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ENTRY OF PHOSPHATE INTO YEAST CELL[1]

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Baker's yeast, *Saccharomyces cerevisiae*, has often been selected for study of active, or metabolically associated, salt accumulation. Particular attention has been devoted to the esterification of phosphate in this process and to the nature of coupling with a source of energy. The accumulation of phosphate has been considered as coupled to: A, the general reactions of glycolysis (10); B, the action of glyceraldehyde–3–phosphate dehydrogenase (6,16); C, a redox pump (4,5) and D, oxidations in the respiratory chain under aerobic conditions (14).

With these concepts in mind, the manner of phosphate entry into yeast was examined by the methods of quantitative enzyme kinetics applied by C. E. Hagen and his associates (7) to analysis of phosphate entry into barley roots. The initial objective was to compare phosphate accumulation by Baker's yeast and barley roots, particularly with respect to aerobic and anaerobic conditions, in the hope of more clearly understanding the aerobic requirement of barley roots. Results obtained indicate that phosphate entry into yeast is coupled with three distinct reactions of oxidative phosphorylation, one of which is aerobic and the other two anaerobic.

METHODS

A *Saccharomyces cerevisiae* Meyer ex Hansen was isolated from Baker's yeast (Standard Brands, Inc.) and grown aseptically. The original yeast contained cell fragments and a bacterial contaminant which confounded all experiments until it was eliminated. Cultures were grown with aeration on glucose-bactopeptone medium containing beef extract and 0.1 M in NaCl for 24 hours at 30° C. Cells were removed by centrifuging, followed by washing and resuspension in 0.15 M NaCl. Concentrations of cells were measured turbidmetrically and adjusted to contain 10 mg dry weight/5 ml.

The cells (5 ml) were pretreated in 30 ml of a requisite solution for 60 minutes to establish steady-state conditions with respect to orthophosphate uptake (fig 1). Steady-state persisted for 60 minutes, after which the rate decreased slightly. Constant uptake is reached in shorter times at lower glucose concentrations. Uptake of P^{32} was followed for periods of 30 to 300 seconds and was terminated by centrifugation. Cells were washed once with 0.15 M NaCl. Prolonged washing did not further reduce the amount of phosphate associated with the yeast cells. The phosphate remaining with the cells is described as that accumulated. Radioactivity was measured after drying under infra-red lamps. The amount of P^{32} associated with the cells was converted to moles per mg of cells from the known specific activity which was constant during the absorption period. Oxygen consumption was measured polarographically. Anaerobic conditions were established by bubbling oxygen-free nitrogen through a cell suspension in a 35 ml volume of small surface area.

KINETIC INTERPRETATION: Active accumulation of ions by cells is considered to arise from initial combination with a specific binding compound (carrier) at an external surface, followed by breakdown delivering the ion inside the cell. The reaction

$$X \text{ (carrier)} + R_o^- \text{ or } R_o^+ \text{ (external ions)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}}$$

$$XR^{\pm} \text{ (carrier compound)} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} X' + R_i^- \text{ or } R_i^+ \text{ (inside)}$$

[1] Received August 15, 1960.

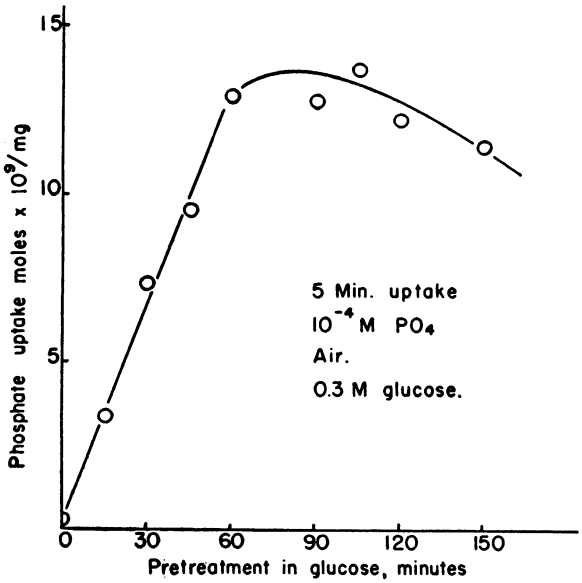


FIG. 1. Dependence of phosphate uptake by yeast in 5 minute periods on the time of incubation on 0.3 M glucose under aerobic conditions at pH 4.0.

Under steady-state conditions the back reaction involving k_4 is negligible. The amount, m, of an ion accumulated by the cell in time, t, is

$$m = k_3 [XR]t + [XR]$$

where [XR] is the concentration of the carrier compound. Under steady-state conditions the uptake follows the Michaelis-Menten equation,

$$m = -\frac{K_m m}{[C_o]} + m_{max}$$

where $[C_o]$ is the constant external ion concentration of an infinite reservoir. The maximum uptake, m_{max}, is obtained by extrapolation of $[C_o]$ to infinite concentrations.

A linear plot of m against $\frac{m}{[C_o]}$ indicates a single reaction with the Michaelis-Menten constant $K_m = \frac{k_2 + k_3}{k_1}$ being the negative slope and m_{max} the intercept on the axis of ordinates. A curvilinear relationship results when two or more first-order reactions occur simultaneously but independently (8). These curves can be resolved into linear components with a reasonably critical attitude toward precision and extent of measurements and possible departure from first-order kinetics.

The uptake of phosphate, m, is measured as a function of phosphate concentration under various conditions of the environment (pH & temperature, aerobiosis & anaerobiosis), and with various substrates, inhibitors, and competing materials. Analysis of the results indicates the number of rate-limiting processes and the corresponding values of K_m and m_{max}. Values of k_3 and [XR] can be found from the phosphate uptake as a function of time.

RESULTS

I. INITIAL CONDITIONS: Yeast showing the maximum rate of phosphate uptake (fig 1) and respiration can readily be depleted of endogenous substrates by suspending in 0.15 M NaCl for 60 minutes. Respiration of these cells responds immediately to additions of glucose, glutamate, and other substrates. It is constant from zero time and responds without lag to the various inhibitors.

Rates of oxygen consumption referred to the rate with 0.3 M glucose as 100 are shown in table I. The concentrations of the several compounds include those used during measurement of phosphate uptake.

II. PHOSPHATE UPTAKE UNDER AEROBIC & ANAEROBIC CONDITIONS: The ratio of the slopes of the time curves to their intercepts at zero time, k_3 [XR]/[XR] are constant with varying phosphate concentrations and constant substrate. This indicates the absence of non-specific adsorption in the measurement of [XR]. The amount of phosphate held at zero time, [XR], is increased with anaerobiosis (fig 2 & 3). Irrespective of the phosphate concentration, the rates of phosphate uptake, $\frac{dm}{dt} = k_3$ [XR], after zero time are the same under aerobic and anaerobic conditions as shown by the parallel displacement of the time curves.

If glucose is withheld the rate of phosphate uptake drops to zero (figs 2 & 3) and the value of [XR] is

TABLE I

OXYGEN CONSUMPTION IN AIR BY YEAST WITH VARIOUS SUBSTRATES AT pH 4.0 & 30°

SUBSTRATE	MOLAR CONC	OXYGEN CONSUMPTION 2.3×10^{-8} moles O_2/min/mg
Glucose 0.3 M		
Without inhibitor		100
5,5-diethyl barbituric acid	2×10^{-3}	85
Sodium azide	10^{-3}	0.01
Dinitrophenol	10^{-4}	70
Without glucose		
β-hydroxy butyrate	0.1	100
Ethanol	0.9	100
Sodium chloride	0.15	0.01
Glutamic acid	10^{-3}	80
Succinic acid	2×10^{-2}	0.02
p-phenylene diamine (reduced)	1×10^{-2}	70

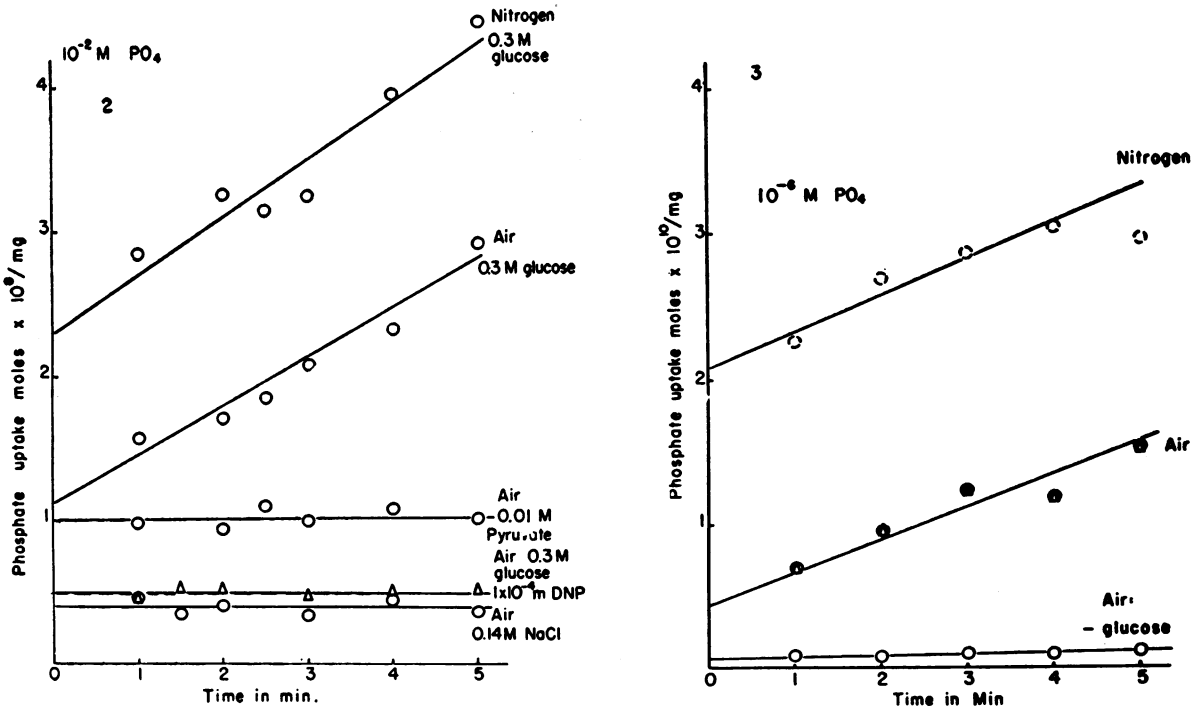


FIG. 2. Phosphate uptake by yeast from 10^{-2} M phosphate at pH 4 as a function of time.
FIG. 3. Phosphate uptake by yeast from 10^{-6} M phosphate, pH 4, as a function of time on 0.3 M glucose. Aerobic and anaerobic.

reduced. A similar result is obtained in the presence of glucose and 1×10^{-4} 2,4–dinitrophenol (DNP) (figs 2 & 10). The rate is also reduced to zero with 0.01 M pyruvate as the substrate (fig 2), however, without changing [XR].

Plots of uptake, m, against $m/\Sigma[PO_4]$ are similar under aerobic and anaerobic conditions (figs 4 & 5). The curves can each be resolved into two linear portions, having K_m values of 10^{-3} and 10^{-5}, respectively. Values of m_{max} for both systems decrease if the

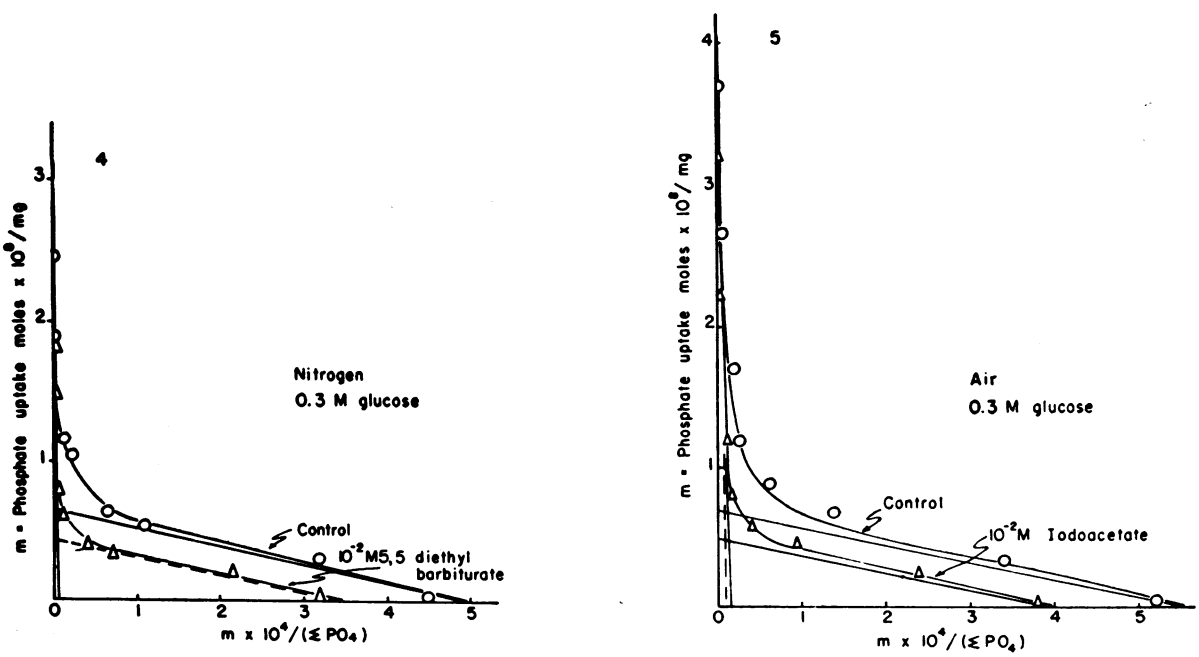


FIG. 4. Hofstee plot of the anaerobic uptake of phos phate by yeast as influenced by 5,5–diethylbarbituate.
FIG. 5. Hofstee plot of the aerobic uptake of phosph ate by yeast as influenced by iodoacetate.

glucose concentration is reduced below 0.3 M without change in values of K_m. Intermediate concentrations of glucose (10^{-3}–10^{-2} M) primarily decrease the value of [XR] while at 10^{-4} M both [XR] and k_3 decrease as shown by comparisons of the time curves. In the absence of glucose the constant, k_3, is not measurable. Thus the K_m value can be determined and expressed solely as a function of k_1 and k_2, i.e., $K_m = k_2/k_1$. The K_m values are constant at all glucose concentrations with the implication that even at the highest turnover rate, the k_3 is insignificant relative to k_2 and K_m is a dissociation constant.

A third reaction with a value of K_m of 10^{-6} is observed only with low glucose concentrations under aerobic conditions (fig 6). When the glucose concentration is increased this reaction is inseparable from the intermediate reaction having $K_m = 10^{-5}$. The increase in glucose increases m_{max} far more for the latter than for the former reaction.

III. Action of Respiratory Inhibitors on Reaction With $K_m = 10^{-6}$: In the absence of glucose and with 2×10^{-6} M PO_4, the value of k_3 is negligible (fig 7). Addition of 0.1 M *p*-phenylenediamine increases the intercept [XR] by about four-fold. A glucose concentration of 10^{-4} M produces an observable value of k_3. The slope/intercept ratio is not changed by addition of *p*-phenylenediamine. This indicates an increase of [XR] with a constant value of k_3.

This reaction is inhibited by 5,5–diethyl barbituric acid (barbital) primarily by decrease of [XR] and by azide with resulting decrease in [XR] and k_3.

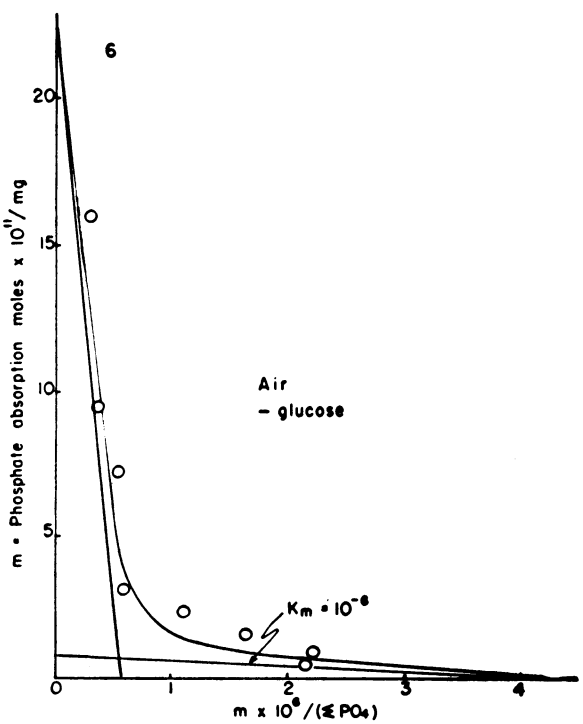


Fig. 6. Hofstee plot of the uptake of phosphate by yeast in the absence of a substrate. Aerobic.

IV. Action of Glycolytic Inhibitors on Reaction With $K_m = 10^{-5}$: Iodoacetate, 5,5–diethyl barbituric acid and Na_2SO_3 are equally effective as inhibitors under aerobic and anaerobic conditions. Each effects a decrease in [XR] and, at high concentrations, causes k_3 values to approach zero (figs 4, 5, & 8). Inhibition of phosphate uptake is of the uncompetitive type; that is, m_{max} is changed by the inhibitor while K_m remains constant.

The action of Na_2SO_3 differs from that of the other two inhibitors. In the presence of Na_2SO_3 the curve on the plot of m against $m/\Sigma[PO_4]$ is resolvable into three components. This might be a result of more effective inhibition of the reaction with $K_m = 10^{-5}$, allowing the reaction with $K_m = 10^{-6}$ to be displayed. However, iodoacetate and the barbituate might decrease [XR] for both reactions so that they are inseparable.

High concentrations of *p*-phenylenediamine are without effect on phosphate uptake in the presence of 0.3 M glucose.

V. Action of Inhibitors on Reactions With $K_m = 10^{-3}$: This reaction has $m_{max} = 27 \times 10^{-8}$, $k_3 = 3.2 \times 10^{-3}$, and [XR] $= 13.8 \times 10^{-8}$ moles/mg of yeast. The k_3 for this reaction is smaller than for the other two. The glycolytic inhibitors at the concentrations indicated in table I have little effect (figs 4 & 5) but moderate changes would be difficult to note.

A concentration of 10^{-4} M DNP decreases [XR] and blocks k_3 (fig 2). Thus, the reaction must be connected with a metabolic step rather than with diffusion or other mode of passive entry.

VI. Action of ATP, DNP, Succinate, & β–Hydroxybutyrate: All reactions are inhibited in a competitive manner by ATP and are blocked by DNP. Succinate supports some respiration (table I) but is without effect on phosphate uptake (fig 10). Beta hydroxybutyrate, on the other hand, markedly inhibits phosphate uptake in the presence of 0.3 M glucose by decreasing [XR] without changing the slope/intercept ratio of the time curve (fig 10). Respiration is sustained to the same level by beta-hydroxybutyrate and 0.3 M glucose (table I).

VII. Effect of Change in pH & Presence of KCl: The optimum pH for phosphate uptake depends upon the concentration of the external solution. As phosphate decreases from 10^{-2} to 10^{-6} M the optimum shifts from pH 5.0 to 6.5 in both anaerobic and aerobic systems. The utilization of glucose from 0.3 M solution is also optimal at pH 6.5.

Potassium chloride is without effect on the pH dependence of phosphate uptake. At pH 4.0 in the presence of 0.3 M glucose and 10^{-6} M PO_4 a concentration of 0.1 M KCl enhances phosphate uptake by increase of [XR] corresponding to a constant ratio of slope/intercept of phosphate uptake (fig 9).

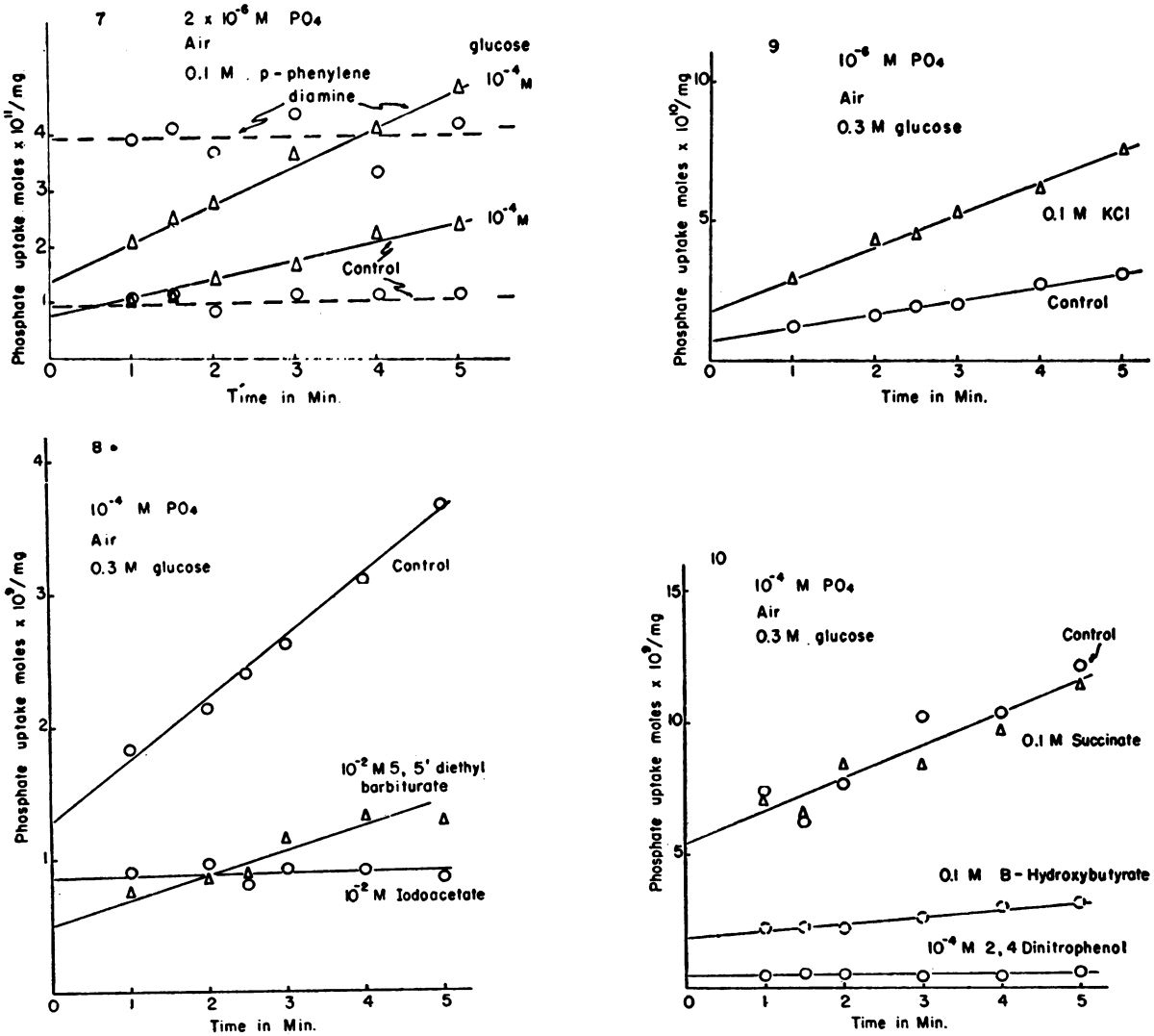


FIG. 7. The effect of *p*-phenylenediamine on the uptake of phosphate by yeast in the presence and absence of glucose. Aerobic.

FIG. 8. The effect of barbital and of iodoacetate on the uptake of phosphate by yeast. Aerobic.

FIG. 9. The effect of KCl on the uptake of phosphate by yeast. Aerobic.

FIG. 10. The effects of succinate, β-hydroxybutyrate, and 2,4-dinitrophenol on the uptake of phosphate in the presence of 0.3 M glucose. Aerobic.

DISCUSSION

Absorption of orthophosphate by Baker's yeast appears to be coupled with the oxidative formation of ATP chiefly by the action of glyceraldehyde-3-phosphate-dehydrogenase under both aerobic and anaerobic conditions, as was earlier concluded in part by Goodman and Rothstein (6). The reaction with $K_m = 10^{-6}$, however, has characteristics indicating coupling with the oxidative formation of ATP associated with cytochrome b of the respiratory chain. The nature of the reaction with $K_m = 10^{-3}$ and of the varied pattern of phosphate uptake by yeast and barley are of particular interest.

The general pattern for the coupling mechanism in oxidative phosphorylation associated with the respiratory chain has been formalized by Lehninger (13), Slater (19), Martius (15), and Lardy (12), [also note Chance & Williams (2)] as follows:

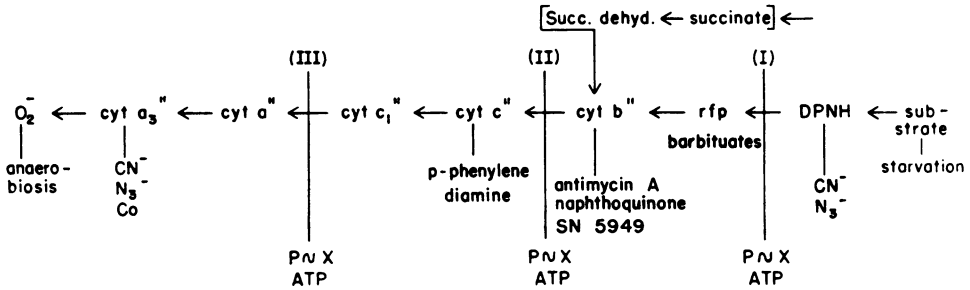
$$\text{Carrier} + \text{X} \xrightarrow[\text{transfer}]{\text{electron}} \text{carrier} \sim \text{X} \qquad [1]$$

$$\text{Carrier} \sim \text{X} + \text{P}_i \rightleftharpoons \text{carrier} + \text{P} \sim \text{X} \qquad [2]$$

$$\text{P} \sim \text{X} + \text{ADP} \rightleftharpoons \text{X} + \text{ATP} \qquad [3]$$

$$\text{ATP} + \text{glucose} \rightleftharpoons \text{glucose-6-phosphate} + \text{ADP} \qquad [4]$$

Three positions of oxidative phosphorylations in the series of oxidations and reductions of the respiratory chain as established by Slater (19) and others (1) are indicated by (I), (II), and (III) in the following scheme:



Positions of action of various inhibitors are indicated below the reaction sequence. Because succinate does not support much oxygen consumption in Baker's yeast (table I fig 10), under the conditions used its action is not further considered.

Several features of interaction of compounds influencing uptake of phosphate by intact cells by action as inhibitors or otherwise must be held in mind. Several respiration inhibitors were found by Chance (1) to have the same action on intact cells and mitochondria of ascites cells. The inhibitor studies are of short duration, which minimizes overall metabolic effects and permits use of compounds which eventually might be toxic. The conditions of use require the inhibitor to be effective as quickly as is phosphate uptake, which is essentially from time zero.

The inhibitor and phosphate both act as if their concentrations are the same or are linearly connected with those of the external solution. This implies that diffusion processes and pathways requiring appreciable time for establishment of steady-state conditions are not limiting. The uptake system can be expected to have a spatial separation of sequential reactions differing from that of a homogenous system such that an inhibitor might be more specific for a particular reaction. This is particularly true for respiration which might, in part, be closely associated with uptake but also arising, in part, from deep in the cell where it is not subject to immediate inhibitor action.

Uptake of phosphate with low concentrations of glucose and phosphate gives a linear component on a Hofstee plot (8) with $K_m = 10^{-6}$ (fig 6). This component reaction is strictly aerobic. Paraphenylenediamine increases the site concentration [XR] without changing the k_3 of [XR]. The maximum site increase is in the absence of glucose (fig 7). The changes are consistent with enhanced reduction of cytochrome b in the presence of the inhibitor, and are consistent with the in vitro observation that the site of action of *p*-phenylenediamine is between cytochromes c and b (11). If this is the case one should be able to follow the oxidation and/or reduction of phenylenediamine in the presence of the yeast cells. This has been observed, using the oxygen electrode. In the absence of exogenous substrate, reduced phenylenediamine supports respiration (table I). On the other hand, yeast will reduce oxidized phenylenediamine at a rate (2×10^{-8} moles/min/mg) equivalent to oxygen uptake by cells in a glucose medium. On the basis of the potential, one would predict that phenylenediamine could function in the proposed region of the respiratory chain. At the present time spectral changes of cytochromes c and b induced by *p*-phenylenediamine were not observed due to its absorption in the region of 560 mμ.

Barbital inhibits the uptake of phosphate at low concentrations of phosphate and glucose by decreasing [XR]. Pyruvate and ethanol, in the absence of glucose, under aerobic conditions increase the site concentration without leading to a measurable rate of turnover of [XR] (fig 2). Failure of turnover possibly arises from depletion of adenosine diphosphate (ADP) in reaction [3] because of the absence of reaction [4]. The several actions are consistent with phosphate uptake associated with change in oxidation of cytochrome b. Uptake of phosphate associated with change in oxidation of DPNH coupled to the respiratory chain could not be examined because of predominant coupling of DPN reduction with an anaerobic reaction ($K_m = 10^{-5}$).

The component of phosphate uptake with $K_m = 10^{-5}$ is present under both aerobic and anaerobic conditions (figs 4 & 5). It is inhibited by iodoacetate (figs 5 & 8) and sulfite and is unaffected by acetate. Iodoacetate probably is effective by reaction with the —SH group of glyceraldehyde–3–phosphate dehydrogenase while sulfite combines with the aldehyde and with acetaldehyde. It is of particular interest that while iodoacetate is inhibitory in both anaerobic and aerobic systems, acetate is effective only in the latter. These results are in agreement with Prankerd and Altman (16) and with the conclusions of Goodman and Rothstein (6) that the phosphate uptake is associated with the action of glyceraldehyde–3–phosphate dehydrogenase.

The anaerobic uptake of phosphate is inhibited by 2,4–dinitrophenol at 1×10^{-4} M and by 5,5,diethyl-

barbituate (figs 4 & 8). The barbituate changes the site concentration and m_{max} without changing K_m (fig 4) or k_3 (fig 8). Both are somewhat inhibitory to respiration. Dinitrophenol when used aerobically with separated mitochondria (2) uncouples respiration and oxidative phosphorylation, while with root disks it uncouples respiration and salt uptake (17). It is widely considered to be without effect on substrate level phosphorylations where most attention has been devoted to α-ketogluterate decarboxylation and subsequent oxidation to succinate (3). Barbituates are used as inhibitors of flavin mononucleotide (FMN) action in the respiratory chain. Neither, accordingly, would be anticipated to affect anaerobic formation of adenosine triphosphate (ATP) and, in fact. were used with this initial expectation.

Dinitrophenol when used in studies of phosphate uptake by barley roots (9) decreased uptake competitively with phosphate. This possibility has not been suggested in the extensive work with separated mitochondria.

The reason for a possible oversight might be that in the work with mitochondria the phosphate level is usually greater than 10^{-3} M and that of DNP is low (1×10^{-5} M or less) so that inhibition would be masked. The K_I value with intact barley roots found by Hopkins is 1.14×10^{-6} moles. DNP then, irrespective of uncoupling action, appears to be competitive with phosphate and this would seem to be its action on the anaerobic components of phosphate uptake with $K_m = 10^{-5}$ and 10^{-3}.

The action of the barbituates in inhibiting anaerobic phosphate uptake by yeast is not understood in terms of reactions immediately associated with action of glyceraldehyde–3–phosphate dehydrogenase. It might be that in the uptake action an FMN protein is connected with the reduction of diphosphopyridine nucleotide (DPN).

The values of k_3 for the aerobic and anaerobic reactions with $K_m = 10^{-5}$ are the same. This indicates that only one reaction is involved which, accordingly, must be in the glycolytic pathway. The reaction with $K_m = 10^{-6}$ also has this same value of k_3 under aerobic conditions. The indication is that this is a result of the sequential nature of the reactions running from glucose to oxygen.

Two steps are recognized for ATP formation resulting from dehydrogenation of phosphoglyceraldehyde. The first is connected with reduction of DPN mediated by glyceraldehyde–3–phosphate dehydrogenase, and the second by reaction with ADP and 1,3 phosphoglyceric acid and release of ATP mediated by 3, phosphoglyceric 1–kinase. The inhibitory action of ATP on the reaction with $K_m = 10^{-3}$ indicates that the last step is involved. A chance of confusion exists if ATP dilutes the added P^{32} either by hydrolysis or exchange, but with the high phosphate concentrations involved the dilution even with complete hydrolysis would have been negligible.

The component of phosphate uptake with $K_m = 10^{-3}$ is markedly effective only at phosphate concentrations $> 10^{-4}$ M in the presence of 0.3 M glucose. It would have appreciably contributed to the phosphate uptake by yeast observed by Goodman and Rothstein (6) and would have been greatly predominant in some of their experiments. Neither iodoacetate nor barbital apparently inhibits the reaction at concentrations of 10^{-1} M, although precision is so low that a one and a half-fold change would be uncertain (figs 4 & 5). The value of k_3 for the reaction is lower than that for the other two, which indicates that the rate-limiting reactions must be different.

At high phosphate concentrations the amount of ADP available for phosphorylation is relatively low. Furthermore, glucose is optimal for respiration, which suggests that hexokinase must be limiting for entrance of glucose into the glycolytic pathway. Under these conditions the hexokinase is also limiting for phosphate uptake. Inhibitors would not be adequately effective to put the controlling rate in earlier reactions by limiting either glycolysis or electron flow in the respiratory chain.

The general concepts of phosphate uptake by Baker's yeast can now be formulated in accord with the preceding analysis and compared with phosphate uptake by barley roots as previously formulated by Hagen, Leggett, and Jackson (7). Only a small fraction of the oxidative phosphorylation associated with the respiratory chain is coupled to phosphate uptake because the respiratory system is present only to a small extent at the surface of the limiting membrane. None of the experiments reported here bears on the extent to which respiration is coupled with ATP formation by turnover of endogenous phosphate, but the results of Chance and Williams (2) indicate that the P/O ratio might approach 3.0. In barley roots, on the other hand, the mitochondria are apparently embedded in the limiting membrane, possibly by the vacuolating of the cell. In yeast the oxidative phosphorylation associated with glyceraldehyde–3–phosphate dehydrogenase is present at the membrane surface, as also is the hexokinase reaction which utilizes ATP and introduces glucose into the cell. Glucose, in the absence of phosphate, can still enter through use of endogenous ATP. These deductions are in accord with those of Rothstein (18) and others. Barley roots, in contrast, while having a glycolytic pathway for utilization of glucose (20), apparently do not have the oxidative phosphorylation reaction coupled to glyceraldehyde–3–phosphate dehydrogenase at the cell surface to any considerable extent. In short, the barley root has the components of the respiratory chain at the surface of the limiting membrane while the yeast has the oxidative phosphorylation step of glycolysis.

Summary

Phosphate uptake by Baker's yeast, *Saccharomyces cerevisiae*, was measured in pure cultures under aerobic and anaerobic conditions. Effects of various substrates and respiratory chain and glycolytic inhibitors were examined.

Analysis of the results by means of formulation of first-order kinetics indicate three components of phosphate uptake. The minor one of the three is associated with oxidative phosphorylation coupled to cytochrome b of the respiratory chain. It is effective only under aerobic conditions. The other two components which constitute the main course of phosphate uptake at concentrations above 10^{-5} molar are effective under both aerobic and anaerobic conditions. Action of inhibitors indicate that they are both associated with oxidative phosphorylation coupled to the action of glyceraldehyde–3–phosphate dehydrogenase. The rate of one of the two components is interpreted as being limited by the phosphorylation of adenosine diphosphate. The rate of the other component, which is predominant at high phosphate concentrations ($> 10^{-3}$ M) and optimal glucose concentrations (0.3 M), appears to be limited by the action of hexokinase.

Acknowledgment

This investigation was started in association with the late C. E. Hagen. Demitros A. Soulides isolated the pure cultures of *Saccharomyces cerevisiae* Meyer ex Hansen, and advised on sterile technique. Advice in experimental design and the critical interest of P. C. Jackson and S. B. Hendricks are gratefully acknowledged.

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