The Function of Phosphate in Alcoholic Fermentation.[1]

By Prof. ARTHUR HARDEN, F.R.S.

THE discovery that phosphates play an essential part in alcoholic fermentation arose out 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 carbon dioxide 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 coenzyme, now often known at the suggestion of 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 (Fig. 1, curves 1, 2, and 3) with such preparations.

(1.) A relatively rapid fermentation (Fig. 1, curve 1) in which sugar is decomposed into carbon dioxide and alcohol and simultaneously inorganic phosphate is converted into an ester (or esters) of a sugar which accumulates. The rate rises to a maximum, and when the 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 (Fig. 1, curve 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 (Boyland, *Biochem. J.*, **23**, 219; 1929), 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.

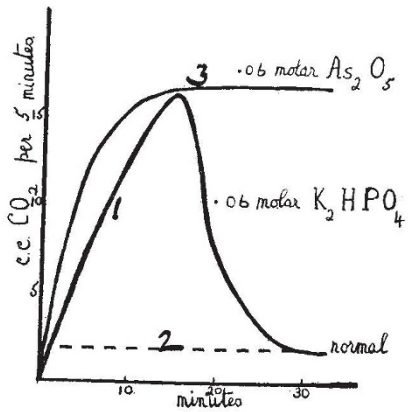

FIG. 1.

(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

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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 (Fig. 1, curve 3) 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 this rate of fermentation, as the rate of fermentation is controlled in these circumstances by the concentration of the fermenting complex (enzymes + co-enzyme). Arsenate, on the other hand, does not increase the maximum rate in fermentation 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 hexosediphosphate is formed, the first representing the evolution of carbon dioxide 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.

I. $2C_6H_{12}O_6 + 2Na_2HPO_4 = C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O + 2CO_2 + 2C_2H_6O$

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

Equation I. represents the condition of affairs in a fermentation of Type 1; Equation II. that in a fermentation of Type 2. In the presence of arsenate, the hydrolysis of hexosephosphate according to Equation II. proceeds sufficiently rapidly to supply phosphate at such a rate that Equation I. 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 in favourable circumstances some ten to twenty 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 making of preparations from yeast it is the phosphate-supplying mechanism that is thrown out of gear, it becomes an object of inquiry in what way this is brought about.

Several possibilities present themselves. As suggested for the fermenting complex itself by 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 localised and that disorganisation of the cell leads to less favourable conditions (for example, 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 disorganisation 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

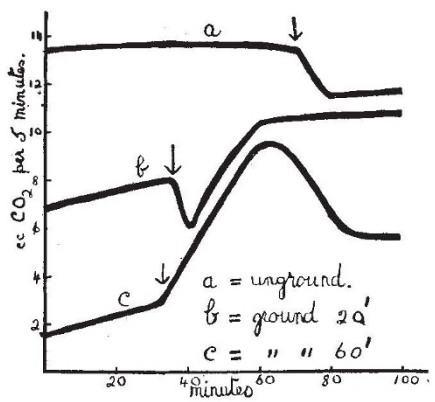

FIG. 2.

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 activate such an accelerating substance is probably that of 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 gm. of yeast + 2 gm. of sand in 20 c.c. of 10 per cent 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*M* potassium hydrogen phosphate 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 substituted for the kieselguhr used by Buchner, the most active juice, for example, being obtained by the use of calcium carbonate, whereas barium carbonate yielded totally inactive material. Further investigation may possibly throw more light on this aspect of the question.

I have assumed up to now 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.

(*To be continued.*)

Hybrid Vigour and Fibre Production.

MAN may not consume more vegetable food per capita now than in days past, but it is certain that the amount of vegetable fibre employed to clothe him, and to spread before his eyes the printed word, increases from year to year, and the subject of vegetable sources for our cellulose supplies is frequently canvassed as a result.

In a very interesting and suggestive chapter in their text-book " Genetics in Relation to Agriculture ", Messrs. Babcock and Claussen, two distinguished geneticists of the University of California at Berkeley, point out that rapid vegetable growth, in all kinds of plants is frequently very marked in the first generation hybrid offspring of crosses between species or varieties ; it is impossible to define the procedure too accurately in view of the confusion that surrounds the taxonomists' definition of a species, but these authors were referring to a familiar phenomenon which very early received attention in the history of breeding experiments.

The rapid production of cellulose for the voracious maw of the printing press depends upon the rapid growth of the plant, and in America, following the lead of such geneticists as Babcock and Claussen, the foresters are exploring the possibilities of hybridisation as the source of an F_1 generation of trees which shall grow more quickly than either parent. The intention, of course, is to propagate such a vigorous seedling when procured by vegetative means. In such a 'clone' of vegetatively propagated trees the hybrid vigour of the original plant may be expected to persist, and when, as in the case of the paper pulp industry, the essential point is quantitative yield of wood, to a certain extent regardless of quality, the problem is relatively clear-cut and there seems considerable possibility of attainment of the practical end in view.

Where, however, plants of briefer duration of life are concerned, and where each new generation of plants is raised from seed, at first sight the possibility of obtaining more-vigorously growing plants as the result of hybridisation seems very remote. As is well known, in subsequent generations, the Mendelian growth factors, combined in the F_1 hybrid, usually segregate independently and there is little likelihood of many seedlings of the new F_2 generation containing that happy summation of dominant factors contributing to vigorous growth which were fortunately combined by the cross making the F_1 plant. However, in recent years numerous cases have come to light where many factors, thus first brought together in a cross, have remained together during subsequent generations as the result of that obscure process controlling the fusion and sorting of the constituents of the chromosome which are grouped under the term 'linkage'. It is, therefore, perhaps worth pointing out that at the present time two plants which are or have been under trial as sources of fibre may represent the offspring of a natural hybrid.

Prof. F. W. Oliver, now enjoying his well-earned rest from the Department of Botany, University College, Gower Street, by taking charge for a short period of the Botanical Department of the University of Cairo, has frequently directed attention to the vigorous growth of one of these plants—rice grass or cord grass, *Spartina Townsendii*.

Three articles upon this grass, upon its distribution, its use in reclaiming maritime muds and resisting foreshore erosion, and as a fodder, etc., appeared in the *Journal of the Ministry of Agriculture* recently, and were reprinted by the Ministry as Miscellaneous Publication No. 66 ; whilst, during the War, experiments were made with this plant in paper-making. As Prof. Oliver points out in his contribution in these articles, we are still uncertain of the accuracy of the assumption that this grass arose as a hybrid between the species *S. alterniflora* and *S. stricta*, experiments made in crossing the putative parents having so far been without result. If a hybrid, it is at least remarkable that no segregates have been discovered during the fifty years in which the spread of *S. Townsendii* has now been observed.

In the other case, the plant in question is under much more vigorous exploitation as a fibre plant. 'Brotex' was referred to in NATURE of Mar. 9, 1929. Its botanical history is far less known than