The Kinetics of Enzyme Changes in Yeast under Conditions that Cause the Loss of Mitochondria

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1. Aerobically grown yeast having a high activity of glyoxylate-cycle, citric acid-cycle and electron-transport enzymes was transferred to a medium containing 10% glucose. After a lag phase of 30 min. the yeast grew exponentially with a mean generation time of 94 min. 2. The enzymes malate dehydrogenase, isocitrate lyase, succinate-cytochrome *c* oxidoreductase and NADH-cytochrome *c* oxidoreductase lost 45%, 17%, 27% and 46% of their activity respectively during the lag phase. 3. When growth commenced pyruvate kinase, pyruvate decarboxylase, alcohol dehydrogenase, glutamate dehydrogenase (NADP⁺-linked) and NADPH-cytochrome *c* oxidoreductase increased in activity, whereas aconitase, isocitrate dehydrogenase (NAD⁺- and NADP⁺-linked), α -oxoglutarate dehydrogenase, fumarase, malate dehydrogenase, succinate-cytochrome *c* oxidoreductase, NADH-cytochrome *c* oxidoreductase, NADH oxidase, NADPH oxidase, cytochrome *c* oxidase, glutamate dehydrogenase (NAD⁺-linked), glutamate-oxaloacetate transaminase, isocitrate lyase and glucose 6-phosphate dehydrogenase decreased. 4. During the early stages of growth the loss of activity of aconitase, α -oxoglutarate dehydrogenase, fumarase and glucose 6-phosphate dehydrogenase could be accounted for by dilution by cell division. The lower rate of loss of activity of isocitrate dehydrogenase (NAD⁺- and NADP⁺-linked), glutamate dehydrogenase (NAD⁺-linked), glutamate-oxaloacetate transaminase, NADPH oxidase and cytochrome *c* oxidase implies their continued synthesis, whereas the higher rate of loss of activity of malate dehydrogenase, isocitrate lyase, succinate-cytochrome *c* oxidoreductase, NADH-cytochrome *c* oxidoreductase and NADH oxidase means that these enzymes were actively removed. 5. The mechanisms of selective removal of enzyme activity and the control of the residual metabolic pathways are discussed.

Anaerobically grown yeasts have substantially lower activities of the enzymes concerned with the citric acid cycle, the glyoxylate cycle and respiration, and mitochondria are absent or very few in number (Schatz, 1963; Polakis, Bartley & Meek, 1964). This condition can also be induced by growth on high concentrations of glucose (Tustanoff & Bartley, 1964; Singer, Rocca & Kearney, 1966) and does not necessarily depend on a lack of oxygen. The repressive effect of glucose on the quantity of citric acid-cycle enzymes can be partially overcome by a requirement to synthesize amino acids (Polakis & Bartley, 1965*a*), but the synthesis of respiratory enzymes is inhibited totally by glucose concentrations above 5% (Singer *et al.* 1966). The changes that occur when facultative organisms take up an aerobic form of metabolism are quite well documented (see, e.g., Slonimski, 1956; Gray, Wimpenny, Hughes & Mossman, 1966*a,b*). This paper is concerned with the relatively unexplored

transition from aerobic conditions without glucose to anaerobic metabolism with a high concentration of glucose. During this transition mitochondria are lost.

In most experiments aerobically grown yeast was transferred to anaerobic conditions in a medium with a high glucose content. This was thought to produce conditions where the rate of transition from the aerobic to the anaerobic state of metabolism would be maximal. In some experiments a high glucose concentration was used with aerobic conditions, but with minor exceptions the glucose and amino acid concentrations were the factors determining the metabolic pattern of the cell. Since the yeast cell will not develop mitochondrial and certain other enzymes in the presence of a hexose, and since anaerobic growth requires the presence of a hexose, it is not possible to test the effect of anaerobiosis without simultaneously having a hexose in the medium. Our results must be

viewed with these limitations in mind, but the evidence suggests that the effect of glucose predominates over the presence or absence of oxygen. This agrees with the findings of workers in many Laboratories (see, e.g., Schatz, 1963; Tustanoff & Bartley, 1964; Singer *et al.* 1966).

MATERIALS AND METHODS

Chemicals. Bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex); α -oxoglutaric acid (monosodium salt), cytochrome *c* (type III), NADPH and CoA were from Sigma Chemical Co. (St Louis, Mo., U.S.A.); casein hydrolysate (acid) L41 was from Oxo Ltd. (London, S.E. 1); yeast extract was from Difco Laboratories (Detroit, Mich., U.S.A.); NAD⁺, NADH, NADP⁺, sodium pyruvate, phosphoenolpyruvate (triethylhexylammonium salt), fructose 1,6-diphosphate (barium salt), glucose 6-phosphate (sodium salt), ATP, ADP, AMP and oxaloacetate were from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Monopotassium L-isocitrate from *Bryophyllum crenatum* was a gift from Professor H. B. Vickery.

All other chemicals were A.R. grade or the purest grade available, most often purchased from either British Drug Houses Ltd. (Poole, Dorset) or Hopkin and Williams Ltd. (Chadwell Heath, Essex).

Yeast. *Saccharomyces cerevisiae* strain no. 77 of the National Collection of Yeast Cultures (Brewing Industry Research Foundation, Nutfield, Surrey) was used. It was maintained on agar slopes described by Polakis *et al.* (1964).

Growth of cells. The aerobic growth medium was the same as that described by Polakis *et al.* (1964). The anaerobic medium differed by the omission of sodium lactate. All cells were grown at 35°. The aerobic cultures were inoculated with about 10 mg. dry wt. of cells/l. of medium and grown in rapidly shaken conical (Fernbach) flasks. A comparison of forced aeration with sterile air and simple diffusion of air through a sterile cotton-wool plug showed that the patterns of enzymes obtained were the same, and in most of the experiments the latter simpler method was used. After 24 hr. of aerobic growth cells were spun down at 0° in sterile centrifuge tubes and resuspended in sterile water, and, after removal of a sample, the rest were transferred to the anaerobic medium so that 1.5 mg. dry wt. of cells was added to each millilitre of medium. Anaerobiosis was maintained by flushing with oxygen-free nitrogen. No contamination by other organisms was found as judged by conventional staining and plating techniques.

In short-term anaerobic adaptation (5–150 min.) growth was arrested by pouring the cells into an equal volume of water in a tube immersed in ice and immediately (less than 30 sec. after sampling) centrifuging the suspension at 0°. In long-term experiments (up to 6.5 hr.) samples were taken from a single flask at various times by being expelled under pressure of nitrogen. Fresh sterile medium, when required, was added similarly under anaerobic conditions. The cells from these experiments were collected in a container immersed in ice and immediately transferred to chilled centrifuge tubes and centrifuged at 0°. The time between starting the withdrawal and centrifuging the cells varied between 2 and 4 min.

Cell disruption. A 20% (w/v) suspension of yeast cells in

50 mm-potassium phosphate buffer, pH 6.8, was used. The cells were disrupted in a chilled French pressure cell at 17000–20000 lb./in.². At least 70% of the cells were broken by this procedure, but usually 90% of the cells were ruptured. Unbroken cells and cell debris were removed by centrifugation at 0° and 1000g for 10 min.

The amount of endogenous substrate present was decreased either by passing the cell extract through a Sephadex G-25 column at 0° (Polakis & Bartley, 1965a) or by dialysis at 0° in cellophane bags against 50 mm-phosphate buffer, pH 6.8, for 12–15 hr. The latter method, though much longer, made no significant alteration to the enzyme activities tested for, but gave much lower background interference in several assays. The extracts obtained were divided into 1 ml. samples for storage at –18°. Some enzymes were tested before the extract was frozen but most were assayed after storage at –18°; such extracts were thawed only once.

Analytical methods. Glucose was determined by the glucose oxidase method of Thompson (1966). Ethanol was determined by the method of Bonnichsen (1963), and for this semicarbazide hydrochloride that had been freshly freeze-dried was used. Protein was estimated by the biuret method. Once the colour was developed all samples were repeatedly washed with light petroleum (b.p. 40–60°) until no lipid could be seen at the liquid interface. This required as many as four washes, but without this treatment variations of up to 20% were found with different samples of the same extract. After treatment duplicate samples did not differ by more than 5%. No protein was lost by this treatment.

Measurement of enzyme activities. Enzymes were assayed in cuvettes with a 1 cm. light-path by using a double-beam temperature-controlled recording spectrophotometer (Beckman model DB). The methods used were essentially the same as those described by Polakis & Bartley (1965a,b), although a number of modifications have been made. Experiments to check assays for dependence on enzyme concentration were always done, and in any set of assays on a particular enzyme the same buffer, cofactor and substrate solutions were used and the assays were carried out on the same day. Usually assays were arranged to give changes in extinction of 0.01–0.04/min.

Succinate-cytochrome *c* oxidoreductase (EC 1.3.99.1) and α -oxoglutarate dehydrogenase [2-oxoglutarate-lipoate oxidoreductase (acceptor-acylating), EC 1.2.4.2] were always assayed before the extract was frozen.

All assays were carried out at 35° except NADPH oxidase [NADPH-(acceptor) oxidoreductase, EC 1.6.99.1], NADH-cytochrome *c* oxidoreductase (formerly EC 1.6.2.1) and NADPH-cytochrome *c* oxidoreductase (formerly EC 1.6.2.3), which were found to be unstable at this temperature. These were assayed at 25°, at which temperature only NADPH-cytochrome *c* oxidoreductase was unstable. This instability was minimized by adding the enzyme to the cuvette after NADPH.

The following were assayed in 50 mm-potassium phosphate buffer: aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3], isocitrate dehydrogenase (NAD⁺-linked) [*threo*-D₂-isocitrate-NAD⁺ oxidoreductase (decarboxylating), EC 1.1.1.41], isocitrate dehydrogenase (NADP⁺-linked) [*threo*-D₂-isocitrate-NADP⁺ oxidoreductase (decarboxylating), EC 1.1.1.42], α -oxoglutarate dehydrogenase, fumarase (L-malate hydro-lyase, EC 4.2.1.2), succinate-cytochrome *c* oxidoreductase, NADH oxidase [NADH-(acceptor) oxido-

reductase, EC 1.6.99.3], NADPH oxidase, NADH-cytochrome *c* oxidoreductase, NADPH-cytochrome *c* oxidoreductase and cytochrome *c* oxidase (cytochrome *c*-oxygen oxidoreductase, EC 1.9.3.1), all at pH 7.4; pyruvate decarboxylase (2-oxo acid carboxy-lyase, EC 4.1.1.1) at pH 6.5; isocitrate lyase (*threo*-D₂-isocitrate glyoxylate-lyase, EC 4.1.3.1) at pH 6.9; glutamate dehydrogenase (NAD⁺-linked) [L-glutamate-NAD⁺ oxidoreductase (deaminating), EC 1.4.1.2] and glutamate dehydrogenase (NADP⁺-linked) [L-glutamate-NADP⁺ oxidoreductase (deaminating), EC 1.4.1.4] at pH 8.0. Glutamate-oxaloacetate transaminase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) and alcohol dehydrogenase (alcohol-NAD⁺ oxidoreductase, EC 1.1.1.1) were assayed in 50mM-sodium pyrophosphate buffer at pH 8.8 and pH 8.5 respectively. Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP⁺ oxidoreductase, EC 1.1.1.49) was assayed in 50mM-triethanolamine hydrochloride buffer at pH 7.5. Malate dehydrogenase (L-malate-NAD⁺ oxidoreductase, EC 1.1.1.37) was measured at pH 7.4, usually in 50mM-potassium phosphate buffer, but, when activities were compared at 0.25mM- and 2.5mM-oxaloacetate, 50mM-triethanolamine buffer was used as this gave a better separation of activities.

Glutamate-oxaloacetate transaminase was measured in the direction of glutamate formation (Holzer, Gerlach, Jacobi & Gnoth, 1958) since this gave a higher activity, and endogenous oxidation of nicotinamide nucleotide was less. Even so the enzyme could only be assayed satisfactorily in dialysed extracts, in which the endogenous activity was very low (less than 10% of the test). Alcohol dehydrogenase was assayed by a modification of the method of Racker (1950a). Since Racker's concentration of NAD⁺ and ethanol (0.05mM and 10mM respectively) did not produce maximum activity under the conditions of our test, 1.67mM-NAD⁺ and 100mM-ethanol were used. Alcohol dehydrogenase was found to be very unstable to dilution and assays were therefore arranged to give the minimum of dilution (not more than tenfold); under these conditions the change in extinction was up to 0.5/min.

Aconitase (with 10mM-citrate as substrate) and fumarase (with 15mM-L-malate as substrate) were assayed by the method of Racker (1950b). No difficulty was encountered in measuring these enzymes when activity was high but when it was low the high extinctions in test cuvettes made the measurement difficult or impossible. It was found that ultrasonic treatment of an extract for 30sec. followed by centrifugation for 30min. at 200000g left over 98% of both these enzymes in the soluble fraction and the extinction of the extract was then sufficiently low for accurate measurement of the low activities. In long-term experiments all extracts were subjected to this treatment.

Isocitrate dehydrogenase (NAD⁺-linked) and α -oxoglutarate dehydrogenase were assayed in anaerobic cuvettes because of interference by NADH oxidase. The cuvettes used had a single side arm and had two inlets. Deoxygenated solutions were kept in tubes with a small gas space. Samples of these solutions were pipetted into the cuvette without appreciable aeration. Once the cuvettes were filled, Suba-seal caps were inserted into the cuvette openings and anaerobic conditions were established by flushing with O₂-free N₂ for 2-3min. Deoxygenated solutions could be injected through the cap.

Reproducibility of the measurements. All specific activities

presented are averages of two or more measurements, usually obtained with different concentrations of the same extract. Although the results presented are those from single experiments, the pattern of development of all enzymes has been checked by repeating the experiment at least twice with different cultures. The errors of estimation of the enzymes are the same as those described by Polakis & Bartley (1965a).

RESULTS

Changes in the growth medium during anaerobic adaptation. Under the conditions of aerobic growth with 0.9% glucose all the carbohydrate is removed by 11hr. of growth (Polakis & Bartley, 1965a). The subsequent 13hr. of growth before transfer to the anaerobic medium was therefore in the absence of glucose. This was necessary to ensure full development of mitochondrial enzymes and those of the glyoxylate pathway. Acetate (see Polakis & Bartley, 1965a) is the main substrate of growth during this latter period.

After these cells were transferred to the anaerobic environment with high glucose there was a lag phase of 30min. Subsequently the cells grew at an accelerating rate for about 1hr., after which a steady exponential growth rate was achieved, with a mean generation time of 94min. With an inoculum of 1.5mg. dry wt. of cells/ml., growth was exponential until 4.5hr. after inoculation and ceased after 5.5hr., by which time the dry weight of the cells had increased to 9.5mg./ml. and no glucose could be detected by the glucose oxidase test. At the start of anaerobic growth the concentration of amino acids in the medium was 15mg./ml. The increase in dry wt. after 5.5hr. was 8mg./ml., and of this about 36%, or 3mg., was protein. The concentration of amino nitrogen was therefore sufficient to support five times this increase.

In the long-term experiments the changes in environment were partially overcome by adding an equal volume of fresh medium every 2.5hr. and, once started, exponential growth was maintained throughout these long-term experiments. Fig. 1 gives the changes in glucose, ethanol and dry wt. of the culture during one of these long-term studies. After 3.5hr. the glucose concentration had decreased by only 24% and ethanol concentration had doubled. More drastic changes in these two substrates occurred only after 5hr. of growth, and this was due to the increase in cell numbers.

During the first 2.5hr. of growth the pH of the medium fell from 5.2 to 4.8. With the addition of the fresh medium the pH rose to 5.0, and it subsequently declined to 4.1 by the time the next lot of medium was added at 5.0hr. This again resulted in a rise of pH to 4.95 and this was again followed by a slow decline to 4.5 at 6.5hr. The major enzyme changes occurred within the first 2.5hr. and it

appears unlikely that the small pH change of the medium could have influenced the enzyme adaptation. There was no break in the pattern of enzyme change when fresh medium was added that could be attributed to the consequent restoration of the pH to about 5.0.

Relationship of enzyme activity to cell growth. There are three possibilities that govern the rates at which enzymes change in the growing cell. If there is a cessation of synthesis of enzyme and no active removal, then the specific activity of the enzyme will decline by 50% for every cell division. Such a rate of loss of activity has been termed the dilution rate, which, in these experiments, means a loss of 50% of the activity every 1.5 hr., except during the lag phase. Any loss in activity that is slower than this implies an active synthesis of the particular enzyme, and a loss faster than this means that active removal of the enzyme is taking place. In this paper the term 'rapid loss of activity' refers to any loss that is greater than that accounted for by dilution.

Most results are plotted as specific activity against time. The exception to this is Fig. 8, which concerns enzymes having a period of rapid loss of activity extending well after the lag phase. The results for these enzymes are plotted on the basis of percentage loss of specific activity per generation time against time. On such a graph any point above the 50% mark indicates a loss of activity faster than the dilution rate. This plot does not take

account of the lack of growth during the lag phase and the initial phase of slower growth, but the arguments concerning ratio of loss of specific activity are not affected.

Changes in specific activities of enzymes

Pyruvate kinase, pyruvate decarboxylase and alcohol dehydrogenase. Fig. 2 shows the changes in the activities of these three sequential glycolytic enzymes. Although the absolute pattern changed, the activities more or less kept in step, with pyruvate kinase always about an order of magnitude higher than the other two. After 5.0 hr. the enzymes had increased in specific activity three- to four-fold.

The very high specific activity of pyruvate kinase is noteworthy. Previous experiments in this Department (Polakis & Bartley, 1965a) did not indicate that this enzyme was induced by glucose. These earlier measurements were made before the allosteric activation by fructose diphosphate (Hess, Haackel & Brand, 1966) had been discovered, and these earlier results may have been influenced by the intracellular concentration of fructose diphosphate and may not have represented the true enzyme activity. In the present series of measurements the optimum concentration of fructose diphosphate (1.5 mM) was added to obtain full activity of the enzyme. Under our conditions this concentration of fructose diphosphate gave a 75-fold increase in activity compared with the 20–50-fold increase found by Hess *et al.* (1966) and the 200-fold increase found by Gancedo, Gancedo & Sols (1967) with

Fig. 1. Changes in the dry wt. of cells (▲) and glucose (●) and ethanol (○) concentrations in the medium during adaptation of yeast to anaerobic growth on 10% glucose. The cells were previously grown aerobically on 0.9% glucose for 24 hr. During adaptation fresh medium was added at 2.5 hr. and 5.0 hr.

Fig. 2. Changes in the activities of pyruvate kinase (○, values $\times 10^{-2}$), pyruvate decarboxylase (●, values $\times 10^{-1}$) and alcohol dehydrogenase (Δ, values $\times 10^{-1}$) during adaptation of yeast to anaerobic growth on 10% glucose. For details of growth see the text and Fig. 1.

Fig. 3. Changes in the activities of glutamate dehydrogenase (NAD⁺-linked) (○), glutamate dehydrogenase (NADP⁺-linked) (●) and glutamate-oxaloacetate transaminase (Δ) during adaptation of yeast to anaerobic growth on 10% glucose. For details of growth see the text and Fig. 1.

1 mM-fructose diphosphate. Our results showed that there was a direct relationship between the amount of pyruvate kinase and the initial concentration of glucose in the medium. Hommes (1966) and Gancedo *et al.* (1967) have shown that this enzyme is induced by glucose in *Candida* sp. Our results do not agree with those of Gancedo *et al.* (1967), who suggested that pyruvate kinase activity in *S. cerevisiae* was controlled only by concentrations of fructose diphosphate and was not induced by glucose.

Amino acid-metabolizing enzymes. The changes in specific activities of the glutamate dehydrogenases (NAD⁺- and NADP⁺-linked) and glutamate-oxaloacetate transaminase are shown in Fig. 3. Our results show the reciprocal relationship between the two glutamate dehydrogenases (Polakis & Bartley, 1965a). The NAD⁺-linked enzyme steadily decreased with a half-life of about 3 hr., but the NADP⁺-linked enzyme increased, roughly doubling its specific activity every 3 hr. The transaminase activity showed smaller changes, and after 5 hr., when specific activity had declined by 40%, it appeared to have reached a new steady value.

Isocitrate dehydrogenases and α-oxoglutarate dehydrogenase (Fig. 4). When growth started the NAD⁺-linked isocitrate dehydrogenase lost specific activity at a rate slightly less than the dilution rate for about the first 1.5 hr. The NADP⁺-linked enzyme lost specific activity at an even slower rate. In contrast, α-oxoglutarate dehydrogenase specific

Fig. 4. Changes in the activities of isocitrate dehydrogenase (NAD⁺-linked) (●), isocitrate dehydrogenase (NADP⁺-linked) (○) and α-oxoglutarate dehydrogenase (Δ) during adaptation of yeast to anaerobic growth on 10% glucose. For details of growth see the text and Fig. 1.

Fig. 5. Changes in the activities of fumarase (○) and aconitase (●) during adaptation of yeast to anaerobic growth on 10% glucose. For details of growth see the text and Fig. 1.

activity dropped by over 90% by the end of the experiment.

Fumarase and aconitase (Fig. 5). In the early stages of adaptation the specific activity of both these enzymes decreased at the dilution rate, but fumarase activity was stabilized after 5.0 hr., whereas aconitase activity continued to be lost at the dilution rate. These results are in agreement with the findings of others that the specific activity of aconitase varied more with the growth conditions than that of fumarase (Hirsch, 1952; Gray *et al.* 1966b).

Fig. 6. Changes in the activities of malate dehydrogenase (●) and isocitrate lyase (○) during adaptation of yeast to anaerobic growth on 10% glucose. For details of growth see the text and Fig. 1.

Malate dehydrogenase and isocitrate lyase (Fig. 6). Malate dehydrogenase and isocitrate lyase both began to lose activity during the lag phase. When growth commenced the rapid loss of malate dehydrogenase activity continued for about 1 hr., and subsequently loss of activity was at the dilution rate. By contrast the rapid loss of activity of isocitrate lyase continued until about 2.5 hr., when the activity had fallen to very low values. John & Syrett (1967) have observed a loss of isocitrate lyase in *Chlorella* under similar circumstances.

Witt, Kronau & Holzer (1966a, b) showed that yeast, like many tissues, possesses two malate dehydrogenase isoenzymes. One is cytoplasmic and is completely repressed by glucose, whereas the other, in highly aerobic cells, is mitochondrial, but it also occurs in glucose-grown cells. The 'mitochondrial' isoenzyme is inhibited by concentrations of oxaloacetate above 0.3 mM. The ratio of malate dehydrogenase activity in the presence of 0.25 mM- and 2.5 mM-oxaloacetate was compared in aerobic cells and cells that had been adapted to anaerobic growth with high glucose concentration for 6.5 hr. The activity with 2.5 mM-oxaloacetate was 66% of that with 0.25 mM-oxaloacetate in the aerobic extract and 77% in the adapted extract. These values are about the same as those calculated from the results of Ferguson, Boll & Holzer (1967) with induced and repressed cells. These results are in agreement with a shift in favour of the 'mitochondrial' isoenzyme under the conditions of high glucose concentration.

Glucose 6-phosphate dehydrogenase (Fig. 7). After the lag phase, the specific activity of glucose 6-phosphate dehydrogenase decreased by 50% during the first 2.5 hr. of adaptation, after which it

Fig. 7. Changes in the activities of glucose 6-phosphate dehydrogenase (○), NADPH-cytochrome *c* oxidoreductase (■) and NADPH oxidase (□) during adaptation of yeast to anaerobic growth on 10% glucose. For details of growth see the text and Fig. 1.

remained constant. Since Witt *et al.* (1966a) found the specific activity of this enzyme in yeast grown aerobically on a variety of carbon sources to be invariant, it seemed unlikely that it was repressed or induced by glucose. Witt *et al.* (1966a) grew their cells aerobically on a medium requiring a high synthetic activity, and their measurements of glucose 6-phosphate dehydrogenase activity were five times our maximum activity. This suggests the possibility that this enzyme is controlled by synthetic requirements.

Electron-transport enzymes. Whereas NADPH-cytochrome *c* oxidoreductase (Fig. 7) increased slightly in activity during anaerobic growth at high glucose concentration, NADPH oxidase (Fig. 7) changed little until after 4 hr. of adaptation, after which specific activity was lost at the dilution rate. The results are similar to those found by Schatz & Klima (1964).

The specific activity of both NADH-cytochrome *c* oxidoreductase (Fig. 8) and succinate-cytochrome *c* oxidoreductase (Fig. 8) rapidly decreased during the lag phase and the early stages of growth. Subsequently both enzymes started a cyclic process ranging from loss at the dilution rate to an accelerated loss.

NADH oxidase (Fig. 8) behaved in a similar manner but the onset of loss greater than the dilution rate was delayed for about one generation time. This cyclic behaviour of the three enzymes was confirmed by duplicate experiments, which gave identical patterns of change.

The relationship between NADH oxidase and NADH-cytochrome *c* oxidoreductase is complicated since the capacity to reduce cytochrome *c* is

Fig. 8. Changes in the activities of succinate-cytochrome *c* oxidoreductase (▲), NADH-cytochrome *c* oxidoreductase (○) and NADH oxidase (●) during addition of yeast to anaerobic growth on 10% glucose. For details of growth see the text and Fig. 1. On this type of plot any point above the 50% mark indicates a loss of activity that is faster than dilution by new protein.

influenced by the degree of physical disruption of the respiratory chain. During preparation of the extract NADH oxidase loses activity whereas NADH-cytochrome *c* oxidoreductase gains activity (Singer *et al.* 1966). The fact that the pattern of change of each enzyme is so reproducible indicates that the results can be attributed to something more than physical disruption during preparation, and suggests that the electron-transport system is actively destroyed at a number of points. Since the periods of rapid loss of activity do not oscillate in phase they must be controlled by a number of factors.

The pattern of disappearance of cytochrome *c* oxidase (Fig. 9) varied more than that of any other enzyme tested. Since the initial loss of activity was less than the dilution rate there must have been a delay before synthesis of cytochrome *c* oxidase stopped, as Singer *et al.* (1966) also found. There is some evidence that the duration of this delay may have been influenced by the speed at which residual traces of oxygen were removed from the medium, and this varied somewhat in our experiments.

Effect of oxygen on glycolytic adaptation. Table 1 gives the results from an experiment designed to test the effect of oxygen on adaptation to growth on high concentrations of glucose. Two samples from a batch of aerobically grown cells were put into anaerobic media containing 10% glucose. One was grown in the presence of air and the other in nitrogen, and after 3 hr. of adaptation enzymic

Fig. 9. Changes in the activity of cytochrome *c* oxidase in three separate experiments during the adaptation of yeast to anaerobic growth on 10% glucose. For details of growth see the text and Fig. 1.

Table 1. *Percentage of original specific activity of various enzymes in Saccharomyces cerevisiae after 3.0 hr. of glycolytic adaptation*

A batch of aerobically grown cells was divided into three samples. One sample was grown aerobically on 10% glucose and another was grown anaerobically in an identical medium. (The third sample was analysed for its enzyme content.) The growth rate of both cultures was the same and after 3 hr. the cells were harvested and the specific activities were compared with those in a sample of the original culture; these control activities were taken as 100.

Enzyme	Percentage of original specific activity	
	Aerobic adaptation	Anaerobic adaptation
Pyruvate kinase	186	263
Alcohol dehydrogenase	310	320
Aconitase	29	28
Isocitrate dehydrogenase (NAD ⁺ -linked)	78	65
Isocitrate dehydrogenase (NADP ⁺ -linked)	92	77
α-Oxoglutarate dehydrogenase	45	21
Fumarase	45	43
Malate dehydrogenase	38	33
Isocitrate lyase	35	22
Succinate-cytochrome <i>c</i> oxidoreductase	31	17
NADH oxidase	48	18
Cytochrome <i>c</i> oxidase	67	41

analysis was carried out. The lag phase and the subsequent rate of growth were the same under both conditions. Specific activities of enzymes are therefore directly comparable in the two conditions. The greatest differences were shown in the activities

of the enzymes most closely associated with the electron-transport system.

Although these results are from a single experiment they show that oxygen lowers the effects of glucose.

DISCUSSION

As shown by others (see, e.g., Schatz, 1963; Tustanoff & Bartley, 1964; Singer *et al.* 1966), even when oxygen is available it is the glucose concentration that determines whether the pathway of metabolism is anaerobic (glycolytic) or aerobic (oxygen as final hydrogen acceptor). The results presented here are in agreement with these findings. However, the study of the effect of glucose is not the main purpose of this paper, which is the study of the controlled removal of the enzymes required at a lower activity by the organism because of changed metabolic circumstances.

Group activity changes. In Table 2, enzymes are grouped according to their pattern of change at the early stages of adaptation. Enzymes in group 1(a) are synthesized presumably because a higher activity of them is required in the new environment. Their rate of synthesis will increase until a new activity is reached that will satisfy the new conditions. The slower but continued synthesis of enzymes in group 1(b) may be due to a long-lived messenger RNA that produces unwanted protein, or possibly to a decrease in the rate of enzyme induction. From the rate of disappearance of the enzymes in group 2 it may be seen that after one or more generations times, during which enzyme activity is diluted out by cell division, new synthesis of the enzyme begins. Only the changes of cytochrome *c* oxidase and perhaps the NAD⁺-linked glutamate dehydrogenase would fit in with the idea of a long-lived messenger RNA.

Table 2. *Enzyme grouping according to early patterns of change during glycolytic adaptation*

Group 1. Enzymes synthesized during adaptation. (a) Those increasing in activity: pyruvate kinase, pyruvate decarboxylase, alcohol dehydrogenase, glutamate dehydrogenase (NADP⁺-linked), NADPH-cytochrome *c* oxidoreductase. (b) Those synthesized at a slower rate so that there is a net loss of specific activity: isocitrate dehydrogenase (NAD⁺- and NADP⁺-linked), glutamate-oxaloacetate transaminase, cytochrome *c* oxidase, NADPH oxidase, glutamate dehydrogenase (NAD⁺-linked).

Group 2. Enzymes neither synthesized nor destroyed. Aconitase, fumarase, glucose 6-phosphate dehydrogenase, α -oxoglutarate dehydrogenase.

Group 3. Enzymes that lose activity faster than the dilution rate. Succinate-cytochrome *c* oxidoreductase, malate dehydrogenase, isocitrate lyase, NADH oxidase, NADH-cytochrome *c* oxidoreductase.

Of the enzymes that decrease in activity during adaptation, the fastest loss is in those not required in the new environment. Their loss at first is usually faster than the dilution rate. Enzymes that are required at a much lower activity lose activity at the dilution rate until their new activity is approached, after which synthesis begins to maintain this value. Those that have only a slight fall in activity are continually synthesized at a rate that increases as the new activity is approached.

Enzymes that rapidly lose activity. It is unlikely that inactivation is caused by reversible changes in conformation, induced by the changing intracellular environment, since the methods of assay ensure that an enzyme is measured in an almost constant environment. Neither 15 hr. dialysis of all extracts nor passing them through a Sephadex G-25 column alters the results. We therefore take the view that these results are attributable to specific controlled enzyme inactivation [Ferguson *et al.* (1967) have termed this 'inactivation repression'], which is an important mechanism of metabolic control (Steinberg & Vaughan, 1956; Pollock, 1958; Schimke, 1964).

Fig. 10 shows a more detailed analysis of changes in activity of three of the enzymes that begin a phase of rapid loss of activity in the lag phase. There is a definite difference in time between the start of the phase of rapid loss for each of the three enzymes. Table 3 summarizes the details of the enzymes that have periods of rapid loss of activity. Since the start of rapid loss of activity is different for different enzymes, different factors must control the loss of specific enzymes as parts of metabolic control mechanisms.

Fig. 10. Changes in the activity of succinate-cytochrome *c* oxidoreductase (○), malate dehydrogenase (●) and isocitrate lyase (Δ) during the first 2 hr. of adaptation of yeast to anaerobic growth on 10% glucose. For details of short-term growth see the text.

Table 3. *Enzymes that rapidly lose specific activity during glycolytic adaptation of Saccharomyces cerevisiae*

For those enzymes that have a phase of rapid loss of specific activity after the lag phase, the number in the third column is obtained by subtracting the loss due to dilution (16%) from the total loss during that phase. For those enzymes that have two distinct periods of rapid loss of activity, the phases are distinguished by (i) and (ii).

Enzyme	Time during adaptation when rapid-loss phase began	Decrease during first 30 min. of rapid loss of activity (%)
Malate dehydrogenase	~5-12 min.	46
Isocitrate lyase	~25 min.	40
Succinate-cytochrome <i>c</i> oxidoreductase	(i) Within first 5 min. (ii) ~2.5 hr.	44 12
NADH-cytochrome <i>c</i> oxidoreductase	(i) During lag phase (ii) ~4.0 hr.	34 11
NADH oxidase	(i) ~1.5 hr. (ii) ~4.0 hr.	8 6

Lag phase. During the transition from aerobic to anaerobic growth on high concentrations of glucose two stages can be recognized. The first, caused by alterations in the concentrations of metabolites, is an alteration in the extent to which different metabolic pathways are used, without change in enzyme quality. The second stage is the development of the enzyme systems required by the new conditions and the removal of those that are not used.

In spite of the active glycolytic system of the aerobic cells, there is a 30 min. lag phase before adaptation to high concentrations of glucose in the new environment. During the second 15 min. of the lag phase production of carbon dioxide increases six to eight times over that in the first 15 min., but the three glycolytic enzymes measured (pyruvate kinase, pyruvate decarboxylase and alcohol dehydrogenase) show no significant increase in activity. There is thus an immediate use of spare enzyme capacity. The aerobic inoculum had a high gluconeogenic activity, as judged by the activities of the glyoxylate-cycle enzymes and malate dehydrogenase. The enzymes that are rapidly inactivated during the lag phase are those concerned with gluconeogenesis and the supply of oxaloacetate (isocitrate lyase, malate dehydrogenase and succinate-cytochrome *c* oxidoreductase), as might be expected, since glycolysis and gluconeogenesis do not appear to occur simultaneously at high rates in the same cell, and the conditions for optimum reaction rates shared by the two processes in the two opposite directions are very different (Krebs, 1964).

Role of alcohol dehydrogenase. A number of reports have indicated that alcohol dehydrogenase is repressed by glucose (Epps & Gale, 1942; Witt *et al.* 1966a; Hommes, 1966). Witt *et al.* (1966a) have suggested that the glucose repression indicates

that this enzyme functions in the gluconeogenic pathway from ethanol, whereas our results indicate that alcohol dehydrogenase takes the place of the electron-transport system in the removal of hydrogen. A possible reason for the differing results may be that yeast has two alcohol dehydrogenase isoenzymes, one concerned with the maintenance of glycolysis and the removal of hydrogen and the other concerned with ethanol utilization. The former would be expected to have relatively higher K_m values for ethanol and NAD^+ . Since our assay used substantially higher concentrations of these two substrates than the assays used by others, it would be more likely that we would measure the total alcohol dehydrogenase activity if such isoenzymes exist.

Changes in metabolic pathways. All the citric acid-cycle enzymes decrease in activity during growth on 10% glucose. The already low α -oxoglutarate dehydrogenase activity drops by 90% after 5.0 hr. of adaptation, and in fully adapted cells it may not be present at all. Both Amarsingham & Davis (1965) and Gray *et al.* (1966b) failed to detect any activity in anaerobic *Escherichia coli*. Also, the enzyme next in sequence, succinate dehydrogenase, is lost (Lukins, Tham, Wallace & Linnane, 1966) and is replaced by fumarate reductase (Singer *et al.* 1966). Therefore, as suggested by Amarsingham & Davis (1965) for *E. coli*, the citric acid cycle is modified to become a branched non-cyclic pathway with an oxidative branch to oxoglutarate and a reductive branch to succinate.

At the same time as these events occur the ratio of activity of the NADP^+ -linked isocitrate dehydrogenase to the NAD^+ -linked enzyme increases fourfold. All these changes lower the amount of NADH produced by the citric acid cycle.

The alcohol dehydrogenase pathway cannot

Table 4. *Factors affecting the yeast glutamate dehydrogenases*

Factor	NAD ⁺ -linked enzyme	NADP ⁺ -linked enzyme	Reference
NH ₄ ⁺	Repression	—	Hierholzer & Holzer (1963)
Amino acids	Induction	Repression	Polakis & Bartley (1965a); Bernhardt, Zink & Holzer (1966)
Conditions requiring high rate of synthesis, e.g. lack of amino acids	Repression	Induction	Polakis & Bartley (1965a)
Glucose	Repression	Induction	Present paper

remove hydrogen in excess of that produced by glycolysis, and in fact it does not remove all of this hydrogen (Hommes, 1964; Polakis & Bartley, 1965a). The function of the reductive branch may therefore be to remove this excess of hydrogen.

A supply of oxaloacetate would be essential for the functioning of this reductive pathway. It cannot be supplied by the glyoxylate cycle, since this is repressed, but it could be supplied by pyruvate carboxylase. In yeasts this enzyme has been shown to increase 100-fold after growth on 20% glucose (Hommes, 1966). In mammals pyruvate carboxylase is concerned with supplying oxaloacetate for gluconeogenesis, but in yeasts this function is served by the glyoxylate cycle. Changes in yeast pyruvate carboxylase activity have been shown to follow more closely the pattern of change of pyruvate kinase, a glycolytic enzyme, rather than that of phosphoenolpyruvate carboxykinase, a gluconeogenic enzyme (Ruiz-Amil, de Torrontegui, Palacián, Catalina & Losada, 1965). Such changes would be expected if pyruvate carboxylase were associated with the reductive pathway.

Another enzyme that may be absent anaerobically is pyruvate oxidase. This, like oxoglutarate dehydrogenase, has a very low activity, and Polakis & Bartley (1965a) have shown that these two enzymes change in a similar manner. Condensing enzyme is present in anaerobic cells (see, e.g., Gray *et al.* 1966b) and, if it is to function, a source other than pyruvate oxidase must be present to supply acetyl-CoA. Such a pathway from pyruvate could be acetaldehyde → acetate → acetyl-CoA, and aerobically this pathway feeds the glyoxylate cycle. The anaerobic metabolism of ethanol by yeasts (Hommes, 1964) could be accounted for, at least in part, by this pathway.

In anaerobically adapted cells α -oxoglutarate can only be metabolized to glutamate, and therefore the reason for the change from the NAD⁺- to the NADP⁺-linked enzyme is not obvious. The equilibrium of the reaction is the same with either coenzyme, and is strongly in favour of glutamate synthesis.

The reciprocal changes in the two glutamate

dehydrogenases indicate that the inducer of one enzyme may be closely associated with the repressor of the other. Hierholzer & Holzer (1963) have found that NH₄⁺ represses the NAD⁺-linked enzyme but has no effect on the NADP⁺-linked enzyme, whereas the NADP⁺-linked enzyme is affected more under conditions that require a high rate of synthesis (Polakis & Bartley, 1965a). These results indicate that control of the two glutamate dehydrogenases is exerted by a number of factors, three of which have a reciprocal effect on the two enzymes, as is shown in Table 4.

If a glycolytic intermediate induced the NADP⁺-linked enzyme and repressed the NAD⁺-linked enzyme, this would not only account for the observed changes in activity but would also have the advantage that it would inhibit gluconeogenesis from amino acids at a time when it is not required, at the same time inducing a synthetic pathway when the cell has sufficient energy to support it.

Glutamate-oxaloacetate transaminase in aerobic cells can supply amino acid carbon atoms for gluconeogenesis or aspartate for synthetic requirements. In anaerobic cells growing on high concentrations of glucose it will have only this latter function.

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