XCI. FERMENTATION BY YEAST PREPARATIONS.

I. THE EFFECT OF MONO-iodoacetate ON THE FERMENTATION OF hexosediphosphate.

II. THE ACTION OF ARSENATE ON THE INDUCTION PERIOD OF ZYMIN.

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I. THE EFFECT OF MONO-iodoacetate ON THE FERMENTATION OF hexosediphosphate.

It has been shown [Macfarlane, 1930] that a yeast hexosephosphatase incapable of esterifying and fermenting sugar can be obtained, whose action in liberating inorganic phosphate from hexosediphosphate is accelerated by arsenate only in the presence of the co-enzyme of alcoholic fermentation and with an equivalent production of CO_2 . Fluoride, which inhibits the hydrolysis either in presence or absence of arsenate only by about 10 %, decreases the fermentation of hexosediphosphate in presence of arsenate by 80 %.

According to Lohmann [1930] both muscle extract and yeast maceration juice in the presence of fluoride convert glycogen or starch or added hexosediphosphate into a diphosphoric ester much more resistant to acid hydrolysis than the Harden and Young ester. Lipmann [1930] and Lundsgaard [1930, 1] have shown that the poisoning of muscle by sodium fluoride and by mono-iodoacetate produces similar effects, *i.e.* the muscle can contract without formation of lactic acid but with the formation of a hexosediphosphoric ester, probably of the same type as Lohmann's ester. Moreover mono-iodoacetate prevents the esterification of sugar and phosphate by yeast maceration juice [Lundsgaard, 1930, 2]. The action of this salt was also investigated by Yamazaki [1930], who pointed out that its inhibitory effect on the fermentation of hexosediphosphate by maceration juice was greater in the presence of arsenate, but came to the conclusion that the main effect was an inhibition of the esterifying power with phosphate. It seemed of interest to determine the effect of mono-iodoacetate on fermentation of hexosediphosphate by the hexosephosphatase referred to above, so that the process might be uncomplicated by the presence of zymase.

A hexosephosphatase was prepared by the autolysis of dried baker's yeast,

as previously described in detail [Macfarlane, 1930]. The preparation contained no co-enzyme and did not esterify or ferment sugar with added co-enzyme. A typical experiment was carried out as follows. Exp. 1. 1 cc. of a 20 % solution of the enzyme, 2 cc. 0.1 *M* potassium hexosediphosphate and 1 cc. boiled yeast extract were placed in each of four test-tubes, to which were added:

- (a) 2 cc. H_2O ;
- (b) 0.6 cc. 1 % potassium mono-iodoacetate + 1.4 cc. H_2O ;
- (c) 0.3 cc. 0.1 *M* Na_2HAsO_4 + 1.7 cc. H_2O ;
- (d) 0.3 cc. 0.1 *M* Na_2HAsO_4 + 0.6 cc. potassium mono-iodoacetate
+ 1.1 cc. H_2O .

The tubes were placed in a thermostat at 30°, 1 cc. samples being withdrawn at intervals and the inorganic P estimated by the Briggs method in the trichloroacetic acid filtrates. The quantities of phosphate liberated in 30 minutes, as mg. P per cc., were in (a) 0.175; in (b) 0.160; in (c) 0.984; and in (d) 0.551. The effect is more strikingly shown by plotting the rates of liberation of inorganic P (corrected for the change occurring in the absence of co-enzyme) over short time intervals, as in Exp. 2 (Fig. 1). The curve (b) obtained

Fig. 1. Fermentation of hexosediphosphate.

- Exp. 2. (a) 2 cc. 10 % hexosephosphatase + 5 cc. 0.1 *M* hexosephosphate + 2 cc. yeast extract + 6 cc. 0.1 *M* As_2O_5 + 2.4 H_2O .
 (b) 2 cc. 10 % hexosephosphatase + 5 cc. 0.1 *M* hexosephosphate + 2 cc. yeast extract + 6 cc. 0.1 *M* As_2O_5 + 1.4 cc. 1 % mono-iodoacetate + 1 cc. H_2O .
 (c) 2 cc. 10 % hexosephosphatase + 5 cc. 0.1 *M* hexosephosphate + 2 cc. yeast extract + 6 cc. 0.1 *M* As_2O_5 + 0.6 cc. 1 *M* NaF + 1.8 cc. H_2O .

with mono-iodoacetate is almost identical with (a), the control with arsenate, for the first 5 minutes and then rapidly falls to a low rate coinciding with that reached by the curve (c) representing a similar fermentation in presence of fluoride. The decrease in rate shown in curve (a) after 20 minutes is due to the decreasing concentration of hexosediphosphate, 50 % of which has been decomposed at this point.

The nature of the ester remaining in the present case has not been ascertained but in view of the similarities already noticed between fluoride and mono-iodoacetate it appears probable that the reason for the inhibition of

fermentation lies not so much in a direct effect on the enzyme as in the fact that it is now acting on a less congenial substrate.

The effect of potassium mono-iodoacetate on esterification and fermentation by yeast juice.

Unlike maceration juice, which was used by Lundsgaard, yeast juice contains a fair amount of glycogen and a distinction has therefore to be drawn between the esterification of added sugar and that of the glycogen already present in the medium.

Autofermentation of yeast juice. Flasks containing the desired mixtures were placed in a thermostat at 30°, the CO₂ evolved being measured. Samples for phosphate estimation were taken at zero time and subsequently at intervals. After fermentation had been allowed to proceed for an hour, arsenate was added to make a final concentration of 0.01 *M*. The course of the esterification and fermentation is illustrated in Table I and Fig. 2.

Fig. 2. Autofermentation of yeast juice.

(a) Control. (b) + mono-iodoacetate. The arrow marks addition of 3 cc. 0.1 *M* arsenate.

Table I.

(a) 20 cc. yeast juice + 3 cc. 0.6 <i>M</i> K ₂ HPO ₄ + 3 cc. H ₂ O.				
(b) 20 cc. yeast juice + 3 cc. 0.6 <i>M</i> K ₂ HPO ₄ + 3 cc. 2 % mono-iodoacetate.				
	(a)		(b)	
	mg. P	CO ₂ as mg. P	mg. P	CO ₂ as mg. P
	esterified	(CO ₂ ≡ P)	esterified	(CO ₂ ≡ P)
1st hr.	+ 41.5	21.4	+ 33.6	20.7
2nd hr.	- 89.6	91.5	+ 12.5	10.6
(with arsenate)				

The autofermentation of the juice is considerable, but is slower than the fermentation of sugar and is not appreciably diminished by mono-iodoacetate. A slow esterification of phosphate takes place, which is more than equivalent to the CO₂ produced; in presence of 0.1 *M* iodoacetate 80 % of this esterification still occurs. On adding arsenate the rate of CO₂ evolution in the normal juice

increases considerably for a time and then returns nearly to its original level the excess CO_2 being almost exactly equivalent to the amount of esterified P which disappears during this period. This confirms Meyerhof's [1927] statement that the accelerated rate in presence of arsenate in glycogen fermentation is due entirely to the increased rate of fermentation of accumulated hexosediphosphate and not to an acceleration of the diastatic activity of the juice.

In the presence of mono-iodoacetate no acceleration takes place with arsenate, although the concentration of esterified P is of the same order as that in the control juice at this point; the esterification of phosphate however continues, 12.5 mg. being esterified in the next hour. The iodoacetate has therefore inhibited the acceleration by arsenate without greatly affecting the esterifying power with regard to glycogen.

Fermentation of sugar by yeast juice. On adding phosphate and mono-iodoacetate simultaneously to yeast juice fermenting sugar, an immediate esterification of phosphate occurs, accompanied by an equivalent evolution of CO_2 , 90 % esterification taking place as compared with the control juice. The basal rate of fermentation, however, quickly falls and is not accelerated by the addition of arsenate, while on subsequently adding phosphate no rapid esterification and fermentation take place. A slow esterification of phosphate continues still, corresponding approximately to the rate of glycogen esterification. The esterification of sugar by yeast juice is therefore inhibited by a concentration of mono-iodoacetate which only slightly affects that of glycogen.

The course of a typical experiment is shown in Table II.

Table II.

(a) 20 cc. yeast juice + 3 cc. 0.6 M K_2HPO_4 + 2 g. fructose + 3 cc. H_2O .				
(b) 20 cc. yeast juice + 3 cc. 0.6 M K_2HPO_4 + 2 g. fructose + 3 cc. 2 % mono-iodoacetate.				
	(a)		(b)	
	mg. P esterified	CO_2 as mg. P ($\text{CO}_2 \equiv \text{P}$)	mg. P esterified	CO_2 as mg. P ($\text{CO}_2 \equiv \text{P}$)
30 min.	+ 47.4	45.0	+ 43.3	20.7
1 hr. 10 min. (with arsenate and phosphate)	+ 3.0	555.0	+ 15.2	32.0

The salt acts in a manner similar to fluoride, though its effect is not so immediately apparent. The direct fermentation of hexosediphosphate either by yeast juice or by the hexosephosphatase preparation and co-enzyme is also inhibited as shown by the failure to respond to the presence of arsenate by an increased rate.

The results quoted above afford additional confirmation of the theory of Meyerhof [1927] that a direct fermentation of hexosediphosphate takes place in fermenting yeast preparations and in muscle extracts, in addition to the co-enzyme-free hydrolysis described by Harden and Young [1910]. The possibility is not excluded that a labile sugar may be formed by hydrolysis of

hexosediphosphate in presence of co-enzyme, capable of fermentation without previous re-esterification, rather than a disruption of the molecule with the simultaneous production of phosphate and some intermediate compound, *e.g.* methylglyoxal; moreover, although in the experiments here recorded with the hexosephosphatase preparation the accelerated decomposition of the ester in presence of arsenate was always accompanied by evolution of CO_2 , it is possible that this may not hold for other yeast preparations.

II. THE ACTION OF ARSENATE ON THE INDUCTION PERIOD OF ZYMIN.

It has been shown [Harden, 1925; Harden and Macfarlane, 1928] that mixtures of zymin and sugar solution normally show an induction period varying in duration with the volume of solution added before the onset of rapid fermentation occurs, and that arsenate increases the length of this period during which no evolution of CO_2 takes place. It was noticed that the inorganic phosphate present in trichloroacetic acid filtrates from the mixtures increases during the induction period. If this phosphate arises from a fermentation of hexosediphosphate, the rate of liberation should increase on addition of arsenate and simultaneously, since no CO_2 is evolved, an accumulation of the hexose residue, either unchanged or in the form of an intermediate product, should occur. If, on the other hand, the phosphate is not increased by the addition of arsenate, it must be derived either from a hydrolysis of hexosediphosphate or from some other change in the phosphorus compounds present.

Duplicate series of flasks were prepared containing 2 g. of zymin and known volumes of sugar solution or water; to one arsenate was added in the desired concentration (0.02–0.002 *M*), the corresponding flask of equal volume acting as the control. In some cases hexosediphosphate was added to shorten

Fig. 3. Induction period of zymin fermentation.

(a) 2 g. zymin + 40 cc. H_2O . Induction time, 80 min.

(b) 2 g. zymin + 8 cc. 0.1 *M* Na_2HAsO_4 + 32 H_2O . Induction time, ∞ .

the induction period. The flasks were placed in a thermostat at 30° and the inorganic phosphate was estimated by the Briggs method in the trichloroacetic acid filtrates of samples taken at frequent intervals. Figs. 3 and 4 illustrate typical experiments.

It was found that the maximum liberation of inorganic phosphate took place in the larger volumes employed, that is where the liberation of the available phosphate was completed before any esterification of phosphate prior to fermentation developed, the rate of liberation being linear and unaltered by the addition of arsenate. As the volume decreased, the initial rate of increase was still linear but first gradually and then abruptly decreased as esterification was established, the middle portion of the curve (Fig. 3 *a*) representing the balance between a constant liberation and an increasing esterification. The addition of arsenate did not increase the rate of liberation

Fig. 4. Induction period of zymin fermentation.

- (a) 2 g. zymin + 1 g. fructose + 60 cc. H_2O . Induction time, > 80 min.
- (b) 2 g. zymin + 1 g. fructose + 2.4 cc. 0.5 M Na_2HAsO_4 + 2.5 cc. 0.1 M K hexosediphosphate + 55.1 H_2O . Induction time, > $3\frac{1}{2}$ hr.
- (c) 2 g. zymin + 1 g. fructose + 2.5 cc. hexosediphosphate + 57.5 H_2O . Induction time, 0.

(Fig. 4, *a* and *b*), though by its effect in delaying esterification and thus maintaining the original linear rate, the total amount of the increase in inorganic phosphate was greater than in the control in those cases where the normal induction time was shorter than the time of complete hydrolysis. Estimation of methylglyoxal by distillation and precipitation as the *p*-nitrophenylosazone showed that no accumulation of triose had taken place during the induction period.

It was also found that the total amount of acid-soluble phosphorus present in the trichloroacetic acid filtrate rose during the induction period by an amount approximately equal to the increase in inorganic phosphate. Thus in one case the inorganic phosphate increased from 9.13 to 19.4 mg. during an induction period of 60 minutes, while the total acid-soluble P increased from 23.2 to 32.0 mg. It seems probable that the increase in inorganic phosphate must be due to changes in some other phosphorus-containing substance in zymin during the induction period rather than to the decomposition of hexosephosphoric esters. It must at least be concluded that the action of arsenate in prolonging the induction is not directly due to its specific action in accelerating the rate of fermentation of hexosediphosphate.

It is interesting to note that the same treatment which decreases the rate of fermentation of yeast and enables it to acquire the power of responding to phosphate, such as drying or treating with acetone, also injures the indophenol oxidase-cytochrome system [Keilin, 1929], one of the instances of injury being that cytochrome in dried yeast and zymin is reduced in amount and undergoes oxidation and reduction more slowly than in untreated yeast. In yeast suspensions warmed to 52° the reduction of cytochrome is markedly accelerated by lactate and also by succinate [Keilin, 1929], while arsenate increases the reduction time in suspensions of fresh yeast [Bierich and Rosenbohn, 1929]. The antagonistic action of these two salts in this case is parallel with their effect on the induction period of zymin fermentation, which is decreased and prolonged by lactate and arsenate respectively [Harden and Macfarlane, 1928]. This indicates that the phenomenon may be associated with the cytochrome content of the yeast preparation, and attempts are being made to obtain experimental evidence on this question.

SUMMARY.

1. The fermentation of hexosediphosphate by the hexosephosphatase preparation previously described in presence of co-enzyme and arsenate is inhibited by mono-iodoacetate.
2. The esterification of sugar by yeast juice is inhibited by mono-iodoacetate in concentrations which only slightly inhibit that of glycogen.
3. Arsenate does not accelerate the rate of fermentation by yeast juice in presence of mono-iodoacetate.
4. The rate of liberation of inorganic phosphate during the induction period of zymin fermentation is not accelerated by arsenate, showing that this phosphate does not arise by fermentation of hexosediphosphate.

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