XL. PHOSPHORIC ESTERS IN ALCOHOLIC FERMENTATION.

II. PYROPHOSPHATE IN YEAST PREPARATIONS.

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LOHMANN [1928, 1] has isolated pyrophosphate from plants, the muscles of many animals and from living yeast. He has shown [1928, 2] that about one quarter of the total phosphorus present in both brewer's and baker's yeast occurs as pyrophosphate. More recently Lohmann [1929] has suggested that the pyrophosphate of muscle is combined with adenylic acid and may be concerned with ammonia production.

This idea is supported by the results obtained by Eggleton and Eggleton [1929] on the phosphorus compounds of muscle, and by the work of Embden, Hefter and Lehnartz [1930].

In studying the reaction of orthophosphate with zymin and yeast juice, Harden and Henley [1927] found that often only about 90 % of the theoretical amount of carbon dioxide was evolved. It is probable that the deficiency in carbon dioxide evolution is really due to depression of the basic fermentation to an unknown extent. The deficiency might, however, have been due to formation of pyrophosphate, but the present work shows that not to be the case.

ESTIMATION OF PYROPHOSPHATE.

Lohmann [1928, 1] and Eggleton and Eggleton [1929] have estimated the pyrophosphate of muscle as the phosphate liberated by hydrolysis for 7 minutes in *N* HCl at 100°. This gives reliable results with muscle, where there is no hexosediphosphate, but it cannot be applied to yeast preparations in which most of the phosphorus is usually present as the diphosphoric ester. In *N* HCl at 100° in 7 minutes hexosediphosphate loses about a third of its phosphorus as free phosphate. For this reason the old method of estimation of pyrophosphate could not be applied to yeast preparations.

In order to estimate pyrophosphate and hexosediphosphate it was necessary to find a method of separating these two compounds. Lohmann [1929] suggests that they can be separated as barium salts in slightly acid solution (p_H 4·5), but on trial this method was found to be not quite quantitative. Attempts were made to separate these two phosphates as the uranium, copper, chromium

and iron salts, but largely on account of the complex pyrophosphate compounds, none of these methods gave a reliable separation. Finally the barium salts were used. While both barium ortho- and pyro-phosphate are insoluble in neutral solution, the salt of hexosediphosphate is soluble to the extent of 0·6 % in cold water.

The filtrate from the trichloroacetic acid precipitation was diluted until it contained less than 0·5 mg. phosphorus per cc. and was then treated with cold barium hydroxide solution till neutral to phenolphthalein and kept at 5° overnight. The organic phosphorus (hexosediphosphate and hexosemonophosphates) was estimated as the phosphorus left in solution as barium salt. The precipitate was removed by centrifuging and washed. It was then dissolved in dilute hydrochloric acid, the insoluble matter being removed by centrifuging and estimated as nucleic acid. The hydrochloric acid solution was again precipitated with barium hydroxide and the precipitate was assumed to be a mixture of ortho- and pyro-phosphate. By determination of the orthophosphate and total phosphorus present a figure was obtained for the pyrophosphate.

The phosphorus compounds of living yeast.

The method was first used to estimate the phosphorus compounds of living yeast. Fresh brewer's yeast pressed to ½ ton per square inch was ground with trichloroacetic acid and an extract obtained by centrifuging. The results of analysis of the extract are shown in Table I along with similar figures given by Lohmann [1928, 2]. It should be noted that Lohmann heated his acid extract to 100° which might cause some hydrolysis and increase in the amount of orthophosphate present, and in his method of determination some of the hexosediphosphate would be estimated as pyrophosphate rather than organic phosphorus. Considering this fact the figures are in satisfactory agreement.

Table I. *Phosphorus compounds of yeast.*

	mg. P per g. yeast.		
		From Lohmann [1928, 2]	
	English brewer's yeast	Baker's yeast	Brewer's yeast
Total phosphorus	3·25	3·28	3·00
Orthophosphate	1·37	1·61	1·54
Pyrophosphate	0·68	0·79	0·69
Organic phosphorus	1·17	0·88	0·77
Hexosediphosphate	0·38	—	—
Hexosemonophosphate	0·72	—	—
Nucleic acid*	0·07	—	—

* The precipitate of nucleic acid was washed well with water so that some may have been lost.

The reaction of pyrophosphate with yeast preparations.

The effect of adding pyrophosphate to a zymin fermentation was studied in the hope that it would throw light on the function of pyrophosphate in yeast. Pyrophosphate reacted in the same manner as orthophosphate but the delay in the reaction was a little longer.

The various amounts of phosphorus compounds present during the progress of the reaction were estimated by extension of the methods previously described [Boyland, 1929]. The results of such an experiment (Table II and Fig. 1) clearly indicate that the first change after the addition of pyrophosphate is the conversion of that salt into the orthophosphate, which then reacts in the usual way. Zymin and other preparations must therefore contain an active pyrophosphatase such as Kay [1929] has described as occurring in most mammalian tissues. The necessity for this conversion explains the slower liberation of carbon dioxide as compared with orthophosphate.

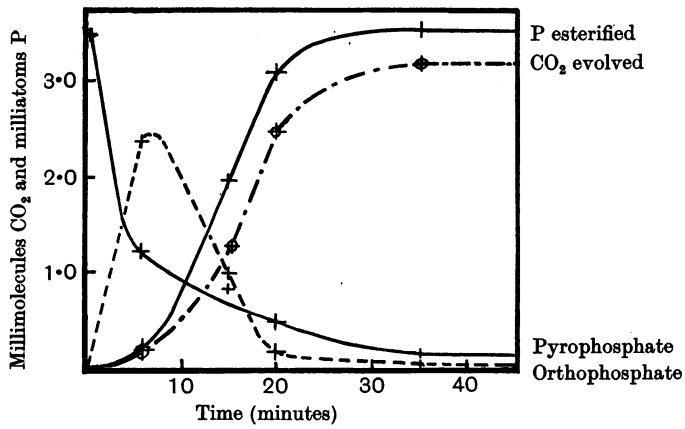

Fig. 1. Fermentation of fructose by zymin in presence of pyrophosphate.

Table II. *The reaction of potassium pyrophosphate with fermenting zymin.*

2 cc. *M* $K_2H_2P_2O_7$ added to 4 g. zymin in 25 cc. 10 % fructose solution.

Time after addition of pyrophosphate (min.)	Pyrophosphate present (milliatoms P)	Orthophosphate present (milliatoms P)	Organic phosphate formed (milliatoms P)	Carbon dioxide liberated (millimols)
½	3·5	0·18	—	—
6	1·2	2·36	0·13	0·10
15	0·8	0·98	1·95	1·24
20	0·5	0·13	3·11	2·50
35	0·1	0·02	3·57	3·20
45	0·1	0·02	3·57	3·20

Pyrophosphate in fermenting yeast juice and zymin.

Yeast juice and zymin fermentations were carried out in the manner described in a previous paper [Boyland, 1929] with and without added phosphate and stopped in equilibrium by the addition of trichloroacetic acid. The amounts of orthophosphate, pyrophosphate and organic phosphate were then estimated in the trichloroacetic acid filtrate (Table III).

It will be seen that no appreciable amount of pyrophosphate is formed in the reaction and that it can be neglected in calculating the ratio of phosphorus esterified to carbon dioxide evolved.

The constant amount of pyrophosphate which occurs and remains unaffected by pyrophosphatase in the reaction is probably combined with adenylic acid in the way that Lohmann [1929] has suggested that it occurs in muscle.

Table III. *Pyrophosphate in yeast preparations.*

A. Exp. 146. 5 cc. 0·5 *M* Na_2HPO_4 added to 2 g. of acetone precipitate of yeast juice in 25 cc. 10 % fructose solution with acetaldehyde (0·1 %) present.

Phosphorus esterified	2·46 milliatoms
Carbon dioxide evolved	2·40 millimols in 40 min.
Pyrophosphate before addition	0·21 milliatoms
„ after reaction	0·24 „

B. Exp. 158. 5 cc. *M* K_2HPO_4 added to 4 g. zymin in 25 cc. 10 % fructose solution (with acetaldehyde).

Phosphorus esterified	5·0 milliatoms
Carbon dioxide evolved	4·9 millimols in 60 min.
Pyrophosphate before reaction	0·04 milliatoms P
„ after „	0·05 „
Nucleic acid before reaction	0·02 „
„ after „	0·03 „

THE ACETONE PRECIPITATE OF YEAST JUICE.

The deficiency in the amount of carbon dioxide liberated when phosphate is esterified by zymin fermentations is probably due to depression of the basic fermentation, owing to inhibition of the phosphatase, to an unknown extent. But when relatively small amounts of orthophosphate are added to fermentations the depression is small and under such conditions the carbon dioxide production approaches the theoretical amount.

Confirmation of the equivalence of esterification and gas formation was obtained with a preparation of yeast juice which had no basic fermentation of its own but reacted to phosphate, if acetaldehyde was present. Such a preparation containing no phosphatase could not have its basic fermentation depressed in the same way as the fermentation of zymin. Although almost 90 % of the phosphorus esterified was in the form of hexosemonophosphate, the carbon dioxide evolved was equivalent to the esterified phosphate. Results of typical experiments with such an enzyme preparation are given in Tables III A (for Na_2HPO_4) and IV. This indicates that the carbon dioxide evolved by esterification of phosphate is exactly equivalent to the phosphate no matter which ester is formed.

Table IV. *The result of the reaction of pyrophosphate with a fermenting preparation of the acetone precipitate of yeast juice.*

Exp. 146 B. 5 cc. 0·6 *M* pyrophosphate added to 2 g. acetone precipitate in 25 cc. 10 % fructose solution. Results expressed in total millimols.

1·68 millimols $Na_4P_2O_7$ (3·36 milliatoms P) were esterified and 3·12 millimols carbon dioxide were evolved in 60 min.

This dry preparation of yeast juice made by precipitation with acetone as described by Buchner, E. and H. and Hahn [1903] was treated with alcohol and ether and finally dried in a desiccator over sulphuric acid. For experimental use the dry powder was ground up with sugar and water; insoluble

material was then removed by centrifuging. This removal of the insoluble material reduced the basic or hydrolysis rate of fermentation to a considerable extent, so that it would appear that hexosephosphatase is more readily removed by this treatment than are the other enzymes of zymase.

A preparation is thus obtained which has no hexosephosphatase but reacts with pyrophosphate in the presence of sugar and acetaldehyde. As pyrophosphate is hydrolysed before it reacts in this way, the preparation must contain a pyrophosphatase, and pyrophosphatase must therefore be a separate enzyme, distinct from hexosephosphatase.

Kay [1929] suggested that the pyrophosphatase action of mammalian tissues was probably due to orthophosphatase, although Lohmann [1928, 3] had shown that pyrophosphatase had a lower optimum p_H (7·0) than has orthophosphatase (p_H 9·0). This difference in optimum hydrogen ion concentration for the two enzymes supports the conclusion drawn above, that pyrophosphatase is a distinct enzyme.

Summary.

1. A method for the separation of hexosediphosphate and pyrophosphate, depending on precipitation of barium pyrophosphate in dilute solution is described.

2. Pyrophosphate occurs in living yeast and forms about a quarter of the total phosphorus of yeast.

3. When pyrophosphate is added to fermenting zymin it is rapidly hydrolysed to orthophosphate which reacts in the usual way, hexosephosphates being formed and carbon dioxide liberated.

4. A preparation of the acetone precipitate of yeast juice did not ferment sugar (having no hexosephosphatase), but in the presence of acetaldehyde reacted with both ortho- and pyro-phosphate to liberate the theoretical amount of carbon dioxide and form 90 % hexosemonophosphate. This preparation must contain pyrophosphatase, which therefore would seem to be distinct from hexosephosphatase.

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