THE ADP AND PHOSPHATE CONTROL OF ETHANOL OXIDATION IN BAKER'S YEAST

P. K. Maitra and R. W. Estabrook*

Johnson Foundation, University of Pennsylvania, Philadelphia 4, Pa.

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The mechanism of control of substrate utilization by intact cells has occupied considerable attention (cf. Ciba Foundation Symposium, 1959). The intracellular levels or localization of adenine nucleotides [ADP or ATP], reduced or oxidized pyridine nucleotides, and inorganic phosphate [Pi] have been suggested as being rate-limiting in the regulation of cellular metabolism. During recent studies on the oxidation of ethanol by yeast cells, associated chemical analyses of these components were carried out in an attempt to clarify the role of nucleotides and phosphate in an integrated metabolic system.

Polarographic measurement of oxygen uptake has shown (Chance, 1959) that the addition of ethanol to starved baker's yeast is associated with a rapid burst of oxygen utilization lasting for 20 to 40 seconds, after which time the respiratory rate decreases by about 40%. Subsequent to the onset of the inhibited rate of respiration there is a gradual increase in the rate of oxygen uptake returning to the level initially observed upon addition of ethanol. This type of control behavior in a microbial system was first reported by Chance (1959), who concluded from associated spectrophotometric measurements of the cytochrome carriers that the onset of respiratory inhibition is caused by an unavailability of ADP. The present report attempts to further identify the control chemical by a more direct measurement of the relevant chemical species

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obtaining during the various transient phases of alcohol oxidation in baker's yeast. The results presented here demonstrate the conditions where ADP control predominates or where the level of inorganic phosphate may be associated with respiratory inhibition and control.

Materials and Methods

Baker's yeast was starved by aeration as described by Chance (1959) and then washed three times in 0.9% saline and finally suspended in 50 mM triethanolamine buffer, pH 7.0 and aerated. Respiration was measured polarographically with the Clark oxygen electrode method, while the intracellular changes in reduced pyridine nucleotides were measured fluorometrically with a modified Eppendorf fluorometer. Measurements of adenine nucleotide concentrations were made as described earlier (Estabrook and Maitra, 1962). Inorganic phosphate was estimated by the procedure of Fiske and SubbaRow (1925).

Results

Dilution of starved baker's yeast in a buffer medium shows a slow rate of oxygen utilization. These cells are characterized by an ATP/ADP ratio of about 0.5 and a relatively high level of inorganic phosphate. As discussed above, addition of ethanol to such cells causes a transient, rapid increase in the rate of oxygen utilization and a concomitant reduction of endogenous pyridine nucleotide. The characteristic pattern of pyridine nucleotide reduction occurring during the observed stimulation and onset of inhibition of respiration is illustrated in Fig. 1 and will be discussed in greater detail in a subsequent publication. Of interest is the question of the type of respiratory control operative during the onset of the inhibited phase of oxygen utilization. When the intracellular levels of inorganic phosphate and adenine nucleotides were determined by analyses of aliquots withdrawn at various time periods before and during ethanol oxidation, it was observed that soon after the addition of

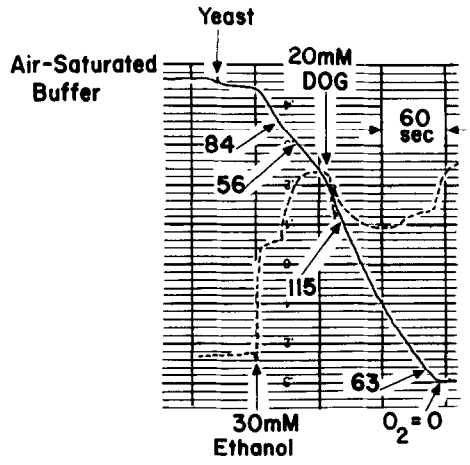

Fig. 1. Kinetics of the utilization of oxygen and reduction of pyridine nucleotide during oxidation of ethyl alcohol by starved baker's yeast and the effect of 2-deoxyglucose (DOG). The solid trace represents respiratory activity with the numbers against the arrows denoting rate of oxygen consumption in μmoles O_2 liter^{-1} min^{-1}. The medium contains 4.6 x 10^8 yeast cells per ml diluted with 50 mM KH_2PO_4 and contained initially 240 μmoles O_2 per liter. The broken trace indicates pyridine nucleotide reduction (upward deflection of the trace) recorded fluorometrically.

ethanol there is a monotonic rise in the level of ATP and a similar decrease in the level of ADP and inorganic phosphate [Pi] (Fig. 2). Establishment of steady state levels of adenine nucleotides and phosphate is associated with the onset of the inhibitory phase of ethanol oxidation as determined polarographically. Associated with the changes observed in pyridine nucleotide and respiratory rate is a dramatic shift in the ratio of ATP/ADP, i.e., from 0.5 prior to ethanol addition to 5 during the inhibited state.

To ensure that inorganic phosphate rather than ADP is not limiting, a substrate such as glucose or 2-deoxyglucose was added to activate the cellular hexokinase and alter the level of ADP and ATP in the cell. [cf. Chance (1959); Kiesow (1961)]. As illustrated in Fig. 1, the addition of 2-deoxyglucose causes about a two-fold stimulation of respiration as in the case of glucose and causes an oxidation of reduced pyridine nucleo-

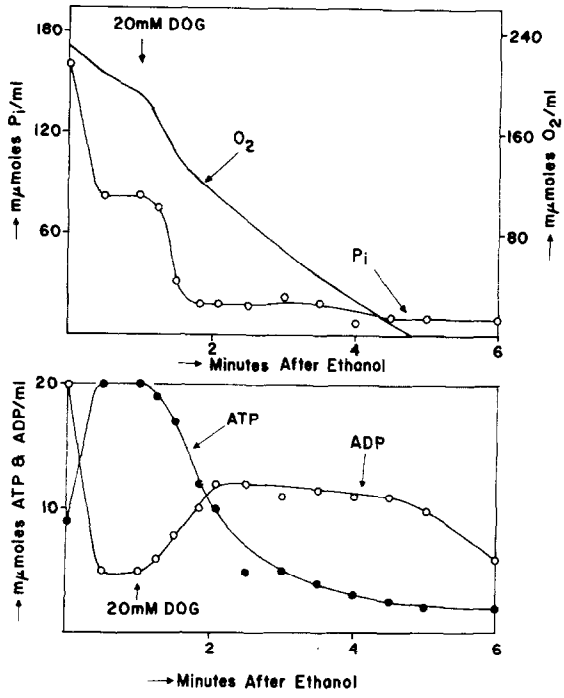

Fig. 2. Changes in the concentrations of dissolved O_2 in the reaction medium and of intracellular adenine nucleotides and inorganic phosphate in baker's yeast during oxidation of ethanol. The reaction mixture contained 2.6 x 10^8 starved washed yeast cells per ml diluted with 50 mM triethanolamine buffer, pH 7.0 at 25° C. At 0 time the reaction was initiated with 30 mM ethanol and 20 mM 2-deoxyglucose (DOG) added after 60 seconds. Aliquots for analysis were withdrawn at the intervals indicated and the reaction terminated by addition to trichloroacetic acid (final concentration, 6%). The curve for oxygen is an exact tracing of the polarographic chart. The ordinates refer to concentration expressed by ml of the reaction mixture.

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tide. During the state of stimulated respiration initiated by 2-deoxyglucose there is a marked decrease in the level of inorganic phosphate and an associated increase in the level of ADP paralleled by a gradual decrease in that of ATP (Fig. 2). After this initial period of accelerated respiratory activity a second inhibitory phase sets in as the rate of respiration starts slowing down gradually with a slow reduction of the steady state level of pyridine nucleotide, continuing till anaerobiosis

(Fig. 1). This inhibited phase of respiration is associated with the establishment of a very low level of inorganic phosphate and a relatively high level of ADP. Independent experiments indicate that this inhibited respiration can be activated to the value obtained immediately after 2-deoxyglucose addition by treatment with a 40 μM concentration of 2:4-dinitrophenol.

The data presented in Figs. 1 and 2, therefore, demonstrate conditions where one may obtain either the ADP control or the phosphate control of oxygen utilization. ADP control is operative during the initial inhibitory phase observed in the first minute of adding alcohol to starved yeast; the ADP pool is converted to ATP and the rate of metabolism is set by the slow rate of ATP-utilizing reactions. Under these conditions, the cells are characterized by high ATP/ADP and Pi/ADP ratios and the addition of 2-deoxyglucose stimulates respiration by increasing the intracellular ADP level. Within a minute thereafter sufficient phosphate has been trapped in 2-deoxyglucose-6-phosphate to cause the Pi/ADP ratio to fall from 17•0 to 1•5; this obviously results from a feedback of the oxidatively produced ATP to the cytoplasm at the site of hexokinase. The simplest explanation of this phenomenon is that the level of inorganic phosphate has fallen sufficiently low to become rate-limiting in respiration, under these special and rather unphysiological conditions. The hypothesis that the ATP inhibition of respiration has occurred (Klingenberg and Schollmeyer, 1961) is unlikely here because of the fact that the quotient $\frac{[ATP]}{[ADP][Pi]}$ is only 430 M^{-1}. The possibility that some other inhibitory phenomenon such as a change in intracellular pH (Wenner, C. E., personal communication) may be involved is eliminated because of the observed reactivation of the respiration by the addition of an uncoupling agent.

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