Biochem. J. (1965) **97**, 284

Changes in the Enzyme Activities of *Saccharomyces cerevisiae* during Aerobic Growth on Different Carbon Sources

By E. S. POLAKIS and W. BARTLEY
Department of Biochemistry, University of Sheffield

(*Received* 5 *March* 1965)

1. The activities of the enzymes of the citric acid cycle, the glyoxylate by-pass and some other enzymes acting on the substrates of these cycles have been measured at the pH of the yeast cell during the aerobic growth of yeast on different carbon sources and in different growth media. 2. Sugars induced an anaerobic type of metabolism as measured by ethanol production. Glucose was much more effective in inducing the anaerobic pathways than was galactose. The production of ethanol by cells grown on pyruvate was very small. 3. Glucose was also a more effective repressor than was galactose of the citric acid-cycle enzymes but both were equally effective in repressing almost completely the enzymes of the glyoxylate by-pass. 4. Disappearance of the sugars from the growth medium resulted in an increase in the activities of the enzymes of the citric acid cycle and in the appearance of substantial activities of the enzymes of the glyoxylate cycle. By contrast, the activities of purely biosynthetic enzymes (glutamate–oxaloacetate transaminase, $NADP^+$-linked glutamate dehydrogenase) and of pyruvate decarboxylase were decreased. 5. The 2-oxoglutarate-oxidase system was found to be the least active enzyme of the citric acid cycle. 6. The regulatory control at the levels of pyruvate and acetaldehyde and the control of the citric acid cycle are discussed.

The rate of oxidation of the intermediates of the citric acid cycle by intact cells of *Saccharomyces cerevisiae* is low, but the experiments of Krebs, Gurin & Eggleston (1952) showed that this low rate of oxidation was largely due to impermeability of the cells to the substrate. The restriction by permeability was confirmed when Nossal (1954*a*,*b*) and Nossal, Hansen & Ladd (1957) showed an oxidation of some of the citric acid-cycle intermediates by cell-free extracts. The study of the distribution of label in citric acid-cycle intermediates, and in those amino acids able to be formed from them by transamination, when yeast utilized labelled acetate, pyruvate, glucose or carbon dioxide (De Moss & Swim, 1957; Wang, Christensen & Cheldelin, 1953; Eaton & Klein, 1957; Stoppani, Conches, de Fevelukes & Sacerdote, 1958) gave chemical confirmation of the studies of the oxidation of the intermediates.

The association of enzymes of the citric acid cycle with a granular fraction by Nossal (1954*b*) suggested that yeast might contain mitochondria, and the later work by Linnane & Still (1955), Utter, Keech & Nossal (1958) and Vitols & Linnane (1961) showed that the isolated granules could oxidize all the intermediates of the citric acid cycle and carry out oxidative phosphorylation. Electron-microscope studies, e.g. by Vitols, North & Linnane (1961) and Polakis, Bartley & Meek (1964), finally gave the anatomical confirmation to the biochemical studies. However, the latter workers also showed that the conditions of growth and especially the carbon sources presented were important in deciding the type of metabolism of the cell, its oxidative activity and the development of true mitochondrial structures. This paper presents a detailed study of the development of the enzymes associated with the citric acid cycle and the glyoxylate by-pass during the aerobic growth of yeast on different carbon sources. In the accompanying paper Polakis, Bartley & Meek (1965) present some more evidence on the effect of the carbon source on the development of mitochondria and a detailed study of the respiratory-chain enzymes in yeast cells grown aerobically in complete and synthetic media on different carbon sources and at different times of growth.

MATERIALS AND METHODS

Chemicals. Bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex); L-aspartic acid was from Roche Products Ltd. (Welwyn Garden City, Herts.); D-galactoside was from T. Kerfoot

and Co. Ltd. (Vale of Bardsley, Lancs.); α-oxoglutaric acid (monosodium salt), sodium glyoxylate, 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.); lipoamide dehydrogenase was from Seravac Laboratories Ltd. (Maidenhead, Berks.); all other enzymes employed, NAD^+, NADH, $NADP^+$, sodium pyruvate, phosphoenolpyruvate (tricyclohexylammonium salt), GSH, ATP, AMP and oxaloacetic acid were from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); *p*-nitroaniline (microanalytical reagent) was from British Drug Houses Ltd. (Poole, Dorset).

Monopotassium L-isocitrate from *Bryophyllum crenatum* was a gift from Professor H. B. Vickery. Succinyl-CoA was prepared by the method of Simon (1957) and acetyl-CoA as described by Stadtman (1957). The potassium salt of ATP was prepared by liberating the free acid from the sodium salt by passing a solution through Amberlite IR-120 (H^+ form) and then bringing the solution to pH 6·4 by adding solid $KHCO_3$. Arylamine transacetylase was prepared by the method of Tabor, Mehler & Stadtman (1953).

All other chemicals were A.R. grade or the purest grade available, most of them purchased either from the British Drug Houses Ltd. 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, Nuffield, Surrey) was used. It was maintained on agar slopes described by Polakis *et al.* (1964) or on agar slopes of the synthetic medium for growth described below.

Growth of aerobic cells. The yeast extract–casein hydrolysate medium for aerobic growth was as described by Polakis *et al.* (1964). The synthetic medium employed contained (per l.): KH_2PO_4, 9g.; $(NH_4)_2SO_4$, 6g.; $MgSO_4,7H_2O$, 1g.; $CaCl_2$ (dried), 0·3g.; NaCl, 0·1g.; $FeCl_3,6H_2O$, 0·02g.; trace-salt solution, 0·1ml.; vitamin mixture, 1ml.; the pH was 4·5.

When sugars were used as carbon source 1ml. of 10N-KOH was added/l. of medium, bringing the pH to about 6.

The trace-salt solution contained (per l.): $MnSO_4,H_2O$, 4g.; $ZnSO_4,7H_2O$, 4g.; $CuSO_4,5H_2O$, 1g.; KI, 1g.; H_3BO_3, 1g.; $(NH_4)_6Mo_7O_{24},4H_2O$, 2g.; $CoCl_2,6H_2O$, 0·4g. The vitamin mixture contained (per l.): *p*-aminobenzoic acid, 1·75g.; biotin, 0·18g.; inositol, 43·2g.; nicotinamide, 10g.; pantothenic acid (calcium salt), 3·5g.; pyridoxine hydrochloride, 0·65g.; riboflavine, 0·4g.; thiamine hydrochloride, 9g.

In all cases the concentration of carbon source employed was 0·9%.

The conditions of growth were as described by Polakis *et al.* (1964) with the only difference that instead of O_2 a stream of air was blown through the culture.

Cell disruption. A 20% (w/v) suspension of yeast cells in 50mM-potassium phosphate buffer, pH 6·45, was used. The cells were disrupted in a Servall Ribi Cell Fractionator model RF–i at 25000lb./in^2. The temperature of the Ribi valve was kept at 0–5° throughout the operation. Unbroken cells and cell debris were removed by centrifugation at 0° and 1000*g* for 10min.

Gel filtration. This was carried out at 0°. The supernatant was carefully removed from the unbroken cells and cell debris and passed through a Sephadex G-25 column pre-equilibrated with 50mM-phosphate buffer, pH 6·45. Protein was eluted with 50mM-phosphate buffer, mixed well, and a sample was taken for protein estimation. The remainder was divided into 1ml. lots in test tubes. Some enzyme activities were tested before freezing. The remaining extracts were frozen and kept at −18°. All other enzyme activities were measured after one freezing and thawing of the extracts.

Measurement of the pH *of the yeast cells.* The pH of the yeast cell was measured by breaking a 20% (w/v) suspension of yeast cells in cold CO_2-free distilled water at 30000lb./in.^{2} and 0° in the Ribi Cell Fractionator and measuring the pH of the broken cell suspension. The pH of cells growing aerobically on glucose was found to change little during growth, i.e. from 6·65 to 6·25 in spite of the great changes in the pH of the growth medium. The direct method of measurement of pH was compared with the method described by Kotyk (1963). Both methods gave essentially the same values. These measurements were responsible for the decision to measure all enzymes at pH 6·45.

Analytical methods. Glucose was determined by the glucose-oxidase method of Huggett & Nixon (1957); galactose by the arsenomolybdate method of Nelson (1944); pyruvate with lactate dehydrogenase as described by Bücher, Czok, Lamprecht & Latzko (1963); glutamate and α-oxoglutarate with glutamate dehydrogenase as described by Bernt & Bergmeyer (1963) and Bergmeyer & Bernt (1963). Before the estimation of glutamate, NH_4^+ ions were removed from the medium by the use of the H^+ form of the cation-exchange resin, Amberlite IR-120. Ethanol was determined with alcohol dehydrogenase by the method of Bonnichsen & Theorell (1951), and protein by the method of Lowry, Rosebrough, Farr & Randall (1951).

Measurement of enzyme activities. Measurements of the activities of enzymes were carried out as described in Table 1 in cuvettes with a 1cm. light-path. A double-beam temperature-controlled recording spectrophotometer (Beckman model DB) was used. All measurements were carried out at 35° in 50mM-potassium phosphate buffer, pH 6·45. Enzyme activities were calculated from the initial rates of the reactions. Each enzyme was measured in triplicate and the values presented are the means of the different measurements. With the exception of phosphoenolpyruvate carboxykinase, different measurements of the activity of the same enzymes agreed within 3% of the mean value. The amount of enzyme added to the cuvette was always such as to give a change in extinction not more than 0·040/min. and not less than 0·010/min.

Pyruvate-oxidase and α-oxoglutarate-oxidase systems, acetyl-CoA kinase and succinate–cytochrome *c* reductase were assayed before freezing the extracts, since experiments showed that changes in activity could result if the solution were frozen.

Malate synthase, isocitrate dehydrogenase (NAD^+-linked), glutamate dehydrogenase (NAD^+-linked) and fumarase, tested before freezing the extracts and after being kept frozen for 5 days at −18°, did not show any loss of activity, but aconitase showed a decline in activity of about 5% in 5 days. The effect of freezing on other enzymes was not tested. The methods described in Table 1 are either essentially the same as described in the references quoted in column (h) or slight modifications have been introduced. A 1min. recording preceded the addition of

Table 1. *Measurement of enzyme activities*

Enzyme (a)	Buffer (b)	Other addition(s) (c)	Substrate(s) (d)	Reference cuvette does not contain (e)	Reaction started by addition of (f)	Wavelength employed and molecular extinction coefficient ϵ ($M^{-1}cm.^{-1}$) (g)	Reference (h)
Pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40)	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (8 mM); ADP (0·8 mM); NADH (0·15 mM); skeletal-muscle lactate dehydrogenase (10 μg.); cell-free extract	Phosphoenolpyruvate (2·5 mM)	Phosphoenolpyruvate	Phosphoenolpyruvate	340 mμ $6·22 \times 10^3$	Bücher & Pfleiderer (1955)
Pyruvate decarboxylase (2-oxo acid carboxy-lyase, EC 4.1.1.1)	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (8·33 mM); cysteine (2 mM); thiamine pyrophosphate (1·67 mM); NADH (0·15 mM); yeast alcohol dehydrogenase (60 μg.); cell-free extract	Pyruvate (33·3 mM)	Pyruvate	Pyruvate	340 mμ $6·22 \times 10^3$	Holzer & Goedde (1957*b*)
Pyruvate-oxidase system	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (2·0 mM); cysteine (10 mM); thiamine pyrophosphate (0·83 mM); NAD^+ (1·0 mM); CoA (0·1 mM); *p*-nitroaniline (0·1 mM); arylamine transacetylase (1–3 mg.); cell-free extract	Pyruvate (1 mM)	Pyruvate	Pyruvate	405 mμ $1·025 \times 10^4$	Alvarez, Vanderwinkel & Wiame (1958)
Acetyl-CoA kinase [acetate–CoA ligase (AMP), EC 6.2.1.1]	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (5 mM); GSH (10 mM); ATP* (10 mM); *p*-nitroaniline (0·1 mM); CoA (0·5 mM); arylamine transacetylase (1–3 mg.); cell-free extract	Potassium acetate (10 mM)	ATP	ATP	405 mμ $1·025 \times 10^4$	Berg (1962); Holzer & Goedde (1957*a*)
Acetyl-CoA deacylase (acetyl-CoA hydrolase, EC 3.1.2.1)	Potassium phosphate buffer, pH 6·45 (50 mM)	Assayed before addition of oxaloacetate in the assay of condensing enzyme and before addition of glyoxylate in the assay of malate synthetase					
Condensing enzyme [citrate synthase, citrate oxaloacetate-lyase (CoA-deacylating), EC 4.1.3.7]	Potassium phosphate buffer, pH 6·45 (50 mM)	Cell-free extract	Acetyl-CoA (0·22 mM); oxaloacetate (0·4 mM)	Oxaloacetate or acetyl-CoA	Oxaloacetate or acetyl-CoA	233 mμ $5·4 \times 10^3$	Srere & Kosicki (1961)
Aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3]	Potassium phosphate buffer, pH 6·45 (50 mM)	Cysteine (6 mM); Fe^{2+} (0·05 mM); cell-free extract	Aconitate (0·3 mM)	Aconitate	Aconitate	240 mμ $2·4 \times 10^3$	Racker (1950)
Isocitrate dehydrogenase (NAD^+-linked) [L$_s$-isocitrate–NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41]†	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (3·33 mM); cysteine (2 mM); semicarbazide (20 mM); AMP (0·17 mM); NAD^+ (0·33 mM); cell-free extract	L$_s$-Isocitrate (0·5 mM)	L$_s$-Isocitrate	L$_s$-Isocitrate	340 mμ $6·22 \times 10^3$	Kornberg (1955*b*)
Isocitrate dehydrogenase ($NADP^+$-linked) [L$_s$-isocitrate–$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42]	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (3·33 mM); cysteine (2 mM); semicarbazide (20 mM); $NADP^+$ (0·17 mM); cell-free extract	L$_s$-Isocitrate (0·5 mM)	L$_s$-Isocitrate	L$_s$-Isocitrate	340 mμ $6·22 \times 10^3$	Kornberg (1955*a*)
α-Oxoglutarate-oxidase system†	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (1·67 mM); thiamine pyrophosphate (1·67 mM); CoA (0·5 mM); NAD^+ (0·5 mM); cysteine (2 mM); cell-free extract	α-Oxoglutarate (1 mM)	CoA	CoA	340 mμ $6·22 \times 10^3$	Holzer, Hierholzer & Witt (1963)
Succinyl-CoA deacylase (succinyl-CoA hydrolase, EC 3.1.2.3)	Potassium phosphate buffer, pH 6·45 (50 mM)	Cell-free extract	Succinyl-CoA (about 0·2 mM)	Succinyl-CoA	Cell-free extract	232 mμ $4·5 \times 10^3$	
Succinyl-CoA synthetase [succinate–CoA ligase (ADP), EC 6.2.1.5]	Potassium phosphate buffer, pH 6·45 (50 mM)	See the text					

Fumarase (L-malate hydro-lyase, EC 4.2.1.2)	Potassium phosphate buffer, pH 6·45 (50 mM)	Cysteine (2 mM); cell-free extract	L-Malate (15 mM)	L-Malate	L-Malate	240 mμ $2·44 \times 10^3$	Racker (1950); Alberty, Massey, Frieden & Fullbrigge (1954)
Malate dehydrogenase (L-malate–NAD^+ oxidoreductase, EC 1.1.1.37)	Potassium phosphate buffer, pH 6·45 (50 mM)	NADH (0·1 mM); cell-free extract	Oxaloacetate (0·25 mM)	Oxaloacetate	Oxaloacetate	340 mμ $6·22 \times 10^3$	Ochoa (1955*a*)
Isocitrate lyase (L$_s$-isocitrate glyoxylate-lyase, EC 4.1.3.1)	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (5 mM); cysteine (2 mM); phenylhydrazine (3·33 mM); cell-free extract	L$_s$-Isocitrate (1·67 mM)	L$_s$-Isocitrate	L$_s$-Isocitrate	324 mμ $1·7 \times 10^4$	Dixon & Kornberg (1959)
Malate synthase [L-malate glyoxylate-lyase (CoA-acetylating), EC 4.1.3.2]	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (3·3 mM); cell-free extract	Acetyl-CoA (0·08 mM); glyoxylate (2·5 mM)	Acetyl-CoA or glyoxylate	Acetyl-CoA or glyoxylate	232 mμ $4·5 \times 10^3$	Dixon & Kornberg (1959)
Citrate-cleavage enzyme [ATP–citrate oxaloacetate-lyase (CoA-acetylating and ATP–dephosphorylating), EC 4.1.3.8]	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (10 mM); mercaptoethanol (10 mM); NADH (0·15 mM); CoA (0·3 mM); malate dehydrogenase (30 μg.); ATP (5 mM)	Potassium citrate (20 mM)	ATP	ATP	340 mμ $6·22 \times 10^3$	Srere (1962)
Citratase (citrate oxaloacetate-lyase, EC 4.1.3.6)	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (1·0 mM); cell-free extract	Potassium citrate (10 mM)	Citrate	Citrate	260 mμ $1·2 \times 10^3$	Daron & Gunsalus (1962)
Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1)	Potassium phosphate buffer, pH 6·45 (50 mM)	Cysteine (2 mM)	Aspartate (20 mM)	Aspartate	Enzyme	240 mμ $2·44 \times 10^3$	Racker (1950)
Glutamate dehydrogenase ($NADP^+$-linked) [L-glutamate–$NADP^+$ oxidoreductase (deaminating), EC 1.4.1.2]	Potassium phosphate buffer, pH 6·45 (50 mM)	EDTA (6 mM); cysteine (2 mM); NH_4^+ (222 mM); NADPH (0·23 mM); cell-free extract	α-Oxoglutarate (6·67 mM)	α-Oxoglutarate (6·67 mM)	α-Oxoglutarate	340 mμ $6·22 \times 10^3$	Holzer & Schneider (1957); Grisolia, Quijada & Fernandez (1964)
Glutamate dehydrogenase (NAD^+-linked) [L-glutamate–NAD^+ oxidoreductase (deaminating), EC 1.4.1.4]	Potassium phosphate buffer, pH 6·45 (50 mM)	EDTA (6 mM); cysteine (2 mM); NH_4^+ (222 mM); NADH (0·23 mM); cell-free extract	α-Oxoglutarate (6·67 mM)	α-Oxoglutarate	α-Oxoglutarate	340 mμ $6·22 \times 10^3$	Holzer & Schneider (1957)
Glutamate–oxaloacetate transaminase (L-aspartate–2-oxoglutamate aminotransferase, EC 2.6.1.1)	Potassium phosphate buffer, pH 6·45 (50 mM)	Cysteine (2 mM); NH_4^+ (8 mM); pyridoxal 5-phosphate (0·027 mM); NADPH (0·26 mM); glutamate dehydrogenase (100 μg.); cell-free extract	Oxaloacetate (0·3 mM); glutamate (3·33 mM)	Glutamate	Glutamate	340 mμ $6·22 \times 10^3$	Holzer, Gerlach, Jacobi & Gnoth (1958)
Glutamate–pyruvate transaminase (L-alanine 2-oxoglutarate aminotransferase, EC 2.6.1.2)	Potassium phosphate buffer, pH 6·45 (50 mM)	Cysteine (2 mM); NH_4^+ (8 mM); pyridoxal 5-phosphate (0·027 mM); NADPH (0·26 mM); glutamate dehydrogenase (100 μg.); cell-free extract	Pyruvate (0·2–3 mM); glutamate (3·33 mM)	Glutamate	Glutamate	340 mμ $6·22 \times 10^3$	Adaptation of the above-described assay
Phosphoenolpyruvate carboxykinase [ATP–oxaloacetate carboxylyase (transphosphorylating), EC 4.1.1.32]	Potassium phosphate buffer, pH 6·45 (50 mM)	See the text					
Malic enzyme [L-malate–$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40)	Potassium phosphate buffer, pH 6·45 (50 mM)	Mg^{2+} (1·0 mM); $NADP^+$ (0·045 mM); cell-free extract	L-Malate (0·5 mM)	L-Malate	L-Malate	340 mμ $6·22 \times 10^3$	Ochoa (1955*b*)

*Potassium salt.
† Measured in anaerobic cells.

the substance that started the reaction and the recording continued for 3–5 min. Owing to the presence of a very active NADH oxidase, those reactions generating NADH, i.e. the α-oxoglutarate-oxidase system and the NAD^+-linked isocitrate dehydrogenase, were tested in anaerobic cuvettes as described by Massey & Singer (1957).

Generally the activities determined are maximum since concentrations of substrate and cofactors used were sufficient to saturate the enzyme at the given pH and temperature, except for the pyruvate kinase, which was not saturated in crude extracts with concentrations of phosphoenolpyruvate up to 3 mM, and the glutamate–oxaloacetate transaminase, which was not saturated with glutamate at concentrations up to 6·7 mM. Glutamate–oxaloacetate-transaminase activity was measured in the direction of aspartate formation because it was thought that, at least in the synthetic medium of growth, this was the direction the enzyme functions in the cell. Coupling with NADPH instead of NADH was chosen because of the much higher NADH-oxidase activity of the crude yeast extracts in comparison with NADPH-oxidase activity and because the yeast malate dehydrogenase functions much more slowly with NADPH than NADH. The oxaloacetate concentrations employed by Holzer *et al.* (1958) were found to inhibit the enzyme very strongly. Oxaloacetate concentrations higher than 0·4 mM were found inhibitory. With a 3·33 mM concentration of glutamate oxaloacetate concentrations 0·4 mM, 0·3 mM and 0·2 mM gave maximum activities.

The activity of fumarase is higher when measured with fumarate (15 mM) than with malate. The high extinction of fumarate at 240 mμ necessitates the use of a longer wavelength (300 mμ) with consequent loss of sensitivity. Because of this difficulty fumarase was tested with malate as substrate.

Succinyl-CoA synthetase was measured by the method described by Kaufman (1955). ATP and CoA were omitted from the blank.

Phosphoenolpyruvate carboxykinase was measured by a radiochemical method essentially as described by Cannata & Stoppani (1963), but the method did not give reproducible results. Duplicates sometimes differed by as much as 35%.

RESULTS

Growth of yeast

The lag phase from the time of inoculation on glucose was about 6 hr., on pyruvate 8 hr. and on galactose 11 hr.

In the exponential phase of growth of yeast on sugars, two stages could be distinguished. In the first stage the sugar was consumed while ethanol and acetate accumulated (see Polakis *et al.* 1964). In the second stage, yeast cells were exclusively growing on the products of sugar metabolism and the growth rate, as measured by the increase in dry weight, was retarded. The rate of proliferation of cells on glucose and galactose in the exponential phase was about the same, but it was much slower on pyruvate. Table 2 gives the amounts of carbon source consumed at the time the cells were harvested and the yield of cells, as well as the amounts of some products of metabolism excreted into the medium.

Changes in amounts of enzymes during growth

In the subsequent sections the changes in the amounts of individual enzymes are presented. Each enzyme is considered separately.

Enzymes undetected by the methods used. Malic enzyme, citratase, citrate-cleavage enzyme, acetyl-CoA deacylase and glutamate–pyruvate transaminase could not be detected by the methods used. The absence of the transaminase was surprising. No attempts were made to clarify the situation. Alanine dehydrogenase was not tested for. The possibility exists, with the test system used, that the $NADP^+$ produced by the reduction of α-oxoglutarate to glutamate was reduced again to NADPH by acetaldehyde dehydrogenase and the acetaldehyde produced by the decarboxylation of pyruvate.

Changes in the activities of some other enzymes are given in Table 3.

Pyruvate kinase. A small increase in the activity of the enzyme was observed in the second stage of growth, i.e. growth on the products of sugar metabolism (ethanol and acetate), but generally enzyme activities did not show great changes.

Pyruvate decarboxylase. The activity of the enzyme in cells grown on glucose was greater during the first stage of growth, i.e. when cells were still growing in the presence of glucose in the medium. There was a sharp decline (about 60%) in the activity at the second stage of growth. Cells grown for $10\frac{3}{4}$ hr. on glucose in the complete medium had at the beginning of the second phase of growth a high activity of decarboxylase, which also subsequently declined by about 40%. Suomalainen & Oura (1959) have also observed a sharp decrease of about 60–80% in the ability of baker's yeast to decarboxylate pyruvate as the yeast cells pass gradually from anaerobic to strictly aerobic conditions. In spite of this sharp decrease in the activity of cells grown on glucose, during the second stage of growth the remaining activity of the enzyme was higher by about 40% than the activity of the same enzyme in galactose-grown cells at the first stage of growth. The decarboxylase activity of glucose-grown cells during the first stage of growth was about 5 times that of galactose-grown cells. Pyruvate-grown cells had about 70% greater decarboxylase activity when grown in the complete medium than in the synthetic one. In general, the highest activity of decarboxylase was obtained in cells grown on glucose in the first stage of growth.

Differences in the activity of this enzyme were reflected in the amount of ethanol produced/μmole

Table 2. *Concentrations of different carbon sources in the growth medium at different stages of growth of yeast cells*

Each medium indicated in column (b) was supplemented with the carbon source indicated in column (a). The test for glutamate in the synthetic medium was negative and α-oxoglutarate was not present in any media.

Carbon source (a)	Medium (b)	Time of growth (hr.) (c)	Turbidity (d)	Yield (g. wet wt./100 ml. of medium) (e)	Concn. of different carbon sources in medium at time of harvesting the cells (μmoles/ml.)			Amount of carbon source originally added to medium consumed at time of harvesting the cells (μmoles/ml.) (i)	Ethanol produced (mμmole/3 μg.-atoms of C of substrate added originally) (j)
					Sugar (f)	Pyruvate (g)	Ethanol (h)		
Glucose (50 μmoles/ml.)	Complete	10¾	93	0·70	0	0·46	33·6	50	0·336*
		16	200	1·29	0	<0·1	3·7	50	—
		24	220	1·43	0	<0·1	0·1	50	—
	Synthetic	12	35	0·21	31·2	0·38	20·1	18·8	0·535
		15	66	0·37	0	<0·1	22·9	50	0·229*
		30	78	0·50	0	<0·1	8·8	50	—
Galactose (50 μmoles/ml.)	Complete	14	40	0·29	31	0·51	7·8	19	0·205
		27	180	1·29	0	<0·1	0·1	50	—
	Synthetic	17	37	0·27	27	0·36	15·6	23	0·339
		27	78	0·68	0	<0·1	8·4	50	—
Pyruvate (82 μmoles/ml.)	Complete	27	45	0·39	—	11·9	5·3	70·1	0·075
	Synthetic	27	12	0·06	—	38·1	0·2	43·9	0·005

* Low estimates because glucose had been consumed for at least 30–60 min. before the harvesting of the cells.

Table 3. *Changes in activities of enzymes during growth of yeast*

For details see the Materials and Methods section and Table 1.

	Enzyme activity (mμmoles/min./mg. of protein)											
Carbon source	Glucose						Galactose				Pyruvate	
Growth medium	Complete			Synthetic			Complete		Synthetic		Complete	Synthetic
Time of growth (hr.)	10¾	16	24	12	15	30	14	27	17	27	27	27
Turbidity of culture	93	200	220	35	66	78	40	180	37	78	45	12
Phase of growth	Early second stage, exponential growth	Second stage, exponential growth	Late second stage, exponential growth	First stage, exponential growth	Second stage, exponential growth	Late second stage, exponential growth	First stage, exponential growth	Late second stage, exponential growth	First stage, exponential growth	Late second stage, exponential growth	Exponential growth	Exponential growth
Enzyme (a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)	(j)	(k)	(l)	(m)
Pyruvate kinase	110	143	133	123	197	128	86	141	121	120	167	208
Pyruvate decarboxylase	3490	2540	2200	6580	2520	2280	1660	1420	1570	1300	2810	1610
Pyruvate-oxidase system	8·6	7·3	7·5	3·8	7·5	6·8	7·5	8·0	10·7	8·0	16·3	13·7
Acetyl-CoA kinase	11·3	38·5	35·6	17·5	20·8	26·1	9·6	48·4	23·9	28·6	67·2	63·2
Condensing enzyme	171	945	951	155	440	506	171	460	178	577	854	694
Aconitase	400	490	480	170	538	460	281	663	229	608	1200	935
Isocitrate dehydrogenase (NAD^+-linked)	16·1	28·6	27·7	17·6	33·4	30·8	20·8	38·3	25·6	36·1	64·3	75·0
Isocitrate dehydrogenase ($NADP^+$-linked)	19·6	71·9	74·6	25·9	72·3	69·8	49·4	106·0	67·3	96·5	202·0	214·0
α-Oxoglutarate-oxidase system	6·0	12·1	11·9	2·0	6·3	6·7	7·1	10·2	8·1	13·0	19·6	23·2
Succinyl-CoA deacylase	Not detectable, but there is spontaneous breakdown of succinyl-CoA at 35° and pH 6·45 of about 20 mμmoles/min.											
Succinyl-CoA synthetase (ATP)	4·3	6·1	6·0	6·8	7·8	9·1	5·1	8·8	5·0	8·2	11·5	16·2
Fumarase	245	890	893	211	407	423	373	674	314	588	1300	987
Malate dehydrogenase	1220	9270	8980	450	2860	3060	1200	7980	697	3700	4910	11300
Isocitrate lyase	2·0	105	103	≪1	41·4	56·2	≪1	90·1	≪1	85·9	31·2	181
Malate synthase	3·0	32·2	30·8	<1	16·9	22·3	<1	63·2	<1	81·2	48·2	82·3
Glutamate dehydrogenase (NAD^+-linked)	24·5	113	184	1·0	30·6	26·5	17·7	279	7·6	39·8	264	53·4
Glutamate dehydrogenase ($NADP^+$-linked)	298	125	98	2910	1600	1630	514	116	2580	1860	90·4	917
Glutamate–oxaloacetate transaminase	84·3	80·4	76·0	148	126	116	114	86·5	133	104	114	366
Aspartase	18·5	65·8	64·6	3·8	26·0	20·3	12·6	36·5	<1	18·1	24·6	15·2
Phosphoenolpyruvate carboxykinase	Approx. 8–17 mμmoles/min./mg. of protein											

of carbon source (Table 2). The amounts of ethanol produced by glucose-grown cells were much higher than the amounts of ethanol produced by galactose-grown cells (column j). The amounts of ethanol found in the complete medium after growth of the cells for 10¾hr. and in the synthetic medium after growth of cells for 15hr. were low, because glucose had completely disappeared for at least 30min. from the complete medium and for about 1hr. from the synthetic medium, and during this time-interval part of the ethanol was being consumed. Notwithstanding, the amount was higher than the amounts produced by galactose-grown cells after 14hr. of growth in the complete medium, in spite of the fact that galactose was still present in the medium. The higher amounts of ethanol (produced from pyruvate) in the pyruvate-grown cells in the complete medium could also be a reflection of the higher decarboxylase activity of the cells.

Because of the differences in the enzyme activity observed under the different conditions, and because there was a possibility of a competition between pyruvate decarboxylase and pyruvate oxidase for pyruvate, the kinetics of the decarboxylase were studied in the yeast extract. In the assay used, the rate of pyruvate decarboxylation was maximum at a pyruvate concentration of 40mM. The K_m of the enzyme was about 17·4mM. At a 1mM concentration of pyruvate, which was optimum for the assay of pyruvate oxidase, the activity of the enzyme was only 2% of the maximum. The enzyme did not follow Michaelis–Menten kinetics and a plot of percentage of maximal activity versus pyruvate concentration resulted in a sigmoid curve. The implications of these results in relation to competition between the two enzymes are discussed below.

Pyruvate-oxidase system. The activity was appreciably (30–100%) higher in the cells grown on pyruvate, but in all media at all stages the activity of the enzyme was low. The presence of glucose in the synthetic medium (Table 3, column e) resulted in less activity, in contrast with the decarboxylase activity. Galactose-grown cells did not show any such effect.

The oxidation of pyruvate by yeast mitochondria via pyruvate oxidase has already been studied by Alvarez *et al.* (1958). They obtained a fraction producing 0·1–0·2μmole of acetyl-CoA from pyruvate/1000sec./mg. of protein. Assuming that mitochondrial protein accounts for 15% of the total protein of the cell, this activity corresponds to about 1–2mμmoles/min./mg. of protein at pH7·3 and room temperature. This activity is only 10–20% of the activity we measured. Holzer & Goedde (1957*a*) have also obtained mitochondria with very low pyruvate-oxidase activity.

Acetyl-CoA kinase. The activity of this enzyme during the first stage of growth was higher in the synthetic medium than in the complete one. At the second stage of growth, the activity of the cells was higher than in the first, but the increase in activity was higher in cells grown in the complete medium (3–5-fold) than in cells grown in the synthetic one (less than 2-fold). This resulted in a higher final activity of the enzyme in the cells grown in the complete medium, in agreement with the higher Q_{O_2} (acetate) values of these cells. [For cells grown on glucose for 24hr. in the complete medium Q_{O_2} (acetate) was 100; for cells grown on glucose in the synthetic medium for 30hr. Q_{O_2} (acetate) was 70.] The activity of the kinase in the cells grown on pyruvate was about twice that of the other cells, but little difference was observed between the complete and synthetic media.

Condensing enzyme (citrate synthase). The enzyme was present at high but variable levels of activity all the time. The amount of the enzyme was about the same in cells grown on sugars, but during the second stage a marked increase in the activity occurred (3–4-fold); this was especially pronounced in cells grown on glucose in the synthetic medium. Cells grown on pyruvate also had high activities.

Aconitase. Like the condensing enzyme, aconitase was also very active at both stages of growth, particularly in the second stage. The activities of pyruvate-grown cells were well above the activities of cells grown on sugars.

High activities of aconitase in yeast have been already shown by Hirsch (1952), who also showed a great increase of activity on aeration of anaerobically grown cells, and Nossal (1954*a*,*b*), who showed aconitase to be a particulate enzyme that was easily solubilized.

Isocitrate dehydrogenases. Like acetyl-CoA kinase and the enzymes of the citric-acid cycle described above, both NAD^+- and $NADP^+$-linked enzymes are very active in pyruvate-grown cells. Again like acetyl-CoA kinase, during the first stage of growth on sugars both enzymes were more active in the cells grown in the synthetic media. In comparison with galactose, glucose exerted an apparent repressive effect on both enzymes, especially on the $NADP^+$-linked isocitrate dehydrogenase. In the second stage of growth, in spite of the bigger increases in activity observed with cells grown on glucose, the final activities of both enzymes in the cells grown on glucose were lower than the activities of the galactose-grown cells.

α-Oxoglutarate-oxidase system. Many similarities of this system to the pyruvate-oxidase system were observed. Both enzymes were present at low activities of about the same magnitude. Glucose apparently repressed the synthesis of both of the enzymes. Cells grown on pyruvate exhibited the

highest activity. At a time when cells had fully developed their oxidative capacities, the α-oxoglutarate-oxidase system was the least active enzyme of the citric acid cycle. Assuming that the enzyme is working at maximum rate and that oxidation is solely through the citric acid cycle, the measured activities of the cells grown on sugars would result at pH 6·45 and 35° in a Q_{O_2} (acetate) value of about 10–15, and a Q_{O_2} (glucose) value of 13–20. Nossal *et al.* (1957), using yeast extract, have found a slow oxidation of α-oxoglutarate coupled with a conversion (sometimes almost complete) into glutamate. Vitols & Linnane (1961) have also observed a very low oxygen uptake by yeast mitochondria, and an accumulation of almost equimolar amounts of α-oxoglutarate when mitochondria were oxidizing citrate. The same workers have shown that the α-oxoglutarate-dehydrogenase activity in their heavy-mitochondrial fraction is low, and all the other fractions were inactive. Their activities, 10–20 mμmoles of oxygen/min./mg. of protein of heavy particles, corresponded in terms of the total yeast extract to about 1·5–3·0 mμmoles of oxygen/min./mg. of protein, or to 3–6 mμmoles of NADH formed/min./mg. of protein at 30° and pH 7·4. Holzer *et al.* (1963) have also extracted from mitochondria an amount of α-oxoglutarate-oxidase activity corresponding to a Q_{O_2} (glucose) value of about 10 at 22° and pH 6·6, but they considered the low activity to be due to losses in the process of preparation. The maximum Q_{O_2} values of cells grown on pyruvate on the basis of the α-oxoglutarate-oxidase activity measured would be 23. Because of the possibility that the low activities observed were due to the dissociation of the enzyme complex, the effect of added hog-heart lipoamide dehydrogenase was tested, but no stimulation was observed.

Tests for excreted α-oxoglutarate and glutamate in the medium were negative, showing that the reactions leading to α-oxoglutarate formation (isocitrate dehydrogenases, transaminases etc.) and the ones leading to its removal (α-oxoglutarate oxidase, glutamate dehydrogenase) were evenly balanced within the cell.

The calculations made on our results were based on the assumption that the pH of the yeast mitochondria was about the same as the pH of the yeast cells, as measured by the methods described. As Holzer *et al.* (1963) have shown, the α-oxoglutarate dehydrogenase of yeast has a broad pH optimum at about 7·5. At pH 6·45 the activity of the enzyme will be about 40% (as calculated from Fig. 2 of Holzer *et al.* 1963). Thus with more favourable pH values in the mitochondria the activity of the oxidase could increase as much as 2·5-fold.

Succinyl-CoA deacylase and succinyl-CoA synthetase. As shown in Table 3, there was a spontaneous breakdown of succinyl-CoA at pH 6·45 and 35° of about 20 mμmoles/min. as calculated from the change in extinction at 232 mμ. The addition of the enzyme preparation to the cuvette did not increase the rate of breakdown.

Succinyl-CoA-synthetase activity was low and followed a similar pattern to that of α-oxoglutarate oxidase. The activity of the succinyl-CoA synthetase is insufficient to account for the rate of respiration observed in the yeast cells. The citric acid cycle must therefore be supplied with succinate by a route not involving the succinyl-CoA synthetase. However, since GTP was not added to the test system, it is possible that the lack of this nucleotide (or some other) might limit the rate of the reaction. If the low activities of succinyl-CoA synthetase measured reflect those within the cell, it becomes probable that the enzymes lie on a synthetic rather than a major degradative pathway.

Fumarase. This was very active at all stages and slightly more active in the complete medium. Highest activities were observed in pyruvate-grown cells and, like the condensing enzyme, in cells grown on glucose in the complete medium, at the second stage of growth (see also Nossal 1954*a*,*b*).

Malate dehydrogenase. At all stages of growth malate dehydrogenase was the most active enzyme of the citric acid cycle (Table 3). It was at least twice as active as fumarase (columns e and j) and three times as active as condensing enzyme (column e) and aconitase (columns e and j). Thus, relative to the other active enzymes of the cycle, the cells grown on sugars in the synthetic media and during the first stage of growth had the lowest malate-dehydrogenase activities. In the second stage of growth there was an increase in activity (about 6–7-fold). The highest increase (about 660%) was observed in cells grown on glucose in the synthetic medium, but it was not as pronounced as the increase in that of the condensing enzyme. Unlike cells grown on sugars, cells grown on pyruvate had higher activities in the synthetic medium. The activity of cells grown in the synthetic medium on pyruvate was the highest recorded and over twice the activity of cells grown in the complete medium.

Isocitrate lyase. The enzyme was almost absent when sugars were still present in the media, but there was a very pronounced increase in activity in the second stage of growth. With the exception of cells grown on glucose in the synthetic medium, which showed about 60% of the activity of other cells grown on sugars, activities reached values of 90–100 mμmoles/min./mg. of protein. In the synthetic medium pyruvate-grown cells had 6 times the activity of the same cells grown in a complete medium; but even in the complete medium isocitrate lyase was always present in amounts comparable with succinate–cytochrome *c* oxidoreductase (Pola-

kis *et al.* 1965). The increase in isocitrate lyase in cells growing on sugars comes at a time when acetate is accumulating in the medium and when the ability to oxidize acetate develops. The role of acetate in the induction of isocitrate lyase has been investigated by Kornberg (1963). The non-competitive inhibition by succinate or glyoxylate of the isocitrate cleavage has been described by Olson (1961). Also, many other substances capable of producing oxaloacetate have been found to inhibit the enzyme. Thus non-competitive inhibition by phosphoenolypyruvate has been described by Ashworth & Kornberg (1963), and inhibition by fumarate, glycollate, α-oxoglutarate and malate has been described by McFadden & Howes (1963). The control of the glyoxylate cycle by this enzyme and the possible role of inhibitors has been reviewed by Kornberg & Elsden (1961). Our observations are consistent with the idea that the isocitrate lyase develops in response to a need for oxaloacetate.

Malate synthase. As with isocitrate lyase, the enzyme was completely repressed during the first stage of growth on sugars. During the second stage of growth there was a marked increase in the activity, which was very much greater in the galactose-grown cells than the glucose-grown cells. If the presence of sugars was repressing the enzyme, then glucose must have a more prolonged effect than galactose. Cells grown in a complete medium on glucose developed only half of the activity of the corresponding galactose-grown cells, whereas cells grown in the synthetic medium achieved about one-fourth. The differences observed in the malate-synthase activities in relation to isocitrate-lyase activities in the different media employed argues against a simple induction of both enzymes by acetate, since the ratios of the two enzymes varied widely. The possibility that more than one enzyme catalyses the same reaction, but under different genetical control, would also not be excluded. Because of differences in the activities of the two enzymes, probably varying proportions of glyoxylate will be metabolized by the available pathways. In the complete medium there is the possibility that amino acids are metabolized concomitantly with acetate. Catabolism of aspartate, for example, in the glucose-complete medium by the aspartase reaction could partially satisfy the need of the cell for oxaloacetate, and this would lessen the demand on the synthesis of oxaloacetate through malate synthetase, and might well result in low activities of malate synthetase.

High isocitrate-lyase activity but low malate-synthetase activity were found when *Bacillus cereus* was grown on glutamate (Megraw & Beers, 1964). In this case it was found that part of the glyoxylate was metabolized through the glycerate pathway. It might be that a similar situation could arise with yeast in the complete medium especially when glucose was the carbon source, since these cells have been found to have high isocitrate-lyase activity but low malate-synthetase activity.

Cells grown on pyruvate in the complete medium had lower glyoxylate-cycle activities than cells grown on pyruvate in the synthetic medium. The difference is possibly due to the lower requirements for oxaloacetate in cells grown in the complete medium.

Glutamate dehydrogenases (NADP⁺- and NAD⁺-linked). The changes in the activities of the two enzymes followed completely opposite patterns. The NAD^+-linked enzyme was always more active in the cells grown in the complete medium, whereas the $NADP^+$-linked enzyme was about 10-fold more active in the cells grown in the synthetic medium. In the first stage of growth the NAD^+-linked enzyme was repressed in the presence of sugars, but in the second stage of growth the activity of the enzyme increased while remaining about 5-fold more active in the cells grown in the complete medium. In contrast, during the second stage of growth when the sugars had been consumed, the activity of the $NADP^+$-linked enzyme decreased. This decrease was concomitant with a decrease in the rate of growth and protein synthesis.

Hierholzer & Holzer (1963) showed that the yeast NAD^+-linked glutamate dehydrogenase was present in small amounts when yeast was grown on ammonia as the sole nitrogen source but in large amounts when glutamate was the nitrogen source. Our results are in agreement with the findings of these authors and suggest a degradative role (i.e. the breakdown of glutamate) for the NAD^+-linked enzyme. Thus in the first stage of growth the development of the NAD^+-linked glutamate dehydrogenase may be controlled by the combined repressive effect of sugars and ammonia, whereas in the second stage ammonia remains the only repressor. It is possible that the higher activities of the enzyme in the complete medium are due to a small inductive effect exerted by glutamate.

In contrast with the NAD^+-linked enzyme, the behaviour of the $NADP^+$-linked enzyme points to a chiefly biosynthetic role for this enzyme.

Glutamate–oxaloacetate transaminase. The activity of the enzyme was higher in the synthetic medium but the differences observed were much smaller (not more than 50%) than for the $NADP^+$-linked glutamate dehydrogenase. During the second stage of growth the enzyme activities fell by about 10%. This could be a reflection of either a smaller demand of cells for aspartate owing to a decreased rate of protein synthesis, or an increase of the intracellular concentrations of the substrates of the enzyme.

Aspartase. The activity of the enzyme is lower

in the first stage of growth, especially in the synthetic medium. Worth noticing are the higher activities of cells grown on glucose in the complete medium, about twice as high as the activities of galactose-grown cells. It is possible that aspartate is degraded by these cells to oxaloacetate, compensating for the low malate-synthetase activity.

Phosphoenolpyruvate carboxykinase. Carbon dioxide fixation in the presence of phosphoenolpyruvate and ADP was always evident. Owing to the great differences observed between duplicates either by the use of the method described or by measuring the exchange of $^{14}CO_2$ with the α-carboxyl group of oxaloacetate in the presence of ATP or by measuring the decarboxylation of radioactive oxaloacetate in the presence of ATP, no quantitative results could be presented. The activity observed was always of the order described.

DISCUSSION

Pathways of utilization of pyruvate. Four main ways exist in yeast for the disposal of pyruvate and oxidation of NADH produced during glycolysis: (*a*) excretion of pyruvate into the medium and oxidation of NADH by the respiratory chain; (*b*) oxidation of pyruvate by the pyruvate-oxidase systems and regeneration of NAD^+ by the respiratory chain; (*c*) the sequence of reactions pyruvate → acetaldehyde → ethanol; (*d*) the sequence of reactions pyruvate → acetaldehyde → acetate and the oxidation of NADH by the respiratory chain. All four pathways seem to operate in cells growing on sugars.

Although small amounts of pyruvate have been found in the media during the growth of cells on sugars, generally this pathway is not very important and it does not present any advantage against pathway (*c*) as to the ultimate amount of energy the cell will obtain, but rather the disadvantage that the subsequent uptake of pyruvate, which does not penetrate the cells as easily as ethanol, will present difficulties to the cell. It is possible that most of the pyruvate is excreted at the beginning of growth when the aerobic cells used for inoculation are poor in pyruvate decarboxylase (Suomalainen & Oura, 1959).

Pathway (*b*) has the advantage that more energy becomes ultimately available but it requires transport of NADH and pyruvate to the mitochondrion and again transport of NAD^+ to the cytoplasm. It also requires a citric acid cycle twice as active as glycolysis and a respiratory chain about 12 times as active as glycolysis to accomplish the regeneration of NAD^+ from the NADH produced. In contrast with the other pathways it makes available to the cell the energy produced by the complete oxidation of 1 mol. of sugar in one continuous process. Owing to the absence of acetyl-CoA deacylase in yeast the cell will be in difficulty in balancing the amounts of energy to its requirements. A very fast glycolysis producing enough energy to satisfy the need of the cell and keeping the concentration of NADH high might be expected to result in lower activities of the enzymes of the citric acid cycle, which would, with this rate of glycolysis, produce more respiratory-chain energy from NADH than required for the set of circumstances.

Pathway (*c*) presents the advantage of being a very quick way by which both purposes of the cell, i.e. disposal of pyruvate and NADH oxidation at the same time and *in situ*, can occur. It has the disadvantage that the cell will ultimately lose energy equivalent to 2 mol. of ATP/mol. of sugar but it makes the energy available in two stages. A fast glycolysis providing high pyruvate and NADH concentrations and enough energy to satisfy the cell will naturally favour this pathway. The high K_m value for pyruvate of the pyruvate decarboxylase and the low value of pyruvate oxidase constitute a built-in regulator channeling pyruvate into pathway (*c*) at high rates of glycolysis, where pyruvate concentration is high, and into pathway (*b*) when glycolysis and the supply of pyruvate is low.

Pathway (*d*) presents the same advantages and disadvantages as far as energy is concerned with pathway (*c*), but the NADH arising is less than by pathway (*b*) and therefore does not require a very active respiratory chain. Since in yeast there are two acetaldehyde dehydrogenases (one exclusively $NADP^+$-requiring) (Black, 1955; Seegmiller, 1955) with K_m values for acetaldehyde of the same order as alcohol dehydrogenase, pathway (*d*) can still function with $NADP^+$ even when the NADH concentration is high. This pathway (*d*) allows the option of either excreting acetate or converting it into acetyl-CoA and thence into intermediates of the citric acid cycle, required for growth, without producing energy beyond that required for immediate use. The fact that all the reactions leading from acetaldehyde to glutamate and which require nicotinamide nucleotides can work with either NAD^+ or $NADP^+$ as well as the activating effect of AMP (produced by the reaction of acetyl-CoA kinase) on the NAD^+-linked isocitrate dehydrogenase could be of primary importance for the regulation of the concentrations of reduced and oxidized nicotinamide nucleotides inside the cell. This extension of pathway (*d*) could supplement pathway (*c*) in a cell satisfied in energy but requiring intermediates of the cycle for growth. The fact that during the first stage of growth, and in spite of the lower rate of growth of cells in the synthetic medium, most of the enzymes of the citric acid

Table 4. *Activity of pyruvate decarboxylase in cells grown on sugars during the first stage of growth, and on pyruvate, and pyruvate decarboxylase/pyruvate oxidase activity ratios at different pyruvate concentrations*

Cells were grown in the medium supplemented with the carbon source indicated in the first column.

Carbon source	Medium	Stage of growth	Concn. of pyruvate (mM)	Pyruvate-decarboxylase activity at concn. of pyruvate employed (mμmoles/min./mg. of protein)	Pyruvate-oxidase activity at concn. of pyruvate employed (mμmoles/min./mg. of protein)	Pyruvate decarboxylase/ pyruvate oxidase activity ratio
Glucose	Synthetic	First	2	263	Max.	69·0
			1	132	Max.	35·0
Galactose	Synthetic	First	2	63	Max.	6·0
			1	31	Max.	3·0
	Complete	First	2	67	Max.	9·0
			1	33	Max.	4·5
Pyruvate	Synthetic	—	2	113	Max.	7·0
		—	1	56	Max.	3·5
	Complete	—	2	65	Max.	4·5
			1	32	Max.	2·5

cycle up to the level of α-oxoglutarate are more active, whereas the opposite is true for the remaining enzymes of the cycle, agrees with the explanation that the cycle is used during the first stage of growth primarily for the production of intermediates to be used for synthetic purposes (see also Krebs *et al.* 1952).

Competition between pyruvate oxidase and pyruvate decarboxylase. Holzer (1961) has discussed the possibility of competition between pyruvate oxidase and pyruvate decarboxylase and has also measured the intracellular concentration of pyruvate in cells metabolizing glucose in the presence of ammonium sulphate as 2·1μmoles/g. of fresh yeast. Table 4 gives the activities of pyruvate decarboxylase at pyruvate concentrations 2mM and 1mM for cells grown on sugars during the first stage of growth and for cells grown on pyruvate, as well as the pyruvate decarboxylase/pyruvate oxidase activity ratios at these concentrations of pyruvate.

If we accept the intracellular concentration of pyruvate as about 2mM, as given by Holzer (1961), we can see that for cells growing in the presence of glucose such a competition, even with optimum NAD^+ concentrations, will be ruled out. With cells grown on galactose, owing to the lower glycolysis rate, the concentration of pyruvate would be expected to be lower. Although the decarboxylase reaction will be still predominant the possibility of effective competition exists. The higher NADH-oxidase activity of galactose-grown cells in comparison with glucose-grown cells (Polakis *et al.* 1965) is another factor that will favour competition more in galactose than in glucose-grown cells. The slow consumption of pyruvate by the cells growing on pyruvate media possibly suggests a slow penetration of pyruvate into the cell and consequently a low intracellular concentration of pyruvate, perhaps lower than 1mM, which will favour the pyruvate-oxidase reaction, considering also the fact that, owing to the high activity of the respiratory chain, NAD^+ concentrations could also be favourable to the reaction of pyruvate oxidase. Ethanol production in galactose-grown cells is lower than in glucose-grown cells; it is very limited in cells grown on pyruvate in the complete medium and almost absent from cells grown on pyruvate in the synthetic medium. This is consistent with the idea of a competition in these cells, either at the pyruvate level or the acetaldehyde level or both.

Oxidation of acetate. The measured activity of α-oxoglutarate dehydrogenase at pH 6·4 is low and, if acetate is oxidized by a pathway utilizing this enzyme, a Q_{O_2} value of not more than 25 would be expected at this pH. Direct measurements of Q_{O_2} (acetate) values of yeast during the first stage of growth are consistent with this value postulated for the Q_{O_2}. At this stage the oxidation of acetate is entirely by the citric acid cycle and the low rate is presumably sufficient for the supply of intermediates for synthetic processes. During the second stage of growth, however, both citric acid cycle and the glyoxylate by-pass, which has now developed, are used for the oxidation of acetate (now mainly yielding a supply of energy) and the supply of intermediates. Krebs & Lowenstein (1960) concluded that the concentrations of citrate found inside the yeast cell by De Moss & Swim (1957) could be explained if the glyoxylate cycle played a major part in acetate assimilation in yeast. It

should be pointed out that the results of our paper are in full agreement with the results of previous studies, especially with labelled isotopes (De Moss & Swim, 1957; Wang *et al.* 1953; Stoppani *et al.* 1958).

In our experiments glutamate and α-oxoglutarate did not accumulate in the media, and Kleinzeller (1941) found no accumulation of succinate in actively growing yeast cells. Presumably then, in such circumstances, as Krebs & Lowenstein (1960) have pointed out, the formation of citrate will be the factor controlling the rate of turning of the citric acid cycle. The high activities of the condensing enzyme present in the yeast cells will result in the control being exerted through the concentration of one or both of the substrates oxaloacetate and acetyl-CoA. Because of the very high malate-dehydrogenase activity, the malate/oxaloacetate concentration ratio and consequently the NADH/NAD^+ concentration ratio will be virtually at the equilibrium value. Any change in the NADH/NAD^+ concentration ratio will be immediately reflected by an appropriate change in the malate/oxaloacetate concentration ratio. The observations described below suggest that the control of the rate at which the citric acid cycle turns in the first stage of growth is the concentration of oxaloacetate. First, acetate oxidation in cells in the second stage of growth, when the glyoxylate-cycle enzymes appear, is much faster although the activity of acetyl-CoA kinase does not increase correspondingly. Secondly, phosphoenolpyruvate carboxylation is low but malate-dehydrogenase activity is very high and, since it is very probable that the malate-dehydrogenase reaction will at all times be near equilibrium, this, at pH 6·45, will be much in favour of malate. Thus the formation of citrate will be controlled by the malate-dehydrogenase reaction. Production of NADH and increase of the NADH/NAD^+ concentration ratio will immediately lower the concentration of oxaloacetate, thus tending to decrease the production of more NADH by the citric acid cycle. The reverse will happen when the NAD^+ concentration increases.

In the second stage of growth, when the glyoxylate enzymes develop, the rate of malate formation is not limited by the sluggish reaction of α-oxoglutarate oxidase, and any consequent high steady-state concentration of malate that develops will raise the standing concentration of oxaloacetate with consequent acceleration in the rate of synthesis of citrate.

The existence of pyruvate carboxylase [pyruvate–carbon dioxide ligase (ADP), EC 6.4.1.1] in yeast has been shown by Losada, Cánovas & Ruiz-Amil (1964). The activity of this enzyme has not been measured in the present work. If a high carbon dioxide fixation is effected by this enzyme, the arguments, based on the assumption of the low carbon dioxide-fixing activity of the crude extracts of yeast cells growing on sugars during the first stage of growth, should be modified. In the use of pyruvate-grown cells, the possibility of net synthesis of oxaloacetate either by carboxylation of pyruvate or by the glyoxylate cycle, or both, should be considered.

We thank Professor J. R. Quayle for helpful criticism and E. S. P. acknowledges the financial support of N.A.T.O.

REFERENCES

Alberty, R. A., Massey, V., Frieden, C. & Fullbrigge, A. R. (1954). *J. Amer. chem. Soc.* **76**, 2485.

Alvarez, A., Vanderwinkel, E. & Wiame, J. M. (1958). *Biochim. biophys. Acta*, **28**, 333.

Ashworth, J. M. & Kornberg, H. L. (1963). *Biochim. biophys. Acta*, **73**, 519.

Berg, P. (1962). In *Methods in Enzymology*, vol. 5, p. 461. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Bergmeyer, H. U. & Bernt, E. (1963). In *Methods of Enzymatic Analysis*, p. 324. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.

Bernt, E. & Bergmeyer, H. U. (1963). In *Methods of Enzymatic Analysis*, p. 384. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.

Black, S. (1955). In *Methods in Enzymology*, vol. 1, p. 508. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Bonnichsen, R. K. V. & Theorell, H. (1951). *Scand. J. clin. Lab. Invest.* **3**, 58.

Bücher, T., Czok, R., Lamprecht, W. & Latzko, E. (1963). In *Methods of Enzymatic Analysis*, p. 253. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.

Bücher, T. & Pfleiderer, G. (1955). In *Methods in Enzymology*, vol. 1, p. 435. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Cannata, J. J. B. & Stoppani, A. O. M. (1963). *J. biol. Chem.* **238**, 1196.

Daron, H. H. & Gunsalus, I. C. (1962). In *Methods in Enzymology*, vol. 5, p. 622. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

De Moss, J. A. & Swim, H. E. (1957). *J. Bact.* **74**, 445.

Dixon, G. H. & Kornberg, H. L. (1959). *Biochem. J.* **72**, 3P.

Eaton, N. R. & Klein, H. P. (1957). *Biochem. J.* **67**, 373.

Grisolia, S., Quijada, C. L. & Fernandez, M. (1964). *Biochim. biophys. Acta*, **81**, 61.

Hierholzer, G. & Holzer, H. (1963). *Biochem. Z.* **339**, 175.

Hirsch, H. M. (1952). *Biochim. biophys. Acta*, **9**, 674.

Holzer, H. (1961). *Cold Spr. Harb. Symp. quant. Biol.* **26**, 277.

Holzer, H., Gerlach, U., Jacobi, G. & Gnoth, M. (1958). *Biochem. Z.* **329**, 529.

Holzer, H. & Goedde, H. W. (1957*a*). *Biochem. Z.* **329**, 175.

Holzer, H. & Goedde, H. W. (1957*b*). *Biochem. Z.* **329**, 192.

Holzer, H., Hierholzer, G. & Witt, I. (1963). *Biochem. Z.* **337**, 115.

Holzer, H. & Schneider, S. (1957). *Biochem. Z.* **329**, 361.

Huggett, A. St G. & Nixon, D. A. (1957). *Biochem. J.* **66**, 12P.

Kaufman, S. (1955). In *Methods in Enzymology*, vol. 1, p. 714. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Kleinzeller, A. (1941). *Biochem. J.* **35**, 495.

Kornberg, A. (1955*a*). In *Methods in Enzymology*, vol. 1, p. 705. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Kornberg, A. (1955*b*). In *Methods in Enzymology*, vol. 1, p. 707. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Kornberg, H. L. (1963). *Biochim. biophys. Acta*, **73**, 517.

Kornberg, H. L. & Elsden, S. R. (1961). *Advanc. Enzymol.* **23**, 401.

Kotyk, A. (1963). *Folia microbiol., Prague*, **8**, 27.

Krebs, H. A., Gurin, S. & Eggleston, L. V. (1952). *Biochem. J.* **51**, 614.

Krebs, H. A. & Lowenstein, J. M. (1960). In *Metabolic Pathways*, vol. 1, p. 129. Ed. by Greenberg, D. M. New York: Academic Press Inc.

Linnane, A. W. & Still, J. L. (1955). *Arch. Biochem. Biophys.* **56**, 264.

Losada, M., Cánovas, J. L. & Ruiz Amil, M. (1964). *Biochem. Z.* **340**, 60.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

McFadden, B. A. & Howes, W. V. (1963). *J. biol. Chem.* **238**, 1737.

Massey, V. & Singer, T. P. (1957). *J. biol. Chem.* **228**, 263.

Megraw, R. E. & Beers, R. J. (1964). *J. Bact.* **87**, 1087.

Nelson, N. (1944). *J. biol. Chem.* **153**, 375.

Nossal, P. M. (1954*a*). *Biochim. biophys. Acta*, **14**, 154.

Nossal, P. M. (1954*b*). *Biochem. J.* **57**, 62.

Nossal, P. M., Hansen, I. A. & Ladd, J. N. (1957). *Biochim. biophys. Acta*, **23**, 103.

Ochoa, S. (1955*a*). In *Methods in Enzymology*, vol. 1, p. 735. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Ochoa, S. (1955*b*). In *Methods in Enzymology*, vol. 1, p. 739. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Olson, J. A. (1961). In *The Enzymes*, 2nd ed., vol. 5, p. 387. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.

Polakis, E. S., Bartley, W. & Meek, G. A. (1964). *Biochem. J.* **90**, 369.

Polakis, E. S., Bartley, W. & Meek, G. A. (1965). *Biochem. J.* **97**, 298.

Racker, E. (1950). *Biochim. biophys. Acta*, **4**, 211.

Seegmiller, E. J. (1955). In *Methods in Enzymology*, vol. 1, p. 511. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Simon, E. (1957). In *Biochemical Preparations*, vol. 5, p. 30. Ed. by Shemin, D. New York: John Wiley and Sons Inc.

Srere, P. A. (1962). In *Methods in Enzymology*, vol. 5, p. 641. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Srere, P. A. & Kosicki, G. W. (1961). *J. biol. Chem.* **236**, 2557.

Stadtman, E. R. (1957). In *Methods in Enzymology*, vol. 3, p. 931. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Stoppani, A. O. M., Conches, L., de Fevelukes, S. L. S. & Sacerdote, F. L. (1958). *Biochem. J.* **70**, 438.

Suomalainen, H. & Oura, E. (1959). *Biochim. biophys. Acta*, **31**, 115.

Tabor, H., Mehler, A. H. & Stadtman, E. R. (1953). *J. biol. Chem.* **204**, 127.

Utter, M. F., Keech, D. B. & Nossal, P. M. (1958). *Biochem. J.* **68**, 431.

Vitols, E. & Linnane, A. W. (1961). *J. biophys. biochem. Cytol.* **9**, 701.

Vitols, E., North, R. J. & Linnane, A. W. (1961). *J. biophys. biochem. Cytol.* **9**, 689.

Wang, C. H., Christensen, B. E. & Cheldelin, V. H. (1953). *J. biol. Chem.* **201**, 683.