Adenosine Phosphates and the Control of Glycolysis and Gluconeogenesis in Yeast

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1. Changes in dry weight, protein, RNA and DNA were measured in yeast during adaptation to glycolytic metabolism. 2. Only RNA increased significantly during the lag phase, but during the exponential phase all these cellular components increased in parallel. 3. The concentrations of ATP, ADP, AMP and glucose 6-phosphate were measured in respiring yeast and during the transition to glycolytic metabolism. 4. In respiring cells the concentration of AMP was at its highest and that of ATP was at its lowest; this relationship was reversed in glycolysing cells. 5. ADP concentration was similar in respiring and glycolysing cells, but glucose 6-phosphate concentration was much higher in the glycolysing cells. 6. A possible reason for mitochondrial repression is suggested. 7. It is concluded that adenosine phosphates do not control the direction of glycolytic flux in yeast and an alternative control of glycolysis and gluconeogenesis by enzyme activation and inactivation is suggested.

Chapman & Bartley (1968) studied the kinetics of enzyme changes after yeast (*Saccharomyces cerevisiae*) that had been grown aerobically on the products of glucose metabolism in a complete medium was transferred to an anaerobic complete medium containing 10% of glucose. Before transfer, the yeast cells were found to have high activities of respiratory, citric acid-cycle and glyoxylate-cycle (gluconeogenic) enzymes and low activities of glycolytic enzymes. After transfer, glucose was found to repress the former enzyme systems and to induce glycolytic enzymes. These results were in agreement with those found by others (see, e.g., Polakis & Bartley, 1965; Jayaraman, Cotman, Mahler & Sharp, 1966). Mitochondria have also been shown to be repressed by glucose (Linnane, Vitols & Nowland, 1962; Polakis, Bartley & Meek, 1964).

In yeast, the transition from a state in which glycolysis is a major metabolic pathway to one in which respiration has a predominant role is in fact the same as the transition from a glycolytic to a gluconeogenic metabolism. In the present work, differences in concentrations of adenosine phosphates and glucose 6-phosphate were determined between respiring yeast and yeast adapting to glycolytic metabolism. The implications of these results for mitochondrial repression and the control of carbohydrate and lipid metabolism are discussed.

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MATERIALS AND METHODS

Chemicals. Bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex). Tris and bovine heart lactate dehydrogenase (type III) were from Sigma Chemical Co. (St Louis, Mo., U.S.A.). All other enzymes, ATP, NADP⁺ and NADH were from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Yeast RNA and salmon-sperm DNA were from Calbiochem (Los Angeles, Calif., U.S.A.). The remaining chemicals used and not listed were purchased from British Drug Houses Ltd. (Poole, Dorset) and were of the purest grade available.

Yeast. *Saccharomyces cerevisiae* strain no. 77 of the National Collection of Yeast Cultures (Brewing Industry Research Foundation, Nutfield, Surrey) was used.

Growth of cells. The aerobic growth medium was the same as that described by Polakis *et al.* (1964). The anaerobic medium was that of Polakis *et al.* (1964) but with sodium lactate omitted. Techniques were those used by Chapman & Bartley (1968). All cells were grown at 35°. The aerobic cultures were inoculated from slopes with about 10 mg. dry wt. of cells/l. of medium, and grown in rapidly shaking conical (Fernbach) flasks. Satisfactory aeration was maintained by simple diffusion of air through a sterile cotton-wool plug. After 24 hr. of aerobic growth, cells were spun down at 0° in sterile centrifuge tubes and resuspended in sterile water. Part of this suspension was used to inoculate the anaerobic medium with 1.5 mg. dry wt. of cells/ml. of medium. Anaerobiosis was maintained by flushing with O₂-free N₂. The time taken for isolation and washing of the cells before transfer to the anaerobic medium was 30 min.

Samples were taken from the anaerobic growth medium

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by expelling them under pressure of N_2 . Fresh sterile medium, when required, was added under anaerobic conditions.

Determination of dry weight. Cells were washed several times at 0° and dried overnight at 105° .

Determination of cell constituents. At the times indicated in Fig. 1 samples were removed from the growth medium. The cells were spun down and washed twice with ice-cold water at 0° . The samples were used for the determination of protein, dry weight and nucleic acids (Polakis & Bartley, 1966a).

Protein, RNA and DNA were fractionated by the procedure of Schmidt & Thannhauser (1945) as described by Hutchinson & Munro (1961). Protein was determined by the biuret method with bovine plasma albumin as a standard (Gornall, Bardawill & David, 1949). Lipids present in the protein fraction were removed as described by Chapman & Bartley (1968).

DNA was determined by the Giles & Myers (1965) modification of the Burton (1956) diphenylamine method. RNA was measured by the orcinol method, as described by Schneider (1957). Salmon-sperm DNA and yeast RNA were used as standards.

Extraction and determination of adenosine phosphates and glucose 6-phosphate. Duplicate samples (10ml.) of yeast suspension were removed from the growth medium at the times indicated in Fig. 2. The other procedures were exactly the same as those used by Polakis & Bartley (1966b). Cells were extracted and substrates measured by the methods of Lowry, Passonneau, Hasselberger & Schulz (1964). All substrates were determined fluorimetrically in a Farrand model A.2 fluorimeter as described by Polakis & Bartley (1966b).

RESULTS

Conditions of growth. Yeast cells were grown aerobically in a complete medium containing 0.9% of glucose for 24 hr. Under the conditions used all glucose was consumed in 11 hr. and subsequent growth was maintained by a respiratory metabolism using the products of glucose fermentation (Polakis & Bartley, 1965). After 24 hr. growth the cells were harvested and transferred to an anaerobic complete medium containing 10% of glucose, and grown anaerobically. Immediately after transfer there was a 30 min. lag phase, after which the cells grew exponentially with a mean generation time of 94 min. Exponential growth was maintained throughout the rest of the experiment by the addition of fresh medium at 2.5 hr. and 5.0 hr. Changes in glucose, ethanol and pH of the medium and also changes of the enzyme pattern of the cells that occur during the transition to glycolytic metabolism were described by Chapman & Bartley (1968).

Changes in cellular components. Fig. 1 gives logarithmic plots of the changes in dry weight, protein, RNA and DNA in the cells during the transition to glycolysis. RNA was the only component to increase significantly during the 30 min.

Fig. 1. Logarithmic plots of changes in the concentration/10ml. of growth medium of dry weight (●), protein (Δ), RNA (○) and DNA (▲) during the transition of aerobically growing yeast to glycolytic metabolism.

lag phase; subsequently all four components increased in parallel.

Changes in adenosine phosphates and glucose 6-phosphate in the cells during both the respiratory metabolism and glycolytic metabolism are presented in Fig. 2. During aerobic growth the concentration of ATP was about one-third of that of AMP. Immediately after transfer of the cells to the anaerobic medium the concentration of total adenosine phosphates increased rapidly, and the relative concentrations of ATP and AMP were reversed. During glycolytic adaptation total adenosine phosphates gradually declined to pre-transfer concentrations. The concentrations of both ATP and AMP fell, but there was a marked increase in the $[ATP]/[AMP]$ ratio. The ADP concentration varied little in either state.

Growth of cells in a complete medium causes extracts prepared from such cells to have a high fluorescence, and so determination of glucose 6-phosphate concentrations below 0.1 mM was not possible. Glucose 6-phosphate was not detected at any stage in the aerobic cells, but its concentration rose rapidly after transfer and remained above 1.0 mM during anaerobic growth on glucose.

Fig. 3 shows the values at different stages of some functions of the concentrations of adenosine phosphates. The $[ATP]/[AMP]$ ratio underwent the greatest change between the respiring yeast and the glycolysing yeast. The function $[ATP][AMP]/[ADP]^2$, although variable, was generally higher in the glycolysing cells. In contrast, the energy charge

$$\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$$

Fig. 2. Intracellular concentrations of total adenosine phosphates (Δ), ATP ($\bullet$), ADP ($\square$), AMP ($\circ$) and glucose 6-phosphate ($\blacktriangle$) in yeast at different stages of respiratory and glycolytic metabolism: (a) aerobic conditions; (b) anaerobic conditions. There was a 30 min. interval between the harvesting of the aerobic cells after 24 hr. growth and their transfer to the anaerobic medium.

Fig. 3. Changes in the values of $[ATP]/[AMP]$ ($\bullet$), $[ADP]/[AMP]$ ($\square$), $[ATP]/[ADP]$ (Δ), the ratio $[ATP][AMP]/[ADP]^2$ catalysed by myokinase ($\circ$) and the energy charge ($\blacktriangle$) in yeast at different stages of respiratory and glycolytic metabolism: (a) aerobic conditions; (b) anaerobic conditions. There was a 30 min. interval between the harvesting of the aerobic cells after 24 hr. growth and their transfer to the anaerobic medium. The energy charge is defined as

$$\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$$

(Atkinson, 1968) was fairly constant for a particular metabolic state, but was higher in the glycolysing cells.

DISCUSSION

Polakis & Bartley (1966b) measured the changes of cofactors in yeast growing aerobically in a synthetic medium after glucose and galactose had been virtually exhausted and the cells were no longer growing exponentially, i.e. during the transition from glycolytic to respiratory metabolism. Their results are comparable with the results from the respiring yeast presented in the present paper, the relative concentrations of adenosine phosphates being $AMP > ADP > ATP$; the glucose 6-phosphate concentration was less than 0.1 mM by the time glucose was exhausted from the medium. On adaptation to glycolytic metabolism the concentrations of glycolytic intermediates were elevated and the relative concentrations of adenosine phosphates were quickly established as $ATP > ADP > AMP$ (see, e.g., Betz & Chance, 1965; Hommes, 1966).

Two noticeable events occurring during the 30 min. lag phase, after the transfer to anaerobic conditions, were the 40% increase in RNA and the 100% increase in total adenosine phosphate. These two events were expected, since before transfer the cells were growing slowly, whereas after transfer the exponential growth was rapid with a mean generation time of 94 min. The increased growth rate is equivalent to a step-up transition, and the events observed are typical of such a transition (Neidhardt & Fraenkel, 1961).

Enzymological differences similar to the differences between glycolysing and respiring yeast have been found in other tissues, e.g. the differences between the livers of well-fed rats and the livers of diabetic or starved rats or of rats fed on a low-carbohydrate diet (Weber, 1963; Ashmore, Wagle & Uete, 1964). Start & Newsholme (1968) showed that in the livers of starved rats the concentrations of ATP and glucose 6-phosphate were lower, and AMP concentration was higher, than in the livers of fed animals; the concentration of ADP differed little between the two states. These differences are similar to the differences between glycolysing and respiring yeast.

The present results suggest that the $[ATP]/[AMP]$ ratio or the concentration of glycolytic intermediates is more likely to control the balance of the glycolytic and respiratory systems than ADP (Polakis & Bartley, 1966b).

The difference between the $[ATP]/[AMP]$ ratios of yeast growing on fermentable and on non-fermentable substrates may help to explain the glucose repression of mitochondria (Linnane *et al.*

1962; Polakis *et al.* 1964). Uyeda & Racker (1965) showed that in reconstructed systems, with limiting concentrations of orthophosphate and ADP, there was competition between the glycolytic system and mitochondria for these two substrates. If this situation exists within a cell, a high glycolytic rate may not be compatible with a functioning respiratory system. In animal tissues it is uncommon for both glycolysis and respiration to contribute substantially to maintaining a high metabolic rate. Skeletal muscle uses glycolysis for its main energy production, whereas heart muscle uses fatty acids, which are metabolized by the respiratory processes of the mitochondria. Insect flight muscle is an exception, but in this case phosphoenolpyruvate is metabolized within the mitochondria, thus eliminating the competition of the glycolytic pathway for ADP (Sacktor, 1955).

Control of glycolysis and gluconeogenesis

Most theories (see, e.g., Newsholme & Gevers, 1967; Horecker *et al.* 1967) relating control of glycolysis and gluconeogenesis to the known controls of pacemaker enzymes, such as phosphofructokinase and fructose diphosphatase, suggest that glycolysis is inhibited and gluconeogenesis stimulated at high $[ATP]/[ADP]$ and $[ATP]/[AMP]$ ratios, and at low $[ATP]/[ADP]$ ratios the reverse obtains. It is concluded therefore that a cell may achieve a balance between these two pathways (see, e.g., Newsholme & Gevers, 1967). Newsholme & Gevers (1967) did, however, point out that the $[ATP]/[ADP]$ and $[ATP]/[AMP]$ ratios did not appear to vary experimentally in the manner predicted from this theory.

Control by adenosine phosphates. Yeast phosphofructokinase is inhibited by ATP and activated by AMP (Betz & Moore, 1967), whereas yeast fructose diphosphatase is inhibited by AMP (Gancedo, Salas, Giner & Sols, 1965). These facts, correlated with the present results, apparently indicate that, as far as control by the $[ATP]/[AMP]$ ratio is concerned, phosphofructokinase ought to be under maximum inhibition when glycolysis is occurring (since the measured $[ATP]/[AMP]$ ratio is high) and fructose diphosphatase ought to be maximally activated, and that the opposite situation ought to exist when gluconeogenesis is occurring. Such results are incompatible with the proposal that the $[ATP]/[AMP]$ ratio acts as a 'flip-flop' control of glycolysis and gluconeogenesis.

Control by glycolytic intermediates. Yeast fructose diphosphatase has a low K_m for fructose diphosphate (Gancedo *et al.* 1965), whereas the operation of yeast phosphofructokinase appears to be dependent on concentrations of fructose 6-phosphate higher than those found in non-glycolysing cells (Viñuela, Salas

& Sols, 1963; Betz & Moore, 1967). If it is assumed that the phosphohexose isomerase reaction is always approximately at equilibrium, the concentration of fructose 6-phosphate (calculated from the measured concentration of glucose 6-phosphate) is much higher in glycolysing cells than in non-glycolysing cells. It is possible, therefore, that in cells that are synthesizing glucose (in which low concentrations of fructose 6-phosphate and fructose diphosphate might be expected) much of the energetically wasteful coupling between fructose diphosphatase and phosphofructokinase would be eliminated. In other situations where fructose 6-phosphate concentration is high this control could not occur.

Control by the quantity of active enzyme. Clearly mechanisms that activated one set of enzymes while simultaneously inactivating the other set of opposing enzymes would be the most efficient way of regulating gluconeogenesis and glycolysis. There is in fact increasing evidence that such mechanisms, similar to the control of glycogen phosphorylase and glycogen synthetase (Krebs & Fischer, 1962), do exist (see Viñuela, Salas, Salas & Sols, 1964; Harris & Ferguson, 1967; Ferguson, Boll & Holzer, 1967; Kopperschlager & Augustin, 1967; Chapman & Bartley, 1968; Mendicino, Prihar & Salama, 1968). Such evidence indicates that the direction of glycolytic flux may be determined by the physiological activation and inactivation of enzymes.

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