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The function of phosphate in alcoholic fermentation

Nobel Lecture, December 12, 1929

The discovery that phosphates play an essential part in alcoholic fermentation arose of an attempt by the late Dr. Allan Macfadyen to prepare an anti-zymase by injecting Buchner's yeast-juice into animals. As a necessary preliminary to the study of the effect of the serum of these injected animals on fermentation by yeast-juice, the action of normal serum was examined. It was thus found that this exerted a two-fold effect: in its presence the action of the proteolytic enzymes of the yeast-juice was greatly diminished, and at the same time both the rate of fermentation and the total fermentation produced were considerably increased. In the course of experiments made to investigate this phenomenon, which it was thought might have been due to the protection of the enzyme of alcoholic fermentation from proteolysis by means of an anti-protease present in the serum, the effect of boiled autolysed yeast-juice was tested, it being thought that the presence of the products of proteolysis might also exert an anti-proteolytic effect. As my colleague Mr. Young, who had by this time joined me, and myself had fortunately decided to abandon the gravimetric method chiefly used by Buchner in favour of a volumetric method which permitted almost continuous observations, we were at once struck by the fact that a great but temporary acceleration of the rate of fermentation and an increase in the CO_2 evolved proportional to the volume of boiled juice added were produced. This was ultimately traced to the presence of two independent factors in the boiled yeast-juice, a thermostable dialysable co-enzyme now often known at the suggestion of von Euler as co-zymase, and inorganic phosphate.

With regard to the phosphate subsequent experiments showed that in all fermentations brought about by preparations obtained from yeast the presence of phosphate is absolutely essential. Leaving aside the question of living yeast for consideration later on, three different types of fermentation can be established (See Curves 1, 2, and 3, in Fig. 1) with such preparations.

1. A relatively rapid fermentation (Curve 1, Fig. 1) in which sugar is decomposed into CO_2 and alcohol, and simultaneously inorganic phosphate is converted into an ester (or esters) of a sugar which accumulates. When the

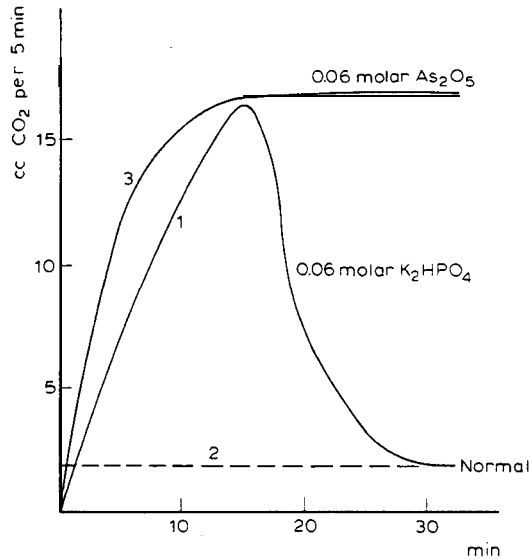


Fig. 1. Fermentation of yeast-juice.

supply of inorganic phosphate ceases, the rate of fermentation falls, the accumulation of ester also naturally ceases, and the fermentation passes into Type 2.

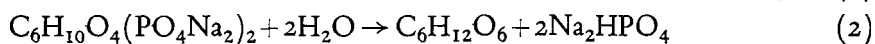
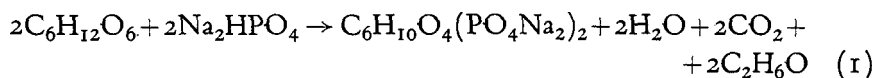
2. A relatively slow fermentation (Curve 2, Fig. 2) in which the rate at which fermentation occurs is controlled by the rate at which inorganic phosphate is supplied by the hydrolysis of the phosphoric esters present in the system by the phosphatase also present. This inorganic phosphate is alternately reconverted into a sugar-phosphoric ester and again liberated by hydrolysis, and thus fermentation proceeds at a steady rate in the presence of available sugar without any permanent increase in the amount of inorganic phosphate or of phosphoric ester present. This is the type of fermentation which goes on when sugar is added to an active preparation from yeast and the process is allowed to proceed until a steady rate is obtained. In some preparations, depending on the amount of phosphatase present, the rate of fermentation is increased to some extent if more of the sugar-phosphoric ester is added or produced (See E. Boyland, *Biochem. J.*, 23 (1929) 219), but this soon reaches a limit. If inorganic phosphate be added, the fermentation passes into Type 1.

If sugar fails, inorganic phosphate appears and ultimately (under favourable conditions) the whole of the sugar-phosphoric ester is hydrolysed, its sugar moiety fermented and the whole of the phosphate liberated in the inorganic form.

3. If now into a fermentation mixture in which a Type 2 fermentation is

proceeding an additional quantity be introduced of a phosphatase, capable of hydrolysing the sugar-phosphoric ester and thus increasing the rate of supply of inorganic phosphate (Harden and Macfarlane, unpublished results), the rate of fermentation also rises. If a sufficiently active preparation of phosphatase could be added, so that the sugar-phosphoric ester was decomposed as rapidly as it was formed, a rapid fermentation would ensue, unaccompanied by accumulation of phosphoric ester. This has not yet been accomplished directly, but an indirect method of attaining the same end is available, inasmuch as arsenates have been found to have the power of greatly stimulating the effect of the phosphatase. This observation was in reality the undeserved reward for thinking chemically about a biochemical problem. In many chemical reactions the type of compound concerned is the main fact of importance, arsenates react like phosphates, potassium may be replaced by sodium, iron by nickel or cobalt. Biochemically the difference between potassium and sodium may be the difference between life and death, and when iron is not used in a respiratory pigment it is not replaced in nature by nickel or cobalt, but by copper or vanadium. So, also, arsenate does not play a similar part to phosphate in fermentation, but acts in an entirely different manner. On the addition of a suitable amount of arsenate, a rapid fermentation (Curve 3, Fig. 1) occurs comparable in rate with that of Type 1, but differing from this in that the rate is permanently raised and that no accumulation of the sugar-phosphoric ester occurs. Under optimal conditions the addition of inorganic phosphate does not produce any significant rise in the rate of fermentation, as the rate of fermentation is controlled under these circumstances by the concentration of the fermenting complex (enzymes + co-enzyme). Arsenate on the other hand does not increase the maximum rate in fermentations of Type 1, as the supply of inorganic phosphate is already optimal.

Without making any assumption as to the exact nature of the phosphoric ester actually produced, the changes so far considered may be illustrated by the two equations, originally proposed by Harden and Young for the case in which only hexose diphosphate is formed, the first representing the evolution of CO₂ and production of alcohol, accompanied by the accumulation of ester, and the second the hydrolysis of this ester with liberation of a hexose and mineral phosphate.



Eq. (1) represents the controlling reaction in a fermentation of Type 1, Eq. (2) that in a fermentation of Type 2. In the presence of arsenate the hydrolysis of hexose phosphate according to Eq. (2) proceeds sufficiently rapidly to supply phosphate at such a rate that Eq. (1) proceeds at maximum velocity.

Fermentation by living yeast

A striking feature of fermentation by yeast preparations is that it proceeds much less rapidly than fermentation by a corresponding amount of living yeast. Thus Buchner's yeast-juice ferments at only about $1/20$ - $1/40$ of the rate of the yeast from which it is derived.

The fact that the rate of fermentation of such a juice can be raised under favourable circumstances some 10-20 times simply by increasing the supply of phosphate seems to me to indicate clearly that a large fraction, at least half, of the fermenting complex of the yeast has escaped injury in the preparation and has passed into the juice, but that the mechanism for the supply of inorganic phosphate has been to a large extent destroyed. Neither arsenate nor phosphate has an accelerating action on the rate of fermentation by living yeast. This may be due to the fact that the supply of inorganic phosphate in the interior of the yeast-cell is already optimal, but some doubt exists as to whether or not these salts freely penetrate the cell. If however, as seems to me probable, it is true that in the preparation of yeast-juice, etc. it is the phosphate-supplying mechanism that is thrown out of gear, it becomes an object of enquiry in what way this is brought about.

Several possibilities present themselves. As suggested for the fermenting complex itself by von Euler and his colleagues, the phosphatase may in large part be combined with the cytoplasm and thrown out of action when the cell is killed. Another possibility is that in the cell the action is localized and that disorganization of the cell leads to less favourable conditions (e.g. concentration, presence of inhibitors, etc.) and to lessened rate of action. There is some evidence for this since the amount of phosphatase present, as judged by the normal rate of fermentation (Type 2), seems to diminish as the disorganization of the cell becomes more complete. Thus dried yeast and yeast dehydrated with acetone ferment sugar (Type 2) more rapidly than yeast-juice, although when phosphate is freely supplied they all cause fermentation at about the same rate. Again some labile substance which acts as an accelerator of the phosphatase may be inactivated by the various modes of treatment (grinding,

drying, treatment with toluene or acetone. etc.) to which the cell is subjected.

The process least likely to inactivate an accelerating substance is probably that used by Buchner, but the possibility also exists that such a substance, if present, might be adsorbed and thus removed from the juice by the large quantity of kieselguhr employed.

Experiments (not yet published) have recently been made in my laboratory by Miss Macfarlane to find out at what stage in the process the change occurs and whether a juice richer in phosphatase could be obtained by modifying the process of grinding and pressing out. It appears, however, that simple grinding with sand produces a change of the same order as that observed in Buchner's yeast-juice. The experiments were made by grinding a mixture of sand and yeast for different times and testing the rate of fermentation and response to phosphate at intervals of the whole mass without pressing out (Fig.2).

These curves show the rate of fermentation of 2 g of yeast + 2 g of sand in 20 c.c. of 10% fructose at 30°: (a) without grinding, (b) after grinding for 20 minutes, and (c) after grinding for 60 minutes. At the point marked with an arrow 0.6 c.c. of 2 MK_2HPO_4 was added.

The curves show that the longer the period of grinding, the lower the rate of fermentation and the greater the response to phosphate. Here again the total loss of fermenting power was only small.

Minor differences were observed when different substances were substi-

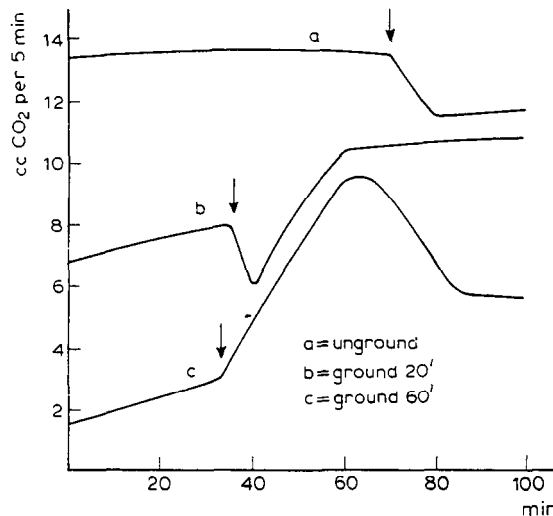


Fig. 2. Fermentation of yeast sand mixture. (At ↓ an addition of phosphate was made.)

tuted for the kieselguhr used by Buchner, the most active juice for example being obtained by the use of CaCO_3 whereas BaCO_3 yielded totally inactive material.

Further investigation may possibly throw more light on this aspect of the question.

I have spoken hitherto on the assumption that the processes in the living cell are essentially of the same kind as those which occur in the various preparations made from the dead cell, but differ from these mainly in the relative intensity of some of the reactions, and I know no valid argument against this assumption.

The cycle undergone by the phosphate in the series of changes which constitutes ordinary fermentation clearly consists in the alternate formation of a phosphoric ester and the hydrolysis of this to free phosphoric acid. A simple calculation based on the phosphorus content of living yeast shows that the whole of this phosphate must pass through the stage of phosphoric ester 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.

Nature and function of the phosphoric esters produced

If we next consider the exact nature of these phosphoric esters and the relation of their formation and hydrolysis to the decomposition of the sugar molecule, we are met with a singularly complex condition of affairs, which cannot yet be interpreted satisfactorily.

The main facts seem to be as follows.

When fermentation of sugar by yeast preparations is carried out under suitable conditions in presence of added inorganic phosphate, a rapid production of CO_2 and alcohol occurs and a phosphoric ester of a sugar accumulates, the amount of phosphate found in this form being approximately proportional in the ratio (CO_2/PO_4) to the increased production of CO_2 and alcohol caused by the addition of the phosphate (Kluyver and Struyk, it is true, have found lower ratios than this, but there is no doubt that high ratios 0.8-1 are often observed).

The phosphoric ester produced, however, may consist mainly of the hexose diphosphate originally described by Young and myself or of the hexose monophosphate described by Robison and myself and subsequently studied by

Robison or it may be a mixture of these in any proportions. In the case of fermentation by dried yeast (and possibly of other preparations) a further complication is afforded by the fact that a disaccharide-phosphoric ester (trehalose monophosphate) may also be present.

This conclusion is founded in the first place on a large amount of experience which has been gained at the Lister Institute in preparing hexose mono- and diphosphate. These preparations are as a rule carried out by making repeated additions of phosphate and sugar to a fermenting mixture of yeast-juice or dried yeast and fructose (or glucose). With dried yeast a large proportion of the diphosphate is usually obtained, and the relatively small amount of monophosphate produced contains a considerable proportion of trehalose monophosphate. With yeast-juice the results are very variable and no trehalose monophosphate has so far been detected among the products. More precise experiments have been made by Lord Henley and myself in which the gas evolved after a single addition of phosphate was carefully measured and the proportions in which mono- and diphosphates were produced were determined as accurately as possible. Unfortunately, the available methods are not very good, as they rest on the solubilities of the different compounds in 10% alcohol, and these are to some extent mutually affected in the presence of both compounds. Further, yeast, like *Africa*, is always yielding something new, and the recently discovered fact that pyrophosphates exist in yeast and by their formation from, or hydrolysis to, orthophosphates may cause disappearance or appearance of "inorganic phosphate" adds another source of inaccuracy to the many previously known.

Allowance has, of course, to be made for the phosphorus compounds existing in the mixture at the moment of addition of inorganic phosphate and for the normal evolution of CO_2 which occurs throughout the experimental period in addition to the enhanced evolution due to the esterification.

In spite of these minor uncertainties the somewhat surprising fact emerges, that whatever the nature of the phosphoric ester which accumulates, the CO_2 is approximately equivalent in the ratio $\text{CO}_2:\text{PO}_4$ to the amount of phosphate which undergoes esterification. The full results are given in two papers published recently by Lord Henley and myself in *The Biochemical Journal* and need not be quoted in detail here. The experiments indicate the wide variation which may occur in the nature of the hexose phosphate produced whilst the ratio of $\text{CO}_2:\text{PO}_4$ esterified remains constant and approximately equal to unity. Two extreme cases may be quoted, in one of which 13.5% of the PO_4 esterified was present as hexose diphosphate and 86.5% as monophosphate

and in the other 97% as diphosphate and only 3% as monophosphate; the CO_2/PO_4 -esterified ratios were 0.98 and 0.86 respectively.

I do not propose to discuss in any great detail the various theories which have been proposed to explain these complicated relationships. It would be natural to assume that the introduction of the phosphoric acid group into the sugar molecule, forming a hexose monophosphate, might render this more accessible to decomposition into the compound (or compounds) containing three carbon atoms which are now accepted as an intermediate stage in the production of CO_2 and alcohol. The phosphate radical from one of these groups might then serve to convert another molecule of the monophosphate into the stable diphosphate (Meyerhof) $2\text{C}_6\text{H}_{11}\text{O}_5\text{P O}_4\text{H}_2 \rightarrow \text{C}_6\text{H}_{10}\text{O}_4(\text{P O}_4\text{H}_2)_2 + 2\text{CO}_2 + 2\text{C}_2\text{H}_6\text{O}$, or two of the three carbon groups containing each one phosphate group might unite with each other, forming the stable diphosphate (Kluyver and Struyk) $2\text{C}_6\text{H}_{11}\text{O}_5(\text{PO}_4\text{H}_2) \rightarrow 2\text{C}_3\text{H}_6\text{O}_3 + 2\text{C}_3\text{H}_5\text{O}_2(\text{PO}_4\text{H}_2) \rightarrow 2\text{CO}_2 + 2\text{C}_2\text{H}_6\text{O} + \text{C}_6\text{H}_{10}\text{O}_4(\text{PO}_4\text{H}_2)_2$. Any monophosphate escaping these reactions would be found as a constituent of the mixed hexose phosphates resulting from the fermentation.

To add further to the difficulty of unravelling this complex tangle it must be remembered that, whether glucose or fructose be fermented, the hexose diphosphate produced is probably a derivative of fructose, or at least yields fructose on hydrolysis, whilst the monophosphate is with equal probability a mixture of about 80% of a glucose monophosphate and 20% of a fructose monophosphate. It is obvious from this that whatever changes occur are not limited to the simple introduction or removal of a phosphoric acid group, fundamental changes occur in the constitution of the molecule of the sugar itself.

Attractive as is the theory of the intermediate character of some one of the hexose phosphates, it seems to me impossible at the moment to bring it into agreement with some of the facts which have just been related. The production of 70-80% of the monophosphate, with an unaltered degree of formation of alcohol and CO_2 , renders it impossible that this ester should be "obviously nothing but a part of the intermediate product which has escaped the coupled decomposition-esterification reaction" (O. Meyerhof and K. Lohmann, *Biochem. Z.*, 185 (1927) 155).

It appears to me that the fundamental idea expressed in the original equation of Harden and Young is nearer the truth than any alternative that has as yet been suggested. A coupled reaction of some kind occurs, as the result of which the introduction of two phosphate groups into certain sugar molecules - either into the same molecule or one each into two different ones - induces the de-

composition of another molecule. The introduction of these phosphate groups in presence of muscle extract and presumably in both muscle itself and yeast, is actually accompanied by a small evolution of heat (O. Meyerhof and J. Suranyi, *Biochem. Z.*, 191 (1927) 106), and it is possible that this may have some significance for the occurrence of the coupled reaction. What are the conditions for the preferential formation of the mono- or di-ester we do not yet certainly know, although the work of Kluyver and Struyk suggests that dilution of the enzyme may be one factor in this.

The lack of exact chemical equivalence among the products (ester on the one hand, CO₂ and alcohol on the other) is probably more easily explicable on this view than on any other.

The mechanism of the fermentation of the monoester has not yet been worked out in sufficient detail to afford valid evidence either for or against the theory, but Dr. Robison and I have made experiments (about to be published) which show that the monophosphate itself reacts with a further quantity of phosphate and that this reaction is accompanied by an enhanced production of carbon dioxide and alcohol.

Sugar metabolism in vegetable and animal organisms

After the establishment of the important part played by phosphates and phosphoric esters in alcoholic fermentation, it was soon found by various workers that these compounds provided the clue to many other biological phenomena. The co-enzyme of alcoholic fermentation was found by Meyerhof to exert an equally important part in the respiration of yeast, and the important observation was made also by Meyerhof that it occurred in muscle and was an essential factor in the carbohydrate metabolism of muscle, in which the intervention of a hexose phosphate had been proved by Embden. This phenomenon was shown to take place on lines quite similar to those of the respiration and fermentation of yeast, and in 1924, before the riddle of lactic acid formation had been completely solved, O. Meyerhof wrote (*Chemical Dynamics of Life Phenomena*, 1924) : "It may indeed be considered a success of general physiology and its mode of experimenting, that the chemical dynamics of a highly differentiated organ like the muscle could be partly revealed by the study of alcoholic fermentation of yeast." But a still greater success was to follow. An astonishing degree of similarity was shown to exist between almost every detail of the production of lactic acid by the muscle enzymes and of

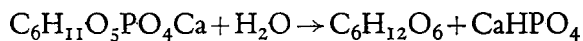
alcohol by the yeast enzyme, which extended to the identity of the phosphoric esters concerned, the accumulation of ester under similar conditions, and even to the effect of arsenate on the process. After the publication of Meyerhof's preliminary papers in which these observations were recorded I wrote the following passage in concluding a short review of the work (*Nature*, 118 (1926) Dec. 18th) which I may perhaps be allowed to quote: "The striking similarity established by Meyerhof between the changes of carbohydrates in muscle and in the yeast cell is seen to be much closer than has been believed. The remarkable phenomena accompanying alcoholic fermentation are now duplicated in the case of lactic acid production, and it may reasonably be expected that most of the fermentative decompositions of the sugars will be found to be initiated in a similar manner"

Direct proof is still wanting in many cases, but some instances are known among bacteria (Virtanen), moulds (von Euler and Kullberg) and higher plants (Ivanoff, Bodnar). It is not too much to say that the fundamental biological mode of attack on carbohydrates is that revealed by the study of alcoholic fermentation.

Ossification

Another biochemical function of the hexose phosphates which is shared by other hydrolysable phosphoric esters is that of being a potential source of phosphate ions. I am happy to say that one of the most beautiful and important developments of this idea has been worked out quite independently at the Lister Institute by Dr. R. Robison as a direct consequence of his work on the hexose monophosphate of yeast-juice.

"During my investigation of the hexose monophosphoric acid isolated from the products of fermentation", he says (*Biochem. J.*, 17 (1923) 286) "the hydrolysis of the ester by enzymes was studied. In some experiments in which the readily soluble calcium and barium salts were used as substrates, the progress of the hydrolysis was shown by the formation of a precipitate of sparingly soluble calcium or barium phosphate



The formation of this precipitate suggested to me the query whether some such reaction might conceivably be concerned in the deposition of calcium phosphate during the formation of bone in the animal body. In the first place I

sought for an enzyme capable of effecting hydrolysis in the bones of growing animals."

The search was successful, a "bone phosphatase" was found in the ossifying cartilage of young animals and a series of interesting and important investigations has followed, as a result of which I have little doubt that their author is on the highway to the chemical explanation of the process of ossification - a good instance of the far-reaching and unexpected results flowing from observations made for quite a different purpose.