inhibits polysaccharide synthesis aerobically, propionitrile does not.

7. The bearing of these results on the mechanism of the Pasteur effect is discussed.

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# Phosphorylations and Dephosphorylations in Yeast and their Inhibition by Various Agents


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Lynen (1941) and Lynen & Koenigsberger (1951) have related the Pasteur effect to phosphorylation processes, attributing the lowered rate of attack on glucose in oxygen to the lowered level of inorganic phosphate which results from the greater aerobic rate of phosphorylation, and which limits the rate of oxidation of triose phosphate.

In the preceding paper (Stickland, 1956) the action of selected inhibitors on the carbohydrate metabolism of yeast, and especially on the Pasteur effect, was studied. The present paper studies more exactly the processes of phosphorylation and dephosphorylation as they appear in one brand of baker's yeast, and the effect of the same inhibitors on these processes.

## MATERIALS AND METHODS

*Yeast.* The same brand of commercial pressed baker's yeast was used as in the previous paper (Stickland, 1956).

*Measurement of the rate of phosphorylation.* Baker's yeast as bought contains about 4 mg./g. dry wt. of P extractable by trichloroacetic acid solution, of which about one-half is present as inorganic orthophosphate. When glucose is added, the total extractable P remains constant, and the inorganic P level falls (Macfarlane, 1936). This fall is rapid, and more rapid under aerobic than under anaerobic conditions (Lynen, 1941). With the yeast used in the present experiments, the fall in inorganic P aerobically had reached its maximum in about 2 min., and was not linear even for 10 sec. To cope with this rapidity of reaction, and with the fact that aeration by bubbling $O_2$ through the solutions was excluded because volatile inhibitors were to be used, the following methods were employed.

(*a*) Aerobic rate. Into each of a series of 4 in. $\times$ 0·5 in. Pyrex test tubes were measured 2·0 ml. of a 15% suspension of yeast (about 80 mg. dry wt.) and 0·5 ml. of 0·1 M aminotrishydroxymethylmethane, pH 6·5. These mixtures were allowed to stand at room temperature for about 30 min. A volume (0·5 ml.) of 0·8% glucose solution was added rapidly, and the tube immediately closed by a thumb and shaken violently for the necessary time. Shaking was stopped 1 sec. before the period was finished, and at the proper time 2·0 ml. of 10% trichloroacetic acid solution was run in from a burette. Enough $O_2$ would be present in solution to keep the respiration going for several seconds. The time of the experiment was taken as that between the commencement of the shaking and the first entry of the trichloroacetic acid, and this interval could be measured with an error of probably 1 or 2 sec. For the control uninhibited rates, duplicate tubes were usually taken at 0, 5, 10, 15 and 20 sec.; the intervals were lengthened when the phosphorylation was inhibited.

Inhibitors, when present, were added, dissolved at the appropriate concentration in 0·5 ml. of tris. The concentration given is that present in the final mixture, after the addition of the glucose solution. The yeast was therefore exposed to a concentration of inhibitor 1·2 times that stated for periods not less than 15 min. and not greater than 60 min. before the addition of the glucose.

(*b*) Anaerobic rate. The same quantities of yeast and solutions were used. Purified $N_2$ was bubbled in a vigorous stream from a glass capillary through the mixture for 15 sec. before the addition of the glucose, and then for a further 30 sec. At this point, if the experiment was going to be of longer duration than 30 sec., the capillary was slowly withdrawn and a cork inserted in the tube; no appreciable quantity of $O_2$ could then reach the yeast. After the necessary period of incubation at room temperature, trichloroacetic acid was run in as before. A fresh capillary was used for each tube. No satisfactory way of measuring the effect of volatile inhibitors (e.g. KCN, propionitrile) on the anaerobic processes has been devised, but such data are not vital to the argument and are omitted.

All the tubes, aerobic and anaerobic, were then left at room temperature for about 1 hr. It was found that the amount of total P extracted from the yeast under these conditions remained constant from 30 to 120 min. after the addition of the trichloroacetic acid but increased if the mixture was left overnight. The acidified mixtures were, in earlier experiments, cooled rapidly in ice and kept at 0° until the samples for the estimations were measured out, but it was found later that this precaution was unnecessary. The tubes were centrifuged and estimations of inorganic P carried out on 2 ml. samples of the supernatants by the method of Fiske & Subbarow (1925). In early experiments estimations of total P in each sample were also carried out, but these were found to be quite constant in any given experiment, and to vary only a little between one sample of yeast and another.

The accuracy of these procedures is open to question on several grounds. First, there is no control of the temperature. All these experiments were done at room temperature, which was almost always 20°, and always lay between 18° and 21°. All the solutions were, if necessary, brought to the prevailing room temperature before use. Shaking by hand was found to increase the temperature of the contents of the tube by about 1° in 1 min., and almost all the measurements of aerobic phosphorylation rates were completed within this time, most of them within 20 sec. Aerobic samples requiring 2 min. or more shaking were shaken in a Towers-Gilson vibratory machine. Secondly, the timing of the shorter intervals is a source of inaccuracy, but the results obtained were perfectly consistent and reproducible, i.e. any errors of timing were constant errors, and comparisons remain valid, even though the details of the course of the reaction may be slightly uncertain.

To make a numerical comparison of the rates of phosphorylation under different conditions is difficult, as the process was not linear even for 10 sec., and the measurement of shorter time intervals than 5 sec. was considered impracticable. The only course possible was to take some arbitrary level of fall in inorganic P, and compare the times taken for this level to be reached under different circumstances. The level chosen was a decrease of 0·40 mg. of P/g. dry wt. of yeast, and this was reached aerobically in approximately 10 sec. and anaerobically in approximately 80 sec. From the average rates over these intervals of time figures for $Q_P$ were evaluated; for the aerobic rate ($Q_P^{O_2}$) this probably gives a minimal value. ($Q_P = \mu$l. of inorganic P esterified/mg. dry wt./hr.)

Determination of P by the Fiske & Subbarow (1925) method is interfered with by arsenate. In experiments in which arsenate was present, special P calibration curves were prepared for the Spekker absorptiometer for each concentration of arsenate used.

*Measurement of level of inorganic* P *in the steady state.* Minimum values of the inorganic P were found to be reached in about 2 min. aerobically and 10 min. anaerobically, and these low levels were maintained as long as some glucose remained unconsumed (see below). Hence, to measure steady-state concentrations under various conditions it was usually sufficient to make in each case two measurements, aerobically after 2 and 4 min., and anaerobically after 10 and 20 min.; if these two values were identical, or nearly so, they were taken as steady-state concentrations.

The yeast and tris or inhibitor solution were treated exactly as in the preceding section, except that a higher glucose concentration had to be used (0·5 ml. of 10%) to ensure that glucose should still be present after 20 min. of anaerobic action. The tubes containing the aerobic samples were closed with rubber stoppers and shaken for the requisite time on a Towers-Gilson shaking machine. The $O_2$ present in the air space in these tubes (about 3 ml. of air) would be enough to maintain the respiration of the yeast for about 5 min. On the few occasions when longer aerobic treatment was required, this was carried out in 6 in. × 0·75 in. test tubes, shaken on the same machine; the $O_2$ here was adequate for 1 hr. or more.

*Measurement of the rate of dephosphorylation.* The steady level of inorganic P set up in respiring or fermenting yeast is the result of opposing esterifying and hydrolysing reactions. It would be of interest to measure directly the rate of the latter processes, and this could be done only by inhibiting completely both respiration and fermentation. In the preceding paper (Stickland, 1956) it was shown that few poisons are capable of doing this, and the only one found suitable was urethane at a concentration of 0·5–1·0 M.

A number of tubes (4 in. × 0·5 in.) was set up with 2·0 ml. of 15% yeast suspension and 0·5 ml. of 0·1 M tris, and 0·5 ml. of 20% (w/v) glucose was added to each; these were left undisturbed, but without passing $N_2$, so that the conditions were almost, but not strictly, anaerobic. After 20 min. had been allowed for the esterification to become maximal, 0·5 ml. of 6 M urethane was added, to one tube at a time, and the solutions were mixed immediately by a rapid stream of purified $N_2$, or else by shaking in air. The samples were then treated with 2·0 ml. of 10% trichloroacetic acid solution after 10 sec., 20 sec., and so on, both aerobically and anaerobically. Every sixth tube was treated first with trichloroacetic acid and later with urethane, and results from these served as a base-line from which to measure the degree of de-esterification. All the tubes were left at room temperature for about 1 hr., centrifuged, and the inorganic P determined in 2·0 ml. of the supernatant.

## RESULTS

### *Phosphorylation in normal yeast*

The baker's yeast used in the experiments to be described had a total P content of about 10 mg. of P/g. dry wt., of which about 40% was extractable by trichloroacetic acid. Of this acid-soluble P rather more than half was present as inorganic orthophosphate (the average of nine determinations gave 3·28 mg. of total acid-soluble P/g. dry wt. (range 2·98–4·10) of which 1·78 mg./g. dry wt. (range

1·50–2·14) was inorganic. This was decreased by esterification during respiration or fermentation to about one-quarter (some 0·5 mg./g. dry wt.), a greater decrease than was found by Macfarlane (1936), Mirski & Wertheimer (1939) or Lynen (1941).

The course of these esterifications with normal yeast is shown in Fig. 1. Anaerobically phosphorylation proceeded approximately linearly for about 2 min., then accelerated slightly before slowing down to give a constant low level of inorganic P after about 10 min. Aerobically, the disappearance of inorganic P was much quicker, began to slow down at once, and ceased after about 2 min., when the level was about four-fifths of that found anaerobically. These figures differ quantitatively from those found in the yeast employed by Lynen & Koenigsberger (1951), chiefly in showing a more rapid and greater uptake of inorganic P.

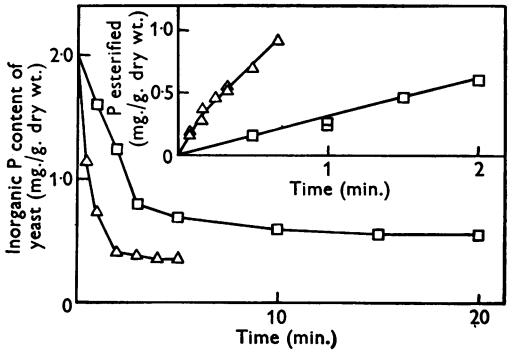


Fig. 1. Changes in level of inorganic phosphate in yeast after the addition of glucose, anaerobically and aerobically. Inset, the initial stages. □, In $N_2$; △, in air.

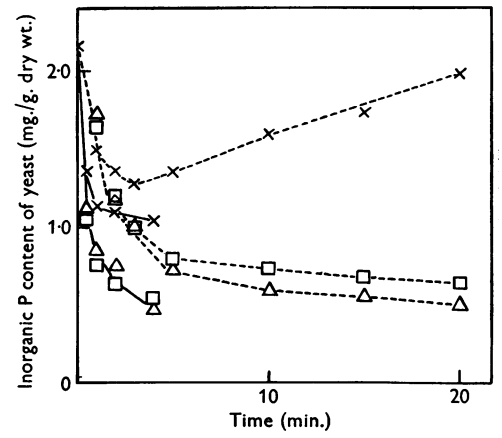


Fig. 2. Changes in the inorganic phosphate content of yeast after the addition of glucose at different initial concentrations. ×—×, 0·2% glucose in air; ×- - -×, 0·2% glucose in $N_2$; □—□, 1·0% glucose in air; □- - -□, 1·0% glucose in $N_2$; △—△, 5·0% glucose in air; △- - -△, 5·0% glucose in $N_2$.

The values of $Q_P$ in the example shown in Fig. 1, derived from the time taken for 0·4 mg. of inorganic P/g. dry wt. to be esterified, were $Q_P^{O_2} = -104$ and $Q_P^{N_2} = -11$. The averages of a large number of control experiments were $Q_P^{O_2} = -126$ (range −82 to −190) and $Q_P^{N_2} = -18$ (range −11 to −24).

The levels of inorganic P reached in the steady states of respiration and fermentation averaged, respectively, 0·45 and 0·55 mg. of P/g. dry wt. of yeast. The average ratio therefore, of steady values, aerobically and anaerobically, was 0·82, the individual values in seven experiments being 0·94, 0·75, 0·94, 1·00, 0·53, 0·70 and 0·90.

*Effect of varying glucose concentration on phosphorylation.* Fig. 2 shows the course of phosphorylation in air and $N_2$ at three glucose concentrations: 0·2, 1 and 5%. The last two showed little difference either in initial rate or in final level reached, either aerobically or anaerobically. At 0·2% of glucose in $N_2$ after 5 min. the glucose concentration plainly began to fall below the optimum, and there followed a progressive increase of the inorganic P concentration until almost complete hydrolysis of the esters had occurred. The aerobic events are not so easily explained, as the glucose should have lasted twice as long, and there is no obvious reason why the level of P should not have fallen as low at the lowest as at the highest glucose concentrations.

*Dephosphorylation.* The rate of liberation of inorganic P after the esterifying systems had been poisoned by urethane was of a similar order of magnitude to the rates of esterification namely, $Q_P = +30$. Fig. 3 shows the whole course of the hydrolysis (including the previous course of the

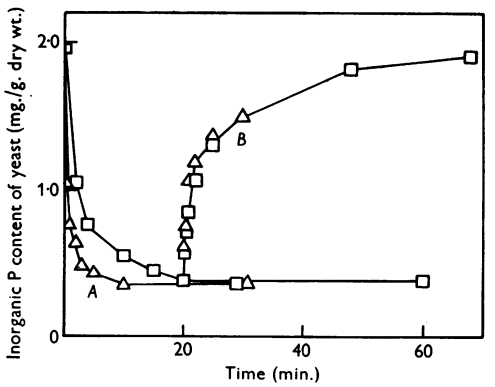


Fig. 3. Changes in inorganic phosphate content of yeast after the addition of 0·5M urethane to fermenting (or respiring) yeast. The course of de-esterification is plotted as if all the measurements were carried out after 20 min. fermentation (or respiration); actually they were done seriatim between 20 min. and 60 min. after the addition of the glucose to the yeast (at zero time). □, In $N_2$; △, in air. *A*, course of esterification; *B*, course of de-esterification.

ester formation for comparison of rates and amount); in this example $Q_P^{O_2} = +25$ and $Q_P^{N_2} = +22$. There is plainly no significant difference of rate of hydrolysis between aerobic and anaerobic conditions; the slightly greater aerobic rate always observed is almost certainly due to a systematic error of timing.

There is no reason to assume that the esters formed in yeast respiring in glucose are the same as those formed in fermenting yeast, and therefore no reason to expect to find the same rate of hydrolysis on the addition of urethane to samples of yeast in which the esters have been formed aerobically or anaerobically. A similar experiment was carried out therefore in which the esterification was allowed to proceed, not under the routine conditions of almost complete anaerobiosis, but, in two parallel sets of tubes, under strictly aerobic and strictly anaerobic conditions respectively. With each set of tubes, containing yeast with a maximum content of esters formed either aerobically or anaerobically, a de-esterification curve was obtained, both in air and in $N_2$. No significant difference was observed between any of the four curves (esters resulting from respiration in glucose, $Q_P^{O_2} = +29$, $Q_P^{N_2} = +26$; esters resulting from fermentation of glucose, $Q_P^{O_2} = +29$, $Q_P^{N_2} = +26$).

### *Effect of inhibitors on phosphorylation*

*Sodium azide.* Table 1 shows the effect of sodium azide on the rates of phosphorylation. Aerobic phosphorylation was inhibited, and at each concentration roughly to the same degree as the respiration (Stickland, 1956), being decreased to about the anaerobic rate at $10^{-3}$M. Anaerobic phosphorylation was scarcely affected. Aerobically, at the lower concentrations the minimum steady value for inorganic P was merely brought up to the anaerobic level, but at $10^{-3}$M the anaerobic level was itself inexplicably increased very much, with the result that approximately the same ratio of aerobic to anaerobic values was maintained.

*Potassium cyanide.* Table 2 shows that the rate of aerobic phosphorylation was reduced to the anaerobic value by $10^{-4}$M-KCN, and appreciably inhibited by lower concentrations, though the extent of inhibition was always less than that of the respiration at the same concentration (Stickland, 1956). Only the very highest concentration of KCN ($10^{-3}$M) was enough to affect appreciably the steady level of inorganic P reached.

*2:4-Dinitrophenol.* Table 3 shows that those concentrations of 2:4-dinitrophenol (DNP) which are effective in modifying the carbohydrate metabolism of yeast and inhibiting the Pasteur effect, inhibit the aerobic phosphorylation but not the anaerobic. The minimum steady value of inorganic P reached aerobically is, as would be expected, considerably raised; less expected is the equally marked increase in the anaerobic level, in spite of the absence of inhibition of the initial rate of esterification.

Table 1. *Effect of sodium azide on aerobic and anaerobic phosphorylation*

| Concn. of $NaN_3$ (M) | $Q_P^{O_2}$ | Inhibition (%) | $Q_P^{N_2}$ | Inhibition (%) | Inorganic P content of yeast (mg. of P/g. dry wt.): Resting value | Minimum steady value reached: In air | Minimum steady value reached: In $N_2$ |
|---|---|---|---|---|---|---|---|
| 0 | −95 | — | −20 | — | 2·07 | 0·45 | 0·50 |
| $3 \times 10^{-5}$ | −69 | 31 | — | — | — | — | — |
| $10^{-4}$ | −30 | 69 | — | — | 2·07 | 0·50 | — |
| $3 \times 10^{-4}$ | −23 | 77 | — | — | — | — | — |
| $10^{-3}$ | −14 | 85 | — | — | 2·07 | 0·79 | — |
| 0 | −138 | — | −24 | — | 2·15 | 0·40 | 0·59 |
| $10^{-4}$ | −69 | 50 | −22 | 8 | 2·15 | 0·62 | 0·74 |
| $10^{-3}$ | −25 | 82 | −22 | 8 | 2·15 | 0·98 | 1·24 |

Table 2. *Effect of potassium cyanide on aerobic phosphorylation*

| Concn. of KCN (M) | $Q_P^{O_2}$ | Inhibition (%) | $Q_P^{N_2}$ | Inorganic P content of yeast (mg. of P/g. dry wt.): Resting value | Minimum steady value reached: In air | Minimum steady value reached: In $N_2$ |
|---|---|---|---|---|---|---|
| 0 | −113 | — | −21 | 1·96 | 0·45 | 0·48 |
| $3 \times 10^{-6}$ | −113 | 0 | — | 1·96 | 0·48 | — |
| $10^{-5}$ | −89 | 21 | — | 1·96 | 0·50 | — |
| $3 \times 10^{-5}$ | −52 | 54 | — | 1·96 | 0·55 | — |
| $10^{-4}$ | −26 | 77 | — | 1·96 | 0·52 | — |
| $3 \times 10^{-4}$ | −14 | 88 | — | 1·96 | 0·52 | — |
| $10^{-3}$ | −9 | 92 | — | 1·96 | 0·67 | — |

Table 3. *Effect of 2:4-dinitrophenol (DNP) on aerobic and anaerobic phosphorylation*

| Concn. of DNP (M) | $Q_P^{O_2}$ | Inhibition (%) | $Q_P^{N_2}$ | Inhibition (%) | Inorganic P content of yeast (mg. of P/g. dry wt.) Resting value | Minimum steady value reached: In air | Minimum steady value reached: In $N_2$ |
|---|---|---|---|---|---|---|---|
| 0 | −124 | — | −18 | — | 1·91 | 0·52 | 0·55 |
| $3 \times 10^{-4}$ | −44 | 64 | — | — | — | — | — |
| $10^{-3}$ | −34 | 72 | −17 | 3 | 1·91 | 0·88 | — |
| $3 \times 10^{-3}$ | −22 | 82 | — | — | 1·91 | 1·10 | — |
| 0 | −89 | — | −14 | — | 2·10 | 0·43 | 0·50 |
| $10^{-3}$ | −19 | 78 | −13 | 7 | 2·10 | 1·00 | 1·33 |
| $3 \times 10^{-3}$ | −16 | 82 | −12 | 11 | 2·10 | 1·33 | 1·50 |

Table 4. *Effect of propionitrile on aerobic phosphorylation*

| Concn. of propionitrile (M) | $Q_P^{O_2}$ | Inhibition (%) | $Q_P^{N_2}$ | Inorganic P content of yeast (mg. of P/g. dry wt.) Resting value | Minimum steady value reached: In air | Minimum steady value reached: In $N_2$ |
|---|---|---|---|---|---|---|
| 0 | −124 | — | −13 | 2·09 | 0·43 | 0·57 |
| 0·1 | −138 | 0 | — | 2·09 | 0·50 | — |
| 0·15 | −113 | 9 | — | 2·09 | 0·45 | — |
| 0·2 | −113 | 9 | — | 2·09 | 0·57 | — |
| 0 | −99 | — | −24 | 2·09 | — | — |
| 0·25 | −99 | 0 | — | — | — | — |

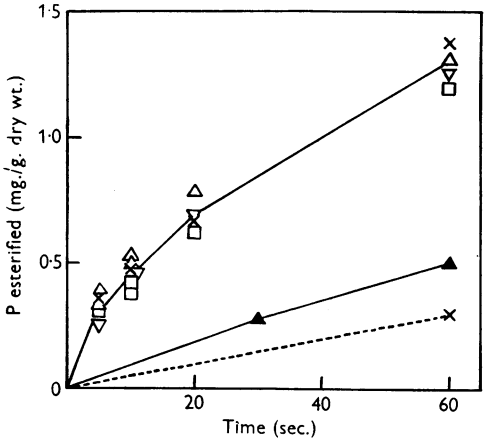


Fig. 4. Absence of inhibition of aerobic phosphorylation in yeast by propionitrile. ×—×, Control in air; △, +0·1 M propionitrile in air; □, +0·15 M propionitrile in air; ▽, +0·2 M propionitrile in air; ▲, $+3 \times 10^{-3}$ M DNP in air; ×- - -×, control in $N_2$.

*Propionitrile.* The effect of propionitrile, at concentrations similar to those required to inhibit the Pasteur effect (Stickland, 1956), on the aerobic phosphorylation process is shown in Table 4. No significant inhibition was observed, and no great change in the steady minimum value found during respiration. These results are of such importance to the argument that a typical set of curves, showing no inhibition by propionitrile, and a high degree of inhibition by DNP, is given in Fig. 4 to illustrate the amount of reliance that may be placed on the figures in the tables.

In view of some experiments with sodium iodoacetate (see below) in which a rapid initial phosphorylation was followed by a much slower hydrolysis of the esters, it was necessary to establish that the low inorganic P level in the yeast in the presence of propionitrile was maintained at least as long as the normal duration of the experiments in which the inhibition of the Pasteur effect had been measured, namely, 1 hr. (Stickland, 1956). Such tests were carried out, and it was found that the inorganic P continued very slowly to decrease up to 1 hr. after the addition of glucose.

In a single experiment with each of two preparations of ethyl carbylamine, at $3 \times 10^{-3}$ and at $10^{-2}$ M respectively, no effect on the rate of phosphorylation and no raising of the minimum inorganic P level were observed (e.g. control, $Q_P^{O_2} = -155$, $Q_P^{N_2} = -19$; with $3 \times 10^{-3}$ M ethyl carbylamine, $Q_P^{O_2} = -155$. Resting inorganic P content was 1·86 mg. of P/g. dry wt., falling to 0·38 in air, to 0·43 in $N_2$, and to 0·33 in air with $3 \times 10^{-3}$ M ethyl carbylamine).

*Urethane.* Urethane inhibits roughly equally the aerobic phosphorylation (Table 5) and the respiration (Stickland, 1956). At concentrations above

about 0·5 M all phosphorylation, in air or $N_2$, is completely suppressed, and the inorganic P content of the yeast actually increases slightly. Urethane at a concentration of, say, M is therefore a suitable agent for stopping the phosphorylations in order to observe the subsequent rate of hydrolysis of the esters, but it must first be shown that its action is immediate. This was done by carrying out parallel phosphorylation measurements, in one of which the reaction was started in the usual way by the addition of 0·5 ml. of 1 % glucose solution, in the other by the addition of the same solution containing also 6 M urethane. In the latter case no phosphorylation was observed, i.e. the inhibition was immediate.

*Sodium arsenate.* Table 6 shows that arsenate, over the whole range of concentrations which inhibits partially both respiration and fermentation (Stickland, 1956), has no inhibitory effect on the phosphorylations, either aerobic or anaerobic, and allows the inorganic P level to reach the same low values as in its absence. The interference of arsenate in the colorimetric method used for P estimations renders these measurements slightly less accurate than most, but they have been repeated several times, and no greater inhibition than the value of 19 % given in Table 6 has been observed (the average inhibition in fifteen measurements on six occasions at concentrations ranging from $10^{-6}$ to $10^{-3}$ M was −3 %, ranging from 19 % to −36 %).

It was also confirmed in this case that the low level of inorganic P was maintained for at least 1 hr., with an arsenate concentration of $10^{-4}$ M, aerobically and anaerobically, although both respiration and fermentation were reduced to about 20 % of those of the control throughout this period (Stickland, 1956).

*Sodium arsenite.* Arsenite also caused no inhibition of the phosphorylation, either aerobically or anaerobically, nor any increase in the minimum steady value of the inorganic P during respiration, except at an extremely high concentration (Table 7).

*Sodium iodoacetate.* The effect of iodoacetate on the rates of phosphorylation was small, and rather more inhibitory anaerobically than aerobically (see Table 8). The steady minimum values of inorganic P showed a more striking difference, there being no effect aerobically, but anaerobically the initial fall in inorganic P, hardly inhibited in rate by iodoacetate, was followed after a few minutes by a reversal of the esterification, the inorganic P returning in one experiment to its initial value after 20 min.

Table 5. *Effect of urethane on aerobic and anaerobic phosphorylation*

| Concn. of urethane (M) | $Q_P^{O_2}$ | Inhibition (%) | $Q_P^{N_2}$ | Inhibition (%) | Inorganic P content of yeast (mg. of P/g. dry wt.): Resting value | Minimum steady value reached: In air | Minimum steady value reached: In $N_2$ |
|---|---|---|---|---|---|---|---|
| 0 | −191 | — | −20 | — | 2·05 | 0·45 | 0·50 |
| 0·3 | −59 | 69 | — | — | 2·05 | 0·95 | — |
| 1·5 | 0 | 100 | — | — | — | — | — |
| 0 | −124 | — | −18 | — | — | — | — |
| 0·75 | 0 | 100 | 0 | 100 | — | — | — |
| 1·5 | 0 | 100 | 0 | 100 | — | — | — |
| 0 | −108 | — | −21 | — | 2·17 | 0·40 | 0·40 |
| 2·0 | 0 | 100 | 0 | 100 | 2·17 | 2·3 | 2·3 |

Table 6. *Effect of sodium arsenate on aerobic and anaerobic phosphorylation*

| Concn. of arsenate (M) | $Q_P^{O_2}$ | Inhibition (%) | $Q_P^{N_2}$ | Inhibition (%) | Inorganic P content of yeast (mg. of P/g. dry wt.): Resting value | Minimum steady value reached: In air | Minimum steady value reached: In $N_2$ |
|---|---|---|---|---|---|---|---|
| 0 | −103 | — | −15 | — | 1·88 | 0·62 | 0·62 |
| $10^{-6}$ | −95 | 8 | — | — | — | — | — |
| $3 \times 10^{-6}$ | −98 | 5 | — | — | — | — | — |
| $10^{-5}$ | −89 | 13 | — | — | — | — | — |
| $3 \times 10^{-5}$ | −83 | 19 | — | — | 1·88 | 0·55 | — |
| $10^{-4}$ | −95 | 8 | — | — | 1·88 | 0·55 | — |
| $3 \times 10^{-4}$ | −103 | 0 | — | — | 1·88 | 0·48 | — |
| $10^{-3}$ | −113 | −10 | — | — | — | — | — |
| 0 | −130 | — | −13 | — | 1·76 | 0·31 | 0·38 |
| $10^{-4}$ | −177 | −36 | −14 | −11 | 1·76 | 0·31 | 0·24 |

Table 7. *Effect of sodium arsenite on aerobic and anaerobic phosphorylation*

| Concn. of arsenite (M) | $Q_P^{O_2}$ | Inhibition (%) | $Q_P^{N_2}$ | Inhibition (%) | Inorganic P content of yeast (mg. of P/g. dry wt.) Resting value | Minimum steady value reached In air | Minimum steady value reached In $N_2$ |
|---|---|---|---|---|---|---|---|
| 0 | −103 | — | −21 | — | 1·62 | 0·31 | 0·45 |
| $10^{-4}$ | −113 | −10 | −21 | 0 | 1·62 | 0·26 | 0·41 |
| $10^{-3}$ | −124 | −20 | −21 | 0 | 1·62 | 0·32 | 0·38 |
| $10^{-2}$ | −124 | −20 | −21 | 0 | 1·62 | 0·55 | 0·38 |

Table 8. *Effect of sodium iodoacetate on aerobic and anaerobic phosphorylation*

| Concn. of iodoacetate (M) | $Q_P^{O_2}$ | Inhibition (%) | $Q_P^{N_2}$ | Inhibition (%) | Inorganic P content of yeast (mg. of P/g. dry wt.) Resting value | Minimum steady value reached In air | Minimum steady value reached In $N_2$ |
|---|---|---|---|---|---|---|---|
| 0 | −225 | — | −34 | — | 2·02 | 0·36 | 0·57 |
| $5 \times 10^{-4}$ | −225 | 0 | −23 | 32 | 2·02 | 0·38 | 0·69* |
| 0 | −155 | — | −19 | — | 1·58 | 0·24 | 0·38 |
| $10^{-3}$ | −138 | 11 | −12 | 37 | 1·58 | 0·24 | 0·74† |

* In 3 min. rising to 2·02 in 20 min.

† In 10 min. rising to 1·27 in 20 min.

*Effect of inhibitors on the rate of dephosphorylation*

The action of several substances on dephosphorylation, at concentrations in the same range as were effective in inhibiting the phosphorylation process, was investigated. The experiments were carried out by incorporating the inhibitor in the 6 M urethane solution which was used to suppress the phosphorylating processes, and thus enable the dephosphorylation to be observed; the inhibitor was not therefore in contact with the yeast for some time before the beginning of the measurement, as was the practice in other types of experiment. No significant effect was observed with KCN, sodium azide, propionitrile, arsenate, or arsenite. The only substance that had any positive effect was DNP, which was moderately inhibitory (at $3 \times 10^{-3}$ M, 54 and 64% inhibition; at $2 \times 10^{-3}$ M, 55% inhibition). It must be remembered that in these experiments the yeast was already treated with M urethane; only effects additional to any that urethane itself may have can be observed.

## DISCUSSION

### *Phosphorylation*

The rates of phosphorylation in yeast measured in the present paper can obviously apply only to the conditions found in the first 10 sec. (aerobically) or first 2 min. (anaerobically) after the addition of glucose. It is known (Kruyk & Klingmüller, 1939) that glucose disappearance during the first few minutes anaerobically is extremely slow, and it is doubtful what relationships these phosphorylation measurements have to the rates of phosphorylation obtaining in the steady states of respiration and fermentation.

These latter rates must necessarily be equal in the steady states to the rates of de-esterification, and that is why the attempt was made to measure these directly, by inhibiting the esterifying reactions with urethane, and observing the rates of hydrolysis of the esters. Unfortunately there is no way of determining what effect this reagent may be exerting on the rates that are measured.

If the difference between aerobic and anaerobic rates which is observed in the initial phase were maintained into the stationary phase, as might reasonably be expected, then the de-esterification rates must differ in the same way. Experimentally this was not found, but it is impossible to say whether this indicates an equality of esterification rates in the two stationary phases, or merely an action of the urethane in slowing down the aerobic rate, or simply that the hydrolysis reactions that are followed are different from the hydrolyses taking place in the stationary phases in the uninhibited yeast.

In the discussion of the effects of inhibitors which follows it must be remembered that the rates are in all cases the rates of the initial processes, which are not necessarily the same processes as those found in the stationary phases. The only reliable information about the stationary phase is the stationary minimum level of inorganic phosphate set up.

*Inhibition of phosphorylation*

Ochoa & Stern (1952) have pointed out that the difference in level of inorganic phosphate in the steady states of respiration and fermentation in the experiments of Lynen & Koenigsberger (1951) is small, and cannot easily be imagined to be the sole cause of the different rates of glucose breakdown. In the experiments described here, the difference was even slighter (on the average the level was 20% higher anaerobically), and the aerobic level was unaffected by concentrations of propionitrile which completely inhibited the Pasteur effect. The automatic control of rates of glucose breakdown through the inorganic phosphate level therefore seems to be ruled out completely.

The effects of some of the inhibitors on the rates of phosphorylation are puzzling, when they are compared, as they must be, with the effects of glucose breakdown and polysaccharide synthesis (Stickland, 1956).

Sodium azide ($10^{-4}$ M) affects respiration, polysaccharide synthesis and phosphorylation roughly equally, and has no effect anaerobically. At $10^{-3}$ M the aerobic results are again consistent, but anaerobically, in spite of the absence of effect on the initial rate of esterification of phosphate, the steady level of inorganic phosphate is greatly raised, and synthesis of polysaccharide is suppressed.

Potassium cyanide at all concentrations up to $10^{-4}$ M causes merely a change-over to the anaerobic type of metabolism, phosphorylation, glucose catabolism, and polysaccharide synthesis behaving as would be expected.

The effect of DNP on aerobic phosphorylation in yeast was observed by Lynen & Koenigsberger (1951). In the present experiments with DNP at $3 \times 10^{-3}$ M, the aerobic results are quite consistent, phosphorylation being inhibited (although of course the respiration is not), the minimum steady value of inorganic phosphate is raised, and polysaccharide synthesis is abolished. Anaerobically no inhibition of rate of phosphorylation was observed, and yet unexpectedly, as with $10^{-3}$ M sodium azide, there was a raised steady inorganic phosphate level and complete inhibition of polysaccharide synthesis.

Sodium arsenate behaved quite anomalously, for at all concentrations from $3 \times 10^{-5}$ M upwards a roughly constant inhibition of 80% of the respiration, and about 70% of the fermentation, was observed, although no effect on the rates of esterification of phosphate or on the final level of inorganic phosphate reached could be detected. At the same time the synthesis of polysaccharide was almost entirely eliminated.

The results with iodoacetate are similar to those of Mirski & Wertheimer (1939), who also observed only a small initial inhibition of phosphorylation, followed by a slower process of hydrolysis of the esters formed (their experiments were not strictly anaerobic, but were presumably almost so). This suggests a slow penetration of the cell by iodoacetate, a penetration initiated by the addition of the glucose; for the yeast and the inhibitor solution were, in the present experiments, always left in contact for at least 30 min. before the reaction was started by the addition of glucose.

In all these experiments, it must be remembered, the phosphorylation measurements were completed within at most 2 min. of the addition of the glucose, whereas glucose breakdown and glycogen synthesis were measured over 1 hr. (they were very nearly linear for that period). The yeast suspensions were in all cases left in contact with the inhibitor solution for 30 min. or longer before the addition of the glucose. There is no reason to postulate, nor any means of excluding, an increase in permeability to some poisons brought about by glucose, as appears to be true for iodoacetate. With one inhibitor at least (urethane, see above) the penetration of the cell was instantaneous.

If the measurements of initial phosphorylation rate with, for example, arsenate were fallacious, owing to the poison not acting in the 20 sec. required for the measurement (which seems unlikely), one would expect some reversal of the esterification to follow when the inhibition began to take effect (and the manometric results show that the inhibition of respiration was effective within a few minutes at most), as was observed in the anaerobic experiments with iodoacetate. Aerobically with arsenate this did not occur; the steady minimum value of inorganic phosphate was even lower than in the control, and remained so for at least 1 hr. By contrast, urethane, at a concentration sufficient to give a comparable degree of inhibition of the respiration, brought about a value of the steady minimum inorganic phosphate level about twice that in the control. The evidence on the whole points to the explanation that the arsenate-stable part of the respiration (about 20%) is responsible for the whole of the aerobic phosphorylation.

## SUMMARY

1. The rates of esterification of inorganic phosphate within the yeast cell during the first minute after addition of glucose have been determined, in nitrogen and in air.

2. The levels of inorganic phosphate in the stationary phases of fermentation and respiration have been determined in the same yeast.

3. The rate of hydrolysis of the esters so formed in yeast, when the further esterification is inhibited with urethane, has been measured.

4. The effect of various poisons on the rates of aerobic and anaerobic phosphorylation and on the

minimum levels of inorganic phosphate in yeast have been measured.

5. An attempt is made to relate these effects to the effect of the same substances on glucose catabolism and polysaccharide synthesis. Various discrepancies are pointed out.

6. The hypothesis of Lynen (1941) and of Lynen & Koenigsberger (1951) that the Pasteur effect is related to the lower inorganic phosphate level obtaining aerobically appears to be untenable, for propionitrile, which inhibits the Pasteur effect, has no effect on phosphorylation phenomena.

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# The Effects of Phosphate Acceptors, *p*-Nitrophenol and Arsenate on Respiration, Phosphorylation and Pasteur Effect in Cell-Free Suspensions

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The first evidence that phosphorylation reactions play a part in the oxidation of pyruvic acid in animal tissues was provided by Banga, Ochoa & Peters (1939) who showed that inorganic phosphate and adenine nucleotide reactivate the pyruvic oxidase system of dialysed dispersions of pigeon brain and of rabbit kidney. Later workers have attributed the restoration of the respiratory activity of deficient systems by 2:4-dinitrophenol to its ability to 'replace' either inorganic phosphate (Loomis & Lipmann, 1948; Teply, 1949) or adenine nucleotide (Judah & Williams-Ashman, 1951). In washed kidney dispersions (cyclophorase), dinitrophenol has been found to abolish phosphorylation without inhibition or with slight stimulation of respiration (Loomis & Lipmann, 1948; Cross, Taggart, Covo & Green, 1949). Lardy & Wellman (1952) observed that dinitrophenol accelerated the respiration of liver mitochondria oxidizing various substrates and that this stimulant action could be reproduced by the addition of phosphate acceptors such as adenylate or glucose plus hexokinase, the effects of the nitrophenol and the phosphate acceptors not being additive.

Since it had been found (Terner, 1954*b*, 1955*a*) that *p*-nitrophenol increased the respiration of mammary-gland homogenates metabolizing fumarate and pyruvate, with and without glucose plus hexokinase, further studies have been made to compare the effects of *p*-nitrophenol and of phosphate acceptors on the oxygen consumption of mitochondrial preparations metabolizing fumarate and pyruvate. The effects of *p*-nitrophenol and of arsenate on aerobic phosphorylation and citric acid formation were studied in suspensions blocked with fluoracetate.

In extension of previous studies of the Pasteur effect in cell-free suspensions (Terner, 1954*a*), it could be shown that the addition of large amounts of adenine nucleotide resulted in a marked increase in the amount of lactic acid accumulating under aerobic conditions, i.e. that the inhibition of the Pasteur effect by *p*-nitrophenol could be reproduced by the addition of adenine nucleotide.

## EXPERIMENTAL

*Material.* Homogenates in 0·154 M-KCl or in 8·55% sucrose were prepared as previously described (Terner, 1955*b*) and used either without further treatment, or after centrifuging twice for short periods at low speed to remove cell debris and nuclei. The resultant mixture of mitochondria and soluble tissue constituents is designated 'suspension'. Washed suspensions were prepared by further centrifuging at 7000 *g* for 30 min. and resuspending the sediment in KCl. After two or three washings the suspension contained almost pure mitochondria, with few if any nuclei, and will be referred to as 'mitochondria'.

The 'supernatant fraction' was the clear solution recovered after the first centrifuging at high speed and was free from nuclei and mitochondria. Nuclei were isolated as described by Hogeboom, Schneider & Striebich (1952).

Unless otherwise stated, the experimental animals were guinea pigs.

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