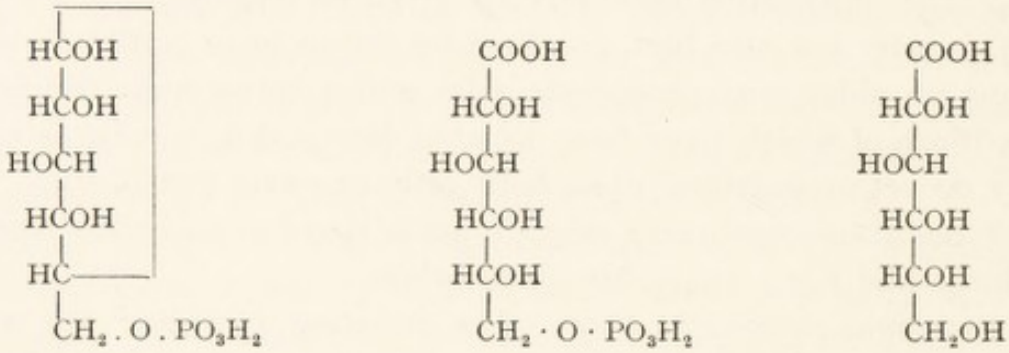
Like hexosediphosphoric acid it is a stronger acid than phosphoric acid. The dissociation constants are given on page 54.

This ester has now been conclusively shown to be a mixture containing an aldosemonophosphoric ester and a ketosemonophosphoric ester (both of which have been isolated (see p. 52), as well as some other, as yet unidentified, phosphoric ester or esters (see p. 53). The properties of the constituent esters, so far as they have been ascertained [Robison and King, 1931], are given below.

Aldosemonophosphoric Ester.—The reducing power of the pure barium salt to sodium hypoiodite corresponds exactly to the glucose equivalent of the hexose present, whilst towards the Hagedorn and Jensen reagent it is about 78 per cent. of the equivalent. The barium salt has $[\alpha]^{20°}_{5461} + 20{\cdot}6°$ ($c = 0{\cdot}84$ per cent.), $+ 21{\cdot}2°$ ($c = 8{\cdot}4$ per cent.), whilst the $[\alpha]^{20°}_{5461}$ of the free acid is $+ 41{\cdot}4°$. This substance yields the same phospho-osazone as Neuberg's monophosphoric ester and as hexosediphosphate (p. 56). The nature of the aldose residue is shown by the fact that on oxidation with bromine a phosphogluconic acid is obtained, which is converted by the action of phosphatase into *d*-gluconic acid. It has been found impossible to prepare a phosphosaccharic acid from the ester by oxidation with nitric acid, and this affords strong evidence that the phosphoric acid group is situated in position 6. This is in agreement with the results obtained by King and Morgan [1929], and King, McLaughlin and Morgan [1931], who methylated the barium salt of aldomonophosphoric acid both at room temperature and at 60°, and found that two different methoxy-compounds were produced, both of which were mixtures of the α- and β-isomerides. That obtained at 18° was very sensitive to acid hydrolysis whereas that obtained at 60° was much more stable.

This indicates that the two methoxy-derivatives corresponded respectively with the furanose and pyranose forms of glucose. If this be accepted it follows that neither position 4 nor 5 in the molecule of the aldomonophosphoric acid is occupied by the phosphoric acid residue.

Levene and Raymond [1930, 1931] are also of opinion that this ester is a 6-phosphoric ester inasmuch as in many of its properties it agrees with those of a phosphoric ester prepared synthetically from monoacetone glucose which there are some grounds for believing to be 6-glucosephosphoric ester. Levene and Raymond have also prepared a 3-glucosephosphoric ester which differs in many respects from Robison's aldosephosphoric ester. If this constitution be accepted the conversion of the ester into gluconic acid would be represented by the following formulæ :—



The ester is very resistant to hydrolysis by acid, the sugar product of the hydrolysis being apparently *d*-glucose. Bone phosphatase rapidly hydrolyses it at room temperature and p_H 7·0, but the resulting sugar contains both glucose and fructose (see p. 68).

The *ketosephosphoric ester* obtained by Robison and King from fermentation hexosemonophosphate appears to be identical with the Neuberg ester obtained by partial acid hydrolysis of hexosediphosphate.

The Neuberg Ester.

A second hexosemonophosphoric acid, which differs from the hexosemonophosphoric acid, described above, isolated by Harden and Robison from yeast-juice [Robison, 1922], has been obtained by Neuberg by the partial hydrolysis of hexosediphosphoric acid. When this is boiled in aqueous solution with oxalic acid for a short time (e.g. 13·7 g. of the calcium salt with 150 c.c. of *N* oxalic acid for half an hour), one of the phosphoric groups is removed by hydrolysis and a hexosemonophosphoric acid is formed, the lead, barium, calcium, magnesium, and zinc salts of which are soluble in water. The calcium

salt is slowly fermented by living yeast, differing in this respect from that of the hexosediphosphoric acid [Neuberg, 1918, 2]. This substance is also fermented by yeast-juice and zymin. The Ba salt has $[a]_D$ + 0·4° and the free acid + 1·5°.

Its dissociation constants are the same as those of the Robison ester (p. 54).

It has approximately the same reducing power to Bertrand's or Hagedorn and Jensen's reagent as the fermentation ester (78 per cent.), but its behaviour to hypoiodite (about 5-10 per cent.) indicates that it consists almost exclusively (90-95 per cent.) of a keto-ester, but appears to contain about 5-10 per cent. of an aldo-ester. The keto-component may possibly be fructo-furanose-6-phosphoric acid, the readily hydrolysable phosphoric group in position 1 of hexosediphosphoric acid (p. 59) having been removed.

The salts of Pb, Ba, Ca, Mg, and Mn are readily soluble in water and those of strychnine, brucine, and cinchonidine are crystalline [Neuberg and Dalmer, 1922].

A remarkable property of this ester is the ease with which it undergoes oxidation at p_H 8 in presence of a trace of copper. Under these circumstances in a phosphate buffer the Neuberg ester absorbs oxygen twice as quickly as fructose, and about five times as rapidly as the Robison monophosphoric ester. The figures given by Meyerhof and Lohmann [1927] are as follows for 0·033 *M* solutions in *M* phosphate or arsenate of p_H 8 at 37° in 1·5 hours.

	mm^3 O_2 Absorbed.	Oxidation Velocity.
Fructose	37·1	100
Glucose	5·3	14
Neuberg ester : (hexosemonophosphoric ester)	80·6	220
Robison ester : (hexosemonophosphoric ester)	16·9	46
Hexosediphosphoric ester . .	1·0	0

This is held by Meyerhof to indicate that in the Neuberg ester the hexose residue is in a condition of peculiar instability, and thus supports his thesis that this substance is intimately concerned in the early stages of fermentation (see p. 136). The oxidation of fructose in presence of phosphate appears not to proceed by way of the intermediate formation of a hexosephosphate [Neuberg and Kobel, 1925].

Trehalosemonophosphoric Ester.

The barium salt of this ester appears to exist in two forms, one amorphous and readily soluble in water, the other crystalline, containing $5H_2O$, and sparingly soluble in water. It has no reducing

power either to Hagedorn and Jensen's solution or to hypoiodite. The $[\alpha]^{20°}_{5461}$ of the barium salt in water is + 132° ($c = 3{\cdot}2$); that of the free acid is + 185° ($c = 2{\cdot}4$). The phosphoric acid group is readily removed by the action of bone phosphatase leaving trehalose. Hydrolysis by acid yields glucose and phosphoric acid as final products, but as the disaccharide linkage is more readily broken than the phosphoric, glucosemonophosphoric ester accumulates for a time in the products. Whether this is identical with the glucosemonophosphoric ester which forms one constituent of the Robison hexosemonophosphoric ester is not yet known.

The sodium salt of the trehalosephosphoric ester is readily fermented by dried yeast, more slowly by zymin and yeast-juice.

The *brucine salt* crystallises with $9H_2O$ in large clusters of needles.

It is not known whether the formation of this ester bears any essential relation to the mechanism either of alcoholic fermentation or of glycogen formation.

The Hexosemonophosphoric Ester of Muscle.

Soon after the discovery of hexosediphosphate, attention was drawn by Embden and his colleagues [Embden, Griesbach, and Schmitz, 1915; Hagemann, 1915; Embden, Griesbach, and Laquer, 1915; see also Foster and Moyle, 1921] to the fact that the addition of this compound to the press juice from muscle, kidney, or uterus caused an increase in the amount of lactic acid and phosphoric acid formed, and Embden concluded that the normal precursor of lactic acid in muscle, which he termed *lactacidogen* was some substance closely connected with hexosediphosphate. Later hexosediphosphoric acid was actually prepared from muscle by a process in which sodium fluoride was added to the muscle press juice [Embden and Zimmermann, 1924], whereas a later preparation [Embden and Zimmermann, 1927] which was carried out without addition of fluoride led to the isolation of a hexosemonophosphoric ester closely corresponding with that obtained by fermentation (Robison ester). By this method no hexosediphosphoric acid could be detected in the muscle pulp, and it is now recognised [see Pryde and Waters, 1929] that living muscle does not contain the diphosphate, but that this is formed in the press juice in the presence of fluoride. Embden's lactacidogen, therefore, is a hexosemonophosphoric ester and this, like the Robison ester which it closely resembles [see Lohmann, 1928, 1], is probably a mixture of an aldo- with a keto-ester, the former being present in large excess.

Lohmann, who took particular precautions to remove all phosphagen [Eggleton, 1927], obtained about 1 g. of the barium salt from 1 kg. of rabbit muscle; Pryde and Waters, working by Embden and Zimmermann's method, obtained 37 g. of the brucine salt (equivalent to 9 g. of acid or 14 of barium salt) from 7 kg. of rabbit muscle. The free acid has $[\alpha]_D^{19°} = +26{\cdot}9°$ ($c = 0{\cdot}34$) (Lohmann); $[\alpha]_{5461} = +33°$ ($c = 0{\cdot}770$) (Pryde and Waters), whilst the brucine salt gave $[\alpha]_{5461} = -29{\cdot}9°$ in water, $-20{\cdot}3°$ in methyl alcohol; it softened at 145°, melted at 155°, and decomposed at 158°-160°. Its second dissociation constant is the same as that of the Robison and Neuberg esters (p. 54). This ester behaves on hydrolysis [Lohmann, 1928, 1] almost precisely like the Robison ester, and its reaction with hypoiodite shows that it contains about 90 per cent. of aldose and 10 per cent. of ketose (Pryde and Waters); 70 per cent. aldose and 30 per cent. ketose (Lohmann), the aldose being probably glucose.

Other Monophosphoric Esters of Sugars.

A hexosemonophosphoric ester which is probably a derivative of fructose has been isolated in small quantity from assimilating *Elodea canadensis* (Barrenscheen and Parry, 1930]. It differs from the other known esters in having a very low reduction, only 6·9 per cent. of that of the hexose contained in it.

Another, different, monophosphoric ester has been obtained by the same workers by the action of taka-diastase on the phosphorylated octo-amylose which occurs in wheat seedlings. This is also a fructose derivative and has a slight negative rotation and a reducing power of 49·8 per cent. (Hagedorn and Jensen). A compound which appears to be a disaccharide-monophosphoric ester has been isolated by Neuberg and Leibowitz [1928] from the products of the action of *B. delbrücki* on sodium hexosediphosphate. It has $[\alpha]_D = +55{\cdot}2°$, forms a soluble barium salt, reduces Fehling's solution and is slowly fermented by yeast.

Synthetic Phosphoric Esters of the Sugars.

Several synthetic phosphoric esters of the sugars have been prepared by the action of phosphorus oxychloride on the sugars and their derivatives. The most interesting of these compounds are the glucose-monophosphoric esters studied by Levene and his colleagues, since they have been obtained by synthetical methods which give some clue as to their constitution. Glucose-3-monophosphoric ester [see Levene

and Raymond, 1930] is obtained by the action of $POCl_3$ on diacetone-glucose. The $[\alpha]_D^{27°}$ of the barium salt is $+ 26{\cdot}5°$, and of the free acid $+ 39{\cdot}5°$. It is fermented by zymin (in presence of arsenate) more slowly than the Robison hexosemonophosphate. With phenylhydrazine it yields an anhydro-osazone, free from phosphorus [Raymond and Levene, 1929]. Still more interesting is a glucose-monophosphoric ester prepared in a similar way from monoacetone-glucose [Levene and Raymond, 1930, 1931]. The $[\alpha]_{5461}^{25°}$ of the barium salt is $+ 20{\cdot}2°$. This corresponds approximately with the value for the pure aldose ester of Robison. This glucosephosphoric ester yields with phenylhydrazine a compound which is apparently identical with that given by hexosediphosphate and by Robison's aldosemono-ester. It is fermented by zymin in presence of arsenate at the same rate as the Robison mono-ester. Its constitution is not yet definitely known, but the mode of preparation suggests that it may be glucose-6-phosphoric ester.

Another interesting derivative is the *sucrosemonophosphoric ester* obtained by the action of $POCl_3$ on sucrose [Neuberg and Pollak, 1910, 1, 2]. The calcium salt of this substance when heated in solution with 1 per cent. oxalic acid [Sabetay and Rosenfeld, 1925], or when treated with invertase free from phosphatase [Neuberg and Sabetay, 1925, 2] yields fructose and a glucosemonophosphoric ester. The latter forms a barium salt, which is more readily soluble in hot water than cold, and has $[\alpha]_D^{25°} = + 8{\cdot}53°(c = 5{\cdot}856)$. The potassium salt is slowly fermented by yeast. On hydrolysis by acid it slowly yields glucose and phosphoric acid. This ester appears to differ from the aldose component of the Robison monophosphoric ester and is also different from the synthetic 1-, 3-, and 6-phosphoric esters of glucose prepared by Komatsu and Nodsu [1925], but its actual constitution is unknown.

Pyrophosphates in Yeast.

Following on the discovery by Lohmann [1928, 2] of the existence of readily hydrolysable pyrophosphoric acid in muscle, the observation was made [Lohmann, 1928, 3, 4,] that this substance also occurred in yeast, to an extent equivalent to 0·79-0·69 mg. P per gram of moist yeast, in baker's and brewer's yeast respectively. Boyland [1930, 1], using a modified method of estimation, found 0·68 mg. of P as pyrophosphate per g. of English brewer's yeast. In this sample of yeast 3·25 mg. of acid-soluble P was present per g. of yeast, and of this 42 per cent. was present as inorganic phosphate, 21 per cent. as pyrophos-

phate, and 36 per cent. as hexosephosphates (approximately 12 per cent. hexosediphosphate and 24 per cent. monophosphate). In muscle the pyrophosphate is combined with adeninenucleotide (adenylic acid), and it is probably present in yeast in some similar form.

Although a pyrophosphate added to a fermenting mixture of zymin and sugar is hydrolysed to phosphate (which is then esterified), the amount present in yeast preparations does not change during a short fermentation [Boyland, 1930, 1]. The acetone precipitate from yeast-juice free from hexosephosphatase (p. 39) which was prepared by Boyland [1930, 1] readily hydrolyses pyrophosphate, so that the hydrolysis must be effected by a specific pyrophosphatase distinct from hexosephosphatase. Both in muscle and yeast, according to Meyerhof [1930, p. 173], adenylic acid pyrophosphate is probably intimately connected with the action of the co-enzyme (see p. 86), although Myrbäck has been unable to confirm this as regards yeast [see Euler, 1930, p. 17].

Action of Phosphatases on the Hexosephosphates.

Phosphatases, capable of hydrolysing the hexosephosphoric esters, are widely distributed [Euler, 1912; Euler and Funke, 1912; Harding, 1912; Plimmer, 1913; Neuberg and Leibowitz, 1927, 2, 4,; Robison, 1923]. The lipase of castor oil seeds, almond emulsin, the leaves of the maple, a glycerol extract of the intestinal mucous membrane of the rabbit and pig, and an aqueous extract of bran have a slow hydrolytic action on hexosediphosphate, whereas pepsin and trypsin are without effect. It is also decomposed *in vitro* by the kidney, spleen, and muscle of the guinea-pig [Takahashi, 1924, 1]. Feeding experiments with rabbits and dogs indicate that the diphosphate is capable of hydrolysis in the animal body, a large proportion of the phosphorus being excreted as inorganic phosphate. The ester is also decomposed and fermented by *Bacillus coli communis* [Manning, 1927].

It is remarkable that hexosediphosphate is not fermented or hydrolysed by living yeast, a fact observed by Euler, Harden and Young, and Ivanov, although according to the experiments of Paine [1911], the yeast cell is, at all events partially, permeable to the sodium salt.

The enzymic hydrolysis of hexosediphosphates proceeds, like the acid hydrolysis, in stages. Hexosemonophosphate can be isolated from the products of incomplete hydrolysis of the diphosphate by yeast in presence of toluene and chloroform [Neuberg and Leibowitz, 1927, 3], both in the absence and presence of arsenate [Neuberg and Leibowitz, 1927, 5], by taka-diastase (which contains a phosphatase)

[Neuberg and Leibowitz, 1927, 2], and by kidney phosphatase [Neuberg and Leibowitz, 1927, 4], and one of the phosphoric acid groups of hexosediphosphate is much more rapidly removed than the other by muscle phosphatase [Robison, 1923, see also Brugsch, Cahen, and Horsters, 1925]. An interesting feature of these changes is that yeast gives a mixture of the Neuberg and Robison mono-esters and kidney diastase almost pure Robison ester, whilst taka-diastase and yeast in presence of arsenate (and chloroform and toluene) give almost pure Neuberg ester. This affords another instance of the labile character of the sugar residue in these compounds.

Similarly the hexose obtained by the hydrolysis of hexosediphosphate with bone phosphatase is much less lævo-rotatory than fructose [Martland and Robison, 1929]. By the action of bottom yeast on the Robison ester Neuberg and Leibowitz [1927, 2] obtained hexosediphosphate which they consider was formed by enzymic synthesis from the monophosphate, but it seems also possible that it may have been produced from a hexose liberated by hydrolysis.

The hexosemonophosphates are themselves hydrolysed (and fermented) by yeast preparations, and the Robison ester and its aldose constituent are readily hydrolysed by bone phosphatase [Robison, 1923] as is the Neuberg ester [Takahashi, 1924, 2; Fujihara and Koken, 1926], which is also hydrolysed by the phosphatase present in many animal organs and tissues [Tomita, 1922].

Some discussion has taken place with regard to the influence of the co-enzyme on the action of the hexosephosphatase of yeast on hexosediphosphate. Raymond [1928], comparing the action of washed and unwashed zymin, concluded that the co-enzyme was involved in the reaction but this has not been confirmed by Macfarlane [1930], who finds that a preparation made by the autolysis of dried baker's yeast and free from co-enzyme and apozymase, i.e. unable to esterify phosphoric acid and sugar and produce CO_2 and alcohol even in presence of added co-enzyme, hydrolyses hexosediphosphate readily, and further that the rate of hydrolysis is not affected by the addition of co-enzyme.

The Equation of Alcoholic Fermentation.

Before it was realised that the hexosediphosphate produced during alcoholic fermentation was accompanied by a hexosemonophosphate, Harden and Young proposed the equation,

$$2C_6H_{12}O_6 + 2PO_4HR_2 = 2CO_2 + 2C_2H_6O + 2H_2O + C_6H_{10}O_4(PO_4R_2)_2,$$

to represent the change which occurs.

This was founded essentially upon the formula of the product and the relation between the phosphate added and the carbon dioxide and alcohol produced. The equation was also supported by experiments [Harden and Young, 1910, 2] with small quantities of sugar in presence of excess of phosphate but no determination was made of the relative proportions of di- and mono-phosphoric esters produced. The same is true of the experiments of Euler and Johansson [1913].

The essential fact that the ratio of CO_2 to phosphorus esterified is usually approximately equal to unity is expressed if a second equation be added representing the relations when hexosemonophosphoric ester is formed :—

$$1\ (a)\ 3C_6H_{12}O_6 + 2PO_4HR_2 = 2C_6H_{11}O_5PO_4R_2 + 2H_2O + 2CO_2 + 2C_2H_6O$$
$$(b)\ 2C_6H_{12}O_6 + 2PO_4HR_2 = C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O + 2CO_2 + 2C_2H_6O.$$

What determines the direction which the reaction will take, 1 (*a*) or 1 (*b*), or in what proportion the di- and mono-esters will be produced is at present unknown. Yeast-juice and maceration extract are very variable in this respect, as much as 60 to 80 per cent. or as little as 30 to 10 per cent. of the monophosphate being produced by various samples. Zymin and dried yeast usually yield 10 to 30 per cent. of monophosphate and 90 to 70 per cent. of diphosphate [Harden and Henley, 1927, 2; 1929; Robison and Morgan, 1930; see also Kluyver and Struyk, 1928].

Cycle of Changes Undergone by Phosphate in Alcoholic Fermentation.

According to the equations 1 (*a*) and (*b*), the free phosphate present is used up in the reaction, and the question at once arises whether there is any source from which a constant supply of free phosphate can be elaborated in the juice, or whether some other change occurs which results in the formation of carbon dioxide and alcohol in the absence of free phosphate. The experimental evidence points in the direction of the former of these alternatives, but the question is a very difficult one to decide with absolute certainty.

When a mixture of a phosphate with yeast-juice and sugar is examined at intervals and the amount of free phosphate estimated, the following stages are observed :—

1. During the initial period of accelerated fermentation following the addition of the phosphate, the concentration of free phosphate rapidly diminishes.

2. At the close of this period, the amount of free phosphate present is very low, and, as long as active fermentation continues, no marked increase in it occurs.

3. As alcoholic fermentation slackens and finally ceases, a marked and rapid rise in the amount of free phosphate occurs at the expense of the hexosephosphates, which steadily diminish in amount, and this change is brought about by an enzyme in the juice and ceases if the liquid be boiled.

This last reaction may be represented by the equations

$$2\ (a)\ C_6H_{11}O_5(PO_4R_2) + H_2O = C_6H_{12}O_6 + PO_4HR_2.$$
$$(b)\ C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2PO_4HR_2.$$

In the light of these equations, together with equations No. 1 (*a*) and (*b*), given above, all these facts can be simply and easily understood.

The rapid diminution in the amount of free phosphate during stage 1 corresponds with the occurrence of reaction (1). During the whole period of fermentation the enzymic hydrolysis of the hexosephosphates is proceeding according to equations 2 (*a*) and (*b*). Up to the end of stage 2 the phosphate thus produced enters into reaction, according to equations 1 (*a*) and (*b*), with the sugar which is present in excess and is thus reconverted into hexosephosphate, so that as long as alcoholic fermentation is proceeding freely, no accumulation of free phosphate can occur.

As soon as alcoholic fermentation ceases, however, it is no longer possible for the phosphate to pass back into hexosephosphate, and hence it accumulates in the free state.

A similar hydrolysis of hexosephosphate and accumulation of phosphate occur when a solution of a hexosephosphate is treated with yeast-juice which has been deprived of the power of fermentation by dialysis, or with zymin freed from co-enzyme by washing (p. 80), and this evidently depends on the presence of a hydrolytic enzyme, which may be provisionally termed *hexosephosphatase*.

It is not yet certain whether more than one hexosephosphatase is present; both hexosemono- and hexosediphosphate are hydrolysed by washed zymin. According to Meyerhof [1930] inorganic phosphate may also be produced by the direct fermentation of hexosediphosphate without preliminary hydrolysis, according to the equation

$$2\ (c)\ C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = 2CO_2 + 2C_2H_6O + 2PO_4HR_2.$$

This process, which is discussed later on (p. 141), apparently proceeds simultaneously with the enzymic hydrolysis just described.

The actual rate of fermentation observed in any particular case in presence of excess of sugar, enzyme, and co-enzyme must on this view depend on the supply of phosphate which is available.

In presence of an adequate amount of phosphate, as well as of sugar,

the highest rate attained represents the maximum velocity at which reactions 1 (*a*) and (*b*) can proceed in that sample of yeast-juice or zymin, and this high rate is characteristic of the initial period of accelerated fermentation which follows the addition of a suitable quantity of phosphate. By the simple expedient of renewing the supply of phosphate as rapidly as it is converted into hexosephosphate, this high rate can be maintained for a considerable time [Harden and Young, 1908, 1]. In this way, for example, an average rate of evolution of carbon dioxide of 15 c.c. in five minutes was maintained for an hour and a quarter, whereas the normal rate in the absence of added phosphate was 3 c.c.

As soon as all the free phosphate has entered into the reaction, however, the supply of phosphate depends in the main on the rate at which the resulting hexosephosphate is decomposed, and the rate of fermentation now attained is conditioned by the rate at which reactions 2*a*, 2*b*, and 2*c* proceed.

The rates attained during the initial period of rapid fermentation and the subsequent period of slow fermentation are thus seen to represent the velocities of entirely different chemical reactions.

These considerations also explain why it is the *extra* carbon dioxide evolved during the initial period, and not the total carbon dioxide, which is equivalent to the added phosphate. As the production of phosphate is proceeding throughout the whole period it is obviously necessary to deduct the corresponding amount of carbon dioxide from the total evolved in order to ascertain the amount equivalent to the added phosphate.

Some uncertainty exists as to the amount of this correction, for the rate of decomposition of hexosephosphate may be (*a*) checked by the presence of inorganic phosphate, (*b*) increased by the presence of an increased concentration of hexosephosphate.

An explanation is also afforded of the fact that a considerable increase in the concentration of hexosephosphate does not materially increase the normal rate of fermentation by yeast-juice. This is probably due to the circumstance that, in accordance with the general behaviour of enzymes in presence of excess of the fermentable substance, approximately equal amounts of hexosephosphate are decomposed in equal times whatever its concentration may be, above a certain limit which for yeast-juice is generally low.

With dried yeast and zymin, which are relatively rich in the enzymes which decompose hexosephosphate, the normal or basal rate is at first considerably increased when the concentration of

hexosephosphate is increased, but a maximum rate is soon reached [Boyland, 1929].

Thus with dried yeast an increase in the concentration of hexosediphosphate from 0·03 to 0·05 *M* increased the basal rate from 3·9 to 6·1 c.c. CO_2 per five minutes, whilst a further increase to 0·07 *M* hexosediphosphate made no additional change.

Effect of Phosphate on the Total Fermentation Produced by Yeast-Juice.

The addition of a phosphate to yeast-juice not only produces the effect already described, but also enables a given volume of yeast-juice to effect a larger total fermentation, even after allowance is made for the carbon dioxide equivalent to the quantity of phosphate added. The increase in the case of ordinary yeast-juice has been found to amount to from 10 to 150 per cent. of the original total fermentation produced by the juice in the absence of added phosphate. The numbers contained in columns 1 and 2 of the table on p. 73 illustrate this effect, the ratio of the total in the presence of phosphate, as well as that of the total in presence of phosphate less the equivalent of the phosphate added, to that obtained in its absence being given. The cause of this increase in the total fermentation is probably to be sought mainly in a protective action of the excess of hexosephosphate on the various enzymes, for, as has been stated above, the rate of fermentation after the termination of the initial period, is practically the same as in the absence of added phosphate (see p. 71).

Now it follows from equations 1 (*a*) and (*b*) (p. 69) that in the total absence of phosphate no fermentation should occur, and the experimental realisation of this result would afford very strong evidence in favour of this interpretation of the phenomenon.

Hitherto, however, it has not been found possible to free the materials employed completely from phosphorus compounds which yield phosphates by enzymic hydrolysis during the experiment, but it has been found that when the phosphate contents are reduced to as low a limit as possible, the amount of sugar fermented becomes correspondingly small, and, further, that in these circumstances the addition of a small amount of phosphate or hexosephosphate produces a relatively large increase in the fermentation produced by the enzyme.

When the total phosphorus present is thus largely reduced, the increase produced by the addition of a small amount of phosphate may amount to as much as eighty-eight times the original, in addition to the

quantity equivalent to the phosphate, whilst the actual total evolved, including this equivalent, may be as much as twenty times the original fermentation. This result must be regarded as strong evidence in favour of the view that phosphates are indispensable for alcoholic fermentation.

The results indicated above were experimentally obtained in three different ways and are exhibited in the following table. In the first place (cols. 3 and 4), advantage was taken of the fact that the residues obtained by filtering yeast-juice through a Martin gelatin filter (p. 76) are sometimes found to be almost free from mineral phosphates, whilst they still contain a small amount of co-enzyme. The experiment then consists in comparing the fermentation produced by such a residue poor in phosphate with that observed when a small amount of phosphate is added. The second method (col. 5) consisted in carrying out two parallel fermentations by means of a residue rendered inactive by filtration and a solution of co-enzyme free from phosphate and hexosephosphate [Harden and Young, 1910, 2].

The third method (col. 6) consisted in washing zymin with water, to remove soluble phosphates, and then adding to it a solution of co-enzyme containing only a small amount of phosphate, and ascertaining the effect of the addition of a small known amount of hexosephosphate upon the fermentation produced by this mixture [Harden and Young, 1911, 1].

	1	2	3	4	5	6
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Gas evolved in absence of added phosphate	369	220	1·4	1·2	20·3	1·5
Gas evolved in presence of added phosphate	629	629	25·8	26·8	92·3	132·7
Increase due to phosphate . .	260	409	24·4	25·6	72·0	131·2
Carbonic acid equivalent to phosphate	63	61	16·9	16·8	16·8	—
Increase after initial period . .	197	348	7·5	8·8	55·2	—
Ratio of totals	1·7	2·9	18·4	21·3	4·5	88
,, ,, increase after initial period to original fermentation . .	0·5	1·6	5·3	7·3	2·7	—

Production of a Fermentable Sugar from Hexosephosphate by the Action of an Enzyme Contained in Yeast-Juice.

The sugar which, according to equations 2 (*a*) and (*b*) accompanies the phosphate formed by the enzymic hydrolysis of hexosephosphate

is under ordinary circumstances fermented by the alcoholic enzyme of the juice and thus escapes detection.

When, however, a solution of a hexosephosphate is exposed to the action of either yeast-juice or zymin, entirely or partially freed from co-enzyme, this sugar, being no longer fermented, accumulates and can be examined. It has thus been found [Harden and Young, 1910, 2] that a sugar is in fact produced in this way from hexosediphosphate which can be fermented by living yeast and exhibits the reactions of fructose, although the presence of other hexoses is not excluded. The products of the enzymic hydrolysis of the hexosediphosphate therefore appear to be the same as, or similar to, those formed by the action of acids [Young, 1909].

A further consequence of these facts is that the hexosephosphates will yield carbon dioxide and alcohol when they are added to yeast-juice or zymin, and this has also been found to be the case [Harden and Young, 1910, 2 ; Ivanov, 1909, 1] (see p. 141).

Hexokinase and the Production of Lactic Acid from Hexoses by the Lactic Acid-forming Enzyme of Muscle.

The name hexokinase has been given by Meyerhof [1927] to a preparation, originally termed yeast activator, which has the properties of an enzyme and is prepared by allowing yeast to autolyse in presence of toluene and water for two to three hours, centrifuging and continuing the autolysis of the residue with an equal weight of toluene water for sixteen hours at 35°. An equal volume of alcohol is then added to the clear extract and the precipitated hexokinase is dissolved in water [see also Brunius and Proffe, 1928]. The hexokinase is readily soluble in water and is thermolabile, being in large part inactivated in one minute at 50°, but may be preserved on ice. It is inactivated both by acid and alkali. When purified by reprecipitation it is free from inorganic phosphate and is active in concentrations of 0·4-1 mg. per c.c.

The characteristic effect of the hexokinase is that it enables muscle enzyme solution, which in its absence can rapidly convert glycogen but not hexoses into lactic acid, to convert added hexoses rapidly into lactic acid, a process accompanied in the presence of phosphate by accumulation of a hexosephosphoric ester. Under these circumstances the conversion of the added sugar into lactic acid is accompanied by the same phenomena as is the conversion of sugar into alcohol and carbon dioxide by yeast preparations.[1] In presence of phosphate a

[1] A full account of this subject will be found in Meyerhof's "Die chemischen Vorgänge im Muskel" (Springer, Berlin), 1930.

rapid production of lactic acid takes place, accompanied by the esterification of two molecules of phosphate (chiefly into hexosediphosphate) for every molecule of sugar converted into lactic acid. When all the phosphate has been esterified the rate falls but, as in yeast-juice, no inorganic phosphate is liberated until the sugar has all been converted into lactic acid, after which the remaining hexosephosphate is slowly converted into lactic acid and inorganic phosphate. As in yeast juice, a small addition of inorganic phosphate after the rate has fallen causes (provided excess of sugar is present) a renewal of the high rate of lactic acid formation and production of hexosediphosphate. Fructose, as with yeast preparations, causes a much more rapid fermentation than glucose.

Experiments indicate that in the presence of the hexokinase the hexoses are probably changed to a more reactive form, which is then acted on by the muscle enzyme, but this is not yet fully established. This function of the hexokinase suggests that it may possibly play a similar part in alcoholic fermentation, assisting to bring about the conversion of the sugar into a more reactive form.

Sugar Metabolism in Animal and Vegetable Organisms.

The fact that phosphate plays an essential part in the decomposition of carbohydrates both in the yeast cell and in the animal organism renders it extremely probable that the fundamental biological mode of attack on carbohydrates is that revealed by the study of alcoholic fermentation.

Direct proof of this is still wanting in many cases, but some instances are already known among bacteria [Virtanen, 1924, 1925, 2], moulds [Euler and Kullberg, 1911, 3], and the higher plants [Bodnár, 1925 ; Barrenscheen and Albers, 1928, Barrenscheen and Parry, 1930], and it is to be expected that more will be revealed by further research.

CHAPTER IV.

THE CO-ENZYME OF YEAST-JUICE (CO-ZYMASE).

In the previous chapter reference was made to the fact that the addition of boiled yeast-juice greatly increases the amount of carbon dioxide and alcohol formed from sugar by the action of a given volume of yeast-juice.

When the boiled juice is dialysed the substance or substances to which this effect is due pass into the dialysate, the residue being quite inactive. In order to ascertain the effect on the process of alcoholic fermentation of the complete removal of these unknown substances from yeast-juice itself, dialysis experiments were instituted with fresh yeast-juice, capable of bringing about an active production of carbon dioxide and alcohol from sugar. It was already known from the experiments of Buchner and Rapp [1898, 1] that dialysis in parchment paper for seventeen hours at 0° against water or physiological salt solution only produced a diminution of about 20 per cent. in the total amount of fermentation obtainable, and in view of the less permanent character of the juice from top yeasts a more rapid method of dialysis was sought. This was found in the process of filtration under pressure through a film of gelatin, supported in the pores of a Chamberland filter candle, which had been introduced by Martin [1896], and is now termed ultrafiltration.

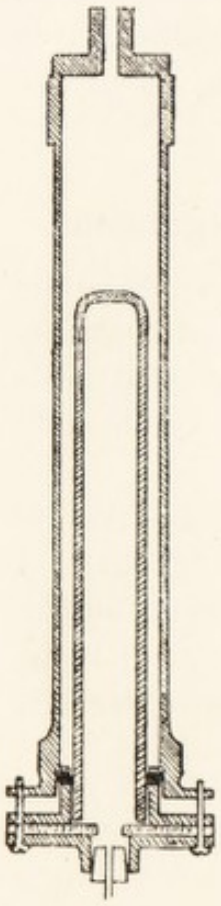


Fig. 5.

In this way it was found possible to divide the juice into a residue and a filtrate, each of which was itself incapable of setting up the alcoholic fermentation of glucose, whereas, when they were reunited, the mixture produced almost as active a fermentation as the original juice [Harden and Young, 1905, 1; 1906, 2].

The apparatus employed for this purpose consists of a brass tube

provided with a flange in which the gelatinised candle is held by a compressed india-rubber ring, and is shown in section in Fig. 5. Two such apparatus are used, each capable of holding about 70 c.c. of the liquid to be filtered. The tubes, after being filled with the yeast-juice, are connected by means of a screw joint with a cylinder of compressed air and the filtration carried out under a pressure of 50 atmospheres, the arrangement employed being shown in Fig. 6. In the earlier experiments 25 to 50 c.c. of yeast-juice were placed in each tube and the

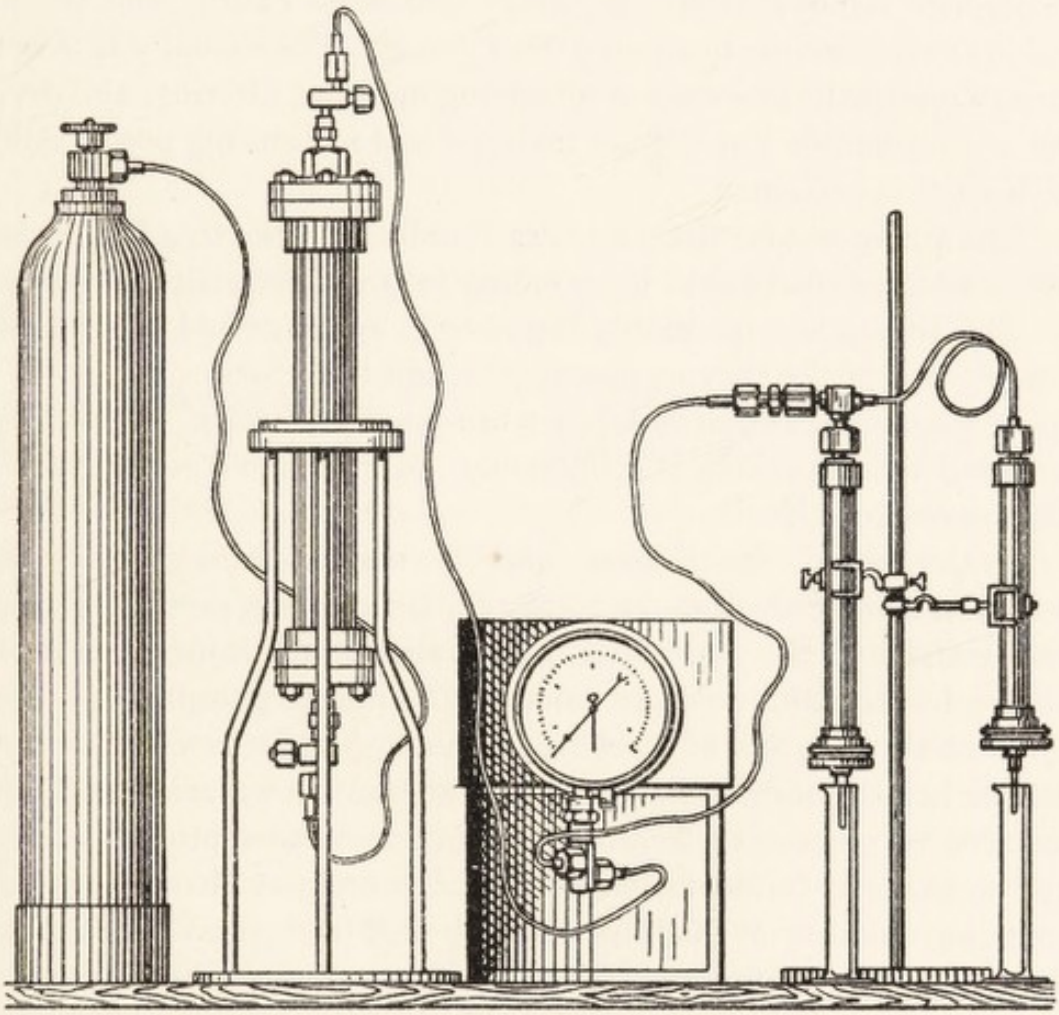


FIG. 6.

filtration continued until no more liquid passed through. The residue was then washed several times *in situ* by adding water and forcing it through the candle. The time occupied in this process varied from six to twelve hours with different preparations of yeast-juice. The candle was then removed from the brass casing and the thick, brown-coloured residue scraped off, dissolved in water, and at once examined. It was subsequently found to be possible to dry this residue *in vacuo* over sulphuric acid without seriously altering the fermenting power, and this led to a slight modification of the method, which is now

conducted as follows. Two quantities of 50 c.c. each of yeast-juice are filtered, without washing, and the residues spread on watch-glasses and dried *in vacuo*. Two fresh quantities of 50 c.c. are then filtered through the same candles and the residues also dried. The 200 c.c. of juice treated in this way give a dry residue of 17 to 24 grams. The residue is then dissolved in 100 c.c. of water and filtered in quantities of 50 c.c. through two fresh gelatinised candles and the residue again dried. A considerable diminution in weight occurs, partly owing to incomplete removal from the candle and brass casing, and the final residue only amounts to about 9 to 12 grams. Occasionally it is necessary to repeat the processes of dissolving in water, filtering, and drying, but a considerable loss both of material and fermenting power attends each such operation.

The sticky residue dries up very rapidly *in vacuo* to a brittle, scaly mass, which is converted by grinding into a light yellow powder.

The filtrate was invariably found to be quite devoid of fermenting power, none of the enzyme passing through the gelatin.

Other methods of ultrafiltration are equally effective. Thus Meyerhof used the apparatus of Zsigmondy, and Bechhold's ultrafilter can also be employed.

Properties of the Filtered and Washed Residue.—The residue prepared as described above consists mainly of the protein, glycogen, and dextrins of the yeast-juice, and is almost free from mineral phosphates, but contains a certain amount of combined phosphorus. It also contains the enzymes of the juice, including the proteoclastic enzyme, and the hexosephosphatase (p. 70). Its solution in water is usually quite inactive to glucose or fructose, but in some cases produces a small and evanescent fermentation. When the original filtrate or a corresponding quantity of the filtrate from boiled fresh yeast-juice is added, the mixture ferments glucose or fructose quite readily. The table opposite shows the quantitative relations observed, the sugar being in all cases present in excess.

These experiments lead to the conclusion that the fermentation of glucose and fructose by yeast-juice is dependent upon the presence, not only of the enzyme, but also of another substance which is dialysable and thermostable.

Precisely similar results were subsequently obtained by Buchner and Antoni [1905, 2] by the dialysis of yeast-juice. One hundred c.c. of juice were dialysed for twenty-four hours at 0° against 1300 c.c. of distilled water, and the dialysate was then evaporated at 40° to 50°

No.	Material.	Volume.	Filtrate added.	Boiled Juice added.	Water added.	CO_2 evolved.
1	Undried and unwashed residue . .	15 c.c.	0 c.c.	0 c.c.	15 c.c.	0 gram.
		15 ,,	15 ,,	0 ,,	0 ,,	0·035 ,,
2	,, ,,	15 ,,	0 ,,	0 ,,	15 ,,	0·024 ,,
		15 ,,	0 ,,	15 ,,	0 ,,	0·282 ,,
3	Undried and washed residue . .	25 ,,	0 ,,	0 ,,	0 ,,	0·4 c.c.
		25 ,,	0 ,,	25 ,,	0 ,,	268 ,,
4	,, ,,	20 ,,	0 ,,	0 ,,	0 ,,	8·3 ,,
		20 ,,	20 ,,	0 ,,	0 ,,	90·3 ,,
5	Washed and dried residue . .	1 gram in 15 c.c.	0 ,,	0 ,,	0 ,,	0 ,,
		,,	0 ,,	12 ,,	0 ,,	108 ,,
6	,, ,,	1 gram in 25 c.c.	0 ,,	0 ,,	0 ,,	0 ,,
		,,	0 ,,	25 ,,	0 ,,	364 ,,

to 20 c.c. The fermenting power of 20 c.c. of the dialysed juice was then determined with the following additions :—

(1) 20 c.c. of dialysed juice + 10 c.c. of water gave 0·02 gram CO_2.
(2) ,, ,, ,, ,, + 10 ,, evaporated dialysate gave 0·52 gram CO_2.
(3) ,, ,, ,, ,, + 10 ,, boiled juice gave 0·89 gram CO_2.

It was shown in the previous chapter that phosphates are essential to fermentation, and hence it becomes necessary to inquire whether the effect of dialysis is simply to remove these. Experiment shows that this is not the case. Soluble phosphates do not confer the power of producing fermentation on the inactive residue obtained by filtration. Moreover, when yeast-juice is digested for some time before being boiled, it is found, as will be subsequently described, that the boiled autolysed juice is quite incapable of setting up fermentation in the inactive residue, although free phosphates are abundantly present [Harden and Young, 1906, 2].

This dialysable, thermostable substance, without which alcoholic fermentation cannot proceed, has been provisionally termed the co-ferment or co-enzyme of alcoholic fermentation. This expression was first introduced by Bertrand [1897], to denote substances of this kind, and he applied it in two instances—to the calcium salt which he considered was necessary for the action of pectase on pectin substances, and to the manganese which he supposed to be essential for the activity of laccase. Without inquiring whether these substances are precisely comparable in function with that contained in yeast-juice, the term may be very well applied to signify the substance of unknown constitution without the co-operation of which the thermolabile enzyme of yeast-juice is unable to set up the process of alcoholic fermentation.

According to the proposal of Euler and Myrbäck [1923] the specific term co-zymase is often used, whilst the enzyme free from co-enzyme has been termed by Neuberg [see Neuberg and Oppenheimer, 1925] apozymase.

The active agent of yeast-juice consisting of both enzyme and co-enzyme may be conveniently spoken of as the *fermenting complex*, and this term will occasionally be employed in the sequel.[1]

The co-enzyme is present alike in the filtrates from fresh yeast-juice and from boiled yeast-juice, and is also contained in the liquids obtained by boiling yeast with water and by washing zymin or dried yeast with water. Well-washed zymin forms a convenient source of the inactivated enzyme (apozymase).

The co-enzyme is capable of being decomposed by a variety of reagents, prominent among which is yeast-juice. This was observed by Harden and Young in the course of their attempts to prepare a completely inactive residue by filtration. In many cases a residue was obtained which still possessed a very limited power of fermentation, only a small amount of carbon dioxide being formed and the action ceasing entirely after the expiration of a short period; on the subsequent addition of boiled juice, however, a very considerable evolution of carbon dioxide was produced. This was interpreted to mean that the residue in question contained an ample supply of enzyme but only a small proportion of co-enzyme, and that the latter was rapidly destroyed, so that the fermentation soon ceased. The boiled juice then added provided a further quantity of co-enzyme by the aid of which the enzyme was enabled to carry on the fermentation. This view was confirmed by adding to a solution of a completely inactive filtration residue and glucose successive small quantities of boiled juice and observing the volumes of carbon dioxide evolved after each such addition. Thus in one case successive additions of volumes of 3 c.c. of boiled juice produced evolutions of 8·2, 6, and 6 c.c. of carbon dioxide. In another case two successive additions of 15 c.c. of boiled juice produced evolutions of 54 and 41·2 c.c. On the other hand, the enzyme itself also gradually disappears from yeast-juice when the latter is incubated either alone or with sugar (p. 81).

The cessation of fermentation in any particular mixture of enzyme

[1] In view of the recent work on the nature of co-zymase, Neuberg and Euler have suggested that the following terms should be used:—

Zymase, the enzymic constituents of the enzyme system of alcoholic fermentation freed from all activators.

Holozymase, the fermenting complex as defined above.

Apozymase, holozymase deprived of co-zymase. Thus the apozymase from dried yeast still contains magnesium (p. 87), and thus differs from zymase.

and co-enzyme may, therefore, be due to the disappearance of either of these factors from the liquid. If the amount of co-enzyme present be relatively small it is the first to disappear, and fermentation can then only be renewed by the addition of a further quantity whilst the addition of more enzyme produces no effect. If, on the other hand, the amount of co-enzyme be relatively large, the inverse is true; the enzyme is the first to disappear, and fermentation can only be renewed by the addition of more enzyme, a further quantity of co-enzyme producing no effect. It has, moreover, been found that the co-enzyme, like the enzyme, disappears more rapidly in the absence of glucose than in its presence, incubation at 25° for two days being as a rule sufficient to remove all the co-enzyme from yeast-juice from top yeasts in the absence of sugar, whilst in the presence of fermentable sugar co-enzyme may still be detected at the end of four days.

In all the experiments carried out by Harden and Young with juice from English top yeast it was found that when a mixture of the juice with glucose was incubated until fermentation had ceased, the further addition of co-enzyme in the form of boiled juice did not cause any renewal of the action; in other words, the whole of the enzyme had disappeared.

On the other hand, Buchner and Klatte [1908], working with juice and zymin prepared from bottom yeast, observed the extremely interesting fact that after the cessation of fermentation the addition of an equal volume of boiled juice caused a renewed decomposition of sugar, and that the processes of incubation until no further evolution of gas occurred and re-excitation of fermentation by the boiled juice could be repeated as many as six times. Thus in one experiment the duration of the fermentation was extended from three to a total of twenty-four days, and the total gas evolved from 0·73 gram to 2·19 grams. The phenomenon has been found to be common to yeast from Munich and from Berlin as well as to zymin and maceration extract, and it was further observed that the boiled juice from one yeast could regenerate the juice from another, although the quantitative relations were different.

In these samples of yeast-juice, therefore, there is present a natural condition of affairs precisely similar to that obtaining in the artificial mixtures of inactive filtration residue and co-enzyme solution made by Harden and Young. The balance of quantities is such that the co-enzyme disappears before the enzyme, leaving a certain amount of enzyme capable of exercising its usual function as soon as sufficient co-enzyme is added. This establishes an interesting point of contrast

with the juice prepared from top yeast in England, in which the enzyme does not outlast the co-enzyme [Harden and Young, 1907]. The difference may be due to some variation in the relative proportions of enzyme and co-enzyme or of the enzymes to which the disappearance of each of these is presumptively due, or to a combination of these two causes. It was, however, found, even in the juice from bottom yeast, that incubation for three days at 22° without the addition of sugar caused the disappearance of the enzyme as well as of the co-enzyme and left a residue alike incapable of being regenerated by the addition of co-enzyme or of restoring the power of producing fermentation to an inactive mixture containing enzyme and sugar.

Nord and Franke [1928] obtained preparations from American bottom yeasts which retained their fermenting powers for two months at 5°-15°, and increased in activity during the first few days. They attribute the loss of fermenting power to increase in peptisation, and find that exposure of the extract to ethylene " protects " the enzyme to some extent.

If the fermenting power of the juice is to be preserved by repeated regeneration for a long period, it is absolutely necessary to add the co-enzyme solution each time as soon as fermentation has ceased, since the enzyme in the absence of co-enzyme rapidly disappears, even in the presence of sugar.

The processes by which the enzyme and co-enzyme are inactivated are still obscure. On the assumption that the alcoholic enzyme of yeast-juice belongs to the class of proteins, the antiprotease of Buchner and Haehn [1910, 1] (see p. 43) may be supposed to lessen the rate at which this enzyme is destroyed by the proteases of the juice. This antiprotease is more stable than the co-enzyme towards hydrolytic agents, and can be obtained free from co-enzyme by boiling the solution for some hours alone or by heating with dilute sulphuric acid. Such a solution possesses no regenerative power, but still retains its power of protecting proteins against digestion and of preserving the fermenting power of yeast-juice.

Kluyver and Struyk [1927 ; 1928, 2] in fact regarded the antiprotease as the essential constituent of the co-zymase (see p. 91), but this has not been confirmed by other workers [see Myrbäck, 1928 ; Stheeman, 1929, 1930].

It must, however, be remembered that the addition of a phosphate alone may greatly prolong the period of fermentation of yeast-juice (p. 72), and sugar is well known to exert a similar action. It appears, therefore, that the existence of the enzyme is prolonged not only by

the presence of the antiprotease but also by that of sugar and of the hexosephosphate into which phosphate is converted in presence of sugar.

Phosphate, however, increases the rate in the early stages of fermentation whereas the antiprotease is more effective in prolonging the process [Haehn and Schifferdecker, 1923].

The extract which contains the antiprotease also exerts a preservative effect on the co-enzyme, but this is probably due to a different agent from that which protects the enzyme [see Haehn and Schifferdecker, 1923]. Sugar and probably hexosephosphate also act as preservatives of the co-enzyme.

The fermenting complex, therefore, in the presence of these substances, either separately or together, falls off more slowly in activity and is present for a longer time, and for both these reasons produces an increased amount of fermentation. It seems probable also that the hexosephosphatase is similarly affected, so that the supply of free phosphate is at the same time better maintained, and the rate of fermentation for this reason decreases more slowly than would otherwise be the case.

It is in this way that an explanation may be found of the remarkable increase in total fermentation which is produced by the addition to yeast-juice and sugar of boiled yeast-juice, containing free phosphate (which passes into hexosephosphate) as well as co-enzyme, of boiled autolysed yeast-juice, containing free phosphate but no co-enzyme, or of phosphate solution alone.

In no case is the original rate of fermentation greatly increased after the initial acceleration has disappeared, but in every case the total fermentation is considerably augmented, and this is no doubt to be attributed, as just explained, mainly to the diminished rate of decomposition of the fermenting complex and probably of the hexosephosphatase.

Although both enzyme and co-enzyme are completely precipitated from yeast-juice, as already described (p. 38), by 10 volumes of acetone, the co-enzyme is less easily precipitated than the enzyme, and a certain degree of separation can therefore be attained by fractional precipitation [Buchner and Duchaček, 1909].

Detection and Estimation of Co-enzyme.

Methods for the detection and estimation have been worked out by Euler and Myrbäck in connection with their work on the isolation

of the co-enzyme (p. 85) [see Myrbäck, 1928]. The fundamental requirement is an apozymase (p. 80) preparation entirely free from co-enzyme. This may be either well-washed ultrafiltration residue as used by Meyerhof [1918, 3], well-washed zymin (Harden and Young), or well-washed dried yeast (Euler and Myrbäck). The conditions of activation of such preparations are discussed later on (p. 89). Myrbäck [1928] lays down the following practical tests which should be fulfilled by the apozymase preparation. (1) There should be no evolution of CO_2 with glucose and phosphate solution at p_H 6·3-6·6. (2) The addition of 1-50 mg. of hexosediphosphate or of acetaldehyde should not cause fermentation. (3) When boiled with water for a minute and kept for a few minutes at 80°, it should yield an extract which gives no fermentation when added to washed yeast, sugar, phosphate, and hexosephosphate. Euler and Myrbäck carry out the test quantitatively on the micro-scale by using the following mixture : 0·2 g. apozymase, 0·1 g. glucose, 0·5 c.c. 10 per cent. phosphate mixture at p_H 6·3-6·6, 5 mg. Na hexosediphosphate, to which are added the solution to be tested for co-enzyme with water sufficient to make the total volume 2 c.c. The mixture is placed in a micro-apparatus (p. 29) at 30° and the evolution of CO_2 measured at intervals. Under these circumstances it is found that the total CO_2 evolved in an hour is proportional to the concentration of the co-enzyme within a certain range of the latter [Myrbäck, 1928]. Hence in order to make a reliable estimation of the concentration of co-enzyme, a series of observations must be made and that part of the range employed in which proportionality between concentration of co-enzyme and CO_2 evolution is proved to exist. Euler and Myrbäck have adopted a convenient nomenclature, defining the unit of co-enzyme (Co) as that amount which under the foregoing conditions produces an evolution of 1 c.c. of CO_2 per hour. The activity of the preparation (ACo) is then given by the ratio

$$\text{ACo} = \frac{\text{c.c. } CO_2 \text{ per hour}}{\text{g. co-enzyme preparation}}.$$

Concentration of the Co-enzyme.

Considerable progress has been made by Euler and Myrbäck towards the isolation of the co-enzyme. Previous to their work it had been shown (Harden and Young) that the co-enzyme was not precipitated from boiled yeast-juice by successive treatment with one volume of alcohol and an excess of lead acetate solution at a p_H of about 7·

Occasionally the precipitate of lead salt retained some of the co-enzyme, apparently by adsorption, but usually the greater part remained in solution. It was also known that the co-enzyme is partially removed from yeast-juice by means of a colloidal solution of ferric hydroxide (Resenscheck). A precipitate is thus obtained which contains phosphorus and resembles boiled yeast-juice in its regenerative action on yeast-juice rendered inactive by fermentation. It has not, however, so far been found possible to isolate any definite compound from this precipitate. There are also indications that when yeast-juice, either fresh or boiled, is electrolysed, the co-enzyme tends to accumulate at the cathode [Resenscheck, 1908, 1, 2].

Euler and Myrbäck [1924, 2, 4; 1928, 1; 1929; Myrbäck and Euler, 1924] start with a yeast extract made by heating yeast with 5-6 parts of water at 90°, and evaporating at a low temperature. This is first dialysed against water in collodion membranes and the dialysate, which is free from colloidal matter, is used. This is treated with lead acetate at p_H 6·0 and the filtrate, which contains the co-enzyme, is precipitated with lead acetate and sodium hydroxide at p_H 10·0. The washed precipitate, containing the co-enzyme, is decomposed by sulphuric acid and from the resulting solution the co-enzyme is precipitated by addition of mercuric nitrate, sufficient sodium hydroxide being added to keep the liquid only slightly acid. The well-washed precipitate is then decomposed by H_2S and the latter removed by a current of air. The double precipitation can be avoided by adding the mercuric nitrate directly to the filtrate from the neutral lead acetate precipitation, but the product is not so pure.

Further purification is effected by adding silver nitrate to the acid solution resulting from the decomposition of the mercuric nitrate precipitate, filtering and to the filtrate adding excess of silver nitrate and then just sufficient dilute ammonia to produce maximum precipitation. The precipitate is washed with water and decomposed by H_2S. This solution (ACo = 20,000) is precipitated with phosphotungstic acid in 2 per cent. sulphuric acid, the precipitate washed with dilute sulphuric acid and extracted with ether and amyl alcohol in presence of very dilute sulphuric acid to remove the phosphotungstic acid, and from this solution the co-enzyme is precipitated, after concentration *in vacuo*, by the addition of a large volume of alcohol or acetone. A perfectly white preparation is obtained of high activity (ACo = 30-50,000). This material is not an individual substance since picric acid precipitates from its concentrated solution the picrate of adenylthiomethylpentose, $C_{11}H_{15}O_3N_5S$, the presence of which in

yeast was first detected by Suzuki, Odake, and Mori [1924], and which has no co-enzymic activity. The material obtained by removing the picric acid from the filtrate from the foregoing picrate is the purest form of the co-enzyme hitherto obtained from yeast. It has an activity (ACo) of 50-80,000 and contains about 15·4 per cent. of N, 40 per cent. C, 5·2 per cent. H, a small amount (about 0·5 per cent.) of S, possibly due to remaining traces of the thiomethylpentose, and about 7·6 per cent. of P. On hydrolysis, it yields adenine and a reducing sugar, the results being consistent with the supposition that the material consists to the extent of at least 90 per cent. of a substance of the nature of an adenine nucleotide, the carbohydrate fraction of which has not yet been identified.

It yields a barium compound, containing 4·82 per cent. of P, in which the atomic ratio Ba : N : P is approximately 1 : 6 : 1. This can be dissolved in water and reprecipitated by 50 per cent. alcohol without change of composition or activity [Euler and Myrbäck, 1929; Euler, 1930]. The molecular weight, as determined by the rate of diffusion is about 486 [Euler, Myrbäck, and Nilsson, 1927].

Some idea of the quantitative relations of the co-enzyme are given below :—

	ACo.
Fresh yeast	170
Dried yeast	85
Yeast extract	200
Filtrate from Pb precipitate at p_H 6	700
From Pb precipitate at p_H 10·0	2000-6000
Purest preparation	83,000

The experiments of Buchner and Klatte [1908] led to the observation that the co-enzyme was destroyed by an emulsion containing the lipase of castor oil seeds, and it was thought that this indicated that the co-enzyme belonged to the class of fats or esters.

It has now been found that in its purest form the co-enzyme is not attacked by purified castor oil lipase, esterase, or trypsin but is inactivated by liver, by kidney, and by a preparation from the latter which also hydrolyses nucleotides.

During the enzymic decomposition of the co-enzyme preparation the activity falls off in proportion to the liberation of inorganic phosphorus [Euler, Myrbäck, and Brunius, 1929].

On the other hand, when boiled in aqueous solution, and in some cases when treated with lime, the preparation becomes inactive when only 30 per cent. of its P has been hydrolysed. This may possibly be due to deamination of the adenine radical [Euler and Myrbäck, 1930].

Against the conclusion that this preparation represents the pure

co-enzyme is the fact that although in muscle the presence of adenylic acid (adenine nucleotide) in the form of a pyrophosphate appears to be essential for the production of lactic acid it does not exert this action unless co-enzyme is also present. A co-enzyme solution free from adenylic acid can be prepared by Euler and Myrbäck's method from muscle which has been allowed to autolyse at 38°. Such a preparation is quite incapable of activating the muscle enzyme unless adenylic acid pyrophosphate (which by itself is likewise inactive) is also added. The adenylic acid therefore acts as a complement to the co-enzyme. The same conditions hold for yeast preparations [Meyerhof 1930, p. 174], but apparently a smaller concentration of adenylic acid suffices than in the case of muscle. Euler and Myrbäck, however, were unable to confirm this [Euler, 1930, p. 17].

According to the experiments of Lohmann [1931] the factor necessary as a complement to adenylic acid or adenylic pyrophosphate is magnesium. The necessity for the presence of magnesium has been confirmed by Euler and Nilsson [1931], who find in agreement with Lohmann that washed dried yeast retains magnesium, which can, however, be removed by means of phosphoric acid. According to their experiments adenyl pyrophosphate + a magnesium salt produces a much smaller acceleration than is produced by co-zymase, whilst the co-zymase cannot be replaced by muscle adenylic acid. They suggest that a further activator, related to Euler's Z factor, is also necessary.

Properties of the Co-enzyme.

Early observations [Buchner and Haehn, 1909] showed that digestion with potassium carbonate solution containing 2·5 grams per 100 c.c. brought about the destruction of the co-enzyme, and that this was also slowly accomplished by the repeated boiling of the juice. The co-enzyme is also destroyed both by acid and alkaline hydrolysis, and by ignition of the residue obtained when the solution is evaporated to dryness.

Experiments with the purified co-enzyme [Myrbäck and Euler, 1924] show that in aqueous solution the decomposition by heat proceeds according to a unimolecular reaction, the constant (k) at p_H 5·5 and 85° being 0·26. The stability varies with the p_H, and is greatest at p_H 2·5, falling off rapidly at p_H values above and below this.

The co-enzyme is remarkably stable to oxidising agents; it is not affected in 24 hours by a current of air at 25° at p_H 4·7, and is not inactivated by bromine or $KMnO_4$ in acid solution at air temperature.

The idea that co-zymase was identical with insulin or that insulin could act as a co-zymase in the fermentations produced by certain organisms [Virtanen, 1925, 1] or in lactic acid production in animal tissues [Virtanen and Karström, 1926; Brugsch and Horsters, 1926] has been shown to be erroneous [Freudenberg and Dirscherl, 1926; Euler and Myrbäck, 1925; Jorpes, Euler, and Nilsson, 1926].

Occurrence of the Co-enzyme in Animal and Vegetable Tissues.

An observation of great interest and importance has been made by Meyerhof [1918, 1, 2], who has found that the co-enzyme of alcoholic fermentation occurs in the muscles and organs of animals, as well as in milk, whereas it is absent from serum.

Meyerhof's experiments with muscle were carried out in the manner already described (p. 78), the well-washed residue from the ultrafiltration of maceration extract being used as the inactive enzyme. The best results are obtained from frog's muscle (hind leg) which is extracted with an equal weight of boiling water. It is remarkable that when the finely minced muscle is treated with cold water the resulting extract does not activate the enzyme, and that when this extract is boiled only slight activation is produced. On the other hand, when the muscle is directly extracted with boiling water a solution possessing a powerful activating effect is at once obtained.

The following example indicates these relations, the numbers being c.c. of CO_2 evolved in two hours in presence of the various additions shown :—

Additions.	CO_2 evolved. c.c.
0·6 c.c. water	0·04
0·6 c.c. muscle extract, made with boiling water	0·69
0·6 c.c. cold water extract of muscle	0·02
0·6 c.c. cold water extract of muscle, subsequently boiled	0·26

The muscle extract made with boiling water is about half as effective as boiled maceration extract and contains only about one-sixth of the mineral phosphate of the latter (boiled maceration extract 0·83 per cent.; muscle extract, 0·14 per cent. P_2O_5).

The co-enzyme is essential for the activity of the lactic acid-forming enzyme of muscle, which like the zymase of yeast can be rendered inactive by washing muscle or by the ultrafiltration or dialysis of the muscle enzyme solution. The co-enzyme of yeast can be used to activate such an inactive preparation, just as the co-enzyme of muscle can activate the apozymase of yeast. Although the substances

acting as co-enzyme are closely similar in both cases and behave alike under all conditions of temperature, alkalinity, etc. [see also Euler and Myrbäck, 1924, 1], certain points of difference exist to which reference has been made above (p. 87).

The cold-water extract of muscle contains an inhibitory substance, inactivated by boiling, which appears to exert its action chiefly on the enzyme, not on the co-enzyme (Meyerhof). This substance can be adsorbed by aluminium hydroxide and kaolin and eluted by phosphate at p_H 6·2 [Myrbäck, 1925] and is non-dialysable. It was found by Myrbäck to diminish the rate of fermentation by three different dried bottom yeasts, but slightly to increase that of two dried top yeasts.

The co-enzyme is present in varying amount in nearly all animal tissues, the retina and grey matter of the brain (of the ox) being especially rich in it [Euler and Runehjelm, 1927 ; Euler and Nilsson, 1927, 1 ; Sym, Nilsson and Euler, 1930]. In the blood, in which it is present in comparatively small amount [Euler, Nilsson, and Jansson, 1927], it is confined to the red corpuscles [Euler and Nilsson, 1927, 1 ; Virtanen and Simola, 1926].

The same co-enzyme is found in germinating peas [Meyerhof, 1918, 2] in the higher fungi and in green leaves and other plant tissues [Euler and Steffenburg, 1928 ; Zaleski and Pissarjewski, 1927 ; Zaleski and Notinka, 1927] ; and appears also to be present in the bacteria which produce propionic and lactic acids [Virtanen, 1924, 1925, 1]. In leaves it is accompanied by a thermostable inhibitory substance, different from the thermolabile inhibitor of muscle.

The Conditions of Activation of Washed Zymin.

Many direct experiments have been made to induce fermentation by adding various substances to an inactivated enzyme preparation, with the precautions already laid down (p. 84). In this way it was shown that soluble phosphates, hexosephosphates and a number of oxidisable and reducible substances, such as quinol, *p*-phenylenediamine, methylene blue, peptone, beef broth, etc. (Harden and Young ; Harden and Norris [1914] ; see also Euler and Bäckström [1912]), and glycerophosphates [Buchner and Klatte, 1908] were inactive.

The first apparent success was obtained by Neuberg [Neuberg and Schwenk, 1915, 3], who found that whereas single α-ketonic acids were without effect, a mixture of α-ketonic acids produced a definite activation, when added along with a phosphate to maceration extract

inactivated by dialysis, or to dried yeast which had been washed with water and then treated with acetone. At a later period Harden [1917] found that washed zymin, prepared by treating top yeast with acetone, could be activated by pyruvates and by acetaldehyde, in presence of phosphate, provided that potassium ions were present, whereas no activation occurred in their absence. Thus when sodium phosphate was used, potassium pyruvate produced activation whereas the sodium salt did not, and acetaldehyde similarly produced no effect in presence of sodium phosphate, whereas in presence of potassium phosphate vigorous fermentation was produced.

Neuberg, however [1918, 1], was unable to obtain this result with preparations from bottom yeast and considered that a mixture of ketonic acids was essential. Meyerhof [1918, 1] found that when thoroughly washed the filtration residue from maceration extract (bottom yeast) was not activated by sodium pyruvate, and several workers [Kluyver and Struyk, 1927; Stheeman, 1929; Myrbäck, 1928] have found that zymin and dried yeast when sufficiently washed cannot be activated by pyruvates or aldehydes whereas they are activated by boiled yeast washings. This has also been confirmed in the author's laboratory [Harden and Macfarlane, 1931].

Meyerhof further observed that in washing ultrafiltration residue a stage is reached at which the residue will no longer ferment sugar but still ferments hexosediphosphate. He at first was inclined to think that the fermentation of hexosediphosphate could be effected without the aid of the co-enzyme but ultimately found that this was not the case, and that the thoroughly washed residue could not ferment hexosediphosphate, a fact confirmed by many workers. On the fact that hexosediphosphate requires a smaller concentration of co-enzyme for its fermentation than does sugar, he has based a theory of the direct fermentation of hexosediphosphate, without re-esterification of the hexose (p. 141).

The whole question has been re-investigated first by Kluyver and Struyk [1927, 1928, 2], and then by Stheeman [1929, 1930], who have found that several stages of inactivation of maceration residue and zymin by washing with water can be distinguished. An important point to bear in mind is that different preparations vary considerably in the ease with which they can be deprived of co-enzyme and other soluble constituents by washing. Zymin or dried yeast from bottom yeast is usually much more easily freed from co-enzyme by washing than are the corresponding preparations from top yeast [see Neuberg and Gottschalk, 1925, 3], but the rule is not invariable.

Using zymin from a Dutch baker's yeast Stheeman found that four distinct stages of inactivation could be successively produced :—

1. The residue produces no fermentation with glucose (or fructose) and phosphate but can be activated by aldehydes and by methylene blue.

2. The residue can no longer be activated by methylene blue but becomes active when a hexosediphosphate is added.

3. The residue cannot be activated either by methylene blue alone or by hexosediphosphate alone but is activated by a mixture of the two.

4. The residue cannot be activated as in 3 but requires the addition of another factor, which is present in the concentrated preparation of the co-enzyme obtained by Euler and Myrbäck (p. 86), and of a hydrogen acceptor, such as methylene blue. If only a small quantity of the co-enzyme preparation be added hexosediphosphate is also necessary, but not if a larger quantity be used.

It is not easy to understand the meaning of all these stages and further work must be awaited. In Stheeman's opinion thoroughly washed zymin requires three factors for activation : a hydrogen acceptor, hexosediphosphate and Euler and Myrbäck's " co-enzyme," but the last of these in large quantity may replace the hexosediphosphate. He considers that the function of the hexosediphosphate is probably to supply esterified phosphoric acid groups (for what purpose is unknown), and it seems probable that the co-enzyme, which contains organic phosphorus, can also do this if present in sufficient quantity.

Kluyver and Struyk [1927] and Stheeman [1929] at one time thought that at stage 3 activation required the presence of an antiprotease, but according to the later work of Stheeman [1930] it seems probable that the antiprotease solution acted mainly, if not entirely, in virtue of its content of hydrogen acceptor.

The Factors Z.

Euler and his colleagues (Euler and Swartz, 1924 ; Euler and Myrbäck, 1924, 5 ; Myrbäck and Euler, 1928 ; Euler, Brunius, and Proffe, 1928 ; Philipson, 1930, 1, 2 ; Euler and Philipson, 1931 ; see also Euler, 1930 ; Meyerhof and Iwasaki, 1930 ; Abderhalden, 1921 , 3 ; 1922, 1] have found that, in addition to the co-enzyme, yeast contains substances which have a strong accelerative action on fermentation by living yeast, whereas the co-enzyme has no effect of this sort. Conversely the new factors do not accelerate fermentation by dried yeast.

These factors also occur in many plants, e.g. sprouted barley, green leaves of cabbage or barley, and in many animal organs [Euler and Johansson, 1928]. They are present abundantly in marmite and to a small extent in beer wort. The best source is the dialysate of autolysed bottom yeast. They are much more stable to heat than the co-enzyme and can be separated from it by boiling the solution for one hour at p_H 7, under which conditions the co-enzyme is completely inactivated. They are not precipitated by metallic salts but appear to be in part absorbed by colloidal ferric hydroxide.

The characteristic effect is greatly to increase the rate of fermentation by living yeast without increasing the number of cells present. Thus the rate of fermentation of pressed yeast may be increased by 100 per cent. and of *Saccharomycodes Ludwigii* by 100 to 200 per cent.

Nothing is known of the chemical nature or mode of action of these factors. They appear to be quite distinct from any of the other "bio-catalysts" of yeast. No pure substance has been found to exert a similar effect on yeast.

CHAPTER V.

CARBOXYLASE AND CARBOLIGASE.

Carboxylase.

An observation of remarkable interest, which has thrown light on several important features of the biochemistry of yeast, was made in 1911 in Neuberg's laboratory.

It was found that yeast had the power of rapidly decomposing a large number of hydroxy- and keto-acids [Neuberg and Hildesheimer, 1911 ; Neuberg and Tir, 1911 ; see also Karczag, 1912, 1, 2]. The most important among these are pyruvic acid, $CH_3 \cdot CO \cdot COOH$, and a considerable number of other aliphatic *a*-keto-acids, which are decomposed with evolution of carbon dioxide and formation of the corresponding aldehyde :—

$$R \cdot CO \cdot COOH = R \cdot CHO + CO_2.$$

The reaction is produced by all races of brewer's yeast which have been tried, as well as by active yeast preparations and extracts and by wine yeasts [Neuberg and Karczag, 1911, 4 ; Neuberg and Kerb, 1912, 2]. The phenomenon can readily be exhibited as a lecture experiment by shaking up 2 g. of pressed yeast with 12 c.c. of 1 per cent. pyruvic acid, placing the mixture in a Schrötter's fermentation tube, closing the open limb by means of a rubber stopper carrying a long glass tube and plunging the whole in water at 38°-40°. A comparison tube of yeast and 1 per cent. glucose may be started at the same time, and it is then seen that the pyruvic acid is decomposed but less rapidly than glucose [Neuberg and Karczag, 1911, 1 ; see also Neuberg and Kerb, 1913, 1 ; Neuberg, 1927]. If English top yeast be used it is well to take 0·5 per cent. pyruvic acid solution and to saturate the liquids with carbon dioxide before commencing the experiment. A similar experiment can be made with dried yeast or zymin, 2 g. of this being gradually added to 15 c.c. *M*/10 pyruvate which is at the same time 1·5 *M* with K_2HPO_4 or 2*M* with potassium acetate and the mixture then placed in the fermentation tube [Neuberg, 1924].

The production of acetaldehyde can be readily demonstrated by distilling the mixture at the close of fermentation and testing for the

aldehyde either by Rimini's reaction (a blue coloration with diethylamine or piperidine and sodium nitroprusside) or by means of *p*-nitrophenylhydrazine, which precipitates the hydrazone, melting at 128·5° [Neuberg and Karczag, 1911, 2, 3].

Pyruvic acid should yield equal weights of carbon dioxide and acetaldehyde, and the acetaldehyde can be almost completely accounted for when allowance is made for the fact that part of it is converted into acetoin (p. 99). Thus, as the result of an experiment with yeast, 2·86 g. of acetaldehyde and 2·07 g. of acetoin were obtained, equivalent to a total of 4·93 g. of acetaldehyde, whilst the carbon dioxide evolved was 4·82 g. [Neuberg and May, 1923].

The salts of the acids are also attacked, the carbonate of the metal, which may be strongly alkaline, being formed. Thus, taking the case of pyruvic acid, the salts are decomposed according to the following equation :—

$$2CH_3 \cdot CO \cdot COOK + H_2O = 2CH_3 \cdot CHO + K_2CO_3 + CO_2.$$

Under these conditions a considerable portion of the aldehyde undergoes condensation to aldol [Neuberg, 1912] :—

$$2CH_3 \cdot CHO = CH_3 \cdot CH(OH) \cdot CH_2 \cdot CHO.$$

This change appears to be due entirely to the alkali and not to an enzyme since the aldol obtained yields inactive *β*-hydroxybutyric acid on oxidation [Neuberg and Karczag, 1911, 3 ; Neuberg, 1912]. The various preparations derived from yeast which are capable of producing alcoholic fermentation also effect the decomposition of pyruvic acid in the same manner as living yeast [see also Neuberg and Czapski, 1914], and this is also true of the precipitate obtained from maceration extract with acetone or alcohol and ether [Neuberg and Rosenthal, 1914 ; Neuberg, 1915, 1]. They are, however, more sensitive to the acidity of the pyruvic acid, and it is therefore advisable to employ a salt of the acid in presence of excess of a weak acid, such as boric or arsenious acid, which decomposes the carbonate formed but has no inhibiting action on the enzyme [Harden, 1913 ; Neuberg and Rosenthal, 1913]. Other buffers, e.g. phosphates or acetates, may be employed, and these, moreover, often have a very beneficial effect on the action of living yeasts [Neuberg 1915, 1 ; Haehn and Glaubitz, 1927, 2], which are moderately sensitive to pyruvic acid ; thus digestion for twenty-four hours with a 2 per cent. solution of the acid completely removes their power of decomposing pyruvates.

As already mentioned the action is exerted on *a*-ketonic acids as a class and proceeds with great readiness with oxaloacetic acid,

$COOH \cdot CH_2 \cdot CO \cdot COOH$, all the three forms of which are decomposed, with *a*-ketoglutaric acid [Mayer, 1913], with *a*-ketobutyric acid, which yields *n*-propyl alcohol [Neuberg and Kerb, 1914, 2], and with α-keto-*n*-capronic acid, which yields *n*-valeraldehyde [Sen, 1923]. Hydroxypyruvic acid, $CH_2(OH) \cdot CO \cdot COOH$, is slowly decomposed yielding glycolaldehyde, $CH_2(OH) \cdot CHO$, and this condenses to a sugar [Neuberg and Kerb, 1912, 3; 1913, 1; Neuberg and Rosenthal, 1914]. Positive results have also been obtained with diketobutyric, phenylpyruvic, *p*-hydroxyphenylpyruvic, phenylglyoxylic and acetonedicarboxylic acids [Neuberg and Karczag, 1911, 5], as well as with methyl-, dimethyl-, and methylethyl-pyruvic acids (p. 97).

It is interesting to note that methylethyl-pyruvic acid is asymmetrically decomposed, the lævo-rotatory form being unattacked, whilst the dextro-rotatory form is decomposed, yielding an active valeraldehyde and, as a secondary product, *d*-amyl alcohol [Neuberg and Peterson, 1914].

Preparations obtained from the potato and sugar beet by Bodnár [1916], which produced alcoholic fermentation, also contained carboxylase, identical in its properties with that of yeast. On the other hand, Neuberg [1915, 2] found that several species of Pseudosaccharomyces, which did not ferment glucose, were also destitute of the power of decomposing pyruvic acid. A carboxylase also occurs in many seeds and etiolated seedlings [Zaleski and Marx, 1912, 1913; Zaleski, 1913], but this appears to differ from yeast carboxylase inasmuch as it is stated only to decompose pyruvic acid and not other *a*-ketonic acids [Zaleski, 1914].

The optimum p_H for the action of carboxylase is 3·5-6·0 for yeast preparations, little action being observed at p_H 7·8, and this range holds for the decomposition of other α-ketonic acids as well as pyruvic acid. Living yeast decomposes pyruvic acid most rapidly at p_H 1·5-4·0 and has little action at p_H 6-7 [Hägglund and Augustsson, 1926; Hägglund and Rosenqvist, 1927; Hägglund and Ringbom, 1927; see also Euler, Myrbäck and Nilsson, 1925].

The molar heat of combustion of pyruvic acid is almost exactly the same as that of acetaldehyde, so that the decomposition is not accompanied by any evolution or absorption of energy [Blaschko, 1925; see Neuberg, 1924].

Relation of Carboxylase to Alcoholic Fermentation.

The relation of carboxylase to the process of alcoholic fermentation is a matter of great interest and importance. As Neuberg points out

[see Neuberg and Kerb, 1913, 1] the universal presence of the enzyme in yeasts capable of producing alcoholic fermentation and the extreme readiness with which the fermentation of pyruvic acid takes place create a strong presumption that the decomposition of pyruvic acid actually forms a stage in the process of the alcoholic fermentation of sugar.

This presumption is raised almost to a certainty by the work of Neuberg on the fixation method of fermentation in the presence of sulphites, which is discussed in Chapter VII. Ehrlich's alcoholic fermentation of the amino-acids (p. 169) provides another function for carboxylase—that of decomposing the *a*-ketonic acids produced by the deamination of the amino-acids. It must be remembered in this connection that carboxylase is not specific in its action, but catalyses the decomposition not only of pyruvic acid but also of a large number of other *a*-ketonic acids, including many of those which correspond to the amino-acids of proteins and are doubtless formed in the characteristic decomposition of these amino-acids by yeast.

A comparison of the conditions of action of carboxylase and zymase has revealed many interesting points of difference, and there can be no doubt that carboxylase is an independent enzyme. Neuberg and Rosenthal [1913] have observed that the fermentation of pyruvic acid by maceration extract commences much more rapidly than that of glucose, and interpret this to mean that in the fermentation of glucose a long preliminary process occurs before sufficient pyruvic acid has been produced to yield a perceptible amount of carbon dioxide. The pyruvic acid fermentation is less affected by antiseptics [Neuberg and Karczag, 1911, 4; Neuberg and Rosenthal, 1913, 1914; Neuberg, 1915, 1]; amounts of chloroform and of many other antiseptics [Neuberg and Ivanov, 1914] sufficient to stop the glucose fermentation brought about by yeast or dried yeast are usually without effect on the fermentation of the pyruvates either alone or in presence of boric or arsenious acid. Further, the action of carboxylase is not affected in the same way as that of zymase by solutions of emulsin or taka-diastase, boiled or unboiled [Lvov, 1912; Palladin, Gromov, and Monteverde, 1914] or by salts [Harden and Henley, 1921, 2]. The carboxylase, moreover, persists much longer than the zymase in maceration extract, when this is preserved in presence of antiseptics or at low temperatures [Neuberg and Rosenthal, 1914; Neuberg, 1915, 1] and in dried yeast [Neuberg, 1913, 3]. Extraction with methyl alcohol renders dried yeast incapable of fermenting sugars, but scarcely affects the carboxylase [Palladin, Gromov, and Monteverde, 1914].

The carboxylase content of yeast is not altered by treating it with glucose in presence of asparagine or Lindner's solution, but is slightly increased when sodium pyruvate is substituted for the glucose at 10° and the liquid aerated, the power of fermenting glucose being simultaneously slightly depressed [Euler and Löwenhamm, 1916].

The zymase of maceration extract is, moreover, inactivated in 10 minutes at 50°-51°, whereas after this treatment the carboxylase is still active. The limits of temperature, on the other hand, between which the carboxylase and zymase of living yeast are active (10°-60°) are almost identical [Neuberg, 1915, 1].

A very important difference between the two enzymes is that carboxylase decomposes pyruvic acid in the absence of the co-enzyme which is necessary for the fermentation of glucose [Harden, 1913; Neuberg and Rosenthal, 1913]. This can readily be demonstrated experimentally by washing dried yeast or zymin with water until it is no longer capable of decomposing glucose (Harden), or by allowing maceration extract to autolyse or dialyse until it is free from co-enzyme [Neuberg and Rosenthal, 1913, Neuberg, 1915, 1].

This highly interesting fact leads to the conclusion that if, as is most probable, the decomposition of pyruvic acid actually be a stage in the alcoholic fermentation of glucose, the soluble co-enzyme is required for some change precedent to this, so that in its absence the production of pyruvic acid cannot be effected.

Chemistry of the Fermentation of Pyruvic Acid.

Some direct evidence has been obtained which renders it probable that it is the enol-form of pyruvic acid, $CH_2 : C(OH) \cdot COOH$ which undergoes fermentation and not the keto-form, $CH_3 \cdot CO \cdot COOH$. This view is strongly upheld by Nord [1929, p. 43] on theoretical grounds. The equilibrium of these two forms *in vitro* depends on the concentration of the acid and on the hydrogen-ion concentration, a 0·1 *M* solution containing 20 per cent. of enol at p_H 11·8, none at p_H 1·7 [Fromageot, 1926; Henri and Fromageot, 1925]. Yeast and yeast preparations ferment not only pyruvic acid itself but also methyl-, dimethyl-, and methylethyl-pyruvic acids, all of which are capable of forming an enolic modification. These preparations are, however, without action on trimethylpyruvic acid, $C(CH_3)_3 \cdot CO \cdot COOH$ which is incapable of forming an enol, and it seems probable that this incapacity may be the cause of the nonfermentability of this compound [Neuberg and Weinmann, 1928].

It is to be observed, however, that the optimum activity of carboxylase is exerted at a p_H at which comparatively little of the enolic form is normally present.

Relative Velocity of Fermentation of Glucose and Pyruvic Acid.

Some discussion has taken place as to the relative velocity with which glucose and pyruvic acid are fermented by yeast and yeast preparations. If the decomposition of pyruvic acid by carboxylase is a stage in the fermentation of glucose, then, since no accumulation of pyruvic acid is observed under normal conditions, the pyruvic acid must be decomposed at least as rapidly as glucose. The ratio of these velocities has been termed the Neuberg coefficient (N) by Euler and Karlsson [1922]. Living yeast ferments the acid more slowly than glucose [Neuberg, 1927 ; Hägglund and Ahlbom, 1927], possibly owing to permeability effects. Yeast preparations on the other hand were found by some to ferment pyruvic acid much more rapidly and by others less rapidly than glucose [Neuberg, 1927 ; Lindberg, 1922 ; see also Nilsson and Sandberg, 1926 ; Lebedev, 1927, I ; Neuberg and Simon, 1927]. It must, however, be remembered on the one hand that the rate of decomposition of pyruvic acid is very sensitive to changes in p_H, and on the other that it was, as a rule, the basal rate of fermentation of glucose by yeast preparations which was used in the comparison by these workers, and not the much higher maximum rate in presence of phosphate, which alone could give any information as to the relative efficiencies of the zymase and carboxylase of the preparation. It can be deduced from the results of Hägglund and Ahlbom [1927] that when the initial rate of fermentation of pyruvic acid is compared with the " phosphate " rate for glucose, the two are found to be approximately equal. These workers, however, have frequently observed that certain preparations (e.g. maceration extract after prolonged fermentation) which could ferment glucose rapidly had no action on pyruvic acid. They suggest the possibility that in sugar fermentation the pyruvic acid exists in some special fermentable modification, and that when pyruvic acid is added to a yeast preparation it must be converted before fermentation into this form by some agent, the stability of which is less than that of zymase.

Carboligase.

An enzyme which has been termed carboligase (from the Latin *ligare*, to unite) is present in yeast and its preparations, the effect of

which is to link together two carbon atoms forming a carbon chain. This effect was first observed by Neuberg and Hirsch [1921] who found that when benzaldehyde was added to a fermenting mixture of yeast with sugar or pyruvic acid, phenylacetylcarbinol, $C_6H_5 \cdot CH(OH) \cdot CO \cdot CH_3$ (in an optically active form [Neuberg and Ohle, 1922, 1, 2]) was produced. The reaction occurs with a number of aldehydes [see Neuberg and Liebermann, 1921]. A particularly interesting case is that of acetaldehyde, which is converted into optically active acetoin or acetylmethylcarbinol, $CH_3 \cdot CO \cdot CH(OH) \cdot CH_3$. This occurs in the fermentation of pyruvic acid (p. 94) [Hirsch, 1922, 1], as well as when acetaldehyde is added to a fermenting mixture of yeast or an active yeast preparation and sugar [Neuberg and Reinfurth, 1923; Neuberg and Rosenthal, 1924], but not when acetaldehyde is acted on by a glycogen-free yeast in absence of sugar [Neuberg and Simon, 1925]. In the presence of a hydrogen acceptor, such as methylene blue or sulphur, some of the acetaldehyde produced as an intermediate compound in the formation of alcohol (p. 123) escapes reduction and is converted into acetoin, part of which is then reduced to 2·3-butylene glycol [Kluvyer, Donker, and Hooft, 1925]. The formation of these products is also favoured by aeration, the oxygen acting as hydrogen acceptor, and they are present in the fermented mash which has been used for the manufacture of pressed yeast (Lufthefe).

Acetoin and 2·3-butylene glycol are formed by many bacteria, and it has been shown that they are produced when the organism is supplied with acetaldehyde [see Harden and Walpole, 1906; Harden and Norris, 1912]. The proof of the existence of carboligase in micro-organisms is of great importance, as it points the way to an understanding of the numerous and essential synthetical reactions which the cell carries out. The reverse change, the enzymic decomposition of acetoin into aldehyde, has not yet been observed.

CHAPTER VI.

THE REDUCING ENZYME OF YEAST.

THE fact that yeast possesses powerful reducing properties has long been known, and de Rey-Pailhade [1888] showed that this reducing power was also possessed by extracts of yeast, which when brought into contact with sulphur produced an evolution of sulphuretted hydrogen. To the active substance by virtue of which this change was produced he gave the name *philothion* and ultimately came to regard it as a labile hydride, capable of being readily converted by the removal of the labile hydrogen atoms into a new compound, termed by him *philothionogen* (Pi), which in its turn readily took up hydrogen again to reproduce the original philothion (PiH_2). Under the influence of Heffter's work [1907, 1908] he regarded these labile hydrogen atoms as associated with sulphur to form -SH groups. It seems probable that the tripeptide of glutamic acid, glycine and cysteine which has been isolated by Hopkins [1921, 1929] from yeast and animal tissues, and termed glutathione, represents the philothion of de Rey-Pailhade, since it is readily oxidised to the corresponding cystine derivative, and this again is readily reduced. Its solution when shaken with sulphur yields sulphuretted hydrogen.

De Rey-Pailhade apparently attributed the whole of the reducing power of yeast to the action of philothion, but it is much more probable that this substance merely acts as an acceptor for oxygen and hydrogen alternately, and that the reducing properties of the yeast are to be explained by the presence of an enzymic system.

The Mode of Action of Reducing Enzymes.

According to Bach's general theory, reduction in living tissues is brought about by the aid of an enzyme (somewhat illogically termed by him *perhydridase*) in presence of which the elements of water are distributed between two substances, one, the hydrogen acceptor, being reduced, and the other, the oxygen acceptor, being oxidised. The whole process is accordingly regarded as a "hydrolytic oxidation-reduction" [see Bach, 1913, 1, 2] now usually termed an oxido-reduction.

The classical example of this type of enzyme action is afforded by Schardinger's enzyme in milk [Schardinger, 1902], which brings about the reduction of methylene blue only when an oxidisable substance such as an aldehyde is present :—

$$R \cdot CHO + \text{Methylene blue} + H_2O = R \cdot COOH + [\text{Methylene blue} + 2H]$$
(Leuco-methylene blue).

Wieland [1914], who is supported by Thunberg [1920], on the other hand, considers that in Schardinger's reaction the enzyme acts rather as a direct dehydrogenating agent, a *dehydrogenase or hydrogen-transportase*, which removes hydrogen from the oxidisable substance and transports it to the reducible substance. If the latter is a dye, like methylene blue, it is reduced to the colourless leuco-compound. It may also be a molecule of aldehyde, either of the same one which provides the hydrogen, or of a different one. In either case this second molecule of aldehyde is reduced to the corresponding alcohol. Finally, it may be molecular oxygen which is reduced, primarily to hydrogen peroxide. These reactions are exemplified below, but it will be noticed that Wieland assumes that the enzyme acts on the hydrated form of the aldehyde, so that, as pointed out by Hopkins [1921], there is, from the chemical point of view, very little difference between Wieland's views and those of Bach, as concerns this special class of compounds :—

$$R \cdot CH(OH)_2 + \text{Methylene blue} \rightarrow R \cdot COOH + \text{Leuco-methylene blue.}$$
$$R \cdot CH(OH)_2 + R_1 \cdot CHO = R \cdot COOH + R_1 \cdot CH_2OH.$$
$$2[R \cdot CH(OH)_2 + O_2] \rightarrow 2[R \cdot COOH + H_2O_2] \rightarrow 2R \cdot COOH + 2H_2O + O_2.$$

In the second case, if R and R_1 are the same we have the well-known reaction of Cannizzaro, now often termed, at Neuberg's suggestion [Neuberg, Hirsch, and Reinfurth, 1920], dismutation, which occurs in the presence of alkalis and, as shown by Parnas [1910], is also brought about by many tissues, which according to him contain a specific enzyme, aldehydemutase. Wieland's view ascribes all these oxidations and reductions to a single type of enzyme (dehydrogenase), but it has been found that some of the dehydrogenases are highly specific, e.g. succinoxidase and xanthine oxidase. For a discussion of the question of biological oxidations, see Dixon [1929].

The Reducing Properties of Yeast.

That the reducing properties of yeast are certainly due, at all events in large part, to an enzyme was shown by Hahn [1903], who found that yeast-juice reduced methylene blue but almost entirely lost

this property when heated. Dried yeast and zymin (yeast treated with acetone) also reduced methylene blue, but more slowly than yeast-juice.

The oxido-reductase system of yeast is probably complex. Up to the present the following enzymes and complex substances concerned with oxidation and reduction have been detected in the yeast cell: glutathione, a dehydrogenase, a thermostable peroxidase, an indophenol oxidase and a cytochrome system containing three components [see Keilin, 1929]. The exact part played by any of these in reduction processes has not been definitely ascertained. The enzymes which cause the reduction of methylene blue (oxido-reductase) and the dismutation of acetaldehyde (dismutase) or of methylglyoxal (glyoxalase) are often spoken of as distinct systems, but there is no certainty that this is the case, for it must be remembered that a dismutation in which one CO or $C(OH)_2$ group acts as a hydrogen donator and another as acceptor is merely a special case of oxido-reduction, although it is possible that a specific catalyst may exist for any particular dismutation or oxido-reduction.

Yeast and its preparations are now known to bring about a very large number of reductions. From the older literature we learn of its reducing action on sulphur, Fehling's solution, iodine, etc. To this list was added methylene blue by Hahn as described above. Many observations establish the fact that yeast has the power of reducing aldehydes with the production of the corresponding alcohols. This was first proved for furfuraldehyde by Lintner and von Liebig [1911] and for acetaldehyde by Kostytschev [1912, 3; Kostytschev and Hübbenet, 1913] who found that pressed yeast, dried yeast, and zymin all reduced acetaldehyde to alcohol, 50 grams of yeast in ten hours producing from 660 mg. of aldehyde, 265 mg. of alcohol in excess of the amount produced by autofermentation in absence of added aldehyde. Maceration extract was found to reduce both in absence and in presence of sugar, whereas Lebedev and Griaznov [1912] obtained no reduction in presence of sugar, and observed that the power of reduction was lost by the extract on digestion. Neuberg and Kerb [1912, 4; 1913, 1] have also been able to show by large scale experiments that alcohol is produced in considerable quantity by the fermentation of pyruvic acid by living yeast in absence of sugar and that the yield is increased by the presence of glycerol. When treated with 22 kilos of yeast, 1 kilo of pyruvic acid yielded 241 grams of alcohol in excess of that given by the yeast alone, whilst in presence of glycerol the amount was 360 grams, the amount theoretically obtainable being 523 grams. The

function of the glycerol is not understood but is probably that of lessening the rate of destruction of the yeast enzymes.

Further experiments, chiefly by Neuberg and his colleagues, have greatly extended our knowledge of this property of the yeast cell. In a long series of papers [Neuberg and Steenbock, 1913, 1914; Neuberg and Welde, 1914, 1, 2, 3, 4, 5, 6; Neuberg and Nord, 1914, 1, 2, 3; Mayer, 1914; Neuberg and Schwenk, 1915, 1, 2; Mayer and Neuberg, 1915; Neuberg and Levite, 1918; Neuberg and Ringer, 1918, 1, 2; Neuberg and Kerb, E., 1918, 1, 2; Nord, 1920, 1; Färber and Nord, 1920; see also Dakin, 1914] it has been shown in the first place that a very large number of aldehydes, both aromatic and aliphatic, are reduced to the corresponding alcohols. The reducing power of the yeast is not, however, limited to this one reaction, and many substances of different constitution also yield reduction products when submitted to its action. Thus the ketones and diketones are reduced to the corresponding secondary alcohols, although much less readily than the aldehydes; both aromatic and aliphatic nitro-compounds yield the corresponding amino-derivatives, and nitrobenzene and β-phenylhydroxylamine also undergo reduction, whereas azobenzene and azoxybenzene are not affected. Unsaturated aldehydes yield in the main unsaturated alcohols, although there is some evidence that the unsaturated ethylenic linkage may be slowly reduced.

The reductions in all these cases are carried out by adding the substance to be reduced to a fermenting mixture of 10 per cent. sucrose solution and 10 per cent. of living yeast, and it is sometimes necessary to add a further amount of yeast and sugar to complete the reduction. Thus the reduction of aldol [Neuberg and Kerb, E., 1918, 1] was effected by gradually adding 50 g. of aldol to a mixture of 250 g. bottom yeast with 2·5 litres of water and 250 g. sucrose, two additional amounts of 100 g. yeast and one of 200 g. together with some water being added before the sugar had all disappeared. Two similar fermentations were carried out simultaneously and after seven days 65 g. of 1·3-butylene glycol were obtained from the 100 g. of aldol used, i.e. 63·5 per cent. of the theoretically possible amount. This glycol was optically active, $a = +10{\cdot}9^\circ$ ($l = 1$).

This particular experiment well illustrates two striking features of these reductions:—

1. The yield is frequently more than 50 per cent. of the theoretical amount, being in some cases as high as 80 per cent. This affords definite proof that the alcohol is not produced from the aldehyde by Cannizzaro's reaction:

$$2R \cdot CHO + H_2O = R \cdot CH_2OH + R \cdot COOH.$$

Further proof is afforded by the fact that the corresponding acid is not formed.

2. The product of reaction, when its constitution admits of it, is often optically active, showing that the reduction is carried out by an asymmetric agent and is therefore, probably, a true enzyme reaction.

Nearly all these reductions have been effected in the foregoing way, but the addition of sugar is not always essential. Thus a yield of over 50 per cent. of butylene glycol was obtained from aldol by the action of a total of 1550 g. of yeast during two months on 100 g. aldol without any addition of sugar. It must be remembered, however, that this quantity of yeast might contain a large proportion of glycogen (10-30 per cent.) which would gradually undergo autofermentation.

Mechanism of the Reductions Effected by Yeast.

It may be regarded as established that the source of the hydrogen used up in the various reductions described above is either water (Bach), in which case some acceptor for the oxygen must also be present, or an oxidisable substance, which is directly capable of losing hydrogen (Wieland) and thus acts as a hydrogen donator, and great interest attaches to the nature of this compound.

Some light is thrown on this problem by the experiments of Harden and Norris [1914, 1915, see also Harden and Macfarlane, 1931] who showed that dried yeast and zymin lost the power of reducing methylene blue when they were thoroughly washed, but that this power was restored by the addition of the washings, of ordinary bouillon (peptone-beef-broth) and of lactic acid. It is to be presumed that the active substances which restored the power of reduction were capable of acting as oxygen acceptors (or hydrogen donators) and thus enabled the oxido-reduction to proceed. The reaction is evidently highly specific, as many easily oxidisable substances were inactive, including formaldehyde, which acts as the oxygen acceptor in Schardinger's reaction in milk.

These experiments, however, do not indicate what is the actual substance which undergoes oxidation in the various phytochemical reductions enumerated above, and in order to understand this it is necessary to consider the results obtained by Neuberg and his colleagues, which are discussed later on (p. 130).

It has been shown by them that in all probability the production of alcohol and carbon dioxide from yeast proceeds by way of pyruvic acid, which is then decomposed by the carboxylase of the yeast (p. 93)

into carbon dioxide and acetaldehyde, the latter being then reduced to alcohol. The formation of pyruvic acid from glucose involves a dehydrogenation, and an amount of hydrogen equal to that removed in this change is required for the reduction of the aldehyde. These changes probably take place by a series of dismutations (p. 101), but they may be expressed empirically as follows, the final products being italicised :—

(1) $C_6H_{12}O_6 = 2C_3H_6O_3$.
(2) $2C_3H_6O_3 = 2C_3H_4O_3 + 4H$.
(3) $2C_3H_4O_3 = 2\mathit{CO}_2 + 2C_2H_4O$.
(4) $2C_2H_4O + 4H = 2\mathit{C_2H_6O}$.

It has further been shown by Neuberg (p. 130) that it is possible, in several different ways, partially to protect the acetaldehyde from reduction and that the hydrogen thus rendered available is capable of effecting another reducing action, inasmuch as equivalent quantities of glycerol ($C_3H_8O_3$) are then produced, presumably by the reduction of an intermediate substance of the formula $C_3H_6O_3$ (see equation 1). The production of pyruvic acid, according to equation 2, is thus rendered possible and the fermentation continues. This suggests the possibility that the various substances capable of reduction by yeast might be supplied with the necessary hydrogen by deviation of some of the available hydrogen from the acetaldehyde, an equivalent amount of which would thus be protected from reduction and would be found among the products of fermentation.

So far this possibility seems only to have been tested in one instance, and with a positive result [Neuberg and Levite, 1918]. Methylheptenone, $(CH_3)_2C : CH \cdot CH_2 \cdot CH_2 \cdot CO \cdot CH_3$, was reduced by yeast in presence of sugar to the corresponding secondary alcohol and at the same time it was found that an equivalent amount of acetaldehyde was formed and could be isolated.

It is therefore probable that in all these phytochemical reductions, a portion of the acetaldehyde is protected from reduction and the hydrogen thus rendered available effects the reduction of the added substance. This does not exclude the possibility that other oxidisable substances present in yeast, such as Hopkins's glutathione, may also take some part in the reaction. Neuberg has, however, found that for the reduction of considerable amounts of material it is essential that a vigorous fermentation of sugar should be simultaneously proceeding. Only in isolated cases [see Neuberg and Kerb, E., 1918, 1] has extensive reduction been obtained without the addition of sugar, and even in these the participation of the glycogen present in the large amount of yeast found necessary is highly probable (p. 104). The function of the

reducing enzyme of yeast in the alcoholic fermentation of sugar will be discussed in connection with the chemical changes involved in fermentation (Chapter VII).

Dismutation by Yeast.

Yeast is also capable of bringing about the Cannizzaro reaction with aldehyde (see p. 101). It has been found by Nord [1920, 2] that this reaction can occur in the presence of alkalis, not only between two molecules of the same aldehyde, but also between molecules of different aldehydes, one of these being reduced to alcohol and the other oxidised to acid, and it seems probable that similar mixed dismutations are catalysed by enzymes.

It was shown by Kostytschev [1914, 1] that living yeast converts acetaldehyde into a mixture of alcohol and acetic acid, and later Neuberg and Hirsch [1919, 2] were able to show that in presence of alkalis the products of fermentation were so modified that instead of alcohol a molecular mixture of alcohol and acetic acid was formed, presumably by a reaction of this kind, from aldehyde (see p. 134). Dried yeast also removes acetaldehyde from solution [Myrbäck and Jacobi, 1926], but according to Nilsson [1930] this is not a simple dismutation of the aldehyde, no acetic acid being simultaneously produced, but a complex reaction, possibly of the nature of mixed dismutation or oxido-reduction in which 90 per cent. of the aldehyde is reduced to alcohol.

A second dismutation which is brought about by yeast is the conversion of methylglyoxal into lactic acid, a reaction which may be regarded as an intramolecular Cannizzaro reaction :—

$$\begin{array}{l} CH_3 \\ | \\ CO + H_2O \\ | \\ CHO \end{array} \quad = \quad \begin{array}{l} CH_3 \\ | \\ CH(OH) \\ | \\ COOH \end{array}$$

This reaction is catalysed by an enzyme known glyoxalase which occurs in many animal tissues as well as in yeast and other micro-organisms [Dakin and Dudley, 1913 ; see also Neuberg, 1913, 1, 2,]. According to Neuberg and Kobel [1927, 1] methylglyoxal is readily converted into lactic acid both by living yeast, dried yeast, and maceration extract.

The Question of the Existence of a Co-enzyme for Reduction and Dismutation.

It has been suggested that in the oxido-reductions and dismutations produced by yeast some factor of the nature of a co-enzyme is concerned

together with the hydrogen donator, hydrogen acceptor, and dehydrogenase. The question is a difficult one to decide, for it is necessary to be certain that the " co-enzyme " is not accompanied by and does not give rise to substances capable of acting as hydrogen donators or acceptors, and it cannot be said that the evidence so far adduced is entirely satisfactory, either in the case of dismutase or oxido-reductase.

The necessity for a co-enzyme in the process of dismutation of aldehydes, which is maintained by Euler and his colleagues, is deduced from the observation that whereas acetaldehyde rapidly disappears from solution in presence of dried yeast (as well as of living yeast) [Myrbäck and Jacobi, 1926 ; Neuberg and Hirsch, 1919, 2 ; Josephson and Euler, 1924], no disappearance is observed in the presence of washed dried yeast, unless yeast washings or boiled yeast extract are added [Myrbäck and Jacobi, 1926 ; Euler and Myrbäck, 1927, 1] ; and the rate of disappearance under these circumstances runs parallel (up to a certain limit) to the concentration of co-zymase contained in the extract, even when the latter has been submitted to a considerable degree of purification. Euler in fact regards the " co-mutase " as identical with the co-zymase of alcoholic fermentation and considers that the acceleration of a reaction of the nature of dismutation is the main function of the latter (p. 137). In this connection it is important to note that, as mentioned on p. 106, Nilsson [1930] has shown that the change produced by the action of dried yeast on aldehyde is not a simple dismutation to alcohol and acetic acid, but a complex reaction, in which at least 90 per cent. of the aldehyde is reduced to alcohol, and the occurrence of which depends on the presence of glycogen in the dried yeast. This action then appears to be an oxido-reduction, the hydrogen donator of which is not yet known. Proof is still required that the activating substance added as co-zymase was not simply acting as a hydrogen donator.

The case for the existence of a co-reductase, which was first suggested by Lebedev and Griaznov [1912] but has now been abandoned by Lebedev [1927, 2, 3] while it is maintained by Euler [Euler and Nilsson, 1925, 2], rests on similar evidence. Thus Euler and Nilsson [1926, 2 ; 1927, 2, 3] found that a co-zymase preparation which had been submitted to a considerable degree of purification greatly diminished the time required for the reduction of methylene blue by washed dried yeast in presence of hexosediphosphate, but not in its absence. This result may possibly be influenced by the fact that in presence of co-zymase the hexosediphosphate undergoes fermentation [see Nilsson and Jansson, 1927], and it is possible that some of the substances

produced in this change may be more readily oxidisable than hexose-diphosphate. Against these observations must be set those of Harden and Norris [1914; 1915; see also Harden and Macfarlane, 1931], who found that the power of reducing methylene blue could be restored to washed zymin by definite chemical substances, such as lactic acid (see p. 104), and the more recent work of Lebedev [1927, 3], who has shown that dialysed preparations of the oxido-reductase of maceration extract are devoid of reducing power to methylene blue, but become active when crotonaldehyde is added, without the need for a co-enzyme.

Similarly, unsatisfactory evidence of the existence of a co-enzyme of glyoxalase, the enzyme which converts methylglyoxal into lactic acid and occurs in yeast (p. 106), has been recorded by Gottschalk [1928, 2], who found that washed dried yeast did not affect added methylglyoxal. Neuberg and Kobel [1929, 1] working with bacterial glyoxalase, found that an extract of an alcohol-ether preparation of a lactic acid-producing bacillus gave a good yield of methylglyoxal from magnesium hexosediphosphate, but that on addition of yeast extract containing co-enzyme the methylglyoxal underwent further change, presumably dismutation to lactic acid. Neuberg and Kobel attribute this change to the action of a co-glyoxalase, but do not regard this as identical with co-zymase.

A solution potentially of high reducing power and incapable of producing fermentation has been prepared by Lebedev [1926; 1927, 2] by heating maceration extract to 65°, precipitating with 2·5 volumes of saturated ammonium sulphate solution and dialysing the solution of the precipitated enzyme. This, in contradistinction to the washed dried yeast and zymin of Harden and Norris [1914; 1915], is activated towards methylene blue by acetaldehyde as well as by methylglyoxal.

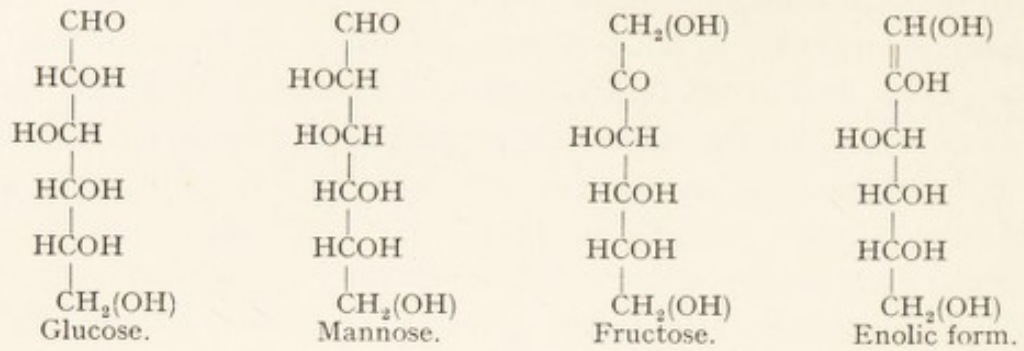
CHAPTER VII.

THE CHEMICAL CHANGES INVOLVED IN FERMENTATION.

It has long been the opinion of chemists that the remarkable and almost quantitative conversion of sugar into alcohol and carbon dioxide during the process of fermentation is most probably the result of a series of reactions, during which various intermediate products are momentarily formed and then used up in the succeeding stage of the process. Many attempts have been made to obtain evidence of such a series of reactions, and numerous suggestions have been made of probable directions in which such changes might proceed. In making these suggestions, investigators have been guided mainly by the changes which are produced in the hexoses by reagents of known composition. The fermentable hexoses, glucose, fructose, mannose, and galactose, appear to be relatively stable in the presence of dilute acids at the ordinary temperature, and are only slowly decomposed at 100°, more rapidly by concentrated acids, with formation of ketonic acids, such as levulinic acid, and of coloured substances of complex and unknown constitution.

In the presence of alkalis, on the other hand, the sugar molecule is extremely susceptible of change. In the first place, as was discovered by Lobry de Bruyn [1895 ; Bruyn and Ekenstein, 1895 ; 1896 ; 1897, 1, 2, 3, 4], each of the three hexoses, glucose, fructose, and mannose, is converted by dilute alkalis into an almost optically inactive mixture containing all three, and probably ultimately of the same composition whichever hexose is employed as the starting-point.

This interesting phenomenon is most simply explained on the assumption that in the aqueous solution of any one of these hexoses, along with the molecules of the hexose itself, there exists a small proportion of those of an enolic form which is common to all the three hexoses, as illustrated by the following formulæ, the aldehyde formulæ being employed instead of the oxide formulæ for the sake of simplicity :—



This enolic form is capable of giving rise to all three hexoses, and the change by which the enolic form is produced and converted into an equilibrium mixture of the three corresponding hexoses is catalytically accelerated by alkalis, or rather by hydroxyl ions. In neutral solution the change is so slow that it has never been experimentally observed ; in the presence of decinormal sodium hydroxide solution at 70° the conversion is complete in three hours. Precisely similar effects are produced with galactose, which yields an equilibrium mixture containing talose and tagatose, sugars which appear not to be fermentable.

The continued action even of dilute alkaline solutions carries the change much further and brings about a complex decomposition which is much more rapidly effected by more concentrated alkalis and at higher temperatures. This change has been the subject of very numerous investigations [for an account of these see E. v. Lippmann, 1904, pp. 328, 713, 835], but for the present purpose the results obtained by Meisenheimer [1908] may be quoted as typical. Using normal solutions of sodium hydroxide and concentrations of from 2 to 5 grams of hexose per 100 c.c., it was found that at air temperature in 27 to 139 days from 30 to 54 per cent. of the hexose was converted into inactive lactic acid, $C_3H_6O_3$, from 0·5 to 2 per cent. into formic acid, CH_2O_2, and about 40 per cent. into a complex mixture of hydroxy-acids, containing six and four carbon atoms in the molecule. Usually only about 74 to 90 per cent. of the sugar which had disappeared was accounted for, but in one case the products amounted to 97 per cent. of the sugar. About 1 per cent. of the sugar was probably converted into alcohol and carbon dioxide. No glycollic acid, oxalic acid, glycol, or glycerol was produced.

The fact that alcohol is actually formed by the action of alkalis on sugar was established by Buchner and Meisenheimer [1905], who obtained small quantities of alcohol (1·8 to 2·8 grams from 3 kilos of sucrose) by acting on sucrose with boiling concentrated caustic soda solution. It is evident that under these conditions an extremely

complex series of reactions occurs, but the formation of alcohol and carbon dioxide and of a large proportion of lactic acid deserves more particular attention.

The direct formation of alcohol from sugar by the action of alkalis appears first to have been observed by Duclaux [1886], who exposed a solution of glucose and caustic potash to sunlight and obtained both alcohol and carbon dioxide. As much as 2·6 per cent. of the sugar was converted into alcohol in a similar experiment made by Buchner and Meisenheimer [1904]. When the weaker alkalis, lime water or baryta water, were employed instead of caustic potash no alcohol was formed, but 50 per cent. of the sugar was converted into inactive lactic acid [Duclaux, 1893, 1896]. Duclaux therefore regarded the alcohol and carbon dioxide as secondary products of the action of a comparatively strong alkali on preformed lactic acid. Ethyl alcohol can, in fact, be produced from lactic acid both by the action of bacteria [Fitz, 1880] and of moulds [Mazé, 1902], and also by chemical means. Thus Duclaux [1886] found that calcium lactate solution exposed to sunlight underwent decomposition, yielding alcohol and calcium carbonate and acetate, whilst Hanriot [1885, 1886], by heating calcium lactate with slaked lime, obtained a considerable quantity of a liquid which he regarded as ethyl alcohol, but which was shown by Buchner and Meisenheimer [1905] to be a mixture of ethyl alcohol with isopropyl alcohol.

It appears, therefore, that inactive lactic acid can be quite readily obtained in large proportion from the sugars by the action of alkalis, whilst alcohol can only be prepared in comparatively small amount and probably only as a secondary product of the decomposition of lactic acid.

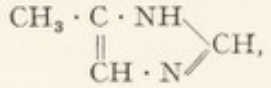
The study of the action of alkalis on sugar has, however, yielded still further information as regards the mechanism of the reaction by which lactic acid is formed. A considerable body of evidence has accumulated, tending to show that some intermediate product of the nature of an aldehyde or ketone containing three carbon atoms is first formed.

Thus Pinkus [1898] and subsequently Nef [1904, 1907], by acting on glucose with alkali in presence of phenylhydrazine obtained the osazone of methylglyoxal, $CH_3 \cdot CO \cdot CHO$. This osazone may be formed either from methylglyoxal itself, from acetol, $CH_3 \cdot CO \cdot CH_2 \cdot OH$, or from lactic aldehyde, $CH_3 \cdot CH(OH) \cdot CHO$ [Wohl, 1908]. Methylglyoxal itself may also be regarded as a secondary product derived from glyceraldehyde, $CH_2(OH) \cdot CH(OH) \cdot CHO$, or dihydroxyacetone,

$CH_2(OH) \cdot CO \cdot CH_2(OH)$, by a process of intramolecular dehydration, so that the osazone might also be derived indirectly from either of these compounds [see also Neuberg and Oertel, 1913]. Methylglyoxal moreover readily pasess into lactic acid when it is treated with alkalis, a molecule of water being taken up :—

$$CH_3 \cdot CO \cdot CHO + H_2O = CH_3 \cdot CH(OH) \cdot COOH.$$

Further evidence in the same direction is afforded by the interesting discovery of Windaus and Knoop [1905], that glucose is converted by ammonia in presence of zinc hydroxide into methyliminazole,



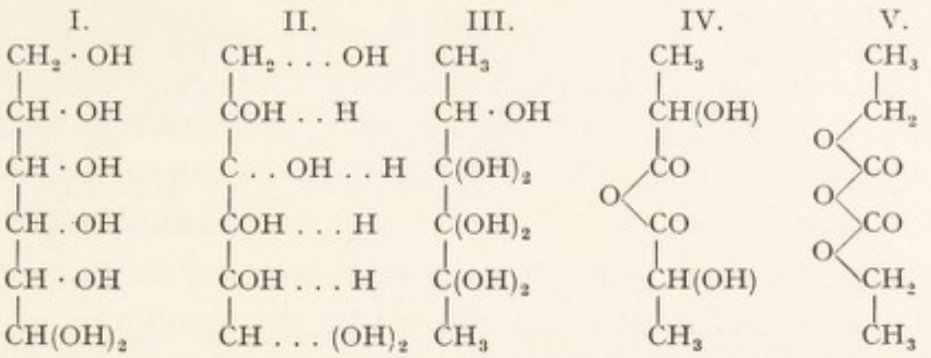
a substance which is a derivative of methylglyoxal.

The idea suggested by Pinkus that acetol is the first product of the action of alkalis on sugar has been rendered very improbable by the experiments of Nef, and the prevailing view (Nef, Windaus and Knoop, Buchner and Meisenheimer) is that the first product is glyceraldehyde, which then passes into methylglyoxal, and finally into lactic acid :—

(1) $C_6H_{12}O_6 = 2CH_2(OH) \cdot CH(OH) \cdot CHO.$
(2) $CH_2(OH) \cdot CH(OH) \cdot CHO = CH_3 \cdot CO \cdot CHO + H_2O.$
(3) $CH_3 \cdot CO \cdot CHO + H_2O = CH_3 \cdot CH(OH) \cdot COOH.$

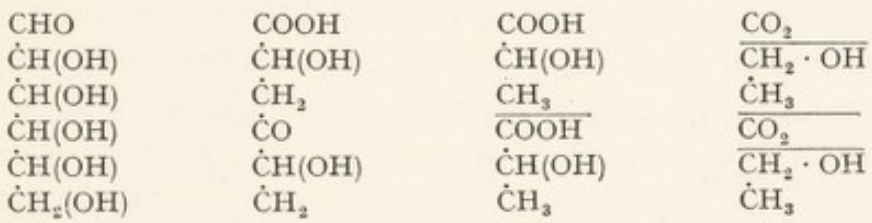
All these changes may occur at ordinary temperatures in the presence of a catalyst, and in so far resemble the processes of fermentation by yeast and bacteria.

The first attempt to suggest a scheme of chemical reactions by which the changes brought about by living organisms might be effected was made in 1870 by Baeyer [1870], who pointed out that these decompositions might be produced by the successive removal and readdition of the elements of water. The result of this would be to cause an accumulation of oxygen atoms towards the centre of the chain of six carbon atoms, which, in accordance with general experience, would render the chain more easily broken. Baeyer formulated the changes characteristic of the alcoholic and lactic fermentations as follows, the intermediate stages being derived from the hydrated aldehyde formula of glucose by the successive removal and addition of the elements of water :—



The immediate precursor of alcohol and carbon dioxide is here seen to be the anhydride of ethoxycarboxylic acid (V), whilst that of lactic acid is lactic anhydride (IV). (Baeyer does not appear, as stated by Meisenheimer [1907, p. 8], Wohl [1907, 2], and Buchner and Meisenheimer [1909], to have suggested that lactic acid was an intermediate product in alcoholic fermentation, but rather to have represented independently the course of the two different kinds of fermentation, the alcoholic and the lactic.)

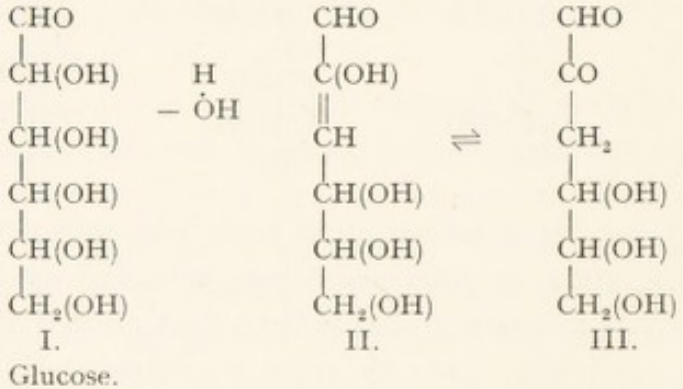
It was subsequently pointed out by Buchner and Meisenheimer [1904] that Baeyer's principle of oxygen accumulation might be applied in a different way, so that a ketonic acid would be produced, the decomposition of which, in a manner analogous to that of acetoacetic acid, would lead to the formation of two molecules of lactic acid, from which the final products alcohol and carbon dioxide might be directly derived, as shown in the following formulæ :—



A scheme based on somewhat different principles was propounded by Wohl [Lippmann, 1904, p. 1891], and was accepted by Buchner and Meisenheimer [1905] as more probable than that quoted above. Wohl and Oesterlin [1901] were able to trace experimentally the various stages of the conversion of tartaric acid (I) into oxaloacetic acid (III), which can be carried out by reactions taking place at the ordinary temperature, and they found that the first stage consisted in the removal of the elements of water leaving an unsaturated hydroxy-derivative (II) which in the second stage underwent intramolecular change into the corresponding keto-compound (III) :—

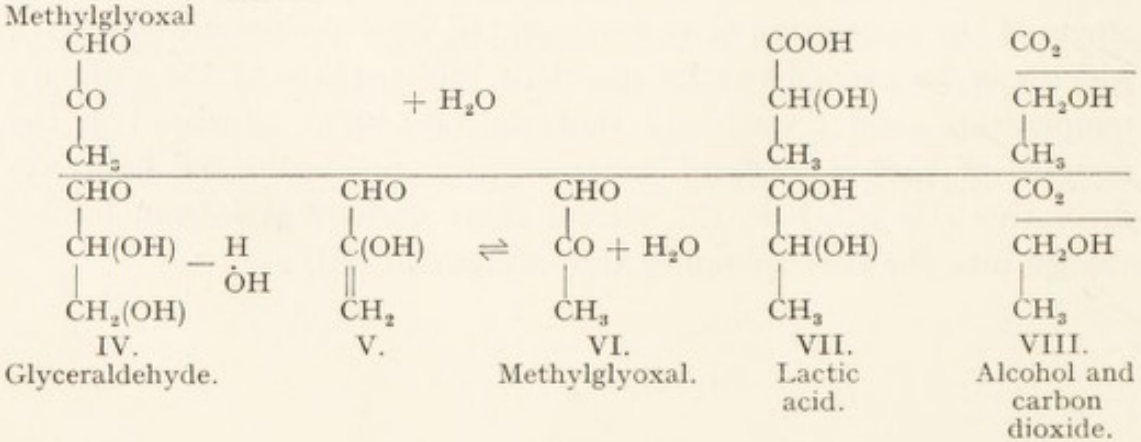
I. Tartaric acid. II. III. Oxaloacetic acid.

This change differs in principle from that assumed by Baeyer, inasmuch as the second stage is not effected by the re-addition of water, but by the keto-enol transformation, which is now usually ascribed to the migration of the hydrogen atom, although the same result can theoretically be arrived at by the addition and removal of the elements of water. The analogy of this process to what might be supposed to occur in the conversion of sugar into carbon dioxide and alcohol was pointed out by Wohl and Oesterlin, and subsequently Wohl developed a theoretical scheme of reactions by which the process of alcoholic fermentation could be represented. In the first place the elements of water are removed from the α and β carbon atoms of glucose (I) and the resulting enol (II) undergoes conversion into the corresponding ketone (III), which has the constitution of a condensation product of methylglyoxal and glyceraldehyde, and hence is readily resolved by hydrolysis into these compounds (IV). The glyceraldehyde passes by a similar series of changes (V, VI) into methylglyoxal, and this is then converted by addition of water into lactic acid (VII), a reaction which is common to all ketoaldehydes of this kind. Finally, the lactic acid is split up into alcohol and carbon dioxide (VIII) :—



I. Glucose. II. III.

Methylglyoxal



IV. Glyceraldehyde. V. VI. Methylglyoxal. VII. Lactic acid. VIII. Alcohol and carbon dioxide.

This scheme agrees well with the current ideas as to the formation of lactic acid from glucose under the influence of alkalis (p. 112). It postulates the formation as intermediate products of no less than three compounds containing a chain of three carbon atoms—glyceraldehyde, methylglyoxal, and lactic acid.

The Lactic Acid Theory of Alcoholic Fermentation.

A practical interest was given to these various schemes by the fact that Buchner and Meisenheimer adduced experimental evidence in favour of the view that lactic acid is an intermediate product in the formation of alcohol and carbon dioxide from sugar by fermentation [1904, 1905, 1906, 1909].

These observers proved by a series of very careful analyses that yeast-juice frequently, but not invariably, contains small quantities of lactic acid, not exceeding 0·2 per cent. When yeast-juice is incubated alone or with sugar the amount of lactic acid may either increase or decrease. Moreover, lactic acid added to the juice is sometimes diminished and sometimes increased in quantity. On the whole, it appears that the addition of a considerable quantity of sugar or of some lactic acid favours the disappearance of lactic acid. Juices of low fermenting power produce a diminution in the lactic acid present, those of high fermenting power an increase.

In all cases the amounts of lactic acid either produced or destroyed are very small in relation to the volume of the yeast-juice employed.

Throughout the whole series of experiments the greatest increase amounted to 0·47 per cent. on the juice employed, and the greatest decrease to 0·3 per cent. [See also Oppenheimer, 1914, 1.] Buchner and Meisenheimer at one time regarded these facts as strong evidence that lactic acid is an intermediate product of alcoholic fermentation. It was thought probable that the production of alcohol and carbon dioxide from sugar occurred in at least two stages and under the influence of two distinct enzymes. The first stage consisted in the conversion of sugar into lactic acid, and for the enzyme which brought about this decomposition was reserved the name zymase or yeast-zymase. The lactic acid was then broken down into alcohol and carbon dioxide by the second enzyme, lactacidase.

This theory, which is quite in harmony with the current ideas as to the mode of decomposition of sugars by alkalis and is also consistent with Wohl's scheme of reactions, is open to adverse criticism from several points of view. In the first place, it is noticeable that the total

amount of lactic acid used up by the juice is extremely small, even in the most favourable cases, relatively to the amount of the juice [Harden, 1905], and it may be added to the sugar-fermenting power of the juice. Moreover, as pointed out by Buchner and Meisenheimer themselves [1909], no proof is afforded that the lactic acid which disappears is converted into alcohol and carbon dioxide. It is not even certain, although doubtless probable, that the lactic acid which occurs or is produced in the juice is really derived from sugar.

Slator [1906 ; 1907 ; 1908, 1, 2] has based a criticism of this theory on the consideration that if lactic acid be an intermediate product of alcoholic fermentation the reaction by which it is fermented must proceed at least as rapidly as that by which it is formed, in order to prevent accumulation of lactic acid. The fermentation of lactic acid by yeast should therefore proceed at least as rapidly as that of glucose. So far is that from being the case that it has been experimentally demonstrated that lactic acid is not fermented at all by living yeast. This was rendered extremely probable by Slator, who showed that lactic acid, even in concentrations insufficient to prevent the fermentation of glucose, is not fermented to any considerable extent. The final proof that lactic acid is neither formed nor fermented by pure yeast was brought by Buchner and Meisenheimer in a series of very careful quantitative experiments carried out with a pure yeast and with strict precautions against bacterial contamination [1909, 1910].

As already mentioned the fermentation of added lactic acid by yeast preparations is irregular and very restricted in amount.

At first sight these facts appear decisive against the validity of the lactic acid theory, and they were recognised as such by Buchner and Meisenheimer. Wohl, however, suggested that the non-fermentability of lactic acid by yeast was not really conclusive [1907, 1 ; see also Franzen and Steppuhn, 1912, 1]. The production of lactic acid from glucose is attended by the evolution of a considerable amount of heat (22 cal.), and it is possible that at the moment of production the molecule of the acid is in a condition of activation corresponding with a much higher temperature than the average temperature of the fermenting liquid. Under these circumstances the molecule would be much more susceptible of chemical change than if brought into the solution at a lower effective temperature.

This " activation " theory of chemical reaction is now generally accepted, so that the possibility of the intervention of lactic acid in alcoholic fermentation still remains. It is noteworthy in this connection that lactic acid restores the power of reducing methylene blue

to washed zymin and under these conditions is fermented to acetaldehyde and carbon dioxide [Harden and Norris, 1914; 1915; Harden and Macfarlane, 1931].

$$C_3H_6O_3 + M.B = M.B \cdot H_2 + CO_2 + C_2H_4O.$$

This reaction does not occur with acetaldehyde as hydrogen acceptor under normal conditions, but it is not excluded that the activated molecules might react in this way. It is to be remembered that methylglyoxal, like lactic acid, is not fermented readily by yeast or yeast preparations, although it is generally accepted as an intermediate product in alcoholic fermentation (p. 123).

It has also been suggested, as in the case of methylglyoxal (see below), that an unknown isomeride of lactic acid is formed as an intermediate product and fermented [Luther, 1907], and that traces of lactic acid are formed by a secondary reaction from this, but no satisfactory evidence for this view is forthcoming.

If, as is now generally assumed, methylglyoxal is an intermediate product of alcoholic fermentation it is somewhat remarkable that lactic acid is not formed in larger amount during fermentation by the powerful glyoxalase which is present in yeast (p. 106).

Fermentation of Methylglyoxal, Dihydroxyacetone, and Glyceraldehyde.

As regards the fermentability by yeast of compounds containing three carbon atoms, which may possibly appear as intermediate products in the transformation of sugar into carbon dioxide and alcohol, many experiments have been carried out, with somewhat uncertain results. Care has to be taken that the substance to be tested is not added in such quantity as to inhibit the fermenting power of the yeast or yeast-juice, and that the conditions are such that the substance in question, often of a very unstable nature, is not converted by some chemical change into a different, fermentable compound. It must further be remembered that some of these substances may exist in several tautomeric forms and that the stable form in which they can be isolated may not be that which is fermentable. Thus Neuberg [1913, 2] points out that there are many possible formulæ for such a substance as methylglyoxal, which moreover has a strong tendency to polymerisation. It is also possible that the substance to be tested may accelerate the rate of autofermentation in a similar manner to arsenates (p. 156) and many other substances. These are all points which have not up to the present received sufficient attention. In the case of living yeast the further question arises of the permeability of the cell.

Methylglyoxal, $CH_3 \cdot CO \cdot CHO$, has been tested by Mayer [1907] and Wohl [1907, 2] with yeast, and by Buchner and Meisenheimer both with acetone-yeast [1906] and yeast-juice [1910], in every case with negative results, but it may be noted that the concentration employed in the last-mentioned of these experiments was such as considerably to diminish the autofermentation of the juice.

Glyceraldehyde, $CH_2(OH) \cdot CH(OH) \cdot CHO$, was also tested with yeast with negative results by Wohl [1898] and by Emmerling [1899], who employed a number of different yeasts. The same negative result attended the experiments of Piloty [1897] and Emmerling [1899] with pure dihydroxyacetone. Fischer and Tafel [1888, 1889], however, had previously found that glycerose, a mixture of glyceraldehyde and dihydroxyacetone prepared by the oxidation of glycerol, was readily fermented by yeast, agreeing in this respect with the still older observations of Van Deen and of Grimaux. The reason for this diversity of result has not been definitely ascertained, but it has been supposed by Emmerling to lie in the formation of some fermentable sugar from glycerose when the latter is subjected to too high a temperature during its preparation.

On the other hand, Bertrand [1904] succeeded in fermenting pure dihydroxyacetone by treating a solution of 1 gram in 30 c.c. of liquid with a small quantity of yeast for ten days at 30°, the best result being a fermentation of 25 per cent. of the substance taken. Moreover, Boysen-Jensen [1908, 1910, 1914] stated that he had also observed both the formation of this substance from glucose by living yeast and its fermentation, but the amounts of alcohol and carbon dioxide produced were so minute and the evidence for the production of dihydroxyacetone so inconclusive that the experiments cannot be regarded as in any way decisive [see Chick, 1912 ; Euler and Fodor, 1911 ; Karauschanov, 1911 ; Buchner and Meisenheimer, 1912].

A careful investigation by Buchner [1910] and Buchner and Meisenheimer [1910] has led them to the conclusion that both glyceraldehyde and dihydroxyacetone are fermentable. Glyceraldehyde exerts a powerful inhibiting action both on yeast and yeast-juice and was only found to give rise to a very limited amount of carbon dioxide, quantities of 0·15 to 0·025 gram being treated with 1 gram of yeast or 5 c.c. of yeast-juice and a production of 4 to 12 c.c. of carbon dioxide being attained.

When 0·1 gram of dihydroxyacetone in 5 c.c. of water was brought in contact with 1 gram of living yeast, about half was fermented, 17 c.c. of carbon dioxide (at 20° and 600 mm.) being evolved in excess of the

autofermentation of the yeast (13 c.c.). A much greater effect was obtained by the aid of yeast-juice, and the remarkable observation was made that whilst yeast-juice alone produced comparatively little action a mixture of yeast-juice and boiled yeast-juice was much more effective, quantities of 20 to 50 c.c. of yeast-juice mixed with an equal volume of boiled juice, which in some experiments was concentrated, yielding with 0·4, 1, and 2 grams of dihydroxyacetone almost the theoretical amount of carbon dioxide and alcohol in excess of that evolved in the absence of this substance. It was further observed that the fermentation of this substance commenced much more slowly than that of glucose. No explanation of either of these facts has at present been offered. The conclusion drawn from their experiments by Buchner and Meisenheimer, that dihydroxyacetone is readily fermentable, was confirmed by Lebedev [1911, 1], who further made the important observation that during the fermentation of dihydroxyacetone the same hexosephosphoric acid is produced as is formed during the fermentation of the hexoses. Lebedev accordingly propounded a scheme of alcoholic fermentation according to which the hexose was first converted into two molecules of triose, the latter being first esterified to triosephosphoric acid and then condensed to hexosediphosphoric acid, which then underwent fermentation, being hydrolysed to phosphoric acid, and some unidentified substance, probably an unstable modification of a hexose, much more readily attacked by an appropriate enzyme than the original glucose or fructose [1911, 1, pp. 2941-2].

The idea that the sugar is first converted into triose and this into triosemonophosphoric acid had been previously suggested by Ivanov, who postulated the agency of a special enzyme termed *synthease* [1909, 1] and supposed that this triosemonophosphoric acid was then directly fermented to alcohol, carbon dioxide, and phosphoric acid.

It has been shown that the arguments adduced by Ivanov in favour of the existence of his synthease are not valid [Harden and Young, 1910, 1].

The fermentation of dihydroxyacetone was moreover proved by Harden and Young [1912] to be effected by yeast-juice and maceration extract at a much slower rate than that of the sugars, in spite of the fact that the addition of dihydroxyacetone did not inhibit the sugar fermentation. The same thing has been shown for living yeast by Slator [1912] in agreement with the earlier results of Buchner [1910] and Buchner and Meisenheimer [1910].

The logical conclusion from Lebedev's experiments would appear rather to be that dihydroxyacetone is slowly condensed to a hexose

which is then fermented in the normal manner [Harden and Young, 1911, 2; 1912; Buchner and Meisenheimer, 1912; Kostytschev, 1912, 2]. Buchner and Meisenheimer, however, regarded this as improbable on the ground that dihydroxyacetone, being symmetric in constitution, would yield an inactive hexose of which only at most 50 per cent. would be fermentable. Against this it may be urged that enzymic condensation of dihydroxyacetone might very probably occur asymmetrically yielding an active and completely fermentable hexose. Buchner and Meisenheimer never abandoned the view that dihydroxyacetone formed an intermediate stage in the fermentation of glucose, and adduced as confirmatory evidence of the probability of such a change the observation of Fernbach [1910] that this compound is produced from glucose by a bacillus, Tyrothrix tenuis, which effects the change both when living and after treatment with acetone.

Yeasts have since been found which act more vigorously on dihydroxyacetone than those used by Buchner. Such are, e.g. a bottom yeast from the Schultheiss-Patzenhofer brewery [Fischer and Taube, 1927; Neuberg and Gottschalk, 1924, 1], and especially *Saccharomycodes Ludwigii* [Haehn and Glaubitz, 1927, 1]. With the latter organism the rate of fermentation of dihydroxyacetone is less than that of glucose [Haehn and Leibowitz, 1927, 1; Neuberg and Kobel, 1928, 2; Iwasaki, 1928; see also Meyerhof, 1928], but after cultivation of the organism in presence of dihydroxyacetone may ultimately attain the same velocity. Esterification of phosphoric acid occurs and the product consists of a mixture of hexosediphosphate and hexosemonophosphate (Neuberg and Robison esters). No triosephosphoric acid has been detected. These experiments confirm the conclusion that dihydroxyacetone is fermented by way of hexose. The heat of combustion [Kobel and Roth, 1928] and the heat of fermentation [Iwasaki, 1928] of dihydroxyacetone (2 mols.) are both about 12-14 kg.-cal. greater than the corresponding values for glucose (1 mol.), and this provides a further argument against the possibility of dihydroxyacetone being an intermediate product in the fermentation of glucose.

Lebedev subsequently [1912, 3; Lebedev and Griaznov, 1912] extended his experiments to glyceraldehyde and modified his theory very considerably. Using maceration extract it was found in general agreement with the results of Buchner and Meisenheimer (p. 118) that 20 c.c. of juice were capable of producing about half the theoretical amount of carbon dioxide from 0·2 gram of glyceraldehyde, whereas 0·4 gram caused coagulation of the extract and a diminished evolution fo carbon dioxide. The addition of phosphate diminished rather than

increased the fermentation. Even in the most favourable concentration, however (0·2 gram per 20 c.c.), the glyceraldehyde is fermented much more slowly than dihydroxyacetone or sucrose, as is shown by the following figures :—

20 c.c. Extract + 0·2 gram.	CO_2 in grams in successive periods of			Duration of fermentation.	Total CO_2.
	6 hours.	18 hours.	24 hours.	Hours.	Gram.
Sucrose	0·050	0·000	0·000	6	0·05
Dihydroxyacetone .	0·042	0·000	0·000	6	0·042
Glyceraldehyde . .	0·008	0·022	0·005	48	0·035

Further, during an experiment in which 0·129 gram of CO_2 was evolved in 22·5 hours from 0·9 gram of glyceraldehyde in presence of phosphate, no change in free phosphate was observed, whereas in a similar experiment with glucose a loss of about 0·2 gram of P_2O_5 would have occurred. Hence the fermentation takes place without formation of hexose-diphosphate. This was confirmed by the fact that the osazone of hexosephosphoric acid was readily isolated from the products of fermentation of dihydroxyacetone (0·259 gram of CO_2 having been evolved in twenty hours), but could not be obtained from those of glyceraldehyde (0·138 gram CO_2 in twenty hours).

This result is extremely interesting, although it is not impossible that the rate of fermentation of the glyceraldehyde is so slow that any phosphoric ester produced is hydrolysed as rapidly as it is formed.

Lebedev regards the experiments as proof that phosphate takes no part in the fermentation of glyceraldehyde and bases on this conclusion and his other work the following theory of alcoholic fermentation :—

1. The sugar is split up into equimolecular proportions of glyceraldehyde and dihydroxyacetone :—

$$(a)\ C_6H_{12}O_6 = C_3H_6O_3 + C_3H_6O_3.$$

2. The dihydroxyacetone then passes through the stages previously postulated (p. 119) :—

$$(b)\ 4C_3H_6O_3 + 4R_2HPO_4 = 4C_3H_5O_2PO_4R_2 + 4H_2O.$$
$$(c)\ 4C_3H_5O_2PO_4R_2 = 2C_6H_{10}O_4(R_2PO_4)_2.$$
$$(d)\ 2C_6H_{10}O_4(R_2PO_4)_2 + 4H_2O = 2C_6H_{12}O_6 + 4R_2HPO_4.$$

After which the hexose, $C_6H_{12}O_6$, re-enters the cycle at (*a*).

3. The fermentation of the glyceraldehyde occurs according to the scheme developed by Kostytschev (p. 123), pyruvic acid being formed along with hydrogen and then decomposed into carbon dioxide and acetaldehyde, which is reduced by the hydrogen. Lebedev,

however, suggests [1914, 1, 2] that glyceric acid is first formed (1) and then converted by an enzyme, which he terms *dehydratase*, into pyruvic acid (2) :—

(1) $CH_2(OH) \cdot CH(OH) \cdot CHO \rightarrow CH_2(OH) \cdot CH(OH) \cdot CH(OH)_2 \rightarrow$
$+ H_2O.$ $CH_2(OH) \cdot CH(OH) \cdot COOH + 2H.$

(2) $CH_2(OH) \cdot CH(OH) \cdot COOH = CH_3 \cdot CO \cdot COOH + H_2O.$

The experimental basis for this idea is the fact that glyceric acid is fermented by dried yeast and maceration juice [compare Neuberg and Tir, 1911]. Neuberg and Kerb [1914, 1], who have been confirmed by Kostytschev and Jegorova [1929], were unable to observe any fermentation with living yeast.

This scheme has the merit of recognising the fact that the carbon dioxide does not wholly arise from the products of decomposition of hexosephosphate, nor from its direct fermentation. The function assigned to the phosphate is that of removing dihdroxyacetone and thus preventing it from inhibiting further conversion of hexose into triose, according to the reversible reaction,

$$C_6H_{12}O_6 \rightleftharpoons 2C_3H_6O_3.$$

This, however, appears to be quite inadequate, since, on the one hand, the fermentation of glucose proceeds quite freely in presence of as much as 5 grams per 100 c.c. of dihydroxyacetone [Harden and Young, 1912], and on the other hand alcoholic fermentation appears not to proceed at all in the absence of phosphate (see p. 72). This forms the chief objection to the theory. In a later account of the theory [Lebedev, 1924, 1, 2] the function of phosphate is left uncertain. The slow rate at which glyceraldehyde is fermented also affords an argument against the validity of Lebedev's view, but this may possibly be accounted for to some extent by the fact that glyceraldehyde is a strong inhibiting agent so that it might be more rapidly fermented if added in a more dilute condition.

The unfermented glyceraldehyde cannot be recovered from the solution, and nothing is known as to its fate except that it readily gives rise both to lactic acid and glycerol [Oppenheimer, 1914, 1, 2]. Evidently the reaction between glyceraldehyde and yeast-juice is by no means a simple one.

The Pyruvic Acid Theory.

The idea that pyruvic acid was produced as an intermediate stage in the fermentation of sugar immediately suggested itself when it became known that yeast was capable of rapidly decomposing *a*-ketonic

acids with evolution of carbon dioxide [see Neubauer and Fromherz, 1911, p. 350; Neuberg and Kerb, 1912, 4; Kostytschev, 1912, 2].

This scheme has been differently elaborated by different workers. According to the simple form suggested by Kostytschev it involves (1) the production of pyruvic acid from the hexoses, a process accompanied by loss of hydrogen; (2) the decomposition of pyruvic acid into acetaldehyde and carbon dioxide; and (3) the reduction of the acetaldehyde to ethyl alcohol:—

(1) $C_6H_{12}O_6 = 2CH_3 \cdot CO \cdot COOH + 4H$.
(2) $2CH_3 \cdot CO \cdot COOH = 2CH_3 \cdot CHO + 2CO_2$.
(3) $2CH_3 \cdot CHO + 4H = 2CH_3 \cdot CH_2 \cdot OH$.

Neuberg and Kerb [1913, 2] on the other hand propose a more complicated scheme, according to which methylglyoxal is the starting-point for the later stages of the reaction. Moreover, the various oxidations and reductions involved are all assumed to be carried out by Cannizzaro transformations, or as the authors term them *dismutations*, of the aldehydes which are formed as intermediate products.

(*a*) The sugar is split up into two molecules of methylglyoxal, the process probably taking place in two stages:

$$C_6H_{12}O_6 - 2H_2O = \underset{\text{Methylglyoxal aldol.}}{C_6H_8O_4} = \underset{\text{or } 2CH_3 \cdot CO \cdot CHO \text{ Methylglyoxal.}}{2CH_2 : C(OH) \cdot CHO}$$

(*b*) A portion of the methylglyoxal is converted by a Cannizzaro transformation into glycerol and pyruvic acid:

$$\begin{array}{l} CH_2 : C(OH) \cdot CHO + H_2O \\ \qquad + \\ CH_2 : C(OH) \cdot CHO \end{array} \quad \begin{array}{c} H_2 \\ | \\ = \\ | \\ O \end{array} \quad \begin{array}{l} \underset{\text{glycerol}}{CH_2(OH) \cdot CH(OH) \cdot CH_2(OH)} \\ \qquad + \\ \underset{\text{Pyruvic acid.}}{CH_2 : C(OH) \cdot COOH} \end{array}$$

(*c*) The pyruvic acid is then decomposed by carboxylase yielding aldehyde and carbon dioxide:

$$CH_3 \cdot CO \cdot COOH = CH_3 \cdot CHO + CO_2.$$

(*d*) The aldehyde and a molecule of methylglyoxal then undergo a Cannizzaro reaction and yield alcohol and pyruvic acid:

$$\begin{array}{l} CH_3 \cdot CO \cdot CHO \\ \qquad + \\ CH_3 \cdot CHO \end{array} \quad \begin{array}{c} O \\ | \\ = \\ | \\ H_2 \end{array} \quad \begin{array}{l} CH_3 \cdot CO \cdot COOH \\ \qquad + \\ CH_3 \cdot CH_2(OH) \end{array}$$

and the latter then undergoes reaction (*c*).

A small amount of glycerol is thus necessarily formed, as is actually found to be the case.

1. As regards the production of pyruvic acid from the hexoses by yeast, Fernbach and Schoen [1913; 1914; 1920; 1922; Fernbach, 1916]

have shown that calcium pyruvate, accompanied by some calcium lactate [Fernbach and Schoen, 1923], is formed when certain yeasts (a "mycolevure" and a Champagne yeast) are grown in a synthetic medium in presence of calcium carbonate. On the other hand, Kerb [1919], and Kerb and Zeckendorf [1921], who have been confirmed by von Grab [1921], and by Klein and Fuchs [1929], find that the acid is not produced by culture yeasts in this way.

Rapid fermentation of considerable amounts of sugar by pressed yeast in presence of calcium carbonate also yielded no pyruvic acid [Kostytschev and Frey, 1925], whereas acetic, malic, and succinic acids were all formed.

By employing a "fixing" agent (see p. 128), v. Grab [1921] has also succeeded in demonstrating the production of pyruvic acid as an intermediate stage in the alcoholic fermentation of sugar by yeast-juice. The reaction employed was the condensation of pyruvic acid with β-naphthylamine to form α-methyl-β-naphthocinchoninic acid (Döbner) :

$$2CH_3 . CO \cdot COOH + H_2N \cdot C_{10}H_7 = 2H_2O + CO_2 + H_2 + \begin{matrix} CH_3 \cdot C : N \\ | \quad \diagdown C_{10}H_6 \\ HC : C \cdot COOH. \end{matrix}$$

The experiment was carried out by adding the naphthylamine in ethereal solution and shaking the mixture until all the sugar had been fermented, the condensation product being then isolated by extraction of the evaporated mass with alcoholic ammonia. 18 g. of sucrose when fermented by 1620 c.c. yeast-juice in presence of 3·6 g. β-naphthylamine in 75 c.c. ether yielded in all 0·63 g. of pure condensation product, and a larger scale experiment with 180 g. sucrose gave a yield of 7·3 g.

The demonstration of the formation of pyruvic acid by the aid of other fixation agents such as brucine [Traetta-Mosca, 1926, 1927] and semicarbazide [Kostytschev and Soldatenkov, 1928] has been reported, but in each case the claim has been subjected to severe criticism [Rimini, 1926 ; Neuberg and Kobel, 1927, 3 ; Lebedev, 1928 ; Klein and Fuchs, 1929, where the literature is fully cited].

Demonstration of the Intermediate Formation of Methylglyoxal and Pyruvic Acid.

A more satisfactory result has been obtained [Neuberg and Kobel, 1928, 1 ; 1929, 3 ; 1930, 1, 2 ; Bayo, 1929, 1, 2 ; Kobel and Scheuer, 1930] by the action of dried yeast on magnesium hexosediphosphate under particular conditions of volume of solution per unit weight of dried yeast. The effect of altering the volume of liquid employed is

that whereas at one ratio normal fermentation occurs, with more solution the process to a large extent stops at the formation of pyruvic acid, and with a greater volume at the still earlier stage of formation of methylglyoxal.

Neuberg's explanation of the reaction is that in all these preparations the co-enzyme, by dilution or otherwise, has been rendered more or less completely incapable of aiding the normal reaction, i.e. assisting in the production of a phosphoric ester and the reaction by which the methylglyoxal under normal conditions undergoes further change. Hence it is necessary to employ a phosphorylated sugar on which the apozymase exerts its normal desmolytic effect, converting it into methylgloxal and then (owing to the inefficiency of the co-enzyme) the reaction stops and the methylglyoxal accumulates, or under different conditions yields pyruvic acid and glycerol. It is perhaps more probable that the apozymase exerts its action on the sugar liberated from the hexosediphosphate by hydrolysis, which is possibly a reactive form of fructose (p. 58).

Pyruvic acid.—For the production of pyruvic acid 7·5 g. of the sterile dried yeast, 300 c.c. of 3·4 per cent. magnesium hexosediphosphate solution, and 5 c.c. of toluene were used, and after maintenance at 37° for twenty-four hours the pyruvic acid was obtained in the form of the insoluble 2 : 4-dinitrophenylhydrazone.

The pyruvic acid was accompanied, as was expected, by an equivalent amount of glycerol (see p. 131) and amounted to 52 per cent. of the theoretical yield to be expected, according to the equation

$$C_6H_{12}O_6 = C_3H_4O_3 + C_3H_8O_3,$$

from the hexose liberated by hydrolysis from the magnesium hexosediphosphate, 38 per cent. of which remained unattacked. A certain amount of normal fermentation, accompanied by formation of alcohol and CO_2, also occurred. This is termed by Neuberg the fourth form of fermentation.

The production of glycerol and pyruvic acid from glucose by living yeast in presence of magnesium phosphate, sodium phosphate and magnesia has also been demonstrated by Neuberg and Kobel [1930, 3].

Methylglyoxal.—For this purpose [Neuberg and Kobel, 1928, 1; 1929, 2] a similar method to the above was used. Good yields of methylglyoxal, separated in the form of its *bis*-dinitrophenylhydrazone, were obtained both from the extract of yeast plasmolysed by ether or by a number of other substances [Neuberg and Kobel, 1930, 2],

from the residue from this plasmolysis, from dried yeast, both washed and unwashed, from the washings from dried yeast and from dialysed and diluted maceration extract.

The change is somewhat complex as the hexosediphosphate is partly converted into monophosphoric ester and some of the methylglyoxal undergoes dismutation. In one experiment (extract of plasmolysed yeast) using 500 c.c. of liquid, 579·0 mg. of hexose were liberated by hydrolysis and yielded 53·3 mg. of methylglyoxal hydrate, a gross percentage of 9·2. As 339·0 mg. of sugar were still present as such, the yield on the sugar which had actually undergone change was 22·2 per cent. Some of the methylglyoxal produced had presumably undergone dismutation, as did methylglyoxal in a comparative experiment. Allowing for this the yield amounted to 71·6 per cent. of the theory, according to the equation

$$C_6H_{12}O_6 = 2C_3H_6O_3.$$

Neuberg designates this production of methylglyoxal,

$$C_6H_{12}O_6 = 2C_3H_4O_2 . H_2O,$$

the fifth form of fermentation.

A similar production of methylglyoxal can be demonstrated in the case of lactic acid-forming bacteria [Neuberg and Kobel, 1929, 1]; and during glycolysis by animal tissues [Vogt, 1929; see also Toenniessen and Fischer, 1926].

Pyruvic acid is very closely related to several substances which are intimately connected both chemically and biochemically with the hexoses. Thus lactic acid is its reduction product,

$$CH_3 \cdot CO \cdot COOH \underset{+\,2H}{\rightarrow} CH_3 \cdot CH(OH) \cdot COOH,$$

glyceraldehyde can readily be converted into it by oxidation to glyceric acid followed by abstraction of water (Erlenmeyer),

$$CH_2(OH) \cdot CH(OH) \cdot CHO \underset{+\,O}{\rightarrow} CH_2(OH) \cdot CH(OH) \cdot COOH \underset{-\,H_2O}{\rightarrow} CH_3 \cdot CO \cdot COOH,$$

and finally methylglyoxal, $CH_3 \cdot CO \cdot CHO$, is its aldehyde.

2. The decomposition of pyruvic acid into acetaldehyde and carbon dioxide has already been fully discussed (Chapter V). The universality of the enzyme carboxylase in yeasts and the rapidity of its action on pyruvic acid form the strongest evidence at present available in favour of the pyruvic acid theory. Given the pyruvic acid, there is

no doubt that yeast is provided with a mechanism capable of decomposing it at the same rate as an equivalent amount of sugar.

The question of the occurrence of acetaldehyde as an intermediate product in alcoholic fermentation is discussed below, along with that of its reduction to alcohol.

3. The final step postulated by the pyruvic acid theory is the quantitative reduction to ethyl alcohol of the acetaldehyde formed from the pyruvic acid.

The idea that acetaldehyde is an intermediate product in the various fermentations of sugar has frequently been entertained [Magnus Levy, 1902; Leathes, 1906; Buchner and Meisenheimer, 1908; Harden and Norris, D., 1912; Grey, 1913]. It is a well-known fact that traces of acetaldehyde are invariably formed during alcoholic fermentation [see Ashdown and Hewitt, 1910], and this is of course consistent with the occurrence of acetaldehyde as an intermediate product. Another possible source of acetaldehyde exists in the oxidation of the ethyl alcohol, and it has been suggested [Buchner, Langheld, and Skraup, 1914; Neuberg and Kerb, 1914, 1, 3, 4] that the traces of acetaldehyde observed may sometimes arise in this way. It has however been shown [Neuberg and Kerb, 1914, 4; Neuberg and Schwenk, 1915, 4] that undoubted formation of aldehyde takes place when yeast is preserved under anaerobic conditions or allowed to autolyse under water saturated with carbon dioxide. Important evidence as to the specific capability of yeast to reduce acetaldehyde to alcohol has been obtained by several workers, and has already been discussed in connection with the reducing powers of yeast (Chapter VI).

It thus appears that the requirements of the pyruvic acid theory are fulfilled in so far that acetaldehyde is actually found in small amounts in the products of fermentation and that a mechanism exists in yeast by which the aldehyde can be reduced to alcohol.

It remains to consider the evidence that the normal process of alcoholic fermentation actually occurs in this way.

Function of the Reducing Enzyme of Yeast in Alcoholic Fermentation.

Many investigations have been made as to the relation of the reducing properties of yeast to the process of alcoholic fermentation. Thus Hahn (Buchner, E. and H., and Hahn, 1903, p. 343) found that the power of reducing methylene blue, which was possessed both by yeast and zymin (p. 101), on the whole ran parallel to the fermenting power in the

process of alcoholic fermentation. The intervention of a reducing enzyme was suggested by Grüss [1904, 1908, 1, 2] and was supported by Palladin [1908]. The latter observed that zymin which reduces sodium selenite and methylene blue in absence of sugar almost ceases to do so in presence of a fermentable sugar, and concluded that the great diminution of reduction during fermentation was due to the fact that the reducing enzyme was largely combined with a different substrate arising from the sugar, the reduction of which was necessary for alcoholic fermentation. Grüss, however, found that with living yeast the reduction is greatly increased in presence of a fermentable sugar, while Harden and Norris, R. V. [1914] confirmed the observation of Grüss, but found that the reducing power of zymin is not seriously affected by the presence of a fermentable sugar in concentration less then 20 grams per 100 c.c., whilst its fermenting power for glucose is inhibited by 1 per cent. sodium selenite. Hence Palladin's conclusion cannot be regarded as proved.

The greatest light has been thrown on this problem by the successive attempts which have been made to obtain evidence of the participation of a reducing enzyme in alcoholic fermentation by adding some substance which would be capable either of taking up hydrogen and thus preventing the reduction of the acetaldehyde or of converting the aldehyde into some compound less liable to reduction. Some caution must however be observed in interpreting the results of such experiments. As pointed out by Wo. Ostwald [1919, 1920 ; see also Neuberg, 1919, 2 ; 1920, 2] any process which removes one of the products from the sphere of action disturbs the equilibrium, so that more of that same product is formed until equilibrium is again restored. In this way the importance of a secondary reaction may be greatly increased relatively to that of a primary reaction. For example, if acetaldehyde were not an intermediate product in the main reaction of alcoholic fermentation, but the product of a secondary reaction, the addition of some substance which would remove the aldehyde from the sphere of action might lead, as explained above, to such an increased production of acetaldehyde as would make this one of the chief products and thus entirely alter the nature of the chief chemical change being produced. In such a case it would be wrong to argue that acetaldehyde was an intermediate product in the primary reaction. This forms an inherent weakness in what is now known as the " fixation " method (Neuberg) of attacking such problems, and must be borne in mind when the results obtained by this method are considered.

Kostytschev [1912, 1 ; 1913, 1, 2 ; 1914, 1, 2 ; Kostytschev and

Hübbenet, 1913; Kostytschev and Scheloumov, 1913; Kostytschev and Brilliant, 1913] has examined the effect of the addition of zinc chloride, chosen with the idea that it might polymerise the aldehyde and thus remove it from the sphere of action. Kostytschev's first results were subjected to some criticism [Neuberg and Kerb, 1912, 1 1913, 2; 1914, 3], partly on the ground of faulty analytical methods, and his experiments were subsequently repeated and extended to the action of cadmium salts [Kostytschev and Frey, 1920; Kostytschev and Subkova, 1920]. Salts of both of these metals exert a very marked action on the course of alcoholic fermentation by dried yeast. The effect is similar in both cases and seems to be due, not to any polymerisation caused by the salt, but to the ion of the metal, that of cadmium being the more effective. The addition of zinc chloride, for example, to the extent of 0·4-0·6 g. per 100 c.c. of a mixture containing 15 g. glucose and 20 g. dried yeast or hefanol causes the production of 12-24 mgm. of aldehyde. Moreover, about 40 to 50 per cent. of the sugar which is used up is not accounted for by the alcohol, carbon dioxide, and aldehyde obtained, and cannot be recovered by hydrolysis of the products.

In the absence of any information as to the nature of the products into which this missing sugar is converted, the exact significance of Kostytschev's experiments is not clear.

A similar lack of success has attended attempts to modify the course of the reaction by adding methylene blue, with the object of deviating the hydrogen supposed normally to be used in reducing the alcohol to aldehyde. Lvov [1913, 1, 2, 3; 1914] made quantitative experiments on the effect of methylene blue both on the sugar fermentation and autofermentation of dried yeast and maceration extract. In presence of sugar the methylene blue causes a decrease in the extent of fermentation, the difference during the time required for reduction of the methylene blue being represented by an amount of glucose equimolecular to the latter. In the absence of sugar on the other hand an excess of carbon dioxide equimolecular to the methylene blue is evolved but no corresponding increase in the alcohol production occurs. Lvov [1914] also states that in presence of methylene blue the addition of 2 gram-molecules of a phosphate (which normally causes the extra-evolution of 2 g.-mols. of carbon dioxide) likewise causes the extra-reduction of 1 g.-mol. of methylene blue. In these latter experiments Lvov's attention seems to have been too exclusively directed to the changes in the amount of methylene blue, and the evolution of carbon dioxide was not as a rule measured. He therefore came to

the conclusion stated above, whereas it has subsequently been shown [Neuberg and Ehrlich, 1920, 2; Harden and Henley, 1920, 1921, 1] that in presence of methylene blue the usual phosphate reaction takes place, but more rapidly, so that the alleged protection of a molecule of glucose from fermentation does not in fact occur. The effect of methylene blue is evidently complex, and it is impossible at present to say whether Lvov's contention is correct that the methylene blue actually interferes with the fermentation by taking up hydrogen (2 atoms per molecule of glucose) destined for the subsequent reduction of some intermediate product, or whether the effect is one of general depression of the fermenting power which would be presumably proportional to the concentration of methylene blue and inversely proportional to that of the fermenting complex [see Harden and Norris, R. V., 1914]. In any case it will be noticed that Lvov's interpretation of the results is at variance with the requirements of Kostytschev's theory (p. 123), according to which 4 atoms of hydrogen should be given off by a molecule of glucose.

Kostytschev [1913, 2; Kostytschev and Scheloumov, 1913] has also observed a depression of the extent of fermentation by methylene blue without any serious alteration in the ratio of CO_2 to alcohol, although an increase occurs in the production of acetaldehyde.

On the whole it cannot be said that the evidence gathered from these experiments on the reduction of acetaldehyde and methylene blue is very convincing.

Neuberg's Fixation Method.

The first reallysuccessful attempt to modify the course of fermentation in such a way as to protect the acetaldehyde from reduction was made by Neuberg and Reinfurth [1918, 1], the same result being independently attained by Connstein and Lüdecke [1919; see also Schweizer, 1919] at about the same time. The method has been exhaustively studied by Neuberg [Neuberg and Reinfurth, 1918, 2; 1919; 1920, 1, 2, 3; Neuberg, 1919, 1; Neuberg and Hirsch, 1919, 1; see also Zerner, 1920] and has supplied a very striking confirmation of the pyruvic acid theory, besides throwing some light on the earlier stages of the fermentation process. The starting-point of the experiments seems to have been certain observations made by Neuberg and Färber [1917] who found that in presence of alkalis the course of fermentation was considerably altered. Fermentation still proceeds in maceration extract which is 0·1-0·2 *M* with respect to alkali carbonates, borates, or

triphosphate, or 0·02 *M* to sodium sulphite, provided the alkali be added after the fermentation has become well established. At the same time increased amounts of aldehyde, acetic acid, and glycerol are found among the products. Further investigation revealed the fact that sulphites modified the reaction in a somewhat different way from alkalis as such, and each of these different forms of fermentation has been separately examined.

Neuberg regards the ordinary alcoholic fermentation, the end results of which are expressed by Gay-Lussac's classical equation

$$(1)\quad C_6H_{12}O_6 = 2CO_2 + 2C_2H_6O,$$

as the first form of fermentation, that produced in presence of sodium sulphite as the second form, and that in presence of alkalis as the third.

Neuberg's Second Form of Fermentation.

The essential fact discovered both by Neuberg and Reinfurth and by Connstein and Lüdecke is that when sodium sulphite, Na_2SO_3, is added to a fermenting mixture of yeast and sugar, the yield of alcohol and carbon dioxide diminishes, whilst considerable amounts of glycerol and acetaldehyde are formed, the latter being present as the bisulphite compound. Connstein and Lüdecke adapted the process to the manufacture of glycerol, and it was employed on a large scale in Germany during the war, no less than 1,000,000 kilos of glycerol being manufactured per month, with a yield of about 15 to 20 per cent. of the sugar fermented. Neuberg on the other hand, with various colleagues, has investigated the chemistry of the process in great detail. He has found by careful analytical determinations that the aldehyde finally produced is exactly equivalent to the glycerol [Neuberg and Reinfurth, 1918, 2; 1919], and, moreover, that this equivalence persists throughout the whole period of the fermentation [Neuberg and Hirsch, 1919, 1]. Neuberg propounds the following equation, based upon these experiments, for this second form of fermentation:

$$(2)\quad C_6H_{12}O_6 = C_3H_8O_3 + C_2H_4O + CO_2,$$

or, if the sodium sulphite be included,

$$(2a)\quad C_6H_{12}O_6 + Na_2SO_3 + H_2O = C_3H_8O_3 + C_2H_4O \cdot NaHSO_3 + NaHCO_3.$$

This has not been experimentally realised to its full extent, since a certain amount of the sugar always undergoes the normal fermentation, so that the final result is compounded of those given by the two equations (1 and 2).

The theory of the process is that in the presence of the sulphite the aldehyde combines to form the stable, non-reducible bisulphite complex so rapidly that it escapes reduction. As a consequence of this some other acceptor for the hydrogen must be utilised if the fermentation is to continue, and this condition is satisfied by the production of an equivalent amount of glycerol from some, so far unknown, product, probably of the formula $C_3H_6O_3$, derived from the sugar :

$$C_6H_{12}O_6 = 2C_3H_6O_3.$$

In other words, for the reduction of aldehyde with production of alcohol is substituted the reduction of another substance with production of glycerol, as shown in the equations (2 and 2*a*) above.

The proportion of aldehyde and glycerol obtained increases with the amount of sulphite added, but this cannot be indefinitely augmented since it ultimately interferes with the action of the yeast and sugar then remains unfermented [see Polak, 1929]. Moreover, the aldehyde-bisulphite compound is partially dissociated and hence a certain proportion of aldehyde always escapes protection and undergoes the normal change into alcohol.

The experiments were carried out by dissolving the sugar in water, together with small amounts of nutrient salts, to provide for the growth of the yeast, adding the yeast and allowing the fermentation to commence. The sodium sulphite (Na_2SO_3) dissolved in water was then added and the fermentation continued until all the sugar had been fermented.

For the estimations of aldehyde and glycerol it was necessary to precipitate excess of sulphite by barium chloride. The aldehyde was then estimated by distilling with magnesia or calcium carbonate, which hydrolysed the bisulphite compound, condensing the aldehyde in well-cooled alcohol and estimating its amount by Ripper's method, or by converting it into the *p*-nitrophenylhydrazone. Glycerol was estimated in the filtrate from barium sulphite by boiling off the aldehyde with baryta, precipitating the Ba by CO_2, filtering and evaporating at a low temperature. The residue was repeatedly taken up with alcohol, filtered and evaporated, a mixture of alcohol and ether being finally employed. The resulting syrup was dissolved in water, freed from traces of alcohol by evaporation on the water-bath and the glycerol finally estimated by Zeisel's method. The alcohol was estimated by distilling a portion of the fermentation mixture, removing traces of aldehyde by treatment with *p*-nitrophenylhydrazine and acetic acid, and repeatedly concentrating by distillation from acid, alkaline, and finally acid solutions.

The following table shows some typical results :—

Na_2SO_3.	Sucrose.	Acetaldehyde.	Glycerol	
g.	g.	g.	g.	per cent. of theoretical yield.
33	100	11·90	23·37	43·4
50	100	12·52	24·86	46·2
75	100	13·89	27·61	51·4
100	100	18·65	36·90	68·5

The amount of glycerol normally produced in the absence of sulphite is about 2 to 3 per cent. of the sucrose fermented, whilst acetaldehyde is present, if at all, only in traces, so that there can be no doubt of the enormous increase in the quantity of these substances formed as the result of the addition of the sulphite. Precisely similar results are produced by the addition of the insoluble sulphites of calcium, magnesium and zinc [Neuberg and Reinfurth, 1919].

The fixation method is most satisfactory with living yeast, but also succeeds with yeast preparations [Neuberg and Reinfurth, 1919]. During the fermentation of sugar by dried yeast in presence of phosphate, the production of acetaldehyde can also be demonstrated by the addition of sulphite [Hemmi, 1923].

Both glucose and pyruvic acid, like acetaldehyde, form compounds with sodium bisulphite. That of glucose is unstable and is largely hydrolysed in aqueous solution, so that fermentation, either of the complex or of the free glucose, proceeds as usual. The pyruvic acid intermediately produced, probably forms the stable bisulphite compound, $CH_3 \cdot C(OH)SO_3Na \cdot COOH$, which however is readily fermented. This has been proved by special experiments [Neuberg and Reinfurth, 1920, 1, 2; see also Zerner, 1920] in which it was found that pyruvic acid was fermented even in the presence of excess of sodium sulphite or, better still, of calcium sulphite. Hence it is that the aldehyde is the substance fixed and not the pyruvic acid. Increase of sulphite beyond a certain limit necessitates dilution of the solution and does not increase the yield of glycerol.

A precisely similar influence on the course of the fermentation is observed when the sulphite is replaced by dimethyl-*cyclo*-hexanedione, $(CH_3)_2C_6H_6O_2$ (dimedone), a neutral very sparingly soluble substance, which reacts with aldehyde but not with glucose or pyruvic acid [Neuberg and Reinfurth, 1920, 3]. The aldehyde is then found at the close of the fermentation in the form of anhydroacetaldehyde-*bis*-dimethyl-*cyclo*-hexanedione (aldomedone):

$$(CH_3)_2C_6H_6O_2 + CH_3 \cdot CHO \rightarrow [(CH_3)_2 \cdot C_6H_5O_2]_2 : CH \cdot CH_3.$$

This affords additional and conclusive evidence that it is the specific power of combining with the aldehyde and not merely the alkalinity of the reagent which is the decisive factor in the sulphite fixation method. Thiosemicarbazide [Neuberg and Kobel, 1927, 2] and even charcoal [Abderhalden and Glaubach, 1922] can also be used as fixation agents.

Experiments carried out in presence of sulphite show that the

velocity constants of the two fermentation reactions (the normal or first form and the acetaldehyde-glycerol or second form) are approximately equal [Neuberg and Hirsch, 1919, 1], and this constitutes important evidence in favour of the view that the production of acetaldehyde is part of the normal reaction and is not an independent secondary reaction brought into fictitious prominence by the fixation method [see Ostwald, Wo., 1919, 1920].

Origin of the Glycerol.

The high yield of glycerol renders it certain that the origin of this product is the glucose and not any constituent of the yeast. The amount of glycerol produced is moreover exactly equivalent to the aldehyde as expressed in equation (2), and the most obvious supposition to account for its origin is that one half of the sugar molecule appears in the form of some substance of the empirical formula $C_3H_6O_3$ which then undergoes reduction to glycerol.

What this substance actually is has not yet been ascertained. Direct experiments with dihydroxyacetone in presence of sulphite have shown that, although some aldehyde and glycerol are produced, the reaction is very irregular and the ratio of the two products variable. Glyceraldehyde, on the other hand, which might be supposed to be the most natural source of glycerol, yields neither this substance nor aldehyde in presence of sulphite [Neuberg and Reinfurth, 1919]. Neuberg, in accordance with his general theory, supposes that the compound in question is some form of methylglyoxal, or of a compound of this with the elements of water, but this has not yet been experimentally confirmed.

Neuberg's Third Form of Fermentation.

Alkalis alone, as already mentioned [see also Euler, 1917 ; Wilenko, 1917 ; Euler and Moberg, 1917 ; Euler and Svanberg, 1918], modify the course of the reaction in a somewhat different manner from sulphites. A careful examination of the products of fermentation in their presence [Neuberg and Hirsch, 1919, 2, 3 ; Neuberg, Hirsch, and Reinfurth, 1920 ; Neuberg and Ursum, 1920] has shown that the aldehyde undergoes the Cannizzaro reaction and is converted into equimolecular proportions of acetic acid and alcohol :

$$2C_2H_4O + H_2O = C_2H_6O + C_2H_4O_2.$$

This enzymic dismutation of the aldehyde, which is presumably formed as intermediate product, occurs to a considerable extent when

almost any alkaline salt is added to a sugar solution and the mixture fermented by top yeast. Under the most favourable conditions (in presence of 4 per cent. of ammonium carbonate) as much as 41·3 per cent. of the sugar is fermented in this way. Soluble carbonates, the various alkaline phosphates, potassium pyrophosphate and a number of other alkaline salts, such as sodium sulphydrate, thioantimonate, arsenate, silicate, and oleate are all active. Zinc hydroxide and magnesia are active, aluminium hydroxide and colloidal ferric hydroxide inactive ; guanidine carbonate, diethylamine and *dl*-alanine also show the effect, whereas methylene blue is inactive. All these active substances are termed alkalisers (*alkalisatoren*) by Neuberg. The effect appears to depend mainly on the alkalinity of the medium and increases with the concentration of the alkaline salt employed. The best concentration is about 0·5-1 *M*, but this naturally varies with the nature of the alkaliser.

Precisely as in the fermentation in presence of sulphite, the withdrawal of the aldehyde from the system involves the production of a molecular equivalent of glycerol, so that the equation of the third form of fermentation is

$$(3)\quad 2C_6H_{12}O_6 + H_2O = 2CO_2 + C_2H_6O + C_2H_4O_2 + 2C_3H_8O_3,$$

which may be regarded as involving the two stages,

$$(3a)\quad 2C_6H_{12}O_6 = 2CO_2 + 2C_2H_4O + 2C_3H_8O_3$$
$$(3b)\quad 2CH_3 \cdot CHO + H_2O = C_2H_5 \cdot OH + CH_3 \cdot COOH.$$

Two molecules of glycerol are thus produced for each molecule of acetic acid and alcohol.

During these alkaline fermentations there appears to be at first an accumulation of aldehyde, which subsequently partly disappears. Thus in a 10 per cent. solution of sugar undergoing normal fermentation the concentration of the aldehyde is almost constant throughout the process at about 0·2 per mille (calculated on the original sugar), whereas in presence of *M* K_2HPO_4 it first increases, and then diminishes as shown below :—

Hours.	Aldehyde. ‰
4	0·2
5·5	0·7
7	1·3
10	1·3
14·5	0·3
24	0·3

It will be seen that these highly important investigations of Neuberg and his colleagues afford brilliant confirmation of a considerable part of Neuberg's theory. It may fairly be claimed firstly, that they

show that some substance capable of being reduced to glycerol is undoubtedly formed, and secondly, that they afford a presumption so strong as almost to amount to a proof that acetaldehyde is normally produced as an intermediate product and reduced to alcohol, and that this acetaldehyde is formed by the decomposition of pyruvic acid by the carboxylase of the yeast.

The fixation method has also been successfully applied in the study of the dissimilation of carbohydrates by many bacteria, by the higher plants [see Neuberg and Gottschalk, 1925, 2; Pirschle, 1926], and in the muscles of animals [see Neuberg and Gottschalk, 1925, 1; Hirsch, 1922, 2].

The Early Stages of Alcoholic Fermentation.

As regards the changes which lead up to the fermentation of pyruvic acid or its precursor $C_3H_6O_3$, nothing is definitely known. It is now generally admitted that the intervention of phosphate is essential at this stage, and this conclusion has been greatly strengthened by the discovery that phosphate plays a closely similar part in the conversion of carbohydrate into lactic acid in muscle (see p. 74).

The Function of Phosphate and the Mechanism of Formation of Hexosediphosphate.

No agreement as to the actual function of phosphate in the fermentation process has, however, yet been attained. Meyerhof, Euler, and Kluyver all consider that the introduction of the phosphoric group into the sugar molecule renders the latter unstable and facilitates its decomposition into two groups each containing three carbon atoms, but they differ as to the exact way in which this is brought about.

Meyerhof [1930] considers that an "active" monophosphate is the first product (equation I. (*a*) below). Some of this (equation I. (*c*)) passes without change of composition into a stabilised form, which is the fermentation monophosphoric ester (Robison ester). Of the remainder a portion is fermented at a high rate, according to equation I. (*b*), which results in the decomposition of the sugar residue of one molecule into CO_2 and alcohol, and the esterification of a second molecule to hexosediphosphate by the phosphoric acid thus liberated. Finally, another portion is converted into hexosediphosphate according to equation I. (*d*) by esterification of phosphoric acid contained in the medium.

The hexosediphosphate thus produced undergoes decomposition

in two different ways (see p. 141). Part of it is hydrolysed into hexose and inorganic phosphate (II. (*b*)) which then re-enter the cycle and take part in the reaction I. (*a*); the remainder is directly fermented (very little co-enzyme being required) with formation of CO_2, alcohol and inorganic phosphate (II (*a*)), the sugar not being re-esterified whilst the phosphate re-enters the cycle of reaction (I. (*a*)). Meyerhof's system may then be expressed by the following equations:—

I. Phosphate Period; Rapid Fermentation; High Concentration of Co-enzyme Required.

(*a*) $C_6H_{12}O_6 + R_2HPO_4 = C_6H_{11}O_5(PO_4R_2)^* + H_2O$.
(*b*) $2C_6H_{11}O_5(PO_4R_2)^* = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R_2)_2$.
(*c*) $C_6H_{11}O_5(PO_4R_2)^* = C_6H_{11}O_5(PO_4R_2)$.
(*d*) $C_6H_{11}O_5(PO_4R_2)^* + R_2HPO_4 = C_6H_{10}O_4(PO_4R_2)_2 + H_2O$.

* = "active" form.

II. Ester Period; Slow Fermentation; Low Concentration of Co-enzyme Required for II. (*a*).

(*a*) $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = 2CO_2 + 2C_2H_6O + 2R_2HPO_4$.
(*b*) $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$.

The main criticism which may be directed against this system is that the extra CO_2 evolved (I. (*b*)) should be equivalent to or less than the hexosediphosphate formed, whereas in reality it is nearly equivalent to the total phosphorus esterified—which may greatly exceed that present as diphosphate. Moreover, it must be remembered that hexosediphosphate is a derivative of fructose, whereas the stable monophosphate (the Robison ester) consists to the extent of about 60 per cent. of a glucosemonophosphate and can only yield the diphosphate by direct esterification if there is an accompanying intramolecular change in the sugar molecule.

Euler and Myrbäck [1927, 2; 1928, 2] formulated the equation of fermentation as follows:—

$$C_6H_{12}O_6 + PO_4HR_2 = C_3H_6O_3 + C_3H_5O_2 \cdot PO_4R_2 + H_2O$$
$$2C_3H_5O_2 \cdot PO_4R_2 = C_6H_{10}O_4(PO_4R_2)_2$$
$$C_3H_6O_3 = CO_2 + C_2H_6O.$$

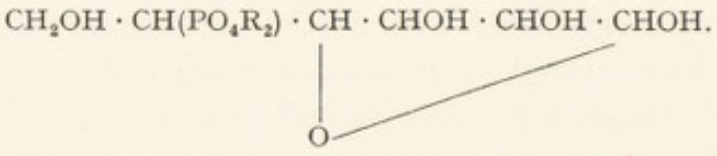
The essential points of this idea were that the introduction of the phosphoric radical gave rise to the possibility of the conversion of the glucose molecule into two 3-carbon atom chains, and that of the two smaller molecules thus produced one was richer and the other poorer in energy than the half-glucose molecule. A reaction of this type they term a dismutation, using the term in a wider sense than was intended by Neuberg, and it is in this reaction that they suppose the co-enzyme to intervene. In view of their own results and those of Meyerhof on the fermentation of hexosemonophosphate (p. 140), they now consider [Euler, 1930] that this dismutation more probably

occurs in the molecule of hexosemonophosphate (Meyerhof's equation I. (*b*), p. 107) than in that of glucose and is due to the action of a mutase in presence of co-zymase (see p. 137).

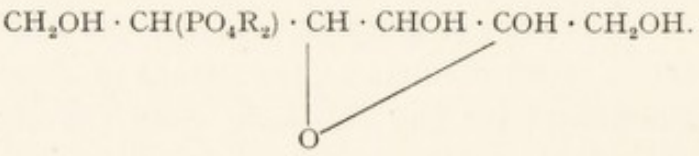
Euler terms the enzyme which aids the esterification of the sugar, hexosephosphat*ese*, to distinguish it from the hexosephosphat*ase* which hydrolyses the ester. The adoption of this nomenclature seems unnecessary, and it is inconsistent with the conception of an enzyme as a catalyst, capable of accelerating both the direct and the reverse reactions.

This idea is liable to the same criticism as that of Meyerhof, and does not explain how large amounts of the monophosphate may be left unfermented.

Kluyver and Struyk [1926] have propounded still a different system, which, however, is as yet to a large extent devoid of experimental foundation. The first product in the fermentation of glucose is a hexosemonophosphate of the constitution



Fructose yields a different ester,



Both these esters are assumed to be derivatives of the unstable reactive γ-forms of their respective sugars. These esters are then decomposed, the glucose derivative yielding glyceraldehyde and a triosephosphoric ester (glyceraldehydephosphoric ester) and the fructose derivative the same phosphoric ester and dihydroxyacetone:

$$C_6H_{11}O_5(PO_4R_2) = C_3H_6O_3 + CH_2OH \cdot CH(PO_4R_2) \cdot CHO.$$

The glyceraldehyde and dihydroxyacetone are fermented,

$$C_3H_6O_3 = CO_2 + C_2H_6O,$$

whilst the triosephosphoric ester condenses to form hexosediphosphoric ester,

$$CH_2(OH) \cdot CH(PO_4R_2) \cdot CHO + CHO \cdot CH(PO_4R_2) \cdot CH_2(OH) \rightarrow$$
$$CH_2OH \cdot CH(PO_4R_2) \cdot CO \cdot CHOH \cdot CH(PO_4R_2) \cdot CH_2OH.$$

The same criticism as to ratio of extra CO_2/P esterified as hexosediphosphate can be made here as in the case of Meyerhof's theory. In addition experimental evidence is still wanting.

1. That fructose and glucose yield different monophosphoric esters.
2. That a triosephosphoric ester is formed.
3. That hexosediphosphoric ester has the constitution of the 2-5 phosphoric ester of a 3-keto-hexose (see p. 58).

The theory further involves the difficulty of supposing that glyceraldehyde and dihydroxyacetone are the intermediate 3-carbon substances which are converted into CO_2 and alcohol (see p. 117).

In Kluyver and Struyk's view the significance of the biochemical phosphorylation of the hexoses is to be found in the conversion of sugars into the active γ-modifications (furanose forms).

As opposed to the view that a phosphorylated sugar is an intermediate compound in the decomposition of sugar, the present author has pointed out [1929] that the essential fact which has been demonstrated is that the enhanced fermentation which accompanies phosphorylation is frequently, if not invariably, equivalent to the amount of P esterified, irrespective of the proportions in which mono- and di-phosphoric esters are produced. This would seem to indicate that it is the act of phosphorylation which gives the impulse to the decomposition of a molecule of sugar, so that an induced or coupled reaction occurs.

I. (*a*) $2[C_6H_{12}O_6 + H_3PO_4 = C_6H_{11}O_5(PO_4H_2) + H_2O]$
(*b*) $C_6H_{12}O_6 + 2H_3PO_4 = C_6H_{10}O_4(PO_4H_2) + 2H_2O.$
II. $C_6H_{12}O_6 = 2C_3H_6O_3 = 2CO_2 + 2C_2H_6O.$

It is to be noted that the schemes of Meyerhof and Euler, in which two molecules of the reactant simultaneously undergo different reactions, also involve a reaction of this kind, namely the decomposition of one molecule of a hexosemonophosphoric acid into CO_2, alcohol, and phosphoric acid and the simultaneous phosphorylation of a second molecule of the monophosphoric acid (reaction I. (*b*), p. 137).

The phenomena which accompany the action of yeast preparations on the hexosemonophosphates (the Neuberg ester and the Robison ester) cannot at present be satisfactorily explained. Meyerhof and Lohmann [1927] consider that these esters "are decomposed at an enhanced initial rate, one part decomposing into carbon dioxide and alcohol, whilst another part takes up phosphate and is converted into diphosphate. At small concentrations of ester the velocity of fermentation only diminishes when the conversion is nearly complete. At higher concentrations (above $M/50$) it diminishes before conversion is complete." The few estimations of inorganic phosphate in the fermenting mixture which have been made indicate that phosphate is esterified during the early stages of the change, whilst hydrolysis may

occur in the later stages. It will be noted that Meyerhof and Lohmann ascribe to the stabilised monophosphate exactly the same fermentation reactions as to the "active" form.

Euler and Myrbäck [1928, 2] consider that in this change half the ester is decomposed and fermented and the other half esterified. Working with washed zymin and purified co-enzyme (+ a little hexose-diphosphate) they found that inorganic phosphate did not influence the fermentation, and that no change in inorganic phosphate occurred until the velocity of fermentation slackened, at which period they suppose that half the monophosphate has been fermented and the other half converted into diphosphate, which was then decomposed in the usual way.

Harden and Robison's (unpublished) experiments, made with zymin, agree with those of Meyerhof and Lohmann, but they have further observed that the ester is only fermented with enhanced velocity when free phosphate is present, so that this stage of the fermentation represents some change in which phosphate plays an active part. Thus 1 c.c. *M* hexosemonophosphate, added to zymin and water, gives a short period of enhanced fermentation, varying with the amount of inorganic phosphate present. When a further addition of hexosemonophosphate is made to this mixture no enhanced fermentation occurs, but if phosphate be then added an enhanced rate of fermentation is again set up. The proportion of ester thus fermented at an enhanced rate is always small ($\frac{1}{4}$-$\frac{1}{5}$) and is approximately the same for both the Neuberg and the Robison ester.

Neuberg and Kobel's [1928, 3] observations agree with those of Harden and Robison and of Meyerhof in that they show that a small proportion of the ester is fermented at a high rate comparable to that of fructose and phosphate. In view of the small proportion which undergoes this rapid reaction they regard their observations as decisive against the idea that the hexosemonophosphate, as isolated, can be regarded as a necessary intermediate product of alcoholic fermentation.

What the explanation of these phenomena is remains so far unknown, but it is obvious that the course of this reaction cannot at the moment be legitimately used as evidence in favour of any one of the various systems of fermentation at present in the field. It must also be remembered that all the hexosemonophosphoric esters hitherto submitted to fermentation contain at least two and sometimes three constituents.

Fermentation of Hexosediphosphate.

Hexosediphosphates are readily fermented by yeast preparations, although they are not attacked by living yeast (p. 67). According to Harden and Young's original view in this fermentation the ester is hydrolysed into a hexose and phosphoric acid,

$$C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O = C_6H_{12}O_6 + 2Na_2HPO_4,$$

which then undergo the normal reactions of fermentation, with the result that the sugar moiety of the molecule is gradually converted into CO_2 and alcohol and the phosphorus liberated as inorganic phosphate. The evolution of CO_2 takes place at first at a steady slow rate, which gradually falls off as the concentration of hexosediphosphate diminishes. In the presence of excess of glucose, on the other hand, as already explained, the whole of the phosphorus is re-esterified so that fermentation continues and no inorganic phosphate accumulates. The rate thus attained is the normal or basal rate observed during the fermentation of sugars by yeast preparations.

Meyerhof's muscle enzyme solution acts upon hexosediphosphate in an analogous manner, converting the sugar residue nearly completely into lactic acid and liberating the phosphorus as inorganic phosphate [Meyerhof, 1926, 1, 2].

As already explained (p. 89) it has been observed that when apozymase is prepared from yeast preparations (e.g. by ultra-filtration or by washing zymin or dried yeast) a stage is reached at which hexosediphosphate is fermented whereas a mixture of fructose and phosphate is not affected [see Gottschalk, 1927], and a similar preparation can be obtained from muscle which yields lactic acid with hexosediphosphate but not with an activated (p. 74) mixture of fructose and phosphate. Meyerhof [1926, 2] was at first of the opinion that hexosephosphate, in contradistinction to sugar, could be fermented in the absence of the co-enzyme, but further experiment has shown that a preparation can be obtained which is without action on hexosediphosphate, but is activated by the addition of co-enzyme [see for example Euler and Myrbäck, 1927, 1]. Meyerhof [1930], however, considers it probable that in the presence of small concentrations of co-enzyme the hexosephosphate is fermented directly, without previous hydrolysis and re-esterification.

With yeast enzyme, $C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O = 2CO_2 + 2C_2H_6O + 2Na_2HPO_4$.
With muscle enzyme, $C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O = 2C_3H_6O_3 + 2Na_2HPO_4$.

The enzymes of the preparations are able to produce this change, but not to cause esterification, and hence free phosphate may accumulate even in the presence of excess of sugar [see Meyerhof, 1930]. This actually occurs in prolonged fermentation, but in this case it must be remembered that the hexosephosphatase is known to outlast the zymase in autolysis, and the condition may simply be that the controlling enzymic factor is now the concentration of the fermenting complex, and not, as in the earlier stages, the concentration of the enzyme liberating phosphoric acid.

Further evidence in favour of the direct fermentation of hexosediphosphate is afforded by the effect of arsenate on this fermentation (see p. 156). An explanation of the fact that hexosediphosphate is more readily fermented than sugar has been sought in the nature of the sugar liberated by hydrolysis, which may be more susceptible of the necessary reactions than ordinary glucose or fructose and hence be fermented in the presence of a smaller concentration of co-enzyme [Gottschalk, 1927]. This explanation appears to be inconsistent with the effect of arsenate (p. 160) which only accelerates the reaction when fermentation is in progress and has no effect on the simple hydrolysis when the sugar is allowed to accumulate.

According to Gottschalk [1928, 1] hexosemonophosphoric ester (Robison) requires more co-enzyme for its fermentation than the diphosphate, but less than glucose.

Fermentation of Glycogen.

The researches of Meyerhof [1926, 2] have shown that in muscle glycogen serves as the source of the lactic acid which is produced in the course of muscular contraction.

In muscle extract the conversion of glycogen into lactic acid proceeds with great rapidity in the presence of phosphate, hexosediphosphate being simultaneously formed; the extract on the other hand has very little effect on added glucose or fructose unless an activator obtained from yeast is added (p. 74). He has also shown that in the oxidation of lactic acid which occurs during the aerobic recovery of fatigued muscle a fraction of the lactic acid is re-synthesised to glycogen. It is thus evident that glycogen plays a part of fundamental importance in the enzymic changes which accompany the formation of lactic acid in muscle. Meyerhof has extended his work to the relation of glycogen to alcoholic fermentation by yeast and several new observations have been made.

According to him in the fermentation of glycogen or starch (both in muscle extract with formation of lactic acid and in yeast maceration juice with formation of alcohol and carbon dioxide) the relation which holds for glucose, viz. that for each molecule of CO_2 evolved, one molecule of inorganic phosphate is esterified, no longer holds. In the fermentation of glycogen by yeast-juice, for instance, 2-4 molecules of H_3PO_4 are in fact esterified for each molecule of CO_2 evolved. Hence in a normal glycogen fermentation hexosediphosphate accumulates more rapidly than in a glucose fermentation. Thus for example the following results were obtained with maceration extract acting on glycogen and starch [Meyerhof, 1927]:—

	Duration of Experiment.	P Esterified.	CO_2 Evolved.	Ratio.
		mg.	mg.	
Glycogen 8 mg. . .	45 mins.	0·46	0·865	2·7
	1 hr. 45 mins.	1·01	1·18	1·7
,, 4 mg . .	80 mins.	0·16	0·43	3·9
Starch 4 mg. . .	3 hrs.	0·22	0·19	1·24
Glucose 5 mg. . .	2 hrs. 30 mins.	0·78	0·723	0·96

The accelerated fermentation of this accumulated hexosediphosphate accounts for the action of arsenate on glycogen fermentation (see p. 161).

Another remarkable reaction of glycogen (see p. 163) [Nilsson, 1930] is that when dried yeast containing glycogen is treated with phosphate, esterification takes place with formation of the Robison hexosemonophosphoric ester. This occurs even in absence of co-enzyme with washed dried yeast and is not stopped by fluoride but is inhibited by iodoacetate [Nilsson, Zeile, and Euler, 1931]. The apparent inconsistency between the results of Nilsson and Meyerhof may possibly be explained by the secondary conversion of primarily formed monophosphate into hexosediphosphate.

It seems probable that the hexose produced from glycogen, starch, etc., by enzymic hydrolysis is in a more reactive form than ordinary *d*-glucose, and it will be remembered that this is also possibly the case with the hexose derived from hexosediphosphate.

The Function of the Co-enzyme.

It has been shown by Harden [1913] and by Neuberg and Rosenthal [1913] that the decomposition of pyruvic acid into carbon dioxide and acetaldehyde is effected by apozymase in the absence of co-enzyme, so that this reaction is excluded from those for which the co-enzyme

may be essential. As early as 1911 Lebedev [1911, 4] showed that the total fermentation produced by maceration extract in presence of 20 per cent. of sucrose was strictly proportional to the amount of co-enzyme (boiled extract) added, and drew the conclusion, not altogether justified, that the co-enzyme was concerned in the esterification of phosphoric acid. Later Euler and Myrbäck [1924, 3] came to a similar conclusion, that the co-enzyme was concerned in esterification or some earlier stage, from consideration of the more rapid fermentation and esterification of phosphoric acid produced by addition of co-enzyme [see also Neuberg and Gottschalk, 1924, 2; 1925, 3]. This argument is not very convincing, as the breaking of any link in a chain of reactions may destroy the continuity and cause all appreciable change to cease. More recently the view has been advanced by Euler and his colleagues (see p. 107) that the co-enzyme is essential for the dismutation which in their opinion is (p. 137) involved in the production both of lactic acid by muscle and of CO_2 and alcohol by yeast, and that the co-enzymes, which they regard as necessary for reduction and dismutation by yeast, are identical with each other and with co-zymase.

Against these views it may be urged (see p. 108) that substances which restore the power of reducing methylene blue to washed zymin and dried yeast do not necessarily restore the power of producing alcoholic fermentation [see Harden and Norris, 1914], and that it is by no means certain that the oxido-reductions produced by yeast require a co-enzyme [Harden and Macfarlane, 1931].

Nilsson has shown that yeast glycogen (in contradistinction to glucose and fructose) undergoes esterification, unaccompanied by production of CO_2 and alcohol, in presence of washed dried yeast and in the absence of co-enzyme (see p. 143). In view of this difference between the behaviour of glycogen and the hexoses and in the prevailing uncertainty as to the nature of the early stages of the alcoholic fermentation of the latter, it is still impossible to say at what stage the co-enzyme is involved.

Other Theories of Alcoholic Fermentation.

The Formic Acid Theory.

An interesting interpretation of the phenomena of fermentation was attempted by Schade [1906] based upon the conception that glucose under the influence of catalytic agents readily decomposes into acetaldehyde and formic acid. It was subsequently found that the experi-

mental evidence upon which this conclusion was founded had been wrongly interpreted [Buchner, Meisenheimer, and Schade, 1906; Schade, 1907], but Schade has succeeded in devising an interesting series of reactions by means of which alcohol and carbon dioxide can be obtained from sugar by the successive action of various catalysts. The following are the stages of this series. (1) Glucose, fructose, and mannose are converted by alkalis into lactic acid along with other products. (2) Lactic acid when heated with dilute sulphuric acid yields a mixture of acetaldehyde and formic acid :

$$CH_3 \cdot CH(OH) \cdot COOH = CH_3 \cdot CHO + H \cdot COOH.$$

(3) It has long been known that formic acid is catalytically decomposed by metallic rhodium at the ordinary temperature into hydrogen and carbon dioxide, and Schade has found that when a mixture of acetaldehyde and formic acid is submitted to the action of rhodium the acetaldehyde is reduced to alcohol at the expense of the hydrogen, and the carbon dioxide is evolved :

$$CH_3 \cdot CHO + H \cdot COOH = CH_3 \cdot CH_2(OH) + CO_2.$$

Schade suggests [1908] that the fermentation of sugar may proceed by a similar series of reactions catalysed by enzymes, the acetaldehyde and formic acid being derived not from the relatively stable lactic acid but more probably from a labile substance capable of undergoing change either into lactic acid or into aldehyde and formic acid.

It will be noticed that this theory resembles the pyruvic acid theory in postulating the intermediate formation of acetaldehyde but differs from it by supposing that the reduction is effected at the expense of formic acid produced at the same time.

The acetaldehyde question has already been discussed. In view of the fact that formic acid is a regular product of the action of many bacteria on glucose [see Harden, 1901], Schade's theory of alcoholic fermentation may be said to be a possible interpretation of the facts. Formic acid is known to be present in small amounts in fermented sugar solutions and the actual behaviour of yeast towards this substance has been investigated in some detail by Franzen and Steppuhn [1911; 1912, 1, 2], who have obtained results strongly reminiscent of those obtained with lactic acid by Buchner and Meisenheimer (p. 115). Many yeasts when grown in presence of sodium formate decompose a certain proportion of it, whereas in absence of formate they actually produce a small amount of formic acid—the absolute quantities being usually of the order of 0·0005 gram molecule (0·023 gram) per 100 c.c. of medium in 4 to 5 days. Only in the case of *S. validus* did the consumption of

formic acid in 5 days reach 0·0017 gram molecule (0·08 gram). Somewhat similar but rather smaller results were given by yeast-juice, a small consumption of formic acid being usually observed. The possibility thus exists that formic acid may be an intermediate product of alcoholic fermentation, and Franzen argues strongly in favour of this view.

Direct experiment, on the other hand, shows that yeast-juice cannot ferment a mixture of acetaldehyde and formic acid, even when these are gradually produced in molecular proportions in the liquid by the slow hydrolysis of a compound of the two, ethylideneoxyformate, $OHC \cdot O \cdot CH(CH_3) \cdot O \cdot CH(CH_3) \cdot O \cdot CHO$, this method being adopted to avoid the inhibiting effect of free acetaldehyde and formic acid [Buchner and Meisenheimer, 1910]. Nor is the reduction of acetaldehyde assisted by the presence of formate [Neuberg and Kerb, 1912, 4; Kostytschev and Hübbenet, 1912].

Various other theories of alcoholic fermentation have been proposed from time to time, but little evidence in their support has been produced [see Ashdown and Hewitt, 1910; Kohl, 1907, 1909; Kusserov, 1910; Löb, 1906; 1908, 1, 2; 1909, 1, 2, 3, 4; 1910; Löb and Pulvermacher, 1906].

CHAPTER VIII.

ACTION OF SOME INHIBITING AND ACCELERATING AGENTS ON THE ENZYMES OF YEAST PREPARATIONS.

One of the most interesting and at the same time most difficult problems concerning enzyme action in general is the nature of the inhibiting or accelerating effect produced by many substances upon the rate of the chemical processes set up in presence of the enzyme. Inhibition, it is usually supposed, involves either the decomposition of the enzyme, in which case it is usually irreversible, its removal from the sphere of action by some change in its mode of solution, or the formation of an inactive or less active compound between the enzyme and the inhibiting agent. This compound it may sometimes be possible to decompose, with the result that the activity of the enzyme is restored. A striking example of this, to which allusion has already been made, is the effect of hydrocyanic acid on alcoholic fermentation (p. 37).

Acceleration of enzyme action can in some cases be ascribed to the fact that the accelerating substance possesses an assignable chemical function in the reaction, so that an increase in the concentration of this substance causes an increase in the rate of the reaction. As we have seen in Chapter III, this is the explanation of the accelerating effect of phosphates on fermentation by yeast-juice. In many other cases, however, no such chemical function can be traced, as, for example, in the effect of neutral salts on the hydrolytic action of invertase, and it is necessary to fall back on some assumption, such as that the accelerating agent acts by increasing the effective concentration of the enzyme or by combining either with the enzyme or the substrate, forming a compound which undergoes the reaction more readily.

The interest in the following examples of inhibition and acceleration of fermentation by yeast-juice lies not only in their relation to these general problems but also, and perhaps chiefly, in their bearing on the specific problem of the nature and mode of action of the various agents concerned in the production of alcohol and carbon dioxide from sugar in the yeast cell.

I. Effect of Aldehydes and other Reducible Substances on Alcoholic Fermentation.

Oppenheimer [1915] observed that the fermentation of glucose by maceration extract was greatly stimulated by the addition of a pyruvate or pyruvic acid and that acetaldehyde had a similar but less pronounced effect. The estimations were made by weighing at comparatively long intervals, but it was obvious, in the case of acetaldehyde, that the stimulation chiefly occurred at the commencement of the fermentation.

Neuberg somewhat later [1915, 1] observed a similar stimulating action of pyruvates and other *a*-keto-acids on the fermentation of glucose, mannose, fructose, and sucrose and remarked that the activation was most pronounced at the commencement of the fermentation. Experiments continued for 19-20 hours showed little difference in the total fermentation in the presence and absence of pyruvate.

Neuberg subsequently examined the effect of a large number of aldehydes [1918, 1] on alcoholic fermentation and found that they were all vigorous activators. He pointed out that the effect was most marked with glucose and mannose, less so with fructose and sucrose, and suggested that this fact might be related to the observation of Harden and Young [1909] that fructose under certain circumstances can stimulate the fermentation of glucose. The stimulation, like that produced by pyruvate, was most marked at the commencement of fermentation.

As an example of the results observed by Neuberg the effect of cinnamaldehyde, which is one of the more active aldehydes, is given below.

10 c.c. of maceration extract were used + 2 c.c. 5 per cent. glucose solution + 1 c.c. *M*/100 cinnamaldehyde (in 20 per cent. alcohol).

c.c. CO_2 after . .	30 mins.	60 mins.	90 mins.	120 mins.	18 hours.
In presence of aldehyde	6·7 ,,	9·3 ,,	10·9 ,,	11·7 ,,	19·3 ,,
Control . . .	0 ,,	0 ,,	3 ,,	7·5 ,,	17 ,,
Difference . .	6·7	9·3	7·9	4·2	2·3

These experiments have been extended to an immense number of substances [Neuberg and Ehrlich, 1920, 1, 2; Neuberg and Sandberg, 1920; Neuberg, Reinfurth, and Sandberg, 1921], and it has been found that the stimulating effect is a very general one, being produced by almost all reducible substances, whether organic or inorganic. Thus aldehydes (no less than 76 having been tested) thioaldehydes, disulphides, ketones, diketones, quinones, nitro-, nitroso-, hydroxyl- and amino-compounds and colouring matters like methylene blue, together

with such inorganic substances as the thiosulphate, sulphide, tetrathionate and thioantimonate of sodium, colloidal sulphur and selenium, ferric chloride, copper sulphate, ammonium stannichloride, uranyl sulphate and many others all produce the effect. It is remarkable that the power of producing this stimulation extends to the non-fermentable aldoses and ketoses, including those of the triose, pentose, and heptose series, and to many of the polyatomic alcohols, such as erythritol, adonitol, sorbitol, ducitol, and mannitol, and is also possessed by many purine derivatives.

The effect is also shown by living yeast with certain of these substances, but it is much less marked.

Neuberg considers that the stimulation caused by these varied substances is connected with their power of being reduced and acting as acceptors for the hydrogen, which must, according to the pyruvic acid theory, be taken up either by aldehyde or some other substance. It has in fact been shown that in certain cases the activator is used up in the reaction, presumably by being reduced. During normal fermentation a constant low concentration of acetaldehyde is maintained (see p. 135) and is necessary for the continuance of the process [Neuberg and Hirsch, 1919, 3]. It may then well be imagined that at the commencement of the fermentation, when this concentration does not yet exist, but must be produced by the yeast in the act of fermentation, the addition of aldehydes will accelerate the process.

Although this explanation applies to the greater number of the substances which act as stimulators, it is clear that others, such as the polyatomic alcohols do not fall obviously within its limits, and further work is called for in many cases to ascertain what actually happens.

With regard to the nature of this remarkable stimulation experiments have been carried out by Harden and Henley [1920, 1921, 1] to ascertain which of the processes which occur in yeast-juice are chiefly affected. Working with yeast-juice and with zymin ("acetone yeast"), both prepared from English top yeast, they have found that the effect produced by aldehydes or methylene blue is confined to the reaction in presence of phosphate whereas the normal rate of fermentation (p. 71) is not affected. The presence of an aldehyde in a fermenting mixture of yeast-juice and glucose or fructose in which the normal rate of fermentation has been attained causes no acceleration. If now phosphate be added, the maximum rate is attained much more rapidly in presence of the aldehyde than in its absence. An example will make this clear.

25 c.c. of yeast-juice were employed + 1 g. glucose, and to two

of the flasks were added 5 c.c. 1 per cent. aldehyde, to the other two, 5 c.c. water. After the attainment of a steady rate 5 c.c. of 0·3 *M* Na_2HPO_4 were added to two of the flasks and 5 c.c. of water to the others.

Rates of evolution of CO_2 (c.c. in successive periods of 5 min.).

Time (minutes).	With Aldehyde.		Without Aldehyde.	
	Phosphate.	Water.	Phosphate.	Water.
5	29·8	2	5·2	1
10	12·8	2	7·8	3
15, 20	7·4	3·7	28·1	4·2
25, 30	1·2	3·4	7·3	3·7
Total in 30 min. .	51·2	11·1	48·4	11·9

The rate of fermentation and the totals produced in 30 minutes without phosphate are scarcely affected by the presence or absence of aldehyde, whilst in presence of phosphate the aldehyde has greatly accelerated the attainment of the maximum. It is thus seen why Neuberg's effect is always greater towards the beginning of the fermentation, when the phosphate, which occurs so largely in maceration extract, is still present as mineral phosphate. Neuberg's theory of the action of activators can be applied almost without modification to these results. When phosphate is added the opportunity for a much greater rate of reaction (the " phosphate rate ") is afforded, but the necessary hydrogen acceptor must be produced before this can be attained. The addition of an aldehyde (or other activator) provides this and the rate is at once very largely increased. In the absence of added aldehyde, this increase only takes place slowly at the expense of some other acceptor present in the juice or formed from the sugar—probably the precursor of glycerol. In this way the requisite concentration of aldehyde and the corresponding increase in the rate of the reaction are slowly attained.

II. Influence of Concentration of Phosphate on the Course of Fermentation.

When a phosphate is added to a fermenting mixture of a sugar and yeast-juice, the effect varies with the concentration of the phosphate and of the sugar and with the particular specimen of yeast-juice employed. With low concentrations of phosphate in presence of excess of glucose the acceleration produced is so transient that no accurate

measurements of rate can be made. As soon as the amount of phosphate added is sufficiently large, it is found that the rate of evolution of carbon dioxide rapidly increases from five to ten times, and then quickly falls approximately to its original value.

As the concentration of phosphate is still further increased, it is first observed that the maximum velocity, which is still attained not long after the addition of the phosphate, is maintained for a certain period before the fall commences, and then, as the increase in concentration of phosphate proceeds, that the maximum is more gradually attained after the addition, the period required for this increasing with the concentration of the phosphate. Moreover, with still higher concentrations, the maximum rate attained is less than that reached with

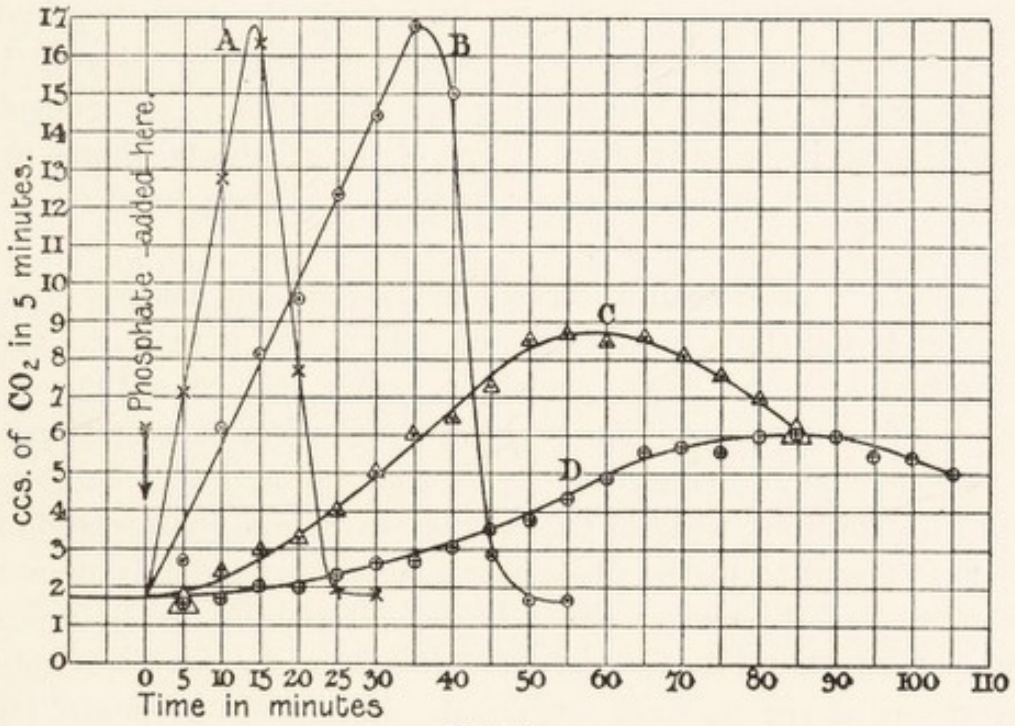


FIG. 7.

lower concentrations, and further, the rate falls off more slowly. The concentration of phosphate which produces the highest rate, which may be termed the optimum concentration, varies very considerably with different specimens of yeast-juice [Harden and Young, 1908, 1].

All these points are illustrated by the accompanying curves (Fig. 7) which show the rate of evolution per five minutes plotted against the time for four solutions in which the initial concentrations of phosphate were (A) 0·033, (B) 0·067, (C) 0·1, and (D) 0·133 molar, the volumes of 0·3 molar phosphate being 5, 10, 15, and 20 c.c. in each case added to 25 c.c. of yeast-juice, and made up to 45 c.c., each solution containing 4·5 grams of glucose. The time of addition is taken as zero, the rate before addition being constant, as shown in the curves.

It will be observed that 5 and 10 c.c. (A and B) give the same maximum, whilst 15 c.c. (C) produce a much lower maximum, and 20 c.c. (D) a still lower one, the rate at which the velocity diminishes after the attainment of the maximum being correspondingly slow in these last two cases. By calculating the amount of phosphate which has disappeared as such from the amount of carbon dioxide evolved, it is found that the maximum does not occur at the same concentration of free phosphate in each case.

These phenomena have been examined by Meyerhof [1918, 3] with interesting results. He finds in the first place that a similar effect on the course of the fermentation in presence of phosphate is exerted by other salts, these acting in the same way as an increase in the phosphate concentration and not only increasing the time required for the attainment of the maximum velocity (termed by Meyerhof the " Gäranstieg ") but also diminishing this maximum. The phosphate, in addition to its specific effect as one of the reactants, shares in this general salt effect, which, however, passes off as the phosphate is converted into hexosephosphate.

These facts have been confirmed by Harden and Henley [1921, 2] who have found that the chlorides of sodium and potassium exert an approximately equal effect, which is rather less than that of the corresponding sulphates. An analysis of the effect of neutral salts on the various enzymes concerned shows that the action of carboxylase is but little affected, whereas that of the hexosephosphatase and of the enzymes concerned in the phosphate reaction are strongly depressed. Moreover, this depressing effect is not altered by the addition of hydrogen acceptor in the form of acetaldehyde and is therefore probably a direct one on the enzymes concerned. Excess of phosphate, on the other hand, while sharing in this general salt effect, also exerts a specific effect which is to a large extent removed by the addition of acetaldehyde and is therefore probably specifically directed against the production from the sugar of the reducible substance which is required to act as acceptor during the attainment of the high velocity of reaction rendered possible by the presence of the phosphate (see p. 150).

As regards the nature of the salt effect little is known. It is possible, as suggested by Buchner for the analogous case of arsenite (p. 159), that the addition of increasing amounts of salt causes a progressive but reversible change in the mode of dispersion of the colloidal enzyme, and that this has the secondary effect of altering the rate of fermentation. No decisive evidence is as yet available upon the subject.

According to Meyerhof [1918, 3] the rate of attainment of the maximum is also greatly affected, in the same way as the period of induction, by the presence of hexosediphosphate. These results were obtained with maceration extract, whereas Harden and Henley [1920] failed to observe a similar phenomenon with their yeast-juice, possibly owing to its greater original content of hexosediphosphate. The cause of the phenomenon is not clear.

III. Reaction of Fructose with Phosphates in Presence of Yeast-Juice.

Although, as has been pointed out (p. 48), glucose, mannose, and fructose all react with phosphate in a similar manner in presence of yeast-juice, there are nevertheless certain quantitative differences between the behaviour of glucose and mannose on the one hand, and fructose on the other, which appear to be of considerable importance. Fructose differs from the other two fermentable hexoses in two particulars : (1) the optimum concentration of phosphate is much greater ; (2) the maximum rate of fermentation attainable is much higher [Harden and Young, 1908, 2 ; 1909].

These points are clearly illustrated by the following results, which all refer to 10 c.c. of yeast-juice and show that the optimum concentration of phosphate for the fermentation of fructose is from 1·5 to 10 times that of glucose, and that the maximum rate of fermentation for fructose in presence of phosphate is 2 to 6 times that of glucose.

Sugar, in Grams.	Total Volume.	Optimum Volume of 0·6 Molar Phosphate in c.c.		Maximum Rate in Cubic Centimetres of CO_2 per Five Minutes.	
		Glucose.	Fructose.	Glucose.	Fructose.
2	35	2	5	7·5	32·2
4	50	1	10	5·4	28·4
1·6	23	2	5	8	17
1	25	1·75	5	5·2	25·9
2	25	5	7·5	16·2	31·2
2	20	2	3·5	7·9	22·6
2	22·5	0·75	2	3·4	22·2

It is interesting to note that the two high rates, 32·2 and 31·2 c.c. per five minutes shown in the table, are equal to about half the rate obtainable with an amount of living yeast corresponding to 10 c.c. of yeast-juice, assuming that about 16 to 20 grams of yeast are required to yield this volume of juice, and that this amount of yeast would give about 56 to 70 c.c. of carbon dioxide per five minutes at 25°, which has

been found experimentally to be about the rate obtainable with the top yeast employed for these experiments.

Even in presence of excess of acetaldehyde this difference between glucose and fructose persists [Harden and Henley, 1921, 1]. Thus, in presence of acetaldehyde and under the optimum conditions of phosphate concentration, glucose gave with zymin a rate of 9 c.c. CO_2 per 5 min., whilst fructose, also under the optimum conditions for its fermentation, gave a rate of 15; with a sample of yeast-juice the corresponding numbers were 21 for glucose and 36 for fructose. It is remarkable that the ratios of these numbers (F/G) are almost constant: $15/9 = 1{\cdot}67$ for zymin; $21/36 = 1{\cdot}7$ for yeast-juice.

These facts point to the conclusion that fructose is specifically more rapidly fermented than glucose, in presence of phosphate. This opens up a wide field of speculation, a number of explanations being possible, between which experiment has not so far decided. Thus it appears possible that glucose may be converted into fructose before being fermented, or that both glucose and fructose undergo a preliminary change into the same intermediate substance, but that fructose is more rapidly changed and is hence more rapidly fermented.

IV. Effect of the Addition of Fructose on the Fermentation of Glucose or Mannose in Presence of a Large Excess of Phosphate.

When the maximum rate of fermentation of glucose or mannose by yeast-juice in presence of phosphate is greatly lowered by the addition of a large excess of phosphate, the addition of a relatively small amount of fructose (as little as 2·5 per cent. of the weight of the glucose) causes rapid fermentation to occur. This induced activity is not due solely to the selective fermentation of the added fructose, since the amount of gas evolved may be greatly in excess of that obtainable from the quantity added.

Another way of expressing the same thing is to say that the optimum concentration of phosphate (p. 151) is greatly raised when 2·5 per cent. of fructose is added to glucose, and that consequently the rate of fermentation rises. The effect is extremely striking, since a mixture of glucose and yeast-juice fermenting in the presence of a large excess of phosphate at the rate of less than 1 c.c. of carbon dioxide in five minutes may be made to ferment at six to eight times this rate by the addition of only 0·05 gram of fructose (2·5 per cent. of the glucose present), and to continue until the total gas evolved is at least five to

six times as great as that obtainable from the added fructose, the concentration of the phosphate being the whole time at such a height as would limit the fermentation of glucose alone to its original value.

The effect is not produced when the concentration of the phosphate

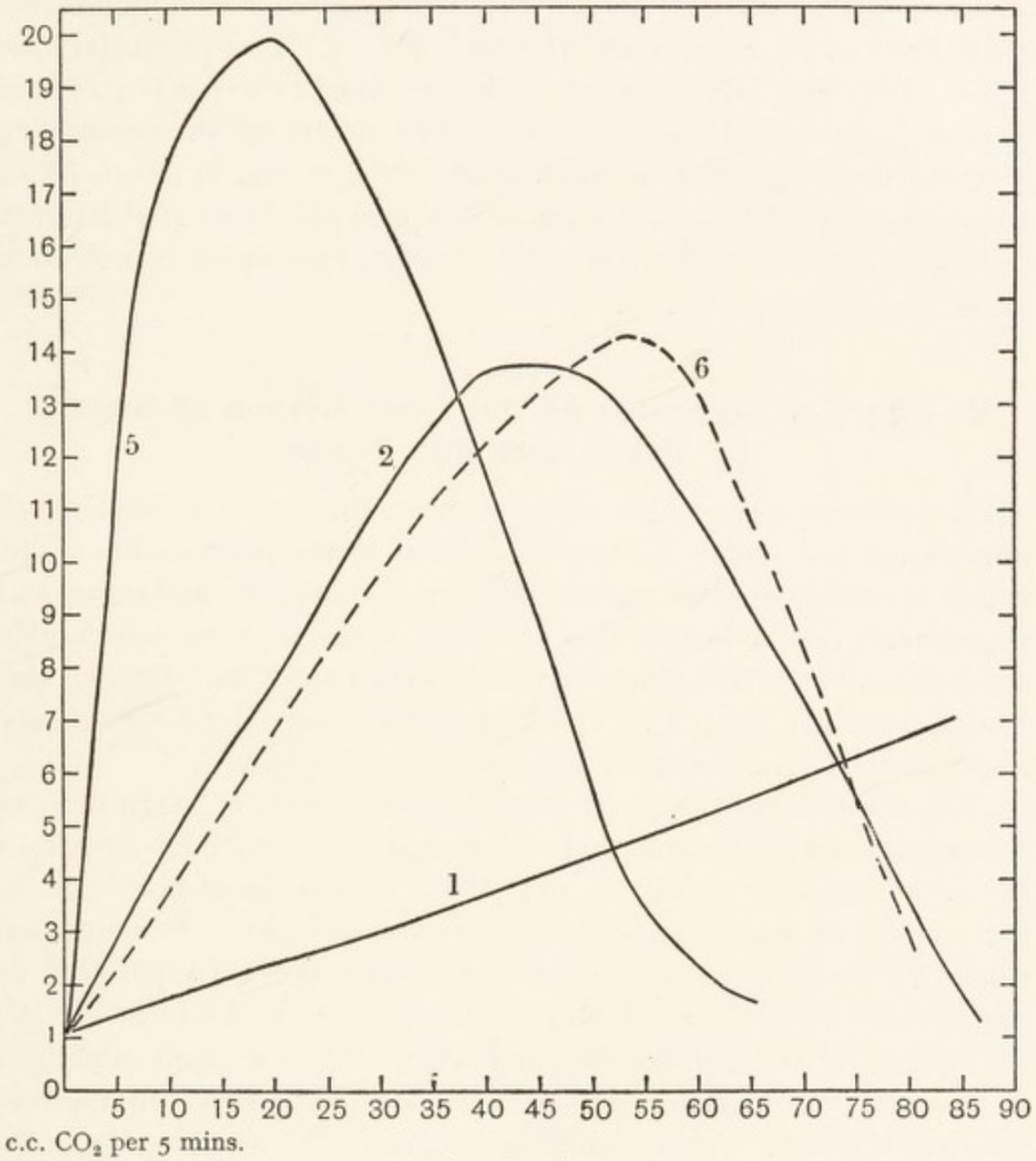


FIG. 8.

(From the Biochemical Journal (1921), **15**, 179. By permission of the Cambridge University Press.)

is so high that the rate of fermentation of fructose is itself greatly lowered.

This remarkable inductive effect is specific to fructose and is not produced when glucose is added to mannose or fructose, or by mannose when added to glucose or fructose, under the proper conditions of concentration of phosphate in each case.

It will be noticed that this effect is precisely like that of acetaldehyde, and direct experiments have shown that almost identical results can be obtained by adding the proper quantities of acetaldehyde or fructose respectively to a mixture of yeast-juice, glucose and such an excess of phosphate that only a slow rate of fermentation is possible (Curve 1, Fig. 8) [Harden and Henley, 1921, 1]. In a particular case it was found that fructose (Curve 6, Fig. 8) equal to 10 per cent. of the glucose present, and amounting to 1 part in 211 of the fermenting mixture, and 1/42,000 of acetaldehyde (Curve 2, Fig. 8) produced an equally rapid rise of rate and induced a typical phosphate reaction. A concentration of acetaldehyde 15 times as great produced a much more rapid rise (Curve 5, Fig. 8).

V. Effect of Arsenates on the Fermentation of Sugars by Yeast-Juice and Zymin.

The close analogy which exists between the chemical functions of phosphorus and arsenic lends some interest to the examination of the action of sodium arsenate upon a mixture of yeast-juice and sugar, and experiments reveal the fact that arsenates produce a very considerable acceleration in the rate of fermentation of such a mixture [Harden and Young, 1906, 3; 1911, 1]. The phenomena observed differ markedly from those which accompany the action of phosphate.

The acceleration produced is of the same order of magnitude as that obtained with phosphate, but it is maintained without alteration for a considerable period, so that there is no equivalence between the amount of arsenate added and the extra amount of fermentation effected. Further, no organic arsenic compound corresponding in composition with the hexosephosphates appears to be formed.

Increase of concentration of arsenate produces a rapid inhibition of fermentation, probably due to some secondary effect on the fermenting complex. An optimum concentration of arsenate therefore exists just as of phosphate, at which the maximum rate is observed, and this optimum concentration and the corresponding rate vary with different samples of juice and are less for glucose than for fructose. The rate of fermentation by zymin is relatively less increased than that by yeast-juice.

Owing to the fact that the rate is permanently maintained the addition of a suitable amount of arsenate increases the total fermentation produced to a much greater extent than phosphate.

The nature of these effects may be gathered from the result of

a few typical experiments. In one case the rate of fermentation of glucose by yeast-juice was raised by the presence of 0·03 molar arsenate from 2 to 23 c.c. per five minutes, and the total evolved in ninety-five minutes from 51 to 459 c.c. The accelerating effect on 20 c.c. of juice of as little as 0·005 c.c. of 0·3 molar arsenate, containing 0·11 mgrm. of arsenic, can be distinctly observed, but the maximum effect is usually produced by about 1 to 3 c.c., the concentration being therefore 0·015 to 0·045 molar. Greater concentrations than this produce a less degree of acceleration accompanied by a shorter duration of fermentation, as shown by the following numbers which refer to 20 c.c. of yeast-juice in a total volume of 40 c.c. containing 10 per cent. of glucose :—

C.c. of 0·3 Molar Arsenate in 40 c.c.	Molar Concentration of Arsenate.	Maximum Rate of Fermentation.
0	0	3·5
0·005	0·0000375	6·3
0·01	0·000075	8
0·02	0·00015	14·2
0·04	0·0003	19·9
0·1	0·00075	29·7
0·2	0·0015	35
0·5	0·00375	34·9
1·0	0·0075	29·5
2·0	0·015	23·2
5·0	0·0375	14·5
10·0	0·075	8·7
15·0	0·1125	5·3
20	0·15	3·2

The contrast between glucose and fructose in their relations to arsenate is well exhibited in the following table, in which the rates of fermentation produced by arsenate in presence of excess of glucose and fructose respectively are given :—

Concentration of Arsenate.	Rate.	
	Glucose.	Fructose.
0·0075 molar	12·1	26·6
0·0225 (opt. for glucose) . .	13·4	—
0·0525 (opt. for fructose) . .	—	45·8
0·1125	5·1	39

Here the optimum concentration for fructose is more than twice that for glucose, whilst the maximum rate of fermentation obtainable

with fructose is between three and four times the maximum given by glucose.

VI. Effect of Arsenites on the Fermentation Produced by Yeast-Juice.

Effects somewhat similar to those produced by arsenates were observed by Buchner [Buchner and Rapp, 1897 ; 1898, 1, 2, 3 ; 1899, 2 ; Buchner, E. and H., and Hahn, 1903, pp. 184-205] when potassium arsenite was added to yeast-juice. This substance, the action of which on yeast had been adduced by Schwann as a proof of the vegetable nature of this organism, was employed by Buchner, on account of its poisonous effect on vegetable cells, as an antiseptic and as a means of testing for the protoplasmic nature of the agent present in yeast-juice. Its effect on the fermentation was, however, found to be irregular, and at the same time it did not act as an efficient antiseptic in the concentrations which could be employed. Even 2 per cent. of arsenious oxide, added as the potassium salt, had in many cases a decided effect in diminishing the total fermentation obtained with sucrose, and this effect increased with the concentration. A number of irregularities were also observed which cannot here be discussed. It was further found that in some cases 2 per cent. of arsenious oxide inhibited the fermentation of glucose but not of sucrose, or of a mixture of glucose and fructose, whilst its effect on fructose alone was of an intermediate character.

The important observation was also made by Buchner that the addition of a suitable quantity of arsenite as a rule caused a greatly increased fermentation during the first sixteen hours even in experiments in which the total fermentation was diminished. By examining the effect of arsenite on fermentation in a similar manner to that of arsenate, Harden and Young [1911, 1] have found that a close analogy exists between the effects and modes of action of these substances, but that arsenite produces a much smaller acceleration than arsenate. An optimum concentration of arsenite exists, just as in the case of arsenate, which produces a maximum rate of fermentation. Further increase in concentration leads to inhibition, and in no case is there any indication of the production of an exactly equivalent amount of fermentation as in the case of phosphate. In various experiments with dialysed, evaporated, and diluted yeast-juice in which 2 per cent. of arsenious oxide was found by Buchner to inhibit fermentation, it is probable that, owing to the small amount of fermenting complex left, this amount

of arsenious oxide was considerably in excess of the optimum concentration, although Buchner ascribes the effect to the removal of some of the protective colloids of the juice, owing to the prolonged treatment to which it had been subjected.

The extent of the action of arsenite appears from the following results. In one case a rate of 1·7 c.c. was increased to 7 c.c. by 0·06 molar arsenite. In another experiment it was found that the optimum concentration was 0·04 molar arsenite, the addition of which increased the rate three-fold. As in the case of arsenate the optimum concentration and the corresponding maximum rate of fermentation are considerably greater for fructose than for glucose. The relative rates produced by the addition of equivalent amounts of arsenate and arsenite (1 c.c. of 0·3 molar solution in each case to 20 c.c. of yeast-juice) were 27·5 and 3·1, the original rate of the juice being 1·7. In general the optimum concentration of arsenite is considerably greater than that of arsenate.

The inhibiting effects of higher concentrations of arsenite and arsenate also present close analogies, but this most interesting aspect of the question has not yet been sufficiently examined to repay detailed discussion. Buchner [Buchner, E. and H., and Hahn, 1903, pp. 199-205] suggested that the inhibition was due primarily to some change in the colloidal condition of the enzyme and showed that certain colloidal substances appeared to protect it, as did also sugar. It seems most probable that the effect is a complex one, in which many factors participate.

Nature of the Acceleration Produced by Arsenate and Arsenite.

In explanation of the remarkable accelerating action of arsenates and arsenites two obvious possibilities present themselves. In the first place the arsenic compound may actually replace phosphate in the reaction characteristic of alcoholic fermentation, the resulting arsenic analogue of the hexosephosphate being so unstable that it undergoes immediate hydrolysis, and is therefore only present in extremely small concentration at any period of the fermentation and cannot be isolated. In the second place it is possible that the arsenic compound may accelerate the decomposition of the hexosephosphate of the juice, and thus by increasing the rate of circulation of the phosphate produce the permanent rise of rate. With this effect may possibly be associated a direct acceleration of the action of the fermenting complex.

The experimental decision between these alternative explanations

is rendered possible by the use of a mixture of enzyme and co-enzyme free from phosphate and hexosephosphate. As has already been described (p. 73) a mixture of boiled yeast-juice, which has been treated with lead acetate, together with glucose or fructose and washed zymin can be prepared which scarcely undergoes any fermentation unless phosphate be added. If now arsenates or arsenites can replace phosphate, they should be capable of setting up fermentation in such a mixture. Experiment shows that they do not possess this power. For fermentation to proceed phosphate must be present and it cannot be replaced either by arsenate or arsenite [Harden and Young, 1911, 1].

If a hexosediphosphate be made the sole source of phosphate in such a mixture as that described above, in which it must be remembered abundance of sugar is present, the rate at which fermentation can proceed will be controlled by the rate at which the hexosediphosphate is decomposed with formation of phosphate. Experiment shows that in the presence of added arsenate or arsenite the rate of fermentation is largely increased, so that the effect of these salts must be to increase the rate of liberation of phosphate.

This conclusion is even more strikingly confirmed by a comparison of the direct action of yeast-juice on hexosediphosphate in presence and in absence of arsenate, as measured by the actual production of free phosphate. In a particular experiment this gave rise to 0·0707 gram of $Mg_2P_2O_7$ in the absence of arsenate and 0·6136 gram of $Mg_2P_2O_7$ in the presence of arsenate.

This increased liberation of inorganic phosphate may be effected in two ways, (*a*) by an increase in the rate of hydrolysis of the compound into hexose and phosphoric acid, and (*b*) by an accelerated fermentation of the compound with production of alcohol, carbon dioxide and phosphoric acid.

Harden and Young expressed the opinion that the former of these processes occurred but the experiments of Meyerhof [1927], Raymond [1928], and Macfarlane [1930] afford strong evidence in favour of the second alternative. They have found that arsenate produces no acceleration in the rate of hydrolysis of hexosediphosphate by yeast preparations free from co-enzyme. On the other hand, when co-enzyme is added rapid fermentation and liberation of phosphoric acid occur, so that it appears that the two processes (enhanced hydrolysis and enhanced fermentation) are under these conditions inseparable.

Macfarlane has further found that a preparation of hexose-phosphatase obtained by the autolysis of dried yeast which was capable of hydrolysing, and, in the presence of co-enzyme, of slowly

fermenting hexosediphosphate, but not sugar, showed no increase in hydrolytic action with arsenate alone, but rapidly fermented hexosediphosphate in presence of both co-enzyme and arsenate.

According to Meyerhof [1927] the effect of arsenate is specific to hexosediphosphate. The fermentation of the hexosemonophosphates (Robison and Neuberg esters) by yeast preparations is accompanied by the conversion of part of the ester into hexosediphosphate (see p. 139), and it is only as this occurs that the accelerating effect of arsenate on the fermentation of these compounds becomes evident.

Arsenates also produce a remarkable effect on the rate of evolution of carbon dioxide during the autofermentation of yeast-juice, etc., or the fermentation of added glycogen. Thus in one case with yeast-juice and added glycogen an initial rate of 1·9 c.c. per 5 minutes was increased by 0·05 *M* arsenate to 10 c.c. and the volume of carbon dioxide evolved in $1\frac{3}{4}$ hours from 32·4 to 141·2 c.c. At the end of this time the rate had fallen to 1·35 in absence of arsenate and 4·45 in its presence. The maximum rate in presence of arsenate (10 c.c. per 5 minutes) falls far short of that attained in presence of sugar, in this case 36 c.c. per 5 minutes.

Since the rate of fermentation of glycogen is probably controlled by the rate of production of a fermentable sugar from it by the action of a glycogenase, this was interpreted by Harden and Young in the sense that the arsenate stimulated not only the hexosephosphatase but also the glycogenase, so that a more rapid supply of glucose from the glycogen was formed, as well as of phosphate from the hexosediphosphate. A study of the phosphate changes during glycogen fermentation has led Meyerhof [1927] to a different explanation, which appears more nearly to represent the true state of affairs. In glycogen fermentation (as explained on p. 143) much more hexosediphosphate is formed relatively to the evolution of CO_2 than in glucose fermentation. Normally this accumulates until the supply of inorganic phosphate is exhausted and is then gradually fermented. When arsenate is added this accumulated hexosediphosphate is rapidly fermented and after this, as the fermentation proceeds, the hexosediphosphate is decomposed as fast as it is produced.

The presence of arsenate does not affect the accelerating action of acetaldehyde on mixtures of yeast-juice and phosphate with glucose or fructose. In the case of zymin (acetone-yeast) and glucose (but not fructose), however, the addition of arsenate produces a much higher rate of fermentation than can be obtained from the optimum mixture of sugar, phosphate, and acetaldehyde [Harden and Henley,

1921, 1]. The reason for this difference between zymin and yeast-juice is not clear.

No other substances have yet been found which share these interesting properties with arsenates and arsenites, and no advance has been made towards an understanding of the mechanism of the accelerating action of these salts on the specific enzymes which are affected by them.

Arsenate has a similar, but less marked, accelerating effect on the production of lactic acid from hexoses in muscle enzyme solution (in presence of hexokinase, p. 74) to that which it has on alcoholic fermentation and also greatly accelerates the decomposition of added hexosediphosphate into lactic acid and an equivalent amount of inorganic phosphate [Meyerhof, 1927].

VII. Effect of Sodium Fluoride on Fermentation by Yeast Preparations.

It has already been pointed out (p. 37) that sodium fluoride has a strongly inhibitory effect on fermentation by yeast-juice; it has been found that this effect is reversible [Lipmann, 1929]. Further study has shown that this substance is highly specific in its action and by its aid much information has been gained as to the changes which occur in alcoholic fermentation and in the production of lactic acid in muscle.

1. *Inhibition of the Action of Phosphatase.*

Both with muscle and yeast preparations sodium fluoride causes a strong inhibition of the hydrolytic action of the phosphatases present [Lipmann, 1928], this having been proved for hexosediphosphate, the Neuberg hexosemonophosphate and glycerophosphate. Using washed preparations free from co-enzyme, the extent of the inhibition varies with different preparations, as much as 50 to 80 per cent. being observed with 0·002 *M* NaF in the case of muscle and 60 to 80 per cent. with 0·1 *M* NaF in the case of washed zymin.

2. *Inhibition of the Phosphorylation and Fermentation of the Hexoses.*

The fermentation and phosphorylation of the hexoses are strongly inhibited by sodium fluoride both with muscle extract in presence of hexokinase (p. 74) and yeast preparations [Meyerhof, 1927; Lipmann, 1928]. Thus with maceration juice acting on glucose 0·025 *M* NaF reduced the fermentation in 2½ hours by 98 per cent. and the esterification by 93 per cent. It will be seen that with yeast preparations the

concentration required to inhibit fermentation is considerably less than that needed to prevent hydrolysis of hexosephosphate.

3. *Effect on the Fermentation of Hexosediphosphate.*

Macfarlane has found [1930] that sodium fluoride inhibits CO_2 production in presence of co-enzyme from hexosediphosphate by a preparation rich in phosphatase in concentrations which hardly affect the rate of hydrolysis. When arsenate is added to this preparation in absence of fluoride rapid production of CO_2 and liberation of inorganic phosphate occur, whereas in presence of fluoride both the enhanced fermentation and liberation of phosphate are greatly inhibited.

4. *Effect of Sodium Fluoride on the Fermentation of Polysaccharides.*

The fermentation of glycogen and other polysaccharides presents many points of difference from that of the hexoses (see p. 142). Notably, more esterification takes place in proportion to the amount of fermentation [Meyerhof, 1927], and esterification can take place in the absence of co-enzyme [Nilsson, 1930, p. 28]. When sodium fluoride is added it is found that the fermentation is strongly inhibited whereas the esterification is much less affected. At the same time the hydrolysis of the resulting hexosephosphate is inhibited as already explained (p. 162). Thus in the case of a fermentation of glycogen by maceration extract it was found that 0·004 *M* NaF reduced the evolution of CO_2 by 70 per cent. and the esterification only by 23 per cent. According to Nilsson [1930] the product of phosphorylation of the glycogen of dried yeast in presence of fluoride is almost exclusively the Robison hexosemonophosphate. Somewhat similar effects are produced by fluoride on the conversion of glycogen to lactic acid by muscle preparations (minced muscle, press juice, and extract). This was first observed by Embden and Zimmermann [1924] who found that in presence of NaF the inorganic phosphate of minced muscle decreased and hexosediphosphate accumulated, the production of lactic acid being at the same time strongly inhibited. These changes are increased by the addition of glycogen but not by that of glucose. Similar phenomena occur in muscle extract not only with glycogen but also with starch and trihexosan [Meyerhof, 1926, 2]. Thus in a muscle extract containing 0·4 per cent. glycogen the amount of lactic acid formed per c.c. in 2 hours was 66 mg. and the amount of sugar esterified 12 mg., whereas in presence of 0·03 *M* NaF only 14 mg. of lactic acid were formed and 67 mg. of sugar were esterified.

5. *Production of Hexosediphosphoric Esters Resistant to Hydrolysis.*

This has already been discussed (p. 59).

Effects similar to those produced by fluorides are also produced by oxalates. They seem to be due to the influence of these salts on the colloidal state of the constituents of the preparations used for the fermentations and not to their well-known de-calcifying properties. They both occupy a position at one end of Hofmeister's lyotropic series [see Meyerhof, 1926, 2].

VIII. Effect of Mono-iodoacetic Acid on Fermentation by Yeast Preparations.

It has been shown by Lundsgaard [1930, 1, 4] that sodium iodoacetate inhibits the formation of lactic acid in muscle. In a muscle contracting in presence of this salt phosphagen is decomposed and the resulting inorganic phosphate esterifies the glycogen (or a sugar derived from it) yielding a hexosephosphate, but no lactic acid is formed.

Added to maceration juice and glucose sodium mono-iodoacetate, like fluoride, inhibits both fermentation and esterification [Lundsgaard, 1930, 2, 3; see also Yamasaki, 1930]. The fermentation of glucose by living yeast is also completely stopped. In addition to this effect the mono-iodoacetate, like fluoride, inhibits the fermentation of hexosediphosphate and the accelerating effect of arsenate on this fermentation at concentrations which have comparatively little effect on the hydrolysis of hexosephosphate [Macfarlane, 1931]. The monobromoacetates act in a similar manner to the iodoacetates. These salts differ from the fluorides in inhibiting the phosphorylation of glycogen in dried yeast during autofermentation [Nilsson, Zeile, and Euler, 1931] although mono-iodoacetate does not inhibit the phosphorylation of glycogen in the autofermentation of yeast-juice [Macfarlane, 1931].

IX. Other Inhibitants and Accelerants.

It is now possible to examine in some detail the effect of inhibitants on the various enzymes which participate in the production of carbon dioxide and alcohol from sugar, e.g. carboxylase, hexosephosphatase, oxido-reductase, etc., as well as on the rate of esterification and rate of evolution of carbon dioxide, but this has hitherto rarely been done.

Cyanides, in addition to prolonging the period of induction with zymin, dried yeast and maceration extract (p. 165), have a slight inhibitory effect on the basal rate, whereas the rate in presence of phosphate is unaltered [Patterson, 1931].

Phenol [Euler and Brunius, 1925, 1926; see also Euler and Westling, 1924] inhibits both esterification and evolution of CO_2 at concentrations of about 0·07 *M* (0·4 g. of dried bottom yeast in 5 c.c. solution containing 0·4 g. glucose); lower concentrations delay the onset of fermentation and lower the rates both of esterification and evolution of carbon dioxide.

An unexplained acceleration has been observed by Abderhalden and Schaumann [1918] when various fractions of an acid extract of yeast are added to yeast-juice.

X. Period of Induction in Alcoholic Fermentation by Zymin, Dried Yeast, and Maceration Extract.

As already mentioned (p. 40) an induction period occurs when zymin or dried yeast is treated with a large volume of sugar solution, the duration of the induction period increasing with the volume of sugar solution added. Further study of the phenomenon [Harden, 1925; Harden and Macfarlane, 1928; Katagiri and Yamagishi, 1929; Raymond and Levene, 1928; Patterson, 1931] has shown that the period of induction can be removed or greatly shortened by the addition of hexosediphosphate, and to a smaller degree by inorganic phosphate or any source from which phosphate can be derived by the action of the yeast enzymes, this being presumably gradually converted into hexosediphosphate. Most salts greatly shorten the period of induction. On the other hand, arsenates and cyanides greatly prolong it. In mixtures each constituent appears to act independently; so that in presence of arsenate, for example, more hexosediphosphate is required than in its absence to reduce the induction period to the same extent. With salts of different metals or acids a hyperbolic relation exists between the relative induction period and the ratio of concentration of the two metal or acid ions [Katagiri and Yamagashi, 1929]. It seems probable that the dilution lowers the concentration of some essential material below the limit at which fermentation can occur. What this substance is and how its concentration can be increased either by the addition of hexosediphosphate or a salt such as sodium acetate are questions to which no satisfactory answer can at present be given. A similar phenomenon is shown by dried yeast.

Some light is thrown on this subject by the observation that during the induction period with zymin the total acid-soluble phosphorus increases, this increase being present as inorganic phosphate. This change is greatly accelerated by salts. The organic phosphorus undergoes little change until the end of the induction period, when

it gradually increases. This is followed by the onset of fermentation, rapid increase of organic phosphorus, decrease of inorganic phosphate and evolution of CO_2.

A similar change occurs in the presence of arsenate, which does not increase the rate of liberation of inorganic phosphate. Hence arsenate does not prolong the induction simply by lowering the concentration of the hexosediphosphate, but must affect some other factor. In presence of cyanide the organic phosphorus decreases until near the end of the induction period.

The induction which occurs when maceration extract is incubated with sugar [Lebedev, 1912, 2] presents certain analogies with that described above, and it is probable that the two phenomena are due to the same cause. It is abolished by the addition of a small concentration of hexosediphosphate [Meyerhof, 1918, 3] and by larger amounts of the other carbohydrate phosphoric esters [Mayer, 1927], and it is prolonged by arsenate or cyanide [Patterson, 1931]; acetate on the other hand does not diminish it, as in the case of zymin, but prolongs it.

Nilsson [1930] has observed that although in the presence of sodium fluoride, co-enzyme and glucose the esterification of phosphoric acid by washed dried yeast is almost completely inhibited, the addition of hexosediphosphate brings about a limited esterification. He suggests that this reaction may play a part in the abolition of the induction period by hexosediphosphate.

No induction period is observed with ordinary yeast-juice, in which autofermentation, accompanied by formation of hexosediphosphate, is constantly in progress. In presence of cyanide, however, yeast-juice also exhibits an induction period. With maceration extract the period is lessened by the addition of sucrose, and by warming the glucose or fructose solution with a neutral phosphate mixture for several hours at 80°. Euler has made the somewhat similar observation that a preliminary partial fermentation of glucose, but not of fructose, by living yeast renders it more readily susceptible of esterification with phosphate [Euler, Ohlsen, and Johanssen, 1917].

CHAPTER IX.

THE BY-PRODUCTS OF ALCHOLIC FERMENTATION.

When pure yeast is allowed to develop in a solution of sugar containing a suitable nitrogenous diet and the proper mineral salts, the liquid at the close of the fermentation contains not only alcohol and some carbon dioxide but also a considerable number of other substances, some arising from the carbonaceous and others from the nitrogenous metabolism of the cell. Prominent among the non-nitrogenous substances which are thus found in fermented sugar solutions are fusel oil, succinic acid, glycerol, acetic acid, aldehyde, formic acid, esters, and traces of many other aldehydes and acids. In addition to these substances which are found in the liquid, there are also the carbonaceous constituents of the newly formed cells of the organism, comprising the material of the cell walls, yeast gum, glycogen, complex organic phosphates, as well as other substances.

The attention of chemists has been directed to these compounds since Pasteur first emphasised their importance as essential products of the alcoholic fermentation of sugar, and his example was generally followed in attributing their origin to the sugar.

The study of cell-free fermentation by means of yeast-juice or zymin has, however, revealed the facts that certain of these substances are not formed in the absence of living cells, and that their origin is to be sought in the metabolic processes which accompany the life of the cell. Their source, moreover, has been traced not to the sugar but to the amino-acids, formed by the hydrolysis of the proteins, which occur in all such liquids as beer wort, grape-juice, etc., which are usually submitted to alcoholic fermentation. This has so far been proved with certainty for the fusel oil and succinic acid, and rendered highly probable for all the various aldehydes and acids of which traces have been detected.

Fusel Oil.

All forms of alcohol prepared by fermentation contain a fraction of high boiling-point, which is termed fusel oil, and amounts to about 0·1 to 0·7 per cent. of the crude spirit obtained by distillation. This

material is not an individual substance, but consists of a mixture of very varied compounds, all occurring in small amount relatively to the ethyl alcohol from which they have been separated. The chief constituents of the mixture are the two amyl alcohols, *iso*amyl alcohol,

$$(CH_3)_2 \cdot CH \cdot CH_2 \cdot CH_2 \cdot OH,$$

and *d*-amyl alcohol,

$$CH_3 \cdot CH(C_2H_5) \cdot CH_2 \cdot OH,$$

which contains an asymetric carbon atom and is optically active. In addition to these, much smaller amounts of propyl alcohol and *iso*-butyl alcohol are present, together with traces of fatty acids, aldehydes, and other substances.

The origin of these purely non-nitrogenous compounds was usually sought in the sugar of the liquid fermented, from which they were thought to be formed by the yeast itself or by the agency of bacteria [Emmerling, 1904, 1905; Pringsheim, 1905, 1907, 1908, 1909], whilst others traced their formation to the direct reduction of fatty acids. Felix Ehrlich has, however, conclusively shown in a series of masterly researches that the alcohols, and probably also the aldehydes, contained in fusel oil are in reality derived from the amino-acids which are formed by the hydrolysis of the proteins.

The close relationship between the composition of leucine,

$$(CH_3)_2 \cdot CH \cdot CH_2 \cdot CH(NH_2) \cdot COOH,$$

and *iso*amyl alcohol,

$$(CH_3)_2 \cdot CH \cdot CH_2 \cdot CH_2 \cdot OH,$$

had previously led to the surmise that a genetic relation might exist between these substances, but the idea had not been experimentally confirmed. In 1903 Ehrlich discovered [1903; 1904, 1, 2; 1907, 2; 1908; Ehrlich and Wendel, 1908, 2] that proteins also yield on hydrolysis an isomeride of leucine known as *iso*leucine, which has the constitution

$$CH_3 \cdot CH(C_2H_5) \cdot CH(NH_2) \cdot COOH,$$

and therefore stands to *d*-amyl alcohol,

$$CH_3 \cdot CH(C_2H_5) \cdot CH_2 \cdot OH,$$

in precisely the same relation as leucine to *iso*amyl alcohol. This suggestive fact at once directed his attention to the problem of the origin of the amyl alcohols in alcoholic fermentation. Using a pure culture of yeast, and thus excluding the participation of bacteria in the change, he found that leucine readily yielded *iso*amyl alcohol, and *iso*leucine

d-amyl alcohol when these amino-acids were added in the pure state to a solution of sugar and treated with a considerable proportion of yeast [1905 ; 1906, 2, 3 ; 1907, 1, 3]. The chemical reactions involved are represented by the following equations :—

(1) $(CH_3)_2 \cdot CH \cdot CH_2 \cdot CH(NH_2) \cdot COOH + H_2O = (CH_3)_2 \cdot CH \cdot CH_2 \cdot CH_2 \cdot OH + CO_2 + NH_3$
Leucine — *iso*-amyl alcohol

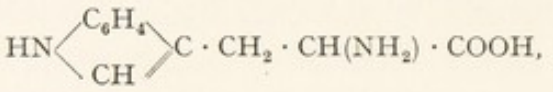
(2) $CH_3 \cdot CH(C_2H_5) \cdot CH(NH_2) \cdot COOH + H_2O = CH_3 \cdot CH(C_2H_5) \cdot CH_2 \cdot OH + CO_2 + NH_3$
iso-leucine — *d*-amyl alcohol

The experiments by which these important changes were demonstrated were of a very simple and convincing character [Ehrlich, 1907, 1]. Two hundred grams of sugar and 3 to 10 grams of the nitrogenous substance to be examined were dissolved in 2 to 2·5 litres of tap-water in a 3 to 4 litre flask ; the liquid was sterilised by being boiled for several hours, and after cooling 40 to 60 grams of fresh yeast were added and the flask was allowed to stand at room temperature until the whole of the sugar had been decomposed by fermentation. In the earlier experiments the amyl alcohols were isolated and identified by conversion into the corresponding valerianic acids, but as a rule the fusel oil as a whole was quantitatively estimated in the filtrate by the Röse-Herzfeld method [Lunge, 1905, p. 571].

The following are typical results. (1) An experiment carried out as above without any addition of leucine gave 97·32 grams of alcohol containing 0·40 per cent. of fusel oil. (2) When 6 grams of synthetic, optically inactive leucine were added, 97·26 grams of alcohol were obtained, containing 2·11 per cent. of fusel oil, which was also optically inactive ; 2·5 grams of leucine were recovered, so that 87 per cent. of the theoretical yield of *iso*amyl alcohol was obtained from the 3·5 grams of leucine decomposed. (3) In the presence of 2·5 grams of *d*-*iso*leucine (prepared from molasses residues), 200 grams of sugar gave 93·99 grams of alcohol, containing 1·44 per cent. of fusel oil, which was lævo-rotatory. This corresponds with 80 per cent. of the theoretical yield of *d*-amyl alcohol from the *iso*leucine added.

This change, which Ehrlich has termed the alcoholic fermentation of the amino-acids, although brought about by living yeast, does not appear to occur at all when zymin [Ehrlich, 1906, 4 ; Pringsheim, 1906] or yeast-juice [Buchner and Meisenheimer, 1906] is substituted for the intact organism, nor is it effected even by living yeast in the absence of a fermentable sugar [Ehrlich, 1907, 1]. The reaction appears indeed to be intimately connected with the nitrogenous metabolism of the cell, and the whole of the ammonia produced is at once assimilated, and does not appear in the fermented liquid. Other α-amino-acids undergo

a corresponding change, and the reaction appears to be a general one. Thus tyrosine, $OH \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, yields *p*-hydroxyphenylethyl alcohol, or tyrosol [Ehrlich, 1911, 1 ; Ehrlich and Pistschimucka, 1912, 2], $OH \cdot C_6H_4 \cdot CH_2 \cdot CH_2OH$, a substance of intensely bitter taste, which was first prepared in this way and is probably one of the most important factors in determining the flavour of beers, etc. Phenylalanine, $C_6H_5 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, in a similar way yields phenylethyl alcohol, $C_6H_5 \cdot CH_2 \cdot CH_2OH$, one of the constituents of oil of roses, whilst tryptophan,



yields tryptophol,

which was also first prepared in this way [Ehrlich, 1912] and has a very faintly bitter, somewhat biting taste.

The extent to which the amino-acids of a medium in which yeast is producing fermentation are decomposed in this sense depends on the amount of the available nitrogen and on the form in which it is present. Thus the addition of ammonium carbonate to a mixture of yeast and sugar was found to lower the production of fusel oil from 0·7 to 0·33 per cent. of the alcohol produced. The addition of leucine alone raised the percentage from 0·7 to 2·78, but the addition of both leucine and ammonium carbonate resulted in the formation of only 0·78 per cent. of fusel oil. The production of fusel oil therefore and the character of the constituents of the fusel oil alike depend on the composition of the medium in which fermentation occurs. This affords a ready explanation of the fact that molasses, which contains almost equal amounts of leucine and *iso*leucine, yields a fusel oil also containing approximately equal amounts of *iso*amyl alcohol and *d*-amyl alcohol [Marckwald, 1902], whilst corn and potatoes, in which leucine preponderates over *iso*leucine, yield fusel oils containing a relatively large amount of the inactive alcohol. The subject is, in fact, one of great interest to the technologist, for as Ehrlich points out " the great variety of the bouquets of wine and aromas of brandy, cognac, arrak, rum, etc., may be very simply referred to the manifold variety of the proteins of the raw materials (grapes, corn, rice, sugar cane, etc.) from which they are derived." Tyrosol and tryptophol have in fact been found to occur in small amounts, chiefly in the form of esters, in beer, wine, and the distillation resdiue from a distillery [Ehrlich, 1917].

Yeast can also form fusel oil at the expense of its own protein, but this only occurs to any considerable extent when the external supply of nitrogen is insufficient. Under these circumstances the amino-acids formed by autolysis may be decomposed and their nitrogen employed again for the construction of the protein of the cell.

The yield is also influenced by the condition of the yeast employed with regard to nitrogen, a yeast poor in nitrogen being more efficacious in decomposing amino-acids than one which is already well supplied with nitrogenous materials. The nature of the carbonaceous nutriment and finally the species of yeast are also of great importance [see Ehrlich, 1911, 2; Ehrlich and Jacobsen, 1911].

A very important characteristic of the action of yeast on the amino-acids is that the two stereo-isomerides of these optically active compounds are fermented at different rates. When inactive, racemic leucine is treated with yeast and sugar, the naturally-occurring component, the *l*-leucine, is more rapidly attacked, so that if the experiment be interrupted at the proper moment the other component, the *d*-leucine, alone is present and may be isolated in the pure state. In an actual experiment 3·8 grams of this component were obtained in the pure state from 10 grams of *dl*-leucine [Ehrlich, 1906, 1], so that the whole of the *l*-leucine (5 grams) had been decomposed but only 1·2 grams of the *d*-leucine. This mode of action has been found to be characteristic of the alcoholic fermentation of the amino-acids by yeast. In all the instances so far observed, both components of the inactive amino-acid are attacked, but usually the naturally occurring isomeride is the more rapidly decomposed, although in the case of β-aminobutyric acid both components disappear at the same rate [Ehrlich and Wendel, 1908, 1]. This reaction therefore must be classed along with the action of moulds on hydroxy-acids [see McKenzie and Harden, 1903], and the action of lipase on inactive esters [Dakin, 1903, 1905], in which both isomerides are attacked but at unequal rates, and differs sharply from the action of yeast itself on sugars [Fischer and Thierfelder, 1894], and of emulsin, maltase, etc., which only act on one isomeride and leave the other entirely untouched.

Succinic Acid.

The origin of the succinic acid formed in fermentation has also been traced by Ehrlich [1909] to the alcoholic fermentation of the amino-acids. It was shown by Buchner and Meisenheimer [1906] that succinic acid like fusel oil was not formed during fermentation by

yeast-juice or zymin, and, in the light of Ehrlich's work on fusel oil, several modes of formation appeared possible for this substance [Ehrlich, 1906, 3]. The dibasic amino-acids might, for example, undergo simple reduction, the NH_2 group being removed as ammonia and replaced by hydrogen. Aspartic acid would thus pass into succinic acid :

$$COOH \cdot CH_2 \cdot CH(NH_2) \cdot COOH + 2H =$$
$$COOH \cdot CH_2 \cdot CH_2 \cdot COOH + NH_3.$$

This change can be effected in the laboratory only by heating with hydriodic acid. Biologically it has been observed [E. and H. Salkowski, 1879] when aspartic acid is submitted to the action of putrefactive bacteria, and almost quantitatively when *Bacillus coli communis* is cultivated in a mixture of aspartic acid and glucose [Harden, 1901]. In this case a well-defined source of hydrogen exists in the glucose, which when acted on by this bacillus yields a large volume of gaseous hydrogen, which is not evolved in the presence of aspartic acid. Some such source is also available in the case of yeast, although it cannot be chemically defined, for this organism is known to produce many reducing actions (see Chapter VI).

A similar action would convert glutamic acid,

$$COOH \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH,$$

into glutaric acid,

$$COOH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH,$$

which also is found among the products of fermentation, whilst the mono-amino-acids would pass into the simple fatty acids.

On submitting these ideas to the test of experiment, however, Ehrlich [1909] found that the addition of aspartic acid did not in any way increase the yield of succinic acid, and that of all the amino-acids which were tried only glutamic acid, $COOH \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, produced a definite increase in the amount of this substance. Further experiments showed that glutamic acid was actually the source of the succinic acid, the relations being quite similar to those which exist for the production of fusel oil.

Succinic acid is formed whenever sugar is fermented by yeast, even in the absence of added nitrogenous matter, and amounts to 0·2 to 0·6 per cent. of the weight of the sugar decomposed, its origin in this case being the glutamic acid formed by the autolysis of the yeast protein. When some other source of nitrogen is present, such as asparagine or an ammonium salt, the amount falls to 0·05 to 0·1. If glutamic acid be added it rises to about 1 to 1·5 per cent., but falls again to about

0·05 to 0·1 when other sources of nitrogen, such as asparagine or ammonium salts, are simultaneously available, either in the presence or absence of added glutamic acid. As in the case of fusel oil, the production does not occur in the absence of sugar, and is not effected by yeast-juice or zymin.

The chemical reaction involved in the production of succinic acid differs to some extent from that by which fusel oil is formed, inasmuch as an oxidation is involved :

$$COOH \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH + 2O = COOH \cdot CH_2 \cdot CH_2 \cdot COOH + NH_3 + CO_2.$$

From analogy with the production of amyl alcohol from leucine, glutamic acid would be expected to yield γ-hydroxybutyric acid :

$$COOH \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH + H_2O = NH_3 + CO_2 + COOH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot OH.$$

As a matter of fact this substance cannot be detected among the products of fermentation, but succinic acid, as already explained, is formed. This acid might, however, possibly be formed by the oxidation of the γ-hydroxybutric acid :

$$COOH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot OH + 2O = COOH \cdot CH_2 \cdot CH_2 \cdot COOH + H_2O,$$

although this change is on biological grounds improbable.

Chemistry of the Conversion of Amino-acids into Alcohols and Carboxylic Acids.

(1) The conversion of the group $-CH(NH_2)-$ into the terminal $CH_2 \cdot OH$ in fusel oil, or COOH in succinic acid, may possibly be effected in several different ways, the most probable of which are the following :

I. Direct elimination of carbon dioxide, followed by hydrolysis of the resulting amine :

$$(1)\ R \cdot CH(NH_2) \cdot COOH = R \cdot CH_2 \cdot NH_2 + CO_2.$$
$$(2)\ R \cdot CH_2 \cdot NH_2 + H_2O = R \cdot CH_2 \cdot OH + NH_3.$$

The reaction (1) is actually effected by many bacteria and has been employed for the preparation of bases from amino-acids [*cf.* Barger, 1914, p. 7], although there is no direct evidence that it can be brought about by yeast. On the other hand, reaction (2) has actually been observed with some yeasts. Thus it has been found [Ehrlich and Pistschimuka, 1912, 1] that many " wild " yeasts produce this change with great readiness in presence of sugar, glycerol, or ethyl alcohol as sources

of carbon and grow well in media in which amines, such as *p*-hydroxyphenylethylamine or *iso*amylamine, form the only source of nitrogen. *Willia anomala* (Hansen) a yeast which forms surface growths, succeeds admirably under these conditions, whereas culture yeasts are much less active in this way, although they produce a certain amount of change. It is therefore possible that this mode of decomposition plays some part in the production of fusel oil, but in the case of culture yeasts it is entirely subordinated to the mode next to be discussed.

II. Oxidative removal of the $-NH_2$ group with formation of an *a*-ketonic acid :—

$$(1)\quad R \cdot CH(NH_2) \cdot COOH + O = R \cdot CO \cdot COOH + NH_3$$

followed by the decomposition of the ketonic acid into carbon dioxide and an aldehyde and the subsequent reduction or oxidation of the aldehyde :—

$$(2)\quad R \cdot CO \cdot COOH = R \cdot CHO + CO_2.$$
$$(3)\ (a)\quad R \cdot CHO + 2H = R \cdot CH_2OH.$$
$$(b)\quad R \cdot CHO + O = R \cdot COOH.$$

The evidence for the occurrence of reaction (1) is supplied by the experiments of Neubauer and Fromherz [1911]. Having previously found that amino-acids undergo a change of this kind in the animal body, Neubauer investigated their behaviour towards yeast. Taking *dl*-phenylaminoacetic acid, $C_6H_5 \cdot CH(NH_2) \cdot COOH$, it was found that the changes produced were essentially the same as in the animal body. The *l*-component of the acid was partly acetylated and partly unchanged, whereas the *d*-component of the acid yielded benzyl alcohol, $C_6H_5 \cdot CH_2 \cdot OH$, phenylglyoxylic acid, $C_6H_5 \cdot CO \cdot COOH$, and the hydroxy-acid $C_6H_5 \cdot CH(OH) \cdot COOH$. Since however this hydroxy-acid was produced in the *l*-form it probably arose by the asymmetric reduction of phenylglyoxylic acid, a reaction which can be effected by yeast as was also found to be the case in the animal body [see Dakin, 1922, pp. 52, 78]. Moreover it was shown that when the effects of yeast on a ketonic acid and the corresponding hydroxy-acid were compared, the alcohol was formed in much better yield from the ketonic acid (70 per cent.) than from the hydroxy-acid (3-4 per cent.), the actual example being the production of tyrosol (*p*-hydroxyphenylethyl alcohol), $OH \cdot C_6H_4 \cdot CH_2 \cdot CH_2OH$, from *p*-hydroxyphenylpyruvic acid, $OH \cdot C_6H_4 \cdot CH_2 \cdot CO \cdot COOH$, and *p*-hydroxyphenyl-lactic acid, $OH \cdot C_6H_4 \cdot CH_2 \cdot CH(OH) \cdot COOH$ respectively.

Neubauer by these experiments established two extremely important points. 1. That the amino-acids actually yield the corresponding *a*-ketonic acids when treated with yeast and sugar solution. 2. That the *a*-ketonic acids under similar conditions give the alcohol containing one carbon atom less in good yield, whereas the corresponding hydroxy-acids only give an extremely small amount of these alcohols.

It is therefore probable that at an early stage in the decomposition of the amino-acids by yeast a ketonic acid is produced, which then undergoes further change.

The source of the oxygen required for this reaction and the mechanism of oxidation have not yet been definitely ascertained. It is possible that hydrated imino-acids of the type $R \cdot C(OH)(NH_2) \cdot COOH$ are first formed [Knoop, 1910], but these have not as yet been isolated.

The spontaneous production of ketonic aldehydes from amino-acids and from hydroxy-acids in aqueous solution, which has been demonstrated by Dakin and Dudley [1913], points to the possibility that the ketonic acid may be a secondary product derived from the corresponding ketonic aldehyde [see also Dakin, 1908; Neuberg, 1908, 1909]. This itself may either arise directly from the amino-acid or from a previously formed hydroxy-acid, the latter alternative being, however, improbable in view of the small yield of alcohol obtained from hydroxy-acids by the action of yeast in the experiments of Neubauer and Fromherz.

$$\begin{array}{ccc} R \cdot CH(NH_2) \cdot COOH & \rightarrow & R \cdot CH(OH) \cdot COOH \\ & \searrow \quad \swarrow & \\ & R \cdot CO \cdot CHO & \\ & \downarrow + \text{Oxygen} & \\ & R \cdot CO \cdot COOH. & \end{array}$$

(2) Whatever be the exact mode by which the ketonic acid is produced, it appears most probable that a compound of this nature forms the starting-point for the next stage in the production of the alcohols. The researches of Neuberg, which have already been discussed in Chapter V, have revealed a mechanism in yeast—the enzyme carboxylase—by which these *a*-ketonic acids are rapidly broken up into an aldehyde and carbon dioxide :—

$$R \cdot CO \cdot COOH = R \cdot CHO + CO_2$$

and it can scarcely be doubted that this is the actual course of the reaction.

(3) The final conversion of the aldehyde into the corresponding alcohol is also a change which it has been proved can be effected by yeast, probably by the aid of the dehydrogenase which is one of the weapons in its armoury of enzymes, and has already been discussed in Chapter VI.

A further possibility exists that in some cases the aldehyde may undergo dismutation or be simultaneously oxidised and reduced, or the molecule of one aldehyde reduced and that of another oxidised with production of the corresponding acid and alcohol by an "aldehydomutase," similar to that which has been observed by Parnas [1910] in many animal tissues.

The intermediate production of an aldehyde would thus be consistent with the production of both alcohols and acids from amino-acids.

Fusel oil would be formed by the reduction of the aldehydes arising from the simple monobasic amino-acids, succinic acid would be produced by oxidation of the aldehyde derived from the dibasic glutamic acid.

In favour of this view is to be adduced the fact that aldehydes such as *iso*butyraldehyde and valeraldehyde have been found in crude spirit, whilst acetaldehyde is a regular product of alcoholic fermentation [see Ashdown and Hewitt, 1910]. Benzaldehyde, moreover, has been actually detected as a product of the alcoholic fermentation of phenylaminoacetic acid, $C_6H_5 \cdot CH(NH_2) \cdot COOH$ [Ehrlich, 1907, 1] Further, the aldehydes so produced would readily pass by oxidation into the corresponding fatty acids, small quantities of which are invariably produced in fermentation.

This view of the nature of the alcoholic fermentation of the amino-acids is undoubtedly to be preferred to that previously suggested by Ehrlich [1906, 3] according to which a hydroxy-acid is first formed and then either directly decomposed into an alcohol and carbon dioxide or into an aldehyde and formic acid, the aldehyde being reduced and the formic acid destroyed.

$$\begin{array}{l} R \cdot CH(NH_2) \cdot COOH \rightarrow R \cdot CH(OH) \cdot COOH \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \text{or} \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \qquad\quad \searrow \\ \qquad\qquad\qquad\qquad R \cdot CH_2OH + CO_2 \quad R \cdot CHO + H \cdot CO_2H \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad R \cdot CH_2OH. \end{array}$$

The most probable course of the decompositions by which *iso*-amyl alcohol and succinic acid are produced from leucine and glutamic acid respectively is therefore the following :—

(*a*) iso*Amyl Alcohol.*

(1) $(CH_3)_2 \cdot CH \cdot CH_2 \cdot CH(NH_2) \cdot COOH$
Leucine

(2) $(CH_3)_2 \cdot CH \cdot CH_2 \cdot CO \cdot COOH$
a-Keto-*iso*valerianic acid

(3) $(CH_3)_2 \cdot CH \cdot CH_2 \cdot CHO + CO_2$
*iso*valeraldehyde

(4) $(CH_3)_2 \cdot CH \cdot CH_2 \cdot CH_2OH$
*iso*amyl alcohol

(*b*) *Succinic Acid.*

(1) $COOH \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$
Glutamic acid

(2) $COOH \cdot CH_2 \cdot CH_2 \cdot CO \cdot COOH$
a-Ketoglutaric acid

(3) $COOH \cdot CH_2 \cdot CH_2 \cdot CHO + CO_2$
Succinic semialdehyde

(4) $COOH \cdot CH_2 \cdot CH_2 \cdot COOH$
Succinic acid

In the special case of succinic acid, evidence strongly confirmatory of this view has been obtained by Neuberg and Ringer [1915, 1, 2 ; 1918, 2] who have found that both *a*-ketoglutaric acid and *β*-aldehydopropionic acid (succinic semialdehyde) are converted by yeast into succinic acid. This transformation is also quantitatively effected by maceration extract (best in the presence of sugar), which, as is well known, does not bring about the characteristic alcoholic fermentation of the amino-acids. It appears therefore that the primary conversion of the amino-acids into ketonic-acids, a change probably accompanied by assimilation of the nitrogen, can only be effected by living, actively fermenting yeast, whereas the subsequent changes occur under the influence of the enzymes which are present in maceration extract, etc. The oxidation of the aldehyde to succinic acid takes place in absence of free oxygen and the source of this oxygen is not yet known.

It is interesting to note that bacteria are also capable of producing succinic acid from *a*-ketoglutaric acid, but it must be remembered that in all probability succinic acid can also be directly produced by many bacteria, e.g. *B. coli communis*) from sugar, as the amount formed (20 per cent. or even more of the sugar) is greatly in excess of that obtainable from the available glutamic acid.

The effect of Ehrlich's work has been clearly to distinguish the chemical changes involved in the production of fusel oil and succinic acid from those concerned in the decomposition of sugar into alcohol and carbon dioxide, and to bring to light a most important series of reactions by means of which the yeast-cell is able to supply itself with nitrogen, one of the indispensable conditions of life.

Glycerol.

Of the three chief by-products of alcoholic fermentation, only glycerol remains at present referable directly to the sugar. This sub-

stance, as shown by the careful experiments of Buchner and Meisenheimer [1906], is formed by the action both of yeast-juice and zymin to the extent of 3·8 per cent. of the sugar decomposed. Glycerol, the origin of which was long debated, is now known by the researches of Neuberg and his colleagues to be derived from the sugar, and the mode and conditions of its formation have already been discussed in Chapter VII.

CHAPTER X.

THE MECHANISM OF FERMENTATION.

The analysis of the process of alcoholic fermentation by yeast-juice and other preparations from yeast which has been carried out in the preceding chapters has shown that the phenomenon is one of a very complex character. Buchner's zymase can no longer be regarded as a single enzyme, but comprises a whole battery of enzymes, including carboxylase, a dehydrogenase, and hexosephosphatase as well as the enzyme or enzymes which bring about the primary changes in the sugar, and the co-enzyme. The substrate consists of sugar, phosphate and the hexosephosphates formed from these. During autofermentation two other factors are involved, the complex carbohydrates of the juice, including glycogen and dextrins, and the diastatic ferment by which these are converted into fermentable sugars. Under special circumstances the rate at which fermentation proceeds may be controlled by the available amount of any one of these numerous factors.

When the juice from well-washed yeast is incubated, the phenomenon of autofermentation is observed. The juice contains an abundant supply of enzyme, co-enzyme, and phosphate or hexosephosphate, and in this case the controlling factor is usually the supply of sugar, which is conditioned by the concentration of the diastatic enzyme or of the complex carbohydrates. When this is the case the measured rate of fermentation cannot be greater than is the rate at which sugar is being produced in the juice. If sugar be now added, an entirely different state of affairs is set up. As soon as any accumulated phosphate has been converted into hexosephosphate, the normal rate of fermentation, which is usually higher than that of autofermentation, is attained, and, provided that excess of sugar be present, fermentation continues for a considerable period at a slowly diminishing rate and finally ceases. During the first part of this fermentation the rate is controlled entirely by the supply of free phosphate, and this depends mainly on the concentration of the hexosephosphatase and of the hexosephosphate, and only in a secondary degree on the decomposition of other phosphorus compounds by other enzymes and on the

concentration of the sugar. The amount of hexosephosphate in yeast-juice is usually such that an increase in its concentration does not greatly affect the rate of fermentation, and hence the measured rate during this period represents the rate at which hexosephosphate is being decomposed, and this in its turn depends on the concentration of hexosephosphatase, or the enzyme which ferments hexosephosphate directly (p. 141), which is therefore the controlling factor. As fermentation proceeds, the concentration of both enzyme and co-enzyme steadily diminishes, as already explained, probably owing to the action of other enzymes, so that at an advanced stage of the fermentation, the controlling factor may be the concentration of either of these, or the product of the two concentrations (see p. 181). The hexosephosphatase appears invariably to outlast the enzyme and co-enzyme. The condition at any moment could be determined experimentally if it were possible to add enzyme, co-enzyme and hexosephosphatase at will and so ascertain which of these produced an acceleration of the rate.

Unfortunately this can at present be only very imperfectly accomplished, owing to the impossibility of separating these factors from each other and from accompanying matter which interferes with the interpretation of the result.

A third condition can also be established by adding to the fermenting mixture of the juice and sugar a solution of phosphate. The supply of phosphate is now almost independent of the action of the hexosephosphatase, and the measured rate represents the rate at which reactions (I), *a* and *b*, and II, p. 139, occur between sugar and phosphate in the presence of the fermenting complex consisting of enzyme and co-enzyme. This change is controlled, so long as sugar and phosphate are present in the proper amounts, by the concentration of the fermenting complex or possibly of either the enzyme or the co-enzyme. If only a single addition of a small quantity of phosphate be made, the rate falls as soon as the whole of this has been converted into hexosephosphate and the reaction then passes into the stage just considered, in which the rate is controlled by the production of free phosphate.

Although these varying reactions have not yet been exhaustively studied from the kinetic point of view, owing to the experimental difficulties to which allusion has already been made, investigations have nevertheless been carried out on the effect of the variation of concentration of yeast-juice and zymin as a whole, as well as of the carbohydrate. Herzog [1902, 1904] has made experiments of this kind with zymin, and Euler [1905] with yeast-juice, whilst many of the

results obtained by Buchner and by Harden and Young are also available.

The actual observations made by these authors show that the initial velocity of fermentation is almost independent of the concentration of sugar within certain limits, but decreases slowly as the concentration increases. When the velocity constant is calculated on the assumption that the reaction is unimolecular [see Haldane, 1930], approximate constancy is found for the first period of the fermentation. This method of dealing with the results is, however, as pointed out by Slator, misleading, the apparent agreement with the law of unimolecular reactions being probably due to the gradual destruction of the fermenting complex.

Experiments with low concentrations of sugar are difficult to interpret, the influence of the hydrolysis of glycogen and of dextrins on the one hand, and the synthesis of sugar to more complex carbohydrates on the other (p. 32), having a relatively great effect on the concentration of the sugar. Unpublished experiments (Harden and Young) indicate that the velocity of fermentation remains approximately constant, until a certain very low limit of sugar concentration is reached, and then falls rapidly. The fall in rate only continues over a small interval of concentration, after which the velocity again becomes approximately constant and equal to the rate of autofermentation. In other words, the rate of fermentation of sugar by yeast-juice and zymin is not proportional to the concentration of the sugar present as required by the law of mass, but, above a certain low limit of sugar concentration, is independent of this and is actually slightly decreased by increase in the concentration of the sugar.

The relations here are very similar to those which have been shown to exist in the case of many enzymes [see Haldane, 1930].

The results of the experiments with yeast-juice therefore indicate that what is being measured is a typical enzyme action, but afford no information as to which of the many possible actions is the controlling one, a fact which must be ascertained for each particular case in the manner indicated above.

Clowes [1909], using washed zymin free from fermenting power and adding various volumes of boiled yeast extract, found that the velocity of reaction was proportional to the product of the concentrations of zymin and yeast extract up to a certain optimum concentration. He interprets these concentrations as representing the concentrations of zymase and co-enzyme, but they also represent the concentrations of hexosephosphatase (present in the zymin) and

phosphate (present in the yeast extract), so that at least four factors were being altered instead of only two.

In a series of kinetic experiments Euler and Kullberg [1911, 3] found that the conversion of phosphate into hexosephosphate in presence of excess of glucose proceeded according to a unimolecular reaction, the temperature coefficient being 1·75 for 10° C.

The rate of fermentation is diminished by dilution of the yeast-juice, but less rapidly than the concentration of the juice. Herzog found that when the relation between concentration of enzyme and the velocity constant of the reaction is expressed by the formula $k_1/k_2 = (C_1/C_2)n$ where k_1 and k_2 are the velocity constants corresponding with the enzyme concentrations C_1 and C_2, the value for n is 2 for zymin, whilst Euler working with yeast-juice obtained values varying from 1·29 to 1·67 and decreasing as k increased.

As, however, the exact significance of k in these ratios is doubtful (see p. 180), that of n is also uncertain.

The temperature coefficient of fermentation by zymin was found by Herzog to be $Q_{24\cdot5/14\cdot5^\circ} = 2\cdot88$, which agrees well with the value found by Slator for yeast-cells (p. 190).

Since Herzog measured the basal rate of fermentation, this coefficient probably applies to the enzyme or enzymes concerned in the liberation of phosphate from hexosephosphate.

Fermentation in the Yeast Cell.

When we endeavour to apply the results of the investigations of the fermentation of sugar by yeast-juice, zymin, etc., to the process which goes on in the living cell, considerable difficulties present themselves. A scheme of fermentation in the living cell can, however, easily be imagined, which is in harmony with these results. According to the most simple form of this ideal scheme, the sugar which has diffused into the cell unites with the fermenting complex and undergoes the characteristic reaction with phosphate, already present in the cell, yielding carbon dioxide, alcohol, and hexosephosphate. The latter is then decomposed, just as it is in yeast-juice, but more rapidly, and the liberated phosphate again enters into reaction, partly with the sugar formed from the hexosephosphate and partly with fresh sugar supplied from outside the cell. The main difference between fermentation by yeast-juice and by the living cell would then consist in the rate of decomposition of the hexosephosphate, for it has been shown that yeast-juice in presence of sufficient phosphate can ferment sugar at a rate

of the same order of magnitude (from 30 to 50 per cent.) as that attained by living yeast.

The difference between the two therefore would appear to lie not so much in their content of fermenting complex as in their very different capacity for liberating phosphate from hexosephosphate and thus supplying the necessary conditions for fermentation.

A simple calculation based on the phosphorus content of living yeast [Buchner and Haehn, 1910, 2] shows that the whole of this phosphate must pass through the stage of hexosephosphate every five or six minutes in order to maintain the normal rate of fermentation, whereas in an average sample of yeast-juice the cycle, calculated in the same way, would last nearly two hours.

Wherein this difference resides is a difficult question, which cannot at present be answered with certainty.

In the first place it must be remembered that a very great acceleration of the fermentation of the hexosephosphate is produced by arsenates (p. 160), and this suggests the possibility that some substance possessing a similar accelerating power is present in the yeast-cell and is lost or altered in the various processes involved in rendering the yeast susceptible to phosphate. The great variety of these processes—extraction of yeast-juice by grinding and pressing, drying and macerating, heating, treating with acetone and with toluene—renders this somewhat improbable, and so far no such substance has been detected.

A comparison of living yeast, zymin, and yeast-juice shows that these are situated on an ascending scale with respect to their response to phosphate. Taking fructose as the substrate in each case, yeast does not respond to phosphate at all (Slator), the rate of fermentation by zymin is approximately doubled (p. 46), and that by yeast-juice increased ten to forty times, whilst the maximum rates are in each case of the same order of magnitude.

The high rate of fermentation by living yeast and its lack of response to phosphate may possibly be explained by supposing that the balance of enzymes in the living cell is such that the supply of phosphate is maintained at the optimum, and the rate of fermentation cannot therefore be increased by a further supply.

A further difference lies in the fact that yeast-juice and zymin respond to phosphate more strongly in presence of fructose than of glucose, whereas yeast ferments both sugars when separately prescribed to it in excess at the same rate (see p. 193). It seems possible that these differences are associated with the gradual passage from the complete living cell of yeast, through the dead and partially disorganised

cell of zymin to yeast-juice, in which the last trace of cellular organisation has disappeared and the contents of the cell are uniformly diffused throughout the liquid.

In confirmation of this it has been found [Harden and Macfarlane, 1930] that when yeast is ground the rate of fermentation of the mass diminishes approximately in proportion to the duration of the grinding and at the same time the mass acquires the property of responding to phosphate (see Fig. 9).

Some light is thrown on these interesting problems by the effect of antiseptics on fermentation by yeast-cells and by yeast-juice. The action of toluene has hitherto been most completely studied, and this substance is an extremely suitable one for the purpose since it has practically no action whatever on fermentation by yeast-juice. The experiments of Buchner have, in fact, shown that the normal rate of fermentation and the total fermentation produced, are almost unaffected by the presence of toluene even in the proportion of 1 c.c. to 20 c.c. of yeast-juice. [See also on this point Buchner and Skraup, 1917; Euler and Kullberg, 1911, 2.] What then is the effect of toluene on the living yeast-cell? When toluene in large excess is agitated with a fermenting mixture of yeast and sugar, the rate of fermentation falls rapidly at first and then more slowly until a relatively constant rate is attained which gradually decreases in a similar manner to the rate of fermentation by yeast-juice. Thus at air temperature (16°) 10 grams of yeast suspended in 50 c.c. of 6 per cent. glucose solution gave the following results when agitated with toluene (see table, p. 185).

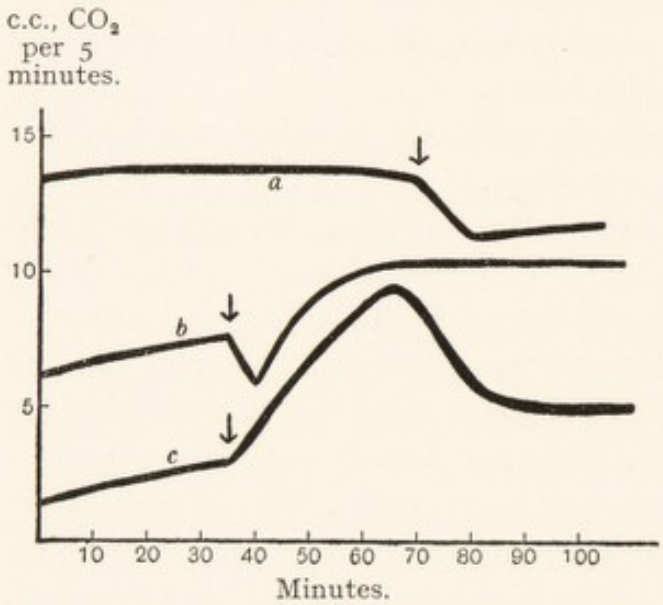


FIG. 9.—Fermentation of yeast after grinding. *a*, 2 g. yeast. *b*, 2 g. yeast ground 20 minutes. *c*, 2 g. yeast ground 60 minutes. ↓ marks addition of 1·2 c.c. *M* K_2HPO_4. (From the Biochemical Journal, **24**, 344. By permission of the Biochemical Society.)

Simultaneously with this, the yeast acquires the property of decomposing and fermenting hexosephosphate and of responding to the addition of phosphate. This last property is only acquired to a small degree in this way, but it becomes much more strongly developed if the pressed yeast be washed with toluene on the filter pump. Thus 10

Time after Addition of Toluene. Minutes.	C.c. of CO_2 per Minute.	Time.	C.c. per Minute.
0	4·6	6	1·6
1	4	8	1·2
2	3·3	12	0·85
3	2·6	24	0·8
4	2	32	0·5
5	1·8		constant

grams of yeast after this treatment fermented fructose at 1·2 c.c. per three minutes; after the addition of phosphate (5 c.c. of 0·6 molar phosphate) the rate rose to 6·9 and then gradually fell in the typical manner [Harden, 1911]. This reaction was in fact adopted by Euler and Johansson [1912, 2] for the preparation of hexosephosphate.

Benzene, xylene, ether, ethyl butyrate, carbon disulphide and light petroleum [Kerr and Young, 1926; Harden and Macfarlane, 1930] all act like toluene, whereas some other antiseptics, *e.g.* formaldehyde, phenol, pyridine, etc., inhibit fermentation without rendering the yeast capable of responding to phosphate. It appears that in these latter cases the fermenting complex itself is attacked and not merely the mechanism for liberating phosphate as in the former.

An explanation of the great decrease in rate of fermentation attending the action of toluene and other antiseptics on living yeast, and following upon the disintegration of the cell, which has been entertained, is that in living yeast the high rate of fermentation is maintained by the continued production of relatively large fresh supplies of fermenting complex, and that when the power of producing this catalytic agent is destroyed by the poison, the rate of fermentation falls to a low value, corresponding to the store of zymase still present in the cell [*cf.* Buchner, E. and H., and Hahn, 1903, pp. 176, 180].

This explanation implies that the rate of fermentation after the action of the toluene represents the amount of fermenting complex present, a supposition which has been shown (p. 182) to be highly improbable. It further necessitates, as also pointed out independently by Euler and Ugglas [1911], a rapid destruction of the fermenting complex both in the process of fermentation and by the action of the antiseptic, as otherwise the store of zymase remaining in the dead cell would be practically the same as that contained in the living cell at the moment when it was subjected to the antiseptic, and this store would therefore suffice to carry out fermentation at the same rate in the dead as in the living cell. No such rapid destruction, however, occurs in yeast-juice, as judged by the rate of fermentation, which falls off slowly and to

about the same extent in the presence or absence of toluene. Moreover, as shown above, it is highly probable that the actual amount of fermenting complex in yeast-juice is a large fraction of that present at any moment in the cell, and is capable under suitable conditions of producing fermentation at a rate comparable with that of the living cell.

This last criticism also applies to the view expressed by Euler [Euler and Ugglas, 1911 ; Euler and Kullberg, 1911, 1, 2] that in the living cell the zymase is partly free and partly combined with the protoplasm ; when the vital activity of the cell is interfered with, the combined portion of the zymase is thrown out of action and only that which was free remains active.

The suggestion made by Rubner [1913] that the action of yeast on sugar is in reality chiefly a vital act, but that a small proportion of the change is due to enzyme action, is similar in its consequences to that of Euler and may be met by the same arguments. Buchner and Skraup [1914, 1917] have moreover shown that the effects of sodium chloride and toluene on the fermenting power of yeast which were observed by Rubner can be explained in other ways.

Some other explanation must therefore be sought for this phenomenon. Great significance must be attached in this connection to the relation noted above between the degree of disintegration and disorganisation of the cell and the fall in the normal rate of fermentation. It seems not impossible that fermentation may be associated in the living cell with some special structure, or carried on in some special portion of the cell, perhaps the nuclear vacuole described by Janssens and Leblanc [1898], Wager [1898, 1911 ; Wager and Peniston, 1910] and others, which undergoes remarkable changes both during fermentation and autofermentation [Harden and Rowland, 1910]. The disorganisation of the cell might lead to many modifications of the conditions, among others to the dilution of the various catalytic agents by diffusion throughout the whole volume of the cell. As a matter of observation the dilution of yeast-juice leads to a considerable diminution of the rate of fermentation of sugar, and it is possible that this is one of the chief factors concerned. That phenomena of this kind may be involved is shown by the remarkable effect of toluene on the autofermentation of yeast [Harden and Paine, 1912]. Whereas the fermentation of sugar is greatly diminished by the action of toluene, the rate of autofermentation, which is carried on at the expense of the glycogen of the cell, is greatly increased. In a typical case, for example, the autofermentation of 10 grams of yeast suspended in 20 c.c. of water amounted to 28 c.c. in 4·8 hours at 25°, whereas the same

amount of yeast in presence of 2 c.c. of toluene gave 97·6 c.c. in the same time.

Many salts produce a similar effect on English top yeasts (in which the autofermentation is large) whereas Neuberg and Karczag in Berlin [1911, 2] were unable to observe this phenomenon.

It is significant that the substances which produce this effect all plasmolyse the yeast cell, whereas urea, which produces no plasmolysis, is inactive.

A necessary preliminary of the fermentation of glycogen is its conversion by a diastatic enzyme into a fermentable sugar, and it is probable that the effect of the disorganisation of the cell by toluene is that this enzyme finds more ready access to the glycogen, which is stored in the plasma of the cell. No such acceleration of autofermentation is effected by the addition of toluene to yeast-juice, and hence the result is not due to an acceleration of the action of the diastatic enzyme on the glycogen.

This effect of toluene is similar in character to the action of anæsthetics on the leaves of many plants containing glucosides and enzymes, whereby an immediate decomposition of the glucoside is initiated [see H. E. and E. F. Armstrong, 1910].

Although as indicated above Euler's theory cannot apply to zymase itself, if applied to the enzymes responsible for the decomposition of hexosephosphate it would afford a consistent explanation of the facts. According to this modified view it would be these enzymes of yeast which existed largely in the combined form, so that in extracts, in dried yeast and in presence of toluene only the small fraction which was free would remain active. It may here be noted that Euler has in fact found that these enzymes are more susceptible to the action of toluene than the other yeast enzymes [Euler and Johansson, 1912, 2]. The zymase on the other hand would have to be regarded as existing to a large extent in the free state so that it would pass into extracts comparatively unimpaired in amount and capable under proper conditions (*i.e.* when supplied with sufficient phosphate) of bringing about a very vigorous fermentation. Further investigation of this question is required.

An entirely different standpoint was taken by Neuberg [1920, 1]. He had found that many yeasts did not produce hexosephosphate in the " fresh " state (i.e. when the yeast is brought into the sugar and phosphate solution in presence of a small amount of toluene, 0·2 c.c. of which was usually employed for 6 g. of yeast in 20 c.c. of medium), whereas these same yeasts when dried gave a quantitative yield. He argued

from this that the production of this compound was not necessarily involved in the fermentation of sugar under normal conditions, but was a pathological phenomenon which only appeared when the cell was placed under abnormal conditions. Weichherz and Nord [1930] on theoretical grounds also express the opinion that hexosephosphates do not play an essential part in fermentation by the living yeast-cell.

Whilst it is admitted that different yeasts vary in regard to their response to phosphate in presence of small quantities of toluene [see Euler, 1918; Euler and Heintze, 1918] it appears highly improbable that Neuberg's view should be correct and it cannot be accepted until more experimental evidence has been obtained of the actual changes which occur in the early stages of alcoholic fermentation.

Fermentation by Living Yeast.

Much important information as to the nature of the processes involved in fermentation has been acquired by the direct experimental study of the action of living yeast on different sugars.

This phenomenon has formed the subject of several investigations from the kinetic point of view, and its general features may now be regarded as well established.

The difficulty, which must as far as possible be avoided in quantitative experiments of this sort with living yeast, is the alteration in the amount or properties of the yeast, due to growth or to some change in the cells. This has been obviated in the work of Slator [1906] by determining in every case the initial rate of fermentation, so that the process only continues for a very short period, during which any change in the amount or constitution of the yeast is negligible. The method has the further advantage that interference of the products of the reaction is to a large extent avoided. The pressure apparatus already described (p. 29) was employed by Slator, the rate of production of carbon dioxide being measured by the increase of pressure in the experimental vessel.

Influence of Concentration of Glucose on the Rate of Fermentation.

With regard to this important factor it is found that the action of living yeast follows the same law as that of most enzymes (p. 181): within certain wide limits the rate of fermentation is almost independent of the concentration of the sugar. This conclusion has been drawn by many previous investigators from their experiments [Dumas, 1874;

Tammann, 1889; Adrian Brown, 1892; O'Sullivan, 1898, 1899] and is implicitly contained in the results of Aberson [1903], although he himself regarded the reaction as unimolecular.

Slator, working with a suspension of ten to twelve yeast-cells per 1/4000 cubic millimetre at 30°, obtained the results which are embodied in the curve (Fig. 10).

This shows that, for the amount of yeast in question, the rate of fermentation is almost constant for concentrations of glucose between 1 and 10 grams per 100 c.c., but gradually decreases as the concentration increases. Below 1 gram per 100 c.c. the rate decreases very rapidly with the concentration.

It follows from this, in the light of what has already been said (p. 181), that the action of living yeast on sugar follows the same

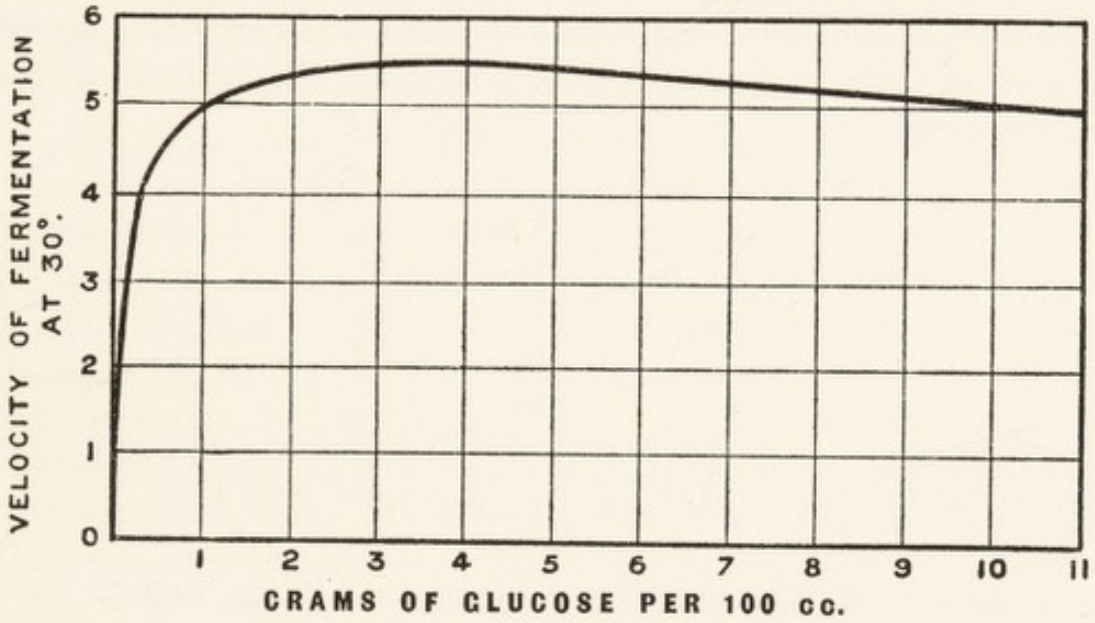


FIG 10.

course as a typical enzyme reaction, although in this case, as in that of yeast-juice, no information is given as to the exact nature of this reaction.

According to Nord and Weichherz [1929], however, the rate of fermentation is by no means so uniform as was supposed by Slator but varies greatly with the rate of stirring of the suspension, increasing in a sufficiently well-stirred mixture up to concentrations of 20 per cent. of sugar.

Influence of the Concentration of Yeast.

It appears to be well established that, when changes in the quantity and constitution of the yeast employed are eliminated, the rate of fermentation is exactly proportional to the number of the yeast-cells

present (Aberson, Slator). This result might be anticipated, as pointed out by Slator, from the fact that the fermentation takes place within the cell, each cell acting as an independent individual.

Temperature Coefficient of Alcoholic Fermentation by Yeast.

The temperature coefficient of fermentation by living yeast has been carefully determined by Slator by measurements of the initial rates at a series of temperatures from 5° to 40° C. The coefficient is found to be of the same order as that for many chemical reactions, but to vary considerably with the temperature, a rise in temperature corresponding with a diminution in the coefficient. The following values were obtained for glucose; they are independent of the concentration of yeast and glucose, the class of yeast, and presence or absence of nutrient salts, and remain the same when inhibiting agents are present. Almost precisely the same ratios are obtained for fructose and mannose :—

t	V_{t+5}/V_t	V_{t+10}/V_t
5	2·65	5·6
10	2·11	3·8
15	1·80	2·8
20	1·57	2·25
25	1·43	1·95
30	1·35	1·6
35	1·20	

Aberson's result, $k_{t+10}/k_t = 2{\cdot}72$, which represents the mean coefficient for 10° between 12° and 33°, agrees well with this.

Action of Accelerating Agents on Living Yeast.

Slator [1908, 1] was unable to find any agent which greatly accelerated the rate of fermentation of living yeast. Small concentrations of various inhibiting agents which are often supposed to act in this way were quite ineffective, and phosphates, which produce such a striking change in yeast-juice, were almost without action On the other hand Euler and Sahlen [1913] find that small concentrations of guaiacol, sodium salicylate and acetaldehyde definitely accelerate fermentation by living yeast, whereas higher concentrations progressively inhibit it.

Similar results have been obtained by Abderhalden with phenol and other substances [1928, 1929] and with charcoal [1921, 1, 2; 1922, 2], which appears to act partly by increasing the small concentration of acetaldehyde present and partly by absorbing alcohol [Ivekovic, 1927].

Euler and Bäckström [1912], moreover, have made the important observation that sodium hexosephosphate causes a considerable acceleration although it is itself neither fermented nor hydrolysed under these conditions. The extent of this is evident from the following numbers :—

20 c.c. of 20 per cent. glucose solution, 0·25 g. Yeast [Yeast H of St. Erik's Brewery].			
Without addition.		+ 0·5 g. Na hexosephosphate	
Time. Min.	CO_2.	Time. Min.	CO_2.
46	10·5	37	8
76	17·5	73	19
197	45	188	52·5
347	74·5	321	123
488	95	450	193·5

The observation has been confirmed with English top yeast (Harden and Young, unpublished experiments), but no explanation of the phenomenon is at present forthcoming.

Euler has also found [Euler and Cassel, 1913 ; Euler and Berggren, 1912] that yeast extract, sodium nucleinate and ammonium formate increase the rate of fermentation of glucose by yeast. These results were criticised by Harden and Young [1913] on the ground that the possibility of growth of the yeast during the experiment had not been excluded, but Euler [Euler and Hammarsten, 1916 ; Euler, 1919] has confirmed his original results by experiments in which this source of error was avoided. The effect of yeast extract is due to the factors Z which have already been discussed (p. 91).

Influence of Hydrogen Ion Concentration on Fermentation by Yeast.

A large amount of research has been devoted to this subject. A detailed account of this, which is beyond the scope of the present work, may be found in Hägglund's summary [1914, see also Hägglund and Augustsson, 1926 ; Hägglund, Söderblom and Troberg, 1926] or in Euler and Lindner's book [1915]. Very briefly, fermentation by living yeast is diminished and finally inhibited both by acids and alkalis, the limiting hydrogen ion concentrations depending on the nature of the acid or alkali employed. The optimum reaction also varies with different acids but is approximately p_H 3·7 for hydrochloric and sulphuric acids, whilst the inhibiting concentrations for the same acids

are approximately p_H 2·3 and 2·4 respectively. In phosphate buffers the optimum reaction varies with the concentration of the buffer solution between p_H 4 and p_H 6. The simple fatty acids, especially formic acid, in the free state have a specific strongly diminishing effect on the rate of fermentation of living yeast [Katagiri, 1926, 1927 ; see also Hägglund and Augustsson, 1925].

In alkaline solutions the course of the fermentation is profoundly modified, and this question has already been discussed (p. 134).

Fermentation of Different Sugars by Yeast.

Many valuable ideas as to the nature of fermentation have been obtained by a consideration of the phenomena presented by the action of yeast on the different hexoses. Of these only glucose, fructose, mannose, and galactose are susceptible of alcoholic fermentation by yeast, the stereoisomeric hexoses prepared in the laboratory being unfermentable, as are also the pentoses, tetroses, and the alcohols corresponding to all the sugars. The yeast cell is therefore much more limited in its power of producing fermentation than such an organism as, for example, *Bacillus coli communis*, which attacks substances as diverse as arabinose, glucose, glycerol and mannitol, and yields with all of them products of the same chemical character, although in varying proportions.

A careful examination of a number of different genera and species of the Saccharomycetaceae and allied organisms by E. F. Armstrong [1905] has shown that all yeasts which ferment glucose also ferment fructose and mannose. Armstrong grew his yeasts in a nutrient solution containing the sugar to be investigated, and his experiments are open to the criticism that the organisms were hereby afforded an opportunity for becoming acclimatised to the sugar. His results, therefore, only demonstrate the fact that the organisms in question when cultivated in presence of the sugars examined brought about their fermentation, and do not exclude the possibility that the same organism when grown in presence of a different sugar might not be capable of fermenting the one to which it had in the other type of experiment become acclimatised.

This has actually been shown to be the case for galactose by Slator [1908, 1] and it is possible that this circumstance explains the negative results obtained by Lindner [1905] with *S. exiguus* and *Schizosaccharomyces Pombe* upon mannose, a sugar which, according to Armstrong, is fermented by both these organisms.

The same problem has been attacked quantitatively by Slator, who has shown that living yeast of various species and genera ferments glucose and fructose at approximately the same rate. Moreover, when the yeast is acted upon by various inhibiting agents, such as heat, iodine, alcohol or alkalis, the crippled yeast also ferments glucose and fructose at the same rate.

With mannose the relations are somewhat different. The relative rate of fermentation of mannose and glucose by yeast is dependent on the variety of the yeast and the treatment which it has received. Fresh samples of yeast ferment mannose more quickly than glucose, but by older samples the glucose is the more rapidly decomposed. This is especially the case with yeast, the activity of which has been partly destroyed by heat, the relative fermenting power to mannose being sometimes reduced by this treatment from 120 per cent. of that of glucose to only 12 per cent. (Slator).

A further difference consists in the fact that with certain yeasts the rate of fermentation of glucose is somewhat increased by monosodium phosphate whilst that of mannose is unaffected [Euler and Lundeqvist, 1911].

Mixtures of glucose and fructose are fermented by yeast at the same rate as either the glucose or the fructose contained in the mixture would be alone. When, however, mannose and glucose are fermented simultaneously interference between the reactions takes place, and this is especially evident when the yeast has comparatively little action on mannose. The following are the results obtained by Slator :—

Yeast.	Relative Rates.		
	2·5 per cent. Glucose.	2·5 per cent. Mannose.	2·5 per cent. Glucose + 2·5 per cent. Mannose.
S. thermantitonum . . .	100	105	92
Brewery yeast, 53 per cent. activity destroyed by heat .	100	21	33
Brewery yeast, 60 per cent. activity destroyed by heat .	100	12	42

Selective Fermentation of Glucose and Fructose.

It has been shown by many workers [see Hopkins, 1928, and Sobotka and Reiner, 1930, where the literature is quoted] that brewer's yeast added to a mixture of equal parts of glucose and fructose ferments the glucose more rapidly than the fructose, so that after a certain time the

latter predominates in the solution. On the other hand certain Sauterne yeasts under these conditions ferment the fructose more rapidly than the glucose [Fernbach and Schoen, 1924; Fernbach, Schoen and Mori, 1927, 1, 2]. The same holds for zymin prepared from these yeasts, but some species yield zymins which differ in their selective fermentation from the fresh organism. The reason for this behaviour is not completely understood. The subject cannot be fully discussed here, but the main facts which have been ascertained may be briefly summarised as follows.

1. Brewer's yeast ferments glucose without any production of fructose.

2. Brewer's yeast ferments glucose and fructose separately at the same rate in concentrations of about 1 to 2 per cent., but at lower concentrations it ferments glucose more rapidly than fructose. Sauterne yeast on the other hand ferments fructose more rapidly than glucose at all concentrations up to 10 per cent. [Hopkins, 1928].

3. When the fermentation of glucose by brewer's yeast is interrupted the residual glucose has a low $[\alpha]_D$ which then changes by mutarotation to the normal value [Willstätter and Sobotka, 1922, 2; Hopkins, 1928]. With Sauterne yeast and glucose the change is in the opposite direction.

4. Brewer's yeast is more selective at lower temperatures.

5. The selectivity does not appear to depend in any way on the nature of the saccharase of the yeast.

Hopkins [1931] suggests that brewer's yeast is specific for the normal stable form of glucose (and mannose), *i.e.* glucose-pyranose or more probably its α-form, but for the unstable γ-form of fructose (fructo-furanose) which is only present in very low proportions in ordinary solutions of fructose (fructo-pyranose), or possibly for the β-form of normal fructose. Sauterne yeast on the other hand is specific for β-glucose and ferments fructose specifically more rapidly than glucose.

Fermentation of Galactose.

The case of galactose merits special attention. Previous investigations [see Lippmann, 1904, p. 734] have shown that the fermentation of galactose by yeast differs greatly from that of the other hexoses. The subject was re-investigated by E. F. Armstrong [1905], and by Slator [1908, 1]. Armstrong carried out his experiments in the manner already described (p. 192), and found that some yeasts had,

and others had not, the power of fermenting galactose, although all were capable of fermenting glucose, fructose, and mannose.

Slator made quantitative experiments on the same subject. He was able to confirm the statement which had previously been made, that certain yeasts which have the property of fermenting galactose possess it only after the yeast has become acclimatised by culture in presence of the sugar. This was shown for brewery yeast and for the species mentioned below. This phenomenon is one of great interest and is strictly analogous to the adaptation of bacteria which has now been quite conclusively established [Neisser, 1906].

Yeast.	Mode of Culture.	Relative Rates.	
		Glucose.	Galactose.
S. carlsbergensis .	Grown in wort . . .	100	< 1
,, .	,, ,, hydrolysed lactose	100	86, 83, 85, 25, 46, 51, 69, 54, 155
S. cerevisiae . .	,, ,, wort . . .	100	< 1
,, .	,, ,, hydrolysed lactose	100	21, 26, 29
S. thermantitonum .	,, ,, wort . . .	100	< 1
,, .	,, ,, hydrolysed lactose	100	77, 53, 35
Sm. Ludwigii . .	,, ,, wort . . .	100	< 1
,, . .	,, ,, hydrolysed lactose	100	< 1

It will be seen that in one case the rate of fermentation of galactose was considerably greater than that of glucose. *Sm. Ludwigii* did not respond to the cultivation in hydrolysed lactose, but, as Slator points out, it is quite possible that repeated cultivation in this medium might effect the change, and this would be strictly analogous to the results obtained with bacteria. Slator's results have been confirmed by Harden and Norris, R. V. [1910], and by Euler and Johansson [1912, 1] who have made an exceedingly interesting study of the progress of the adaptation. As in the case of mannose the rates of fermentation of glucose and galactose are differently affected by agents such as heat and alcohol; moreover, the rate of fermentation of mixtures of glucose and galactose is in no case either the sum or the mean of the rates obtained with the separate sugars. The temperature coefficient of the fermentation of galactose also differs slightly from that of the other hexoses [see also Euler, Laurin and Pettersson, 1921.]

It has further been shown that the process of adaptation only occurs when new yeast cells are produced [Söhngen and Coolhaas, 1925; Euler and Nilsson, 1925, 1; Euler and Jansson, 1927]. Of the Swedish yeasts bottom yeast is more easily trained than top [Euler and Nilsson, 1926, 1]. The fermentation like that of glucose is accelerated by

Yeast.	Relative Rates.		
	Glucose.	Galactose.	Glucose + Galactose.
S. cerevisiae	100	0	103
,, ...	100	34	103
S. carlsbergensis . . .	100	155	119
S. thermantitonum . . .	100	76	124

Euler's Z factors [Euler and Nilsson, 1925, 1]. Some information as to the nature of the change produced is obtained by the examination of preparations made from yeast which has become adapted to galactose fermentation. Yeast-juice [Harden and Norris, 1910], maceration extract and dried yeast [Abderhalden, 1925, 1, 2] prepared from such yeasts all ferment galactose. When such dried yeast is washed it can be reactivated to galactose by co-enzyme from untreated yeast. It thus appears that it is the apozymase which has been altered not the co-enzyme [Euler and Jansson, 1927].

The course of the fermentation of galactose by yeast-juice or dried yeast from " adapted " yeast is very similar to that of glucose, but the fermentation in presence of phosphate is slower. Esterification occurs [Harden and Norris, 1910] and, according to Nilsson [1930], the hexosediphosphate of Harden and Young and a hexosemonophosphate are formed. The latter has not yet been obtained pure ; the crude product has a higher rotation than the Robison ester. According to Nilsson it originates from the polysaccharides of the yeast. It is possible that the galactose may be converted into glucose (or its enolic form) before fermentation [*cf.* Euler, 1920]. Against this is the observation of Willstätter and Sobotka [1922, 1] that in mixtures of glucose and galactose the glucose is relatively much more rapidly fermented than when the two sugars are separately fermented. The formation of the same hexosediphosphate from galactose as is obtained from glucose might possibly occur through the intermediate formation of a triosephosphate, as postulated by Euler and by Kluyver in the fermentation of glucose [Nilsson, 1930].

Theories of the Fermentation of Different Sugars.

Assuming that his conclusion that all yeasts which ferment glucose also ferment fructose and mannose is correct, Armstrong has drawn attention to the fact that these three hexoses are also related by the possession of a common enolic form (p. 110) and has suggested that

this enolic form is the substance actually fermented to carbon dioxide and alcohol [1904].

The idea that such an intermediate form is the direct subject of fermentation has much to recommend it. In the first place it is almost certain, as already pointed out, that the sugars in aqueous solution do exist, although to a very small extent, in this enolic form. The slow rate at which equilibrium is established in aqueous solution, however, must be taken as definite evidence that under these circumstances the enolic form is only produced very slowly [compare Lowry, 1903]. This has been used by Slator [1908, 1] as an argument against the probability of the preliminary conversion of the sugars into the enolic form before fermentation. It appears, however, quite possible that under the influence of the fermenting complex of the yeast cell, or of special enzymes, this change might occur much more rapidly, and at different rates with the different sugars. This reaction might in fact control the observed rate of fermentation. This conception affords a simple explanation of the different rates of fermentation of mannose and glucose, and also of galactose, the enolic form of which is quite different, by yeast under different circumstances, but does not explain the uniformity of rate observed by Slator for glucose and fructose nor the results with mixtures of sugars.

The direct fermentation of a common enolic form is also consistent with the fact that the same hexosediphosphate is produced from all three hexoses by yeast preparations and that a mixture of monophosphates containing both aldo- and keto-esters is formed from both glucose and fructose.

Slator himself prefers the view that the first stage of fermentation consists in the rapid combination of the sugar with the enzyme, producing a compound, which then breaks up at a rate which determines the observed rate of fermentation. This rate will of course vary with the nature of the compound, so that if two sugars form the same compound they will be fermented at the same rate ; if they form different compounds, different rates may result. Slator supposes that glucose and fructose form the same compound with the enzyme. This, however, appears to involve an intramolecular change of the same order as the production of the enolic form. Mannose and galactose, on the other hand, form stereoisomeric compounds, and the capacity of the fermenting complex to form these compounds may be affected by various agents to a different extent from its capacity for combining with glucose or fructose.

A third theory has also been suggested to explain these phenomena,

according to which the various sugars are fermented by different enzymes [see Slator, 1908, 1]. The uniformity of the result obtained with glucose and fructose suggests that these two sugars are fermented by the same enzyme (glucozymase), mannose and galactose by different ones (mannozymase and galactozymase). This would afford a simple explanation of the different rates of fermentation for different sugars and of different degrees of sensitiveness towards reagents.

If, however, a separate and independent mechanism were present for each sugar, the rate of fermentation of mixtures should be the sum of the rates for the constituents. This, as shown above, is not found to be the case, and it is therefore necessary to suppose, either that one sugar influences the fermentation of another in some unknown way, or that only a part of the mechanism of fermentation is specific for the particular sugar. Thus the enzyme may be specific and the co-enzyme non-specific, so that only a certain maximum rate is attainable, or again, the supply of free phosphate may be the controlling factor.

In the prevailing state of ignorance as to the exact function of the co-enzyme and of the conditions upon which the velocity of fermentation in the cell depends, it is at present impossible to decide between these various theories, but they all offer points of attack which justify the hope that much further information can be obtained by experimental inquiry.

It will be seen from the foregoing that Buchner's discovery of zymase has not only led to a more complete, albeit still imperfect, knowledge of the mechanism of alcoholic fermentation, but has also prepared the way to the discovery of the fundamental changes which carbohydrates undergo in the process of metabolism both in vegetable and animal organisms. Much yet remains to be learned and it cannot be doubted that, as in the past, the investigation of the action of the yeast cell will still prove to be of outstanding importance in the attainment of further knowledge.

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Biochem. Z., **188,** 211-216.

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Biochem. Z., **203,** 463-468.

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Biochem. Z., **191,** 460-465.

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Biochem. Z., **67,** 24-27.

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INDEX.

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