# CXCV. PHOSPHORYLATION IN LIVING YEAST.

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(*Received 1 July 1936.*)

Euler & Adler [1935] recently described the preparation from yeast of a cell-free enzyme, "heterophosphatese" which catalyses the transference of phosphate from (muscle) adenosinetriphosphate to glucose or fructose with the formation of a hexosephosphate. Later Meyerhof [1935] showed that preparations of hexokinase contain "heterophosphatese". The natural inference from these experiments is that adenosinetriphosphate may act as a phosphate carrier in alcoholic fermentations by living yeast, in a manner analogous to its role in muscle glycolysis [see Meyerhof & Kiessling, 1935]. This receives material support by the isolation of the triphosphate in crystalline form from the trichloroacetic acid extract of fresh yeast by Wagner-Jauregg [1936] who concluded that it was identical with that of muscle. The amount obtained (60 mg. from 100 g. yeast) contains approximately 2% of the total phosphorus of the yeast.

It seemed possible that the amount of adenosinetriphosphate present in yeast might be increased during the fermentation of sugar by synthesis from adenylic acid, and this question was investigated. Judging by the analysis of the sparingly soluble barium salts no increase in the triphosphate content was found if the yeast were extracted with trichloroacetic acid whilst fermenting sugar rapidly, but it was noticed that such extracts contained less orthophosphate than similar extracts from resting yeast. It is very generally inferred that alcoholic fermentation by living yeast involves esterification of orthophosphoric acid with carbohydrate, similar to that observed in cell-free fermentations, though there is no direct evidence in support of this view. The decrease in orthophosphate has therefore been investigated more fully, and it has been established that at the onset of fermentation of sugar by living yeast the acid-soluble orthophosphate rapidly decreases to a fairly constant level and rises again to the original level when the sugar is consumed.

Whilst this work was in progress Lewitow [1936] showed that as a result of the fermentation of sugar both the (acid-soluble) ortho- and pyro-phosphate contents of living yeast were decreased and came to the conclusion that pyrophosphate acts as a phosphate carrier in alcoholic fermentation in the same manner as adenosinetriphosphate in the glycolysis of animal cells. His experimental findings are open to criticism on several points. The routine deproteinization with trichloroacetic acid was preceded by 5 minutes' boiling of the yeast suspension, which materially alters the phosphorus distribution (see Table IV). The value for pyrophosphate was calculated from the hydrolysis curve in *N* HCl at 100° according to the equation

$$\text{Pyro-P} = P_{7\,\text{min.}} - [P_{0\,\text{min.}} + \tfrac{7}{8}\,(P_{15\,\text{min.}} - P_{7\,\text{min.}})]$$

[Braunstein, 1931] which does not provide an accurate correction for the presence of other phosphoric esters. Finally the implication from the statement "Unter Zuckerzusatz, d.h. in gärender Hefe" that the yeast was still fermenting after 5 hours' incubation is correct only if sufficient sugar were present, which appears

doubtful, and no evidence was offered that the changes induced by the fermentation were reversed by its cessation. His conclusion that during fermentation the orthophosphate decreases in amount has been fully confirmed in the present work, but no evidence was found to support the view that pyrophosphate acts as a phosphate carrier.

## EXPERIMENTAL.

### *Phosphorylation in living yeast.*

The yeast was washed with water, filtered and pressed. 1 g. samples of pressed yeast were weighed into fermentation flasks, water or sugar solution (total volume 12 ml.) being added as desired; the flasks were placed in a thermostat at 30° and connected to a nitrometer for measurement of the $CO_2$ evolved. The enzyme action was stopped by the addition of 3 ml. 25 % trichloroacetic acid. Phosphate estimations were carried out by Briggs's method on aliquot portions of the trichloroacetic acid filtrate, after appropriate treatment, to obtain values for

(*a*) total acid-soluble phosphate, after ashing with $H_2SO_4$ and $H_2O_2$;

(*b*) orthophosphate, by direct estimation;

(*c*) "labile" phosphate by calculation from the hydrolysis curve in *N* HCl at 100° using Lohmann's equation.

$$\text{"Labile" P} = \Delta_{7\,\text{min.}-0\,\text{min.}} - \Delta_{30\,\text{min.}-7\,\text{min.}},$$

which provides a more accurate correction for the presence of other phosphoric esters than that of Braunstein;

(*d*) organic phosphate, calculated as the difference between total phosphate and the sum of the inorganic and labile phosphate values.

Details of the experiments will be found in Tables I and II. It may be emphasized that no phosphate was added to the fermentation flasks. The variation in the values in duplicate experiments due to the sampling of the yeast and the accuracy of the estimations was of the order, for orthophosphate, $\pm 0{\cdot}02$ and for total, labile and organic phosphate, $\pm 0{\cdot}05$ mg. P per g. yeast.

The experiments recorded in Table I were carried out with brewery pressed yeast (mild ale) or with yeast grown four days in yeast extract in the laboratory from an inoculum of brewery yeast. These batches were nominally living yeast, but it was found that rather a large proportion (6–20 %) of the cells were stained after standard treatment with methylene blue (1 drop of 0·1 % methylene blue to 17 drops of yeast suspension containing 40,000 cells per mm.$^3$). Whether or not such cells are to be considered dead in the sense that they will no longer reproduce, their capacity to be stained must reflect a significant difference in condition, whatever the immediate cause. The possibility and even probability thus arises that the fermentations induced by such cells may be as qualitatively different from that of unstainable cells as is the fermentation of yeast juice. So that for example an accumulation of hexosephosphate during $CO_2$ production by a small proportion of the cells might be interpreted as a small but definite change in the phosphorus distribution in all the cells. Moreover, it was realized that a temperature above 25° is generally considered pathological for yeast, so that incubation at 30° might produce fallacious results even though the initial percentage of cells stainable with methylene blue was low.

Through the courtesy of Mr Julian Baker, yeast was obtained from the brewery vats two days after pitching, at a stage of maximum growth and with a very low count (0–5 %) of stainable cells. In order to eliminate from consideration the small changes in phosphorus distribution which take place when yeast

Table I. *Phosphorylation in living yeast at 30°.*

| Treatment of yeast | Total incubation time min. | % cells stained | Type of fermentation | Acid-soluble phosphorus. mg. per g. yeast: Total P | Ortho-P | Labile P | Organic P |
|---|---|---|---|---|---|---|---|
| Y. 9. Brewery press yeast 1 day old. | | | | | | | |
| 1. +10 ml. $H_2O$; not incubated | 0 | 18 | — | 2·48 | 0·71 | 1·18 | 0·59 |
| 2. +10 ml. $H_2O$ 30 min. | 30 | — | NF | 2·59 | 0·90 | 1·15 | 0·54 |
| 3. +10 ml. $H_2O$ 30 min.; +2 ml. 10% glucose 20 min. | 50 | — | F | 2·22 | 0·54 | 0·99 | 0·69 |
| 4. +10 ml. $H_2O$ 30 min.; +2 ml. 10% glucose 60 min. | 90 | — | SF | 2·46 | 0·70 | 1·12 | 0·64 |
| 5. +10 ml. $H_2O$ 30 min.; +2 ml. 10% glucose 20 min. oxygenated throughout | 50 | — | — | 2·10 | 0·43 | 0·91 | 0·76 |
| Y. 10. Brewer's yeast grown in yeast extract in laboratory. | | | | | | | |
| 6. +10 ml. $H_2O$ 25 min. | 25 | — | NF | 4·60 | 0·99 | 3·15 | 0·45 |
| 7. +10 ml. $H_2O$ 25 min. | 25 | — | NF | 4·60 | 0·94 | 3·26 | 0·45 |
| 8. +10 ml. $H_2O$ 25 min.; +2 ml. 10% glucose 25 min. | 50 | — | F | 3·90 | 0·59 | 2·91 | 0·40 |
| 9. +10 ml. $H_2O$ 25 min.; +2 ml. 10% glucose 25 min. | 50 | — | F | 3·97 | 0·59 | 2·95 | 0·41 |
| Y. 11. Similar to Y. 10. | | | | | | | |
| 10. +10 ml. $H_2O$; not incubated | 0 | 6 | — | 2·83 | 0·77 | 1·81 | 0·25 |
| 11. +10 ml. $H_2O$ 15 min. | 15 | — | NF | 3·48 | 0·87 | 2·32 | 0·29 |
| 12. +10 ml. $H_2O$ 15 min.; +1 ml. 10% glucose 15 min. | 30 | — | F | 2·97 | 0·52 | 2·11 | 0·34 |
| 13. +10 ml. $H_2O$ 15 min.; +1 ml. 10% glucose 15 min. | 30 | — | F | 2·85 | 0·49 | 2·02 | 0·34 |
| 14. +10 ml. $H_2O$ 15 min.; +1 ml. 10% glucose 35 min. | 50 | — | SF | 3·12 | 0·78 | 2·06 | 0·28 |
| Y. 12. Brewery press yeast. | | | | | | | |
| 15. +10 ml. $H_2O$; not incubated | 0 | 10 | — | 2·43 | 0·90 | 1·05 | 0·48 |
| 16. +10 ml. $H_2O$ 15 min. | 15 | — | NF | 2·91 | 1·02 | 1·32 | 0·57 |
| 17. +10 ml. $H_2O$ 15 min.; +1 ml. $H_2O$ 15 min. | 30 | — | NF | 2·94 | 1·05 | 1·41 | 0·48 |
| 18. +10 ml. $H_2O$ 15 min.; +1 ml. 10% glucose 15 min. | 30 | — | F | 2·61 | 0·66 | 1·35 | 0·60 |
| 19. +10 ml. $H_2O$ 15 min.; +1 ml. 10% glucose 30 min. | 45 | — | <F | 2·88 | 0·75 | 1·53 | 0·60 |
| 20. +10 ml. $H_2O$ 15 min.; +1 ml. 10% glucose 60 min. | 75 | — | SF | 2·94 | 0·93 | 1·56 | 0·45 |
| 21. +10 ml. $H_2O$ 15 min.; +1 ml. 10% glucose 60 min. +1 ml. glucose 15 min. | 90 | — | F | 2·82 | 0·57 | 1·56 | 0·69 |
| 22. +10 ml. $H_2O$ +1 ml. NaF 15 min.; + 1 ml. $H_2O$ 15 min. | 30 | — | NF | 2·94 | 0·57 | 1·35 | 1·02 |
| 23. +10 ml. $H_2O$ +1 ml. NaF 15 min.; +1 ml. glucose 15 min. | 30 | — | Inhibited | 3·21 | 0·42 | 1·65 | 1·14 |
| 24. +10 ml. $H_2O$ +1 ml. IA 15 min.; +1 ml. $H_2O$ 15 min. | 30 | — | NF | 3·21 | 0·87 | 1·56 | 0·78 |
| 25. +10 ml. $H_2O$ +1 ml. IA 15 min.; +1 ml. glucose 15 min. | 30 | — | Inhibited | 3·21 | 0·90 | 1·50 | 0·81 |
| 26. +10 ml. $H_2O$ +1 ml. toluene 5 min. | 5 | — | — | 3·41 | 1·26 | 1·32 | 0·83 |
| 27. +10 ml. $H_2O$ +1 ml. toluene 5 min.; +1 ml. $H_2O$ 15 min. | 20 | — | NF | 4·08 | 2·14 | 0·77 | 1·17 |
| 28. +10 ml. $H_2O$ +1 ml. toluene 5 min.; +1 ml. glucose 15 min. | 20 | — | <F | 3·93 | 2·09 | 0·71 | 1·13 |

Solutions: 10% glucose. 1 ml. = 25 ml. $CO_2$.
0·1 *M* sodium fluoride.
0·1 *M* sodium iodoacetate.
Rate of fermentation ml. $CO_2$ per 5 min. per g. yeast at 30°: Autofermentation <0·2
Maximum with sugar 5·0.

Symbols: NF, no sugar added.
F, fermenting at maximum rate.
SF, sugar completely fermented.

is incubated with water alone, the experiments were so arranged that comparison could be made between samples incubated for equal times which (*a*) had received no sugar, (*b*) were fermenting sugar at the maximum rate and (*c*) had completely fermented the sugar added. The experiments are recorded in Table II and for convenience the differences in the various fractions between fermenting and non-fermenting samples are summarized in Table III.

The results show that during autofermentation of living yeast, the orthophosphate content is not appreciably changed but that there is some variation in the organic and labile phosphate contents. In yeast fermenting sugar the orthophosphate is decreased in amount (approximately 0·2–0·3 mg. P per g. yeast) but returns to the original level as fermentation ceases. This decrease persists in the presence of fluoride but not in that of iodoacetate, the fermentation being inhibited in each case. The slight increase in the organic phosphorus occasionally found in no case balances the decrease in orthophosphate as might be expected

Table II.

| Treatment | Temp. ° C. | Total incubation time min. | % cells stained | Type of fermentation | Acid-soluble phosphorus mg. P per g. yeast: Total P | Ortho-P | Labile P | Organic P |
|---|---|---|---|---|---|---|---|---|
| Y. 13. Brewery vat yeast. | | | | | | | | |
| 1. +12 ml. $H_2O$; not incubated | — | 0 | 0 | — | 2·76 | 0·96 | 1·29 | 0·54 |
| 2. +11 ml. $H_2O$ 15 min. | 18 | 15 | — | NF | 2·67 | 0·96 | 1·20 | 0·51 |
| 3. +11 ml. $H_2O$ 30 min. | ,, | 30 | — | NF | 2·46 | 0·96 | 1·05 | 0·48 |
| 4. +11 ml. $H_2O$ 45 min. | ,, | 45 | — | NF | 2·76 | 0·96 | 1·29 | 0·51 |
| 5. +11 ml. $H_2O$ 60 min. | ,, | 60 | — | NF | 2·76 | 0·99 | 1·20 | 0·57 |
| 6. +11 ml. $H_2O$ 60 min.; +1 ml. 5 % glucose 20 min. | ,, | 80 | — | F | 2·40 | 0·72 | 1·05 | 0·63 |
| 7. +10 ml. $H_2O$ +1 ml. 5 % glucose 10 min. | ,, | 10 | — | F | 2·91 | 0·66 | 1·59 | 0·66 |
| 8. +10 ml. $H_2O$ +1 ml. 5 % glucose 30 min. | ,, | 30 | — | F | 2·67 | 0·69 | 1·38 | 0·60 |
| 9. +10 ml. $H_2O$ +1 ml. 5 % glucose 60 min. | ,, | 60 | — | <F | 2·70 | 0·74 | 1·43 | 0·53 |
| 10. +10 ml. $H_2O$ +1 ml. 5 % glucose 60 min.; +1 ml. glucose 15 min. | ,, | 75 | — | F | 2·43 | 0·60 | 1·14 | 0·69 |
| 11. +11 ml. $H_2O$ 15 min. | 30 | 15 | — | NF | 3·12 | 0·90 | 1·65 | 0.57 |
| 12. +10 ml. $H_2O$ +1 ml. 5 % glucose 15 min. | ,, | 15 | — | F | 3·03 | 0·72 | 1·83 | 0·48 |
| Y. 14. Brewery vat yeast. | | | | | | | | |
| 13. +12 ml. $H_2O$; not incubated | — | 0 | 3·3 | — | 2·49 | 0·81 | 1·02 | 0·66 |
| 14. +11 ml. $H_2O$ 15 min. | 24 | 15 | — | NF | 2·22 | 0·78 | 0·84 | 0·60 |
| 15. +11 ml. $H_2O$ 60 min. | ,, | 60 | — | NF | 2·28 | 0·81 | 0·84 | 0·63 |
| 16. +11 ml. $H_2O$ 60 min.; +1 ml. 5 % glucose 15 min. | ,, | 75 | — | F | 1·74 | 0·51 | 0·66 | 0·57 |
| 17. +11 ml. $H_2O$ 120 min. | ,, | 120 | 4·5 | NF | 2·52 | 0·87 | 1·08 | 0·57 |
| 18. +11 ml. $H_2O$ 120 min.; +1 ml. 5 % glucose 15 min. | ,, | 135 | — | F | 2·31 | 0·63 | 0·96 | 0·72 |
| 19. +10 ml. $H_2O$ +1 ml. 5 % glucose 15 min. | ,, | 15 | — | F | 2·25 | 0·57 | 1·05 | 0·63 |
| 20. +10 ml. $H_2O$ +1 ml. 5 % glucose 60 min. | ,, | 60 | — | SF | 2·26 | 0·81 | 0·84 | 0·61 |
| 21. +10 ml. $H_2O$ +1 ml. 5 % glucose 120 min. | ,, | 120 | — | SF | 2·30 | 0·81 | 1·11 | 0·38 |
| 22. +10 ml. 5 % glucose +1 ml. $H_2O$ 120 min. | ,, | 120 | 6·6 | F | 2·70 | 0·39 | 1·56 | 0·75 |
| 23. +11 ml. $H_2O$, 24 hr. +1 ml. $H_2O$ 15 min. | ,, | 24 hr. | — | NF | 3·09 | 1·11 | 1·53 | 0·45 |
| 24. +11 ml. $H_2O$, 24 hr. +1 ml. glucose 15 min. | ,, | 24 hr. | — | F | 3·10 | 0·87 | 1·68 | 0·55 |
| 25. +11 ml. $H_2O$ 120 min. | 37 | 120 min. | 6·8 | NF | 3·27 | 1·17 | 1·41 | 0·69 |
| 26. +10 ml. 10 % glucose +1 ml. $H_2O$ 120 min. | ,, | ,, ,, | 19·0 | F | 2·55 | 0·66 | 1·08 | 0·81 |

Symbols as in Table I.
Rates of fermentation. ml. $CO_2$ per 5 min. per g. yeast at 18°. Autofermentation 0. Maximum with sugar 1·5.
Rates of fermentation. ml. $CO_2$ per 5 min. per g. yeast at 24°. Autofermentation <0·1. Maximum with sugar 1·9.

Table III. *Difference in acid-soluble phosphate content between fermenting and non-fermenting yeast (from Table II, F–NF).*

| Yeast | Temp. ° C. | Incubation time | Acid-soluble mg. P per g. yeast: Total | Ortho-P | Labile P | Organic P |
|---|---|---|---|---|---|---|
| Y 13 | 18 | 10–15 min. | +0·24 | −0·30 | +0·39 | +0·15 |
| | | 30 ,, | +0·21 | −0·27 | +0·33 | +0·12 |
| | | 60–80 ,, | −0·33 | −0·39 | −0·06 | +0·12 |
| | | | −0·36 | −0·29 | −0·15 | +0·06 |
| Y 14 | 24 | 15 ,, | +0·03 | −0·21 | +0·21 | +0·03 |
| | | 60–75 ,, | −0·54 | −0·30 | +0·18 | −0·06 |
| | | 120–135 ,, | −0·21 | −0·24 | −0·12 | +0·15 |
| | | 24 hours | +0·01 | −0·24 | +0·15 | +0·10 |
| Y 13 | 30 | 15 min. | −0·09 | −0·18 | +0·18 | −0·09 |
| Y 14 | 37 | 120 ,, | −0·72 | −0·51 | −0·33 | +0·12 |

if hexosephosphoric esters were formed. Taking into account the limited accuracy of determination of the labile and organic phosphate values, and the changes during autofermentation, the variation in these fractions could not be consistently related to the onset of fermentation. Lohmann [1928, 2] showed that the amount of phosphate extracted by trichloroacetic acid from baker's yeast was increased by preliminary heating of the yeast in water, or by addition of toluene, and that much of this phosphate was acid-labile. This is true also of the brewery yeast used in these experiments (Table IV). The variation in labile and

Table IV. *Increase in acid-soluble P after preliminary treatment of yeast.*

| Treatment | mg. acid-soluble P per g. yeast: Total P | Ortho-P | Pyro-P |
|---|---|---|---|
| (1) 1 g. + 10 ml. $H_2O$ + 3 ml. 25% trichloroacetic acid | 2·91 | 0·87 | 1·59 |
| (2) 1 g. + 10 ml. $H_2O$ 2·25 min. at 100°, cooled + trichloroacetic acid | 3·84 | 1·02 | 2·13 |
| (3) 1 g. + 10 ml. *N* HCl 7 min. at 100°, cooled + trichloroacetic acid | 6·35 | 4·50 | — |

Total P by ashing 6·35 mg. per g. yeast.

organic phosphate may therefore be due to a shift between acid-soluble and insoluble phosphorus compounds, and the disappearance of orthophosphate may be ascribed as justly to the formation of an acid-insoluble as to that of an acid-soluble compound, especially as in many cases the decrease coincided with a decrease in the total acid-soluble phosphate.

### *Phosphorus compounds of yeast.*

Pressed yeast contains about 6·0 mg. P per g., of which 50% can be extracted with trichloroacetic acid, approximately 15% as orthophosphate, 25% as a labile phosphoric acid shown by Lohmann [1928, 1] to contain inorganic pyrophosphate and 10% in organic combination. The latter probably includes cozymase and Warburg's coferment (about 1%) as well as adenosinetriphosphate (2%) and adenylic acid. Calculated from the yields which have been obtained 8–12% of the phosphorus is present in (acid-insoluble) nucleic acid [see Levene & Bass, 1931] and 2% as lipin-P [MacLean & Daubney, 1927], whilst from the flavin content [Pett, 1935] 0·05% may be present as flavinmonophosphate. The phosphorus compounds enumerated account for 65% of the total phosphorus of yeast; before attempting to identify the unknown compound formed by the orthophosphate disappearing during fermentation, it was necessary to obtain a very precise idea of the nature of the remaining phosphorus.

*Extraction of phosphorus compounds from the yeast cell.*

Yeast obtained from the brewery two days after pitching was washed 3 times with water, filtered and pressed to a cake on Büchner funnels. The cake was then submitted in succession to the following treatments.

*Extract 1.* The yeast was well mixed with 5% trichloroacetic acid (300 ml. per 100 g. yeast), filtered and re-extracted with half the original volume of acid, the filtrates being combined.

*Extract 2.* The residual yeast cake was suspended in five parts of water (*p*H about 2·0) and sufficient 2 *N* NaOH (about 15–20 ml. per 100 g. yeast) added to bring the *p*H to 8·6–9·0. As the sodium hydroxide was added, part of the cell contents passed rapidly into the solution. The latter, which was reddish brown in colour, filtered fairly easily through a No. 3 (Whatman) paper under suction; and on addition of two volumes methcol gave a flocculent precipitate containing >90% of the phosphorus present in the extract; yield, after washing with alcohol and drying, about 1·0 g. per 100 g. yeast.

*Extract 3.* The yeast cake was again suspended in water and re-filtered giving a clear yellow filtrate from which the phosphorus compounds present were precipitated by addition of methcol either as sodium or barium salts.

At this stage the yeast cell membrane was still unruptured. An attempt was made with one batch of yeast to extract the remaining phosphorus by grinding

Table V.

| Yeast no. | Preliminary treatment | % total P of yeast found in: Extract 1. trichloroacetic — Total P | Ortho-P | Labile P | Extract 2 aq. neutral — Total P | Ortho-P | Labile P | Extract 3 2nd aqueous — Total P | Ortho-P | Labile P | Analyses of Na salts precipitated by methcol from extract 2: % of total P of yeast in salt | % composition: C | H | N | P | Na | $[\alpha]_{5461}$ | % P hydrolysed in 7 min. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | None | 61 | 17 | 33 | 23 | 0 | 19·4 | 7·8 | 0 | 4·7 | 20·6 | — | — | 9·3 | 16·0 | 10·3 | +54° | 83 |
| 17 | None | 52 | — | — | 24 | 0 | 22 | 8·9 | 0 | — | 19·0 | — | — | 7·1 | 18·6 | — | +27 | 92 |
| 18 | None | 39 | 14 | — | 36 | 0 | 32 | 7·2 | 0 | — | 33·0 | — | — | 5·0 | 17·9 | — | +36 | 89 |
| 21A | None | 45 | 17 | 21 | 28 | 0 | 26 | 4·4 | 0 | — | — | — | — | — | — | — | — | — |
| 22A | None | 45 | 18 | 22 | 31 | 0 | 28 | 9·0 | 0 | — | 29 | — | — | 6·9 | 16·0 | — | +32 | 85 |
| 14C | None | 45 | 13 | 19 | 36 | 0 | 28 | — | — | — | 36 | Precipitated as very insoluble Ba salts | | | | | | |
| 14B | Autofermentation + toluene + NaF | 68 | 19 | 3 | 25 | 0 | 15 | — | — | — | 12 | Precipitated as very insoluble Ba salts | | | | | | |
| 15C | Autofermentation, no toluene, 4 hr. | 37 | 16 | 17 | 32 | 0 | 25 | 8·4 | — | — | 25 | 19·9 | 3·4 | 8·6 | 16·3 | 10·3 | +43 | 80·5 |
| 15A | Autofermentation, + toluene, 4 hr. | 62 | 46 | 2 | 18 | 0 | 8 | 0 | — | — | 11 | 30·1 | 4·1 | 13·5 | 8·5 | 6·9 | +73 | 43·0 |
| | | | | | | | | | | | | | | | | | $[\alpha]_D$ | |
| | | | | | | | | | | | Theory for tetrasodium nucleate | — | 32·8 | 3·6 | 15·1 | 8·9 | 6·6 | +60 | — |

the yeast cake (200 g.) with equal parts of sand and kieselguhr. The ground mass was then extracted successively with 1 % saline (extract 4), 5 % trichloroacetic acid (extract 5) and water + NaOH at *p*H 9·0 (extract 6), which removed 1·8, 1·5 and 1·5 % of the total phosphorus respectively, together with 2 g. glycogen (extract 4) and 1 g. protein (extract 6). The phosphorus contained in the last four extracts, though small in amount, may be qualitatively important; up to the present attention has been paid chiefly to the phosphorus compounds present in the first two extracts. The distribution in different batches of yeast is shown in Table V.

*Phosphorus compounds present in the trichloroacetic acid extract.*

*Extract 1.* The trichloroacetic acid filtrate was treated with a slight excess of barium acetate, the *p*H adjusted to 8·0 with baryta and 1/10 volume alcohol added. The precipitate of sparingly soluble barium salts was removed by filtering. After estimation of the phosphorus present the filtrate was treated with basic lead acetate in slight excess, any precipitate removed and the phosphorus remaining in solution estimated. It has been generally assumed that hexosediphosphate, which forms a sparingly soluble barium salt, and hexosemonophosphate, which forms a soluble barium and a sparingly soluble basic lead salt, are present in fresh yeast, though no rigorous identification has been made. The percentage of the total phosphorus of yeast allotted to the hexosediphosphate and hexosemonophosphate fractions (Table VI) on the basis of this separation shows that the

Table VI. *Distribution of acid-soluble phosphorus in fresh yeast.*

| | % of total P of yeast. | | | | | |
|---|---|---|---|---|---|---|
| Yeast | Total acid-sol. | Organic P | P in 10 % alcohol filtrate | P in basic Pb filtrate | P as "hexose diphosphate" | P as "hexose monophosphate" |
| 1 | 46 | 7 | 4·7 | 4·2 | 2·3 | 0·5 |
| 2 | 41 | 8 | 6·7 | 4·8 | 1·3 | 1·9 |
| 3 | 39 | 6·3 | 4·7 | — | 1·6 | — |
| 13 | 49 | 10·1 | 5·7 | 5·2 | 4·4 | 0·5 |

concentration of these esters, if they are present at all, is very small [see Boyland, 1930], but some of the phosphate left in solution after treatment with basic lead acetate may be hexosemonophosphate.

As a consequence of the identification of metaphosphoric acid in the acid-insoluble portion of yeast, described in the next section, the sparingly soluble barium salts from the acid extract were tested for the presence of metaphosphate with positive results.

Trichloroacetic acid filtrate from 200 g. fresh yeast (Y 20). Total P 495 mg.; ortho-P 89 mg.; labile P 289 mg. 5 g. barium acetate and 0·25 volume alcohol were added (*p*H 2) giving 1·2 g. barium precipitate (P, 16·7 %; 200 mg. P). 1·17 g. of this salt were extracted three times with 10 ml. $H_2O$.

Analysis of water-insoluble portion. Wt. 0·94 g. 173 mg. P (=60 % recovery of original labile P). P, 18·4 %, of which 96 % was hydrolysed in 7 min. in *N* HCl at 100°. Ba, 41·9 %; N, 0·4 %. Atomic ratio P/Ba = 2·0/1·03. 1·6 mg. of this salt in acid solution gave a white precipitate with 1 ml. of peptone solution, indicating metaphosphoric acid. The analyses of barium metaphosphate and acid barium pyrophosphate are very similar; ($Ba(PO_3)_2$ requires P, 20·99 %; Ba, 46·51 %; $BaH_2P_2O_4$; P, 19·8 %; Ba, 43·7 %). An attempt was made to prepare the acid barium salt of pyrophosphoric acid; after three reprecipitations with alcohol at *p*H 2·0 of a HCl solution of barium pyrophosphate, the precipitate obtained was partly soluble in water and was obviously still a mixture of the neutral and acid salts (P, 16·4 %; Ba, 45·8 %; ratio P/Ba = 2/1·25).

It was concluded that part of the acid-soluble labile phosphoric acid of yeast is present as metaphosphate; it may be recalled that Lohmann [1928, 1] remarked upon the colloidal nature of the labile phosphoric acid fraction in yeast, though he succeeded in isolating sodium pyrophosphate in crystalline form.

*Isolation of a labile phosphoric acid compound from the acid-insoluble portion of the yeast cell.*

*Extract 2*, aqueous extract at *p*H 9·0. The analyses of the sodium salts precipitated by addition of methcol to this extract from different batches of yeast are given in Table V. The salts were light brown in colour, very soluble in water and had a high surface tension. The re-dissolved substance filtered very badly and frequently was not re-precipitated by alcohol at *p*H 8·0, though acidification to *p*H 6·0–7·0 gave immediate flocculation. On acidifying with glacial acetic acid to *p*H 3·0 the aqueous solution (10 %) became opalescent but did not flocculate. All the salts contained iron (1–2 %) and gave a blue colour on boiling with orcinol in 50 % HCl.

The salts obtained from fresh yeast, either resting or after autofermentation, contained 16–18 % P (of which 80–90 % was hydrolysed in 7 min. in *N* HCl at 100°) and approximately 7 % N, the atomic ratio P/N being of the order 12/14. The salt obtained in a similar manner from yeast which had been allowed to autoferment after addition of toluene (Y15A) contained only 8·5 % P (of which 43 % was labile), the P/N ratio being 4/14. The analysis of this salt corresponds closely with that required for the tetrasodium salt of nucleic acid; it has been assumed for the present, without identification of individual nucleotides, that the nitrogenous component of these labile phosphoric compounds is essentially ribonucleic acid on the following grounds:

(*a*) The percentage composition and molecular rotation.

(*b*) The physical properties.

(*c*) The absolute yield (corresponding to about 10 % of the total P) which agrees with the yields of nucleic acid previously obtained.

(*d*) Failure to find the required amount of nucleic acid in other fractions.

It is of course possible that the salts are merely mixtures of a nucleate and a labile inorganic phosphate; but, since the latter might be expected to be soluble in trichloroacetic acid, it was tentatively concluded that the substance containing nucleic acid and labile phosphoric acid was present as a compound in living yeast and could be enzymically dephosphorylated in the presence of toluene with the ultimate production of orthophosphate and nucleic acid.

*Identification of metaphosphoric acid as a component of the nucleic acid complex.*

*Fractionation with HCl and alcohol.* The sodium salt (8·5 g. containing 1320 mg. P of which 1160 mg. were labile) from extract 2 (Y 16 and Y 17) was dissolved in 100 ml. $H_2O$ and 3 ml. conc. HCl were added. A small flocculent precipitate formed, but the bulk of the substance remained in a very fine suspension which did not deposit on centrifuging. The supernatant fluid was decanted and treated with 3 volumes methcol (*p*H 2·0) giving a granular precipitate. The latter was redissolved 5 times in 10 parts of 5 % HCl and reprecipitated with alcohol, each time removing by centrifuging a small acid-insoluble portion, until finally the solution was only faintly opalescent. The precipitate at this stage was white and almost entirely inorganic in nature. (Wt. 2·66 g. (mg. P 670) P, 25·1 %; Na, 8·2 %; Fe approx. 1 %; N < 1·0 %. Atomic ratio P/Na = 2·2/1.) The analysis was unchanged after two more extractions.

For identification the following salts were prepared from the acid sodium salt:

(*a*) Neutral sodium salt. 1·06 g. were dissolved in 10 ml. water (*p*H 2·0) and one equivalent of NaOH added (*p*H 10). The solution failed to crystallize and the neutral salt (1·15 g.) was precipitated with alcohol. It was taken up in 8 ml. water and dissolved on warming to 40°, but suddenly formed a gel on filtering.

(*b*) Neutral barium salt. 0·43 g. acid sodium salt (109 mg. P) was dissolved in 10 ml. water and the *p*H adjusted to 7·0 with a few drops of cold baryta. 1·0 g. barium acetate in solution was added and the resulting white precipitate filtered, washed with water and dried; wt. 0·54 g.; 102 mg. P (=93 % recovery). Analysis, P, 19·7 %: orthophosphate nil; 100 % hydrolysis in 7 min. in *N* HCl at 100°, Ba, 43·7 %; N nil; Fe approximately 0·8 %; atomic ratio P/Ba=1·99/1. ($Ba(PO_3)_2$ requires P, 20·99 %; Ba, 46·51 %. The salt was very insoluble in water and tended to form a colloidal solution in dilute HCl, from which the barium was not precipitated by dilute $H_2SO_4$ in the cold. 1·0 ml. of the free acid, prepared by decomposition with the theoretical amount of $H_2SO_4$, containing 0·1 mg. P, gave a white precipitate with 1·0 ml. 2 % peptone solution, indicating metaphosphoric acid. No precipitate was obtained by addition of $H_2SO_4$, HCl, $BaCl_2$ or sodium pyrophosphate + acetic acid to a peptone solution.

(*c*) Neutral silver salt. Some difficulty was at first met in preparing this salt from dilute solutions, owing to its colloidal nature. 0·2 g. acid sodium salt (50 mg. P) was dissolved in 5 ml. water+NaOH to *p*H 7·0 and 5 ml. 10 % $AgNO_3$ were added, giving a white precipitate. This was separated and washed twice with water at the centrifuge, the supernatant liquid being very opalescent, and dried in a vacuum desiccator. The yield was poor (66 % recovery) and the salt gave a slightly brown solution; wt. 0·22 g.; P, 15 %; Ag, 53·1 %; Ag/P 1·02/1. For comparison a sample of silver pyrophosphate was prepared without difficulty giving on analysis P, 10·7 %; Ag, 66·5 %, ratio Ag/P 1·8/1. (Theory for $Ag_4P_2O_7$ P, 10·2 %; Ag, 70·7 %; for $AgPO_3$ P, 16·5 %; Ag, 57·7 %.)

There seems no reasonable doubt from the analyses and properties of these salts that at least 50 % of the labile phosphoric acid in the nucleic acid complex is metaphosphoric acid.

The various acid-insoluble precipitates from the HCl extraction were collected, dissolved in dilute NaOH and reprecipitated with methcol. The salts were rather more soluble in alcohol than previously and still contained a high proportion of labile P. Analysis, P, 12·3 %; N, 5·9 %; wt. 2·10 g. It is possible that prolonged extraction with 5 % HCl may split the nucleosidic linkage; only 28 % of the nitrogen was recovered in the acid-insoluble fractions, the remaining phosphorus and nitrogen being found in the alcohol filtrates.

In recent years the occurrence of metaphosphoric acid in organic material has been so rarely mentioned that it was thought that this nucleic acid-metaphosphoric acid complex had not previously been described. In fact Kossel [1893] isolated from yeast an acid "Plasminsäure" which he thought was a metaphosphoric acid closely associated within the cell with nucleic acid. Ascoli [1899], a pupil of Kossel, purified the "Plasminsäure" by extraction in hydrochloric acid, prepared the silver and strychine salts[1] and again concluded from the analytical reactions that the acid was a metaphosphoric derivative. There can be no doubt that the nucleic acid complex now described is Kossel's Plasminsäure.

[1] The salts all contained iron. Ascoli remarks the fact that both meta- and pyro-phosphoric acids inhibit the usual colour reactions of ferric iron.

## Discussion.

The fact that the process of fermentation of sugar by living yeast coincides with a decrease in the orthophosphate content of the cells affords strong evidence that phosphorylation is concerned in the glycolysis. The fate of the esterified phosphate is unknown, but the effects of fluoride and iodacetate on the phosphorylation indicate a general resemblance to this process in yeast juice.

The hypothesis that phosphorylation of carbohydrate is obligatory in glycolysis by all living cells is supported by the widespread distribution of phosphorus compounds known to intervene as coenzymes in cell-free glycolysis; by the phosphorylation of carbohydrate during glycolysis in dead or cell-free preparations not only of yeast, muscle and bacteria but also of higher plants [see Tankó, 1936]; and by the necessity for phosphate-carrying coenzymes in lactic acid formation by extracts of animal tissues [Euler *et al.* 1936; Boyland & Boyland, 1935]. A direct comparison between the glycolytic processes of cells and cell-free extracts may be misleading because of the different conditions, e.g. accessibility of substrate, presence of coenzyme, existing in these systems. The view that enzymic glycolysis can take place without phosphorylation is based chiefly on such a comparison [Ashford & Holmes, 1929; Bumm & Fehrenbach, 1931; Ashford, 1933; 1934]. Phosphorylation is not necessarily demonstrable by an accumulation of hexosephosphoric esters; whilst a stoichiometric relation between esterification and decomposition of hexose appears to be peculiar to yeast preparations. Nord *et al.* [1936] are also of the opinion that phosphorylation may be unnecessary, but they do not take into account the possibility that during glycolysis by living cells a change in the phosphorus distribution may be internal and not external to the cell. During glycolysis in the yeast cell the fluctuations in the acid-soluble phosphorus balance are of a small order (5% of the total phosphorus) yet the rate of glycolysis in yeast is comparatively high. It seems possible therefore that a change in the phosphorus distribution during glycolysis in organisms or tissues less favourable in this respect may be so small in amount as to lie within the experimental error, especially if inorganic phosphate is added to the medium.

The occurrence of metaphosphoric acid in close association with nucleic acid and iron in the yeast cell arouses interest in the physical and chemical roles of this acid in the cell metabolism. The ease with which the nucleic acid complex was obtained free from extraneous material is probably due to the resistant nature of the yeast cell membrane whose continuity remains, even if its permeability has been altered, after treatment with trichloroacetic acid. It seems probable that the latter precipitates the nucleic acid complex as a nucleoprotein and that on subsequent neutralization the nucleic acid passes into solution through the cell wall whilst any redissolved protein, glycogen etc. is held back. A single attempt to obtain protein-free nucleic acid from pancreas by this method was unsuccessful; but the technique could probably be applied to the isolation of nucleic acid from yeast or other cells with a cellulosic membrane.

Since maceration extract [Lohmann, 1928, 2] and yeast juice contain only small amounts of "labile" phosphate, it is possible that the presence of this nucleic acid-metaphosphoric acid complex is not obligatory in enzymic glycolysis. It may however prove to be of importance in the economy of cell glycolysis. The modified fermentation brought about by the addition of toluene to the yeast cell is accompanied by decomposition of the nucleic acid complex with formation of orthophosphoric acid [see also Lewitow, 1936], whilst the intact cell may be incubated in the absence of sugar without appreciable change. Preliminary

experiments indicate that during the fermentation of sugar the composition of the nucleic acid fraction is significantly altered; further experiments are now in progress.

SUMMARY.

1. At the onset of fermentation of sugar by fresh brewery yeast the acid-soluble orthophosphate content of the cells decreases in amount to a fairly constant value and regains the original level when fermentation ceases. This fact is evidence that phosphorylation is concerned in the glycolytic process of the yeast cell.

2. The coincident changes in total, labile and organic (acid-soluble) phosphate could not be consistently related to the fermentation process. Nothing is known of the nature of the compound formed by the disappearing orthophosphate, but the phosphorus balance suggests that it may be acid-insoluble.

3. A process is described by which different phosphorus compounds can be successively extracted from fresh yeast.

4. Metaphosphoric acid has been shown to be present in the trichloroacetic acid extract of fresh yeast, but neither hexosedi- nor hexosemono-phosphoric ester has yet been identified.

5. Approximately 30% of the total phosphorus of yeast is present as a complex containing iron, nucleic acid and metaphosphoric acid. By extraction of the nucleic acid complex with hydrochloric acid, inorganic metaphosphate was obtained and identified. The nucleic acid complex is probably identical with the "Plasminsäure" described by Kossel.

6. During the autofermentation of yeast in the presence of toluene the metaphosphoric acid present in the nucleic acid fraction was decomposed, with the ultimate production of orthophosphate.

I wish to record my gratitude to Prof. R. Robison for his invaluable advice and criticism.

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