Changes in the Intracellular Concentrations of Adenosine Phosphates and Nicotinamide Nucleotides during the Aerobic Growth Cycle of Yeast on Different Carbon Sources

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1. Methods for the quantitative extraction of adenosine phosphates and nicotinamide nucleotides from yeast cells are described. 2. The intracellular concentrations of adenosine phosphates and nicotinamide nucleotides were measured during the aerobic growth cycle of yeast on glucose and galactose. 3. When sugars were still present in the media the intracellular concentrations of NADH and AMP were in general higher in glucose- than in galactose-grown cells, whereas ADP concentration was always lower in glucose-grown cells. 4. The adenylate-kinase reaction was found to be far from equilibrium in the glucose-grown cells and when glucose was still present in the growth medium. 5. The significance of the changes in the intracellular concentrations of adenosine phosphates and nicotinamide nucleotides observed during growth on either sugar is discussed in relation to the metabolism and growth of the cells. 6. The differences observed in the concentrations of these cofactors in glucose- and galactose-grown cells are also discussed in relation to the type of metabolism of these cells. Control of glycolysis at the level of phosphofructokinase in galactose-grown cells and at the level of phosphoglycerate kinase in glucose-grown cells is suggested. 7. ADP is suggested to be the inducer of formation of respiratory enzymes.

The measurements of the activities of the enzymes of a pathway at the pH of the cell and with optimum substrate concentrations lead to conclusions about the maximal capacity of this pathway. However, the intracellular concentrations of intermediate metabolites are mostly at suboptimum levels that will not allow enzymes to function at maximal rate. In addition, some metabolites might have an inhibitory or a stimulatory effect on some enzymes. Thus determination of the intracellular concentrations of such metabolites is necessary before any conclusion about the control steps of a pathway can be drawn from data about quantities of enzymes.

Special attention has been focused on the intracellular concentrations of adenosine phosphates and nicotinamide nucleotides because of their special role as compounds in which energy released during the catabolic processes is trapped and transferred to anabolic reactions. Since the same cofactors take part in all metabolic sequences inside the cell, competition of different pathways for the same cofactor could also be envisaged.

In a previous paper (Polakis, Bartley & Meek, 1965) the repression of mitochondrial structure and enzyme activity was ascribed to a compound involved in the transformation of energy inside the cell. During the growth of yeast on sugars, energy produced by glycolysis is directed either to the reduction of NAD^+ or to the phosphorylation of ADP. The availability of these cofactors, as well as of the products of glycolysis to the mitochondrion, is of primary importance in the function of the oxidative metabolism of the yeast cells. It was therefore thought that the determination of the intracellular concentrations of adenosine phosphates and of nicotinamide nucleotides during the growth of yeast on either glucose or galactose could provide further indications about the nature of the repression mechanism.

In the present paper the results of these determinations are presented. Optimum conditions for the extraction of these cofactors from yeast are also described.

MATERIALS AND METHODS

Chemicals. Bovine plasma albumin was obtained from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex). Mercaptoethanol and $\beta\beta$-dimethylglutaric acid were from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). Tris, NADPH, ADP, dihydroxyacetone phosphate (cyclohexylammonium salt, dimethyl-ketal from which free dihydroxyacetone phosphate was liberated according to the

instruction of Sigma Form no. 383) and bovine heart lactate dehydrogenase (type III) were from Sigma Chemical Co. (St Louis, Mo., U.S.A.). All other enzymes, ATP, AMP, NAD^+, NADH, $NADP^+$, glucose 6-phosphate (sodium salt), α-oxoglutaric acid and phosphoenolpyruvate (tricyclohexylammonium salt) were from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). The remaining chemicals used and not listed were purchased from the British Drug Houses Ltd. (Poole, Dorset) and were of the purest grade available.

Maintenance and growth of yeast. Details were as given by Polakis & Bartley (1965). The synthetic medium, supplemented with either glucose (0·9%) or galactose (0·9%), was used.

Inulin space. This was determined essentially by the gravimetric method of Conway & Downey (1950). Determination of the extracellular space with cells harvested at different stages of growth gave values 22–24% of the pellet volume. In all subsequent experiments the 'inulin space' was not determined but it was taken as 23% of the pellet volume.

Cell volume. This was taken as 77% of the volume of the yeast pellet. The latter was determined in haematocrits.

Wet weight of cells. This was obtained by subtracting weight equivalent to 23% of the volume of the yeast pellet from the wet wt. of the yeast pellet. The latter was obtained by weighing the haematocrit after the careful removal of the last traces of water.

Dry weight of yeast cells. Washed yeast cells were killed by heating for 2 min. in a boiling-water bath and then dried *in vacuo* over H_2SO_4.

Intracellular water. This was taken as equal to the wet wt. of cells minus the dry wt. of cells.

Samples for the determinations described and for the extraction of cofactors were removed at the same time.

Determination of glucose and galactose in the growth medium. Glucose was determined with glucose oxidase according to the method of Huggett & Nixon (1957). Galactose was determined by the arsenomolybdate method of Nelson (1944).

Extraction and determination of nicotinamide nucleotides and adenosine phosphates

Sampling of yeast cells. Duplicate 10 ml. samples of yeast suspension were removed from the growth medium at different times of the growth cycle. The cells were spun down in a bench centrifuge at maximum speed for 1 min. The supernatant medium was quickly decanted and the centrifuge tubes with the cells were cooled in liquid N_2. When the cells were frozen to the temperature of liquid N_2 the tubes were removed and stored in solid CO_2 for subsequent treatment. In all cases less than 2 min. elapsed between sampling and complete freezing.

Extraction of yeast cells. For the extraction of adenosine phosphates essentially the method described by Lowry, Passonneau, Hasselberger & Schulz (1964) was used. The tubes containing the yeast cells were transferred to an ethanol–solid CO_2 bath at −10°, and 0·3 ml. of 3 N-$HClO_4$ precooled to the same temperature was added. The samples were left in the bath and were occasionally stirred in a Vortex mixer until the acid had completely penetrated into the pellet. The samples were then frozen and thawed twice, 0·1 ml. of a 0·4 M-tris–10 mM-EDTA solution was added and the cells were centrifuged at 2500*g* for 3 min. The supernatant was removed, the cells were washed with 0·3 ml. of 3 N-$HClO_4$ (−10°) and the combined supernatant was neutralized with 0·9 ml. of 2 M-$KHCO_3$ and kept for 5 min. at 0°. The precipitate was spun down at 0° and the supernatant removed. The precipitate was washed with 0·4 ml. of ice-cold water containing 0·05 ml. of 2 M-$KHCO_3$ and the combined supernatant was made up to 2 ml. and analysed immediately or stored at −80°. The final pH of this solution was always between 7·0 and 8·0.

For the extraction of oxidized forms of nicotinamide nucleotides the method used was a modification of the method described by Burch, Lowry & Von Dippe (1963). The extraction reagent used (0·02 N-H_2SO_4–0·1 M-Na_2SO_4–5 mM-cysteine hydrochloride) was the one recommended by these authors in which cysteine hydrochloride was added (see Neubert, Schulz & Hoehne, 1964). The tubes with the cells were transferred to an ethanol–solid CO_2 bath at −5° and 0·5 ml. of ice-cold extraction reagent was added. The mixture was frozen and thawed twice and then transferred to a water bath at 60° for 10 min. After cooling in an ethanol–solid CO_2 bath 0·1 ml. of 0·10 M-ββ-dimethylglutaric acid–NaOH buffer, pH 5·4, and 0·01 ml. of M-$KHCO_3$ were added (final pH 5·4). The cells were spun down at 2500*g* for 2 min. at 0° and the supernatant was removed. The cells were washed with 0·3 ml. of ice-cold water and spun down, and the combined supernatant was made up to 1 ml. and analysed immediately or stored at −80°.

For the extraction of the reduced forms of the nicotinamide nucleotides the tubes containing the frozen yeast cells were transferred to an ethanol–solid CO_2 bath (−15°) and 0·01 ml. of 0·5 M-mercaptoethanol precooled to 0° was added. The tubes were removed from the cold bath and 0·5 ml. of 0·1 N-KOH–10 mM-EDTA solution preheated to 90° was added while at the same time the tubes were immersed in a 90° water bath. After 1 min. the tubes were removed and quickly cooled to 0° and 0·025 ml. of 2 M-$KHCO_3$ was added. The cells were spun down at 2500*g* for 5 min. at 0° and the supernatant was removed. Cells were washed with 0·4 ml. of 0·05 M-$KHCO_3$–0·05 M-K_2CO_3 and the combined supernatant was made up to 1 ml. and analysed immediately. The pH of the supernatant was always about 10 as recommended by Lowry, Passonneau & Rock (1961).

The choice of the extraction methods is discussed in the Results section.

Determination of the nicotinamide nucleotides and adenosine phosphates in the extract of yeast cells. All cofactors were determined fluorimetrically in a Farrand model A2 fluorimeter. The output of the fluorimeter (ammeter position) was fed to a compensating voltage and sensitivity control device and then to a Vibron model 33B electrometer (Electronic Instruments Ltd., Richmond, Surrey), which in turn was connected through a 1500 Ω resistance to a Varicord model 43 recorder.

The primary filter used was Corning no. 7–37 (5860) and the secondary a combination of Corning nos. 4–70 (4308), 5–61 (5562) and 3–72 (3387).

Reagent mixtures were used as blanks and a solution of quinine sulphate (0·05 μg./ml.) in 0·1 N-H_2SO_4 was used as a standard. Standard curves with known amounts of each cofactor were constructed.

The procedures of determination of different cofactors are outlined in Table 1. Under the conditions employed,

Table 1. *Determination of some cofactors in extracts of yeast cells*

Enzymes used were diluted with 0·01% bovine plasma albumin (B.P.A.) dialysed against water. The final volume in the fluorimeter tube was 1 ml. Each of the additions of columns (*d*) and (*f*) was made in a volume of 0·01 ml. Blanks without extract [to determine the contribution of the additions of columns (*d*) and (*f*) to the fluorescence] and standards were always run in parallel with the sample.

Substance (*a*)	Yeast taken (mg. dry wt.) (*b*)	First additions (*c*)	Second additions (*d*)	Incubation time (min.) (*e*)	Third additions (*f*)	Incubation time (min.) (*g*)	Reference (*h*)
ATP	0·2–1	Tris buffer, pH 7·5 (100 mM); $NADP^+$ (0·03 mM); Mg^{2+} (5 mM); B.P.A. (0·1 mg./ml.); extract	Yeast glucose 6-phosphate dehydrogenase (2 μg./ml.)	2	Glucose (1 mM); yeast hexokinase (5 μg./ml.)	15	Lowry *et al.* (1964)
		Read		*Read**		*Read*	
ADP	0·2–1	Potassium phosphate buffer, pH 7·0 (100 mM); Mg^{2+} (5 mM); B.P.A. (0·1 mg./ml.); phosphoenolpyruvate (tricyclohexylammonium salt) (0·04 mM); NADH as required (usually 0·013 mM); extract	Heart lactate dehydrogenase (8 μg./ml.)	4	Muscle pyruvate kinase (1 μg./ml.)	15	Lowry *et al.* (1964)
		Record	*Record*		*Record*		
AMP	0·05–0·3	Potassium phosphate buffer, pH 7·0 (100 mM); Mg^{2+} (5 mM); B.P.A. (0·1 mg./ml.); phosphoenolpyruvate (tricyclohexylammonium salt) (0·04 mM); NADH as required (usually 0·013 mM); ATP (0·02 mM); extract	Heart lactate dehydrogenase (8 μg./ml.); muscle pyruvate kinase (1 μg./ml.)	10	Muscle adenylate kinase (myokinase) (1 μg./ml.)	15	Lowry *et al.* (1964)
		Record	*Record*		*Record*		
NADH	0·25–1·30	Phosphate buffer, pH 7·0 (100 mM); EDTA (1 mM); B.P.A. (0·1 mg./ml.); extract	Dihydroxyacetone phosphate (0·04 mM); muscle glycerol 1-phosphate dehydrogenase (5 μg./ml.)	3			Holzer, Busch & Kröger (1958)
		Read		*Read*			
NADH+ NADPH	0·25–1·3	Phosphate buffer, pH 7·0 (100 mM); EDTA (1 mM); B.P.A. (0·1 mg./ml.); NH_4^+ (5 mM); extract	α-Oxoglutarate (potassium salt), pH 6·5 (1·25 mM); liver glutamate dehydrogenase (12·5 μg./ml.)	7			Klingenberg (1963*a*)
		Read		*Read*			
NAD^+	0·05–0·5	Pyrophosphate (50 mM)–semicarbazide (22·5 mM) buffer; B.P.A. (0·1 mg./ml.); extract	Ethanol (A.R.) (0·01 ml./ml.); yeast alcohol dehydrogenase (12 μg./ml.)	4			
		Read		*Read*			
$NADP^+$	0·5–2·0	Phosphate buffer, pH 7·0 (100 mM), or tris buffer, pH 7·5 (100 mM); Mg^{2+} (5 mM); B.P.A. (0·1 mg./ml.); extract	Glucose 6-phosphate (sodium salt), pH 7·0 (1 mM); yeast glucose 6-phosphate dehydrogenase (2 μg./ml.)	10			Klingenberg (1963*b*)
		Read		*Read*			

* Determination of glucose 6-phosphate.

Table 2. *Comparison of the recoveries obtained by different methods of extraction of reduced nicotinamide nucleotides*

For the sampling and extraction of cells and determination of nicotinamide nucleotides see the Methods section and Table 1. The reduced forms of nicotinamide nucleotides of yeast cells in 10ml. of growth medium were extracted in a final volume of 1ml. For the determination of NADH or of the sum NADH plus NADPH 0·05ml. of the extract was used. NADPH was calculated from the difference. Each value presented is the mean of two determinations. Duplicates did not differ more than 3–4% from the mean value. For the technique of the addition of the mixtures of reduced and oxidized forms of nicotinamide nucleotide to the yeast cells see the text: mixture A (NAD$^+$ approx. 10mM, NADP$^+$ approx. 3mM); mixture B (NADH approx. 4mM, NADPH approx. 2mM). Recoveries from the alkali-treated solutions (mixture A+mixture B) are expressed as percentages of the amounts present in the untreated mixture [columns (*g*) and (*h*)]. Recoveries of nicotinamide nucleotides after the addition of mixture A and mixture B to the yeast pellet are expressed either as percentages of the sum [untreated (mixture A+mixture B)+alkali-treated yeast] [columns (*i*) and (*j*)] or as percentages of the sum [alkali-treated (mixture A+mixture B)+alkali-treated yeast] [columns (*k*) and (*l*)]. In all cases 0·01ml. of mixture A and 0·01ml. of mixture B were used.

Expt. no. (*a*)	Extraction medium (*b*)	No. of samples (*c*)	Extraction temp. (*d*)	Temp. and time of heating before neutralization: Temp. (*e*)	Time (min.) (*f*)	Recovery (%): Synthetic mixture NADH (*g*)	Synthetic mixture NADPH (*h*)	Reduced forms of nicotinamide nucleotides added to yeast cells: NADH (*i*)	NADPH (*j*)	NADH (*k*)	NADPH (*l*)
1	0·02N-NaOH–0·5mM-cysteine hydrochloride	1	0°	60°	10	99·8	98·3	122·4	44·3	122·6	45·0
2	0·05N-NaOH–10mM-cysteine hydrochloride–10mM-EDTA	1	0	60	10	103·1	95·0	138·9	41·3	137·1	43·1
3	0·1N-NaOH–5mM-cysteine hydrochloride–5mM-EDTA	1	0	60	2	96·2	94·3	129·4	58·2	132·9	61·5
4	0·1N-NaOH–5mM-cysteine hydrochloride–5mM-EDTA	1	0	60	2	99·5	93·6	147·0	35·0	147·4	37·2
5	0·3N-KOH–10mM-cysteine hydrochloride–10mM-EDTA	1	0	60	1	102·8	96·9	83·9	73·3	85·4	75·3
6	0·5N-KOH–10mM-cysteine hydrochloride–10mM-EDTA	1	0	60	0·5	87·7	89·4	84·8	75·3	93·4	83·2
7	0·5N-KOH–10mM-cysteine hydrochloride–10mM-EDTA	1	0	—	—	100·9	95·3	76·5	67·8	75·9	70·6
8	0·5N-KOH–10mM-cysteine hydrochloride–10mM-EDTA	1	60	60	0·5	—	—	—	—	87·6	99·5
9	0·5N-KOH–10mM-EDTA	1	60	60	0·5	91·2	99·4	92·9	94·5	98·9	95·0
10	1·0N-KOH–10mM-EDTA	1	60	60	0·5	83·8	89·5	89·3	79·0	100·8	87·4
11	0·1N-NaOH–10mM-mercaptoethanol–10mM-EDTA	1	90	90	1	99·4	99·4	102·4	81·0	102·8	81·4
12	0·1N-NaOH–10mM-mercaptoethanol–10mM-EDTA	1	90	90	1	94·4	96·2	90·8	91·1	94·5	94·5
13	0·1N-NaOH–10mM-mercaptoethanol–10mM-EDTA	1	90	90	1	100·3	98·1	96·8	97·6	96·6	99·4
14	0·1N-NaOH–10mM-mercaptoethanol–10mM-EDTA	1	90	90	1	98·8	96·1	96·2	93·1	96·8	97·6
15	0·1 N-NaOH–10 mM-mercaptoethanol–10mM-EDTA	1	90	90	1	96·0	94·3	95·0	96·0	96·8	101·2
	Mean recoveries (±S.E.M.)					97·8±1·1	96·8±0·9	96·2±1·8	91·8±2·9	97·5±1·9	94·8±3·5

full deflexion of the electrometer corresponded to 2·5 μM-NADH.

RESULTS

Extraction procedures

In assessing the different extraction procedures two criteria were taken into consideration: (*a*) the ability of the method to recover all the constituents quantitatively and without interconversion from a synthetic mixture of pure cofactors; (*b*) the ability of the method to extract quantitatively and without interconversion known amounts of cofactors added to the yeast at the same time as the extraction mixture, together with the cofactors already existing in cells. The endogenous cofactors were determined in a separate sample.

Recoveries of adenosine phosphates by the method of Lowry *et al.* (1964) were very good when the temperature was not allowed to rise. Both criteria discussed above were satisfied.

Recoveries of the oxidized form of nicotinamide nucleotides when 0·02N-sulphuric acid–0·1M-sodium sulphate–5mM-cysteine hydrochloride was used for extraction were excellent (approx. 98%). When cysteine hydrochloride was omitted the amount of $NADP^+$ extracted increased by about 50% (see Neubert *et al.* 1964).

The amounts of reduced nicotinamide nucleotides extracted from yeast cells by different methods varied widely. Consequently the recoveries of different methods were examined. The results are presented in Table 2. Extraction at 0° (Expts. 1–7) gave unsatisfactory recoveries of the nucleotides added to yeast cells. Thus when mild alkaline conditions were used the recoveries of NADP were about 30% higher, and those of NADPH 40–60% lower, than the theoretically calculated values.

When strong alkaline conditions were employed for the extraction of the reduced forms of nicotinamide nucleotides at 0° or 60°, the recoveries of both NADH and NADPH were not satisfactory (Expts. 5–7 and 8–10).

The best results were obtained when 0·1N-sodium hydroxide–10mM-EDTA–10mM-mercaptoethanol solution was used at 90° (Expts. 11–15).

The observation that the mild alkaline medium employed by Burch *et al.* (1963) for the extraction of NADH and NADPH from rat liver gave interconversion of the different forms of nicotinamide nucleotides when used for extraction of these compounds from yeast should be interpreted as pointing to differences in the optimum conditions for the extraction of reduced nicotinamide nucleotides from different sources.

Utilization of glucose and galactose during the aerobic growth of yeast

The logarithm of the sugar consumed was proportional to the number of generations with a change in slope at about the fifth generation (Fig. 1). The slow and fast rates of utilization of each sugar are shown as intersecting straight lines fitted by the method of least squares, although the transitions may have been gradual.

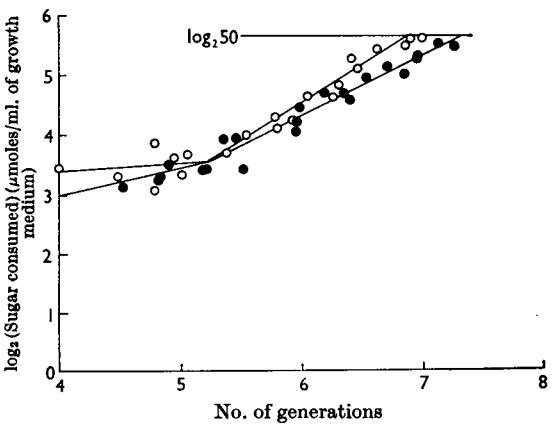


Fig. 1. Consumption of sugar by yeast cells growing aerobically in a synthetic medium on: ○, 0·9% glucose (50 μmoles/ml.); ●, 0·9% galactose (50 μmoles/ml.).

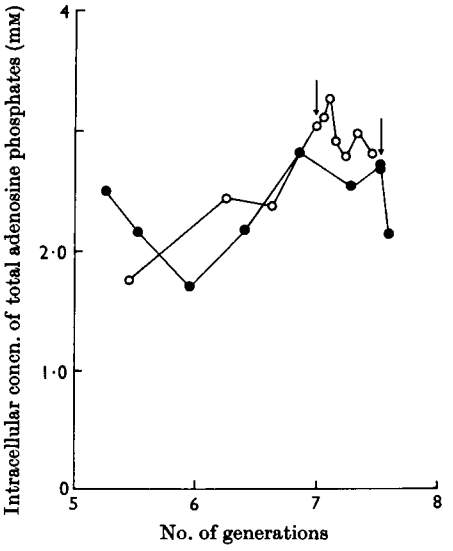


Fig. 2. Changes in the intracellular concentration of total adenosine phosphates during the aerobic growth of yeast cells on 0·9% sugar in a synthetic medium: ○, glucose-grown cells; ●, galactose-grown cells. Glucose and galactose had been consumed at the points indicated by the arrows.

The same amount of yeast was always used for inoculation. Glucose was consumed after 6·86 and galactose after 7·32 generations. If the amount of dry wt. formed/mole of ATP produced by the catabolism of the carbon source is constant, then during the growth of yeast on sugars the catabolism of 1 mole of galactose yielded more ATP than the catabolism of 1 mole of glucose. This, in turn, means that the oxidative metabolism of sugar was higher in galactose-grown cells (see also Polakis & Bartley, 1965; Polakis *et al.* 1965).

Changes in the concentration of adenosine phosphates during the growth of yeast on glucose and galactose

Changes in the total concentration of adenosine phosphates (*Fig.* 2). Total adenosine phosphates were calculated as the sum: ATP plus ADP plus AMP. The fluctuations observed might be due to a slight imbalance between the rate of supply and the rate of utilization of nucleotides.

Changes in the concentration of ATP (*Fig.* 3). As growth proceeded the concentration of ATP fell. Cells of about the same physiological age grown on different sugars had about the same intracellular concentration of ATP. The lowest ATP concentration observed in both cases was about 0·3 mM. At the end of the growth of cells on glucose, a marked rise in the intracellular concentration of ATP was observed. This might be due to a lower rate of growth, or even a transient arrest of growth, during the adaptation of cells on C_2 compounds.

Changes in the concentration of ADP (*Fig.* 4). During the growth of cells on glucose the concentration of ADP remained almost constant at about 0·4 mM. In galactose-grown cells the concentration of ADP fell as low as 0·6 mM at a time that coincided with the fall in the concentration of total adenosine phosphates (Fig. 2). At all other times the ADP concentration in galactose-grown cells was about

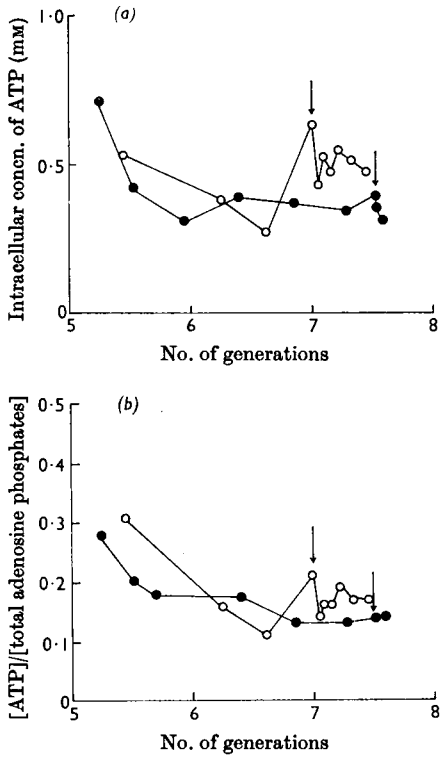


Fig. 3. Changes in the intracellular concentration of ATP (*a*) and in the [ATP]/[total adenosine phosphates] ratio (*b*) during the aerobic growth of yeast cells on 0·9% sugar in a synthetic medium: ○, glucose-grown cells; ●, galactose-grown cells. Glucose and galactose had been consumed at the points indicated by the arrows.

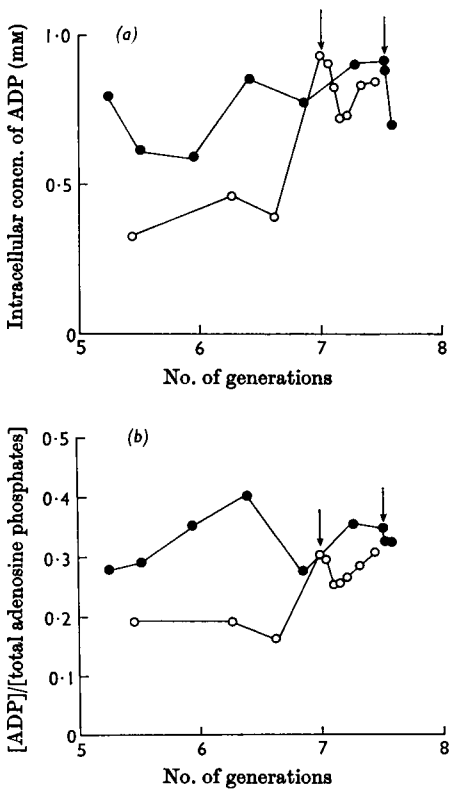


Fig. 4. Changes in the intracellular concentration of ADP (*a*) and in the [ADP]/[total adenosine phosphates] ratio (*b*) during the aerobic growth of yeast cells on 0·9% sugar in a synthetic medium: ○, glucose-grown cells; ●, galactose-grown cells. Glucose and galactose had been consumed at the points indicated by the arrows.

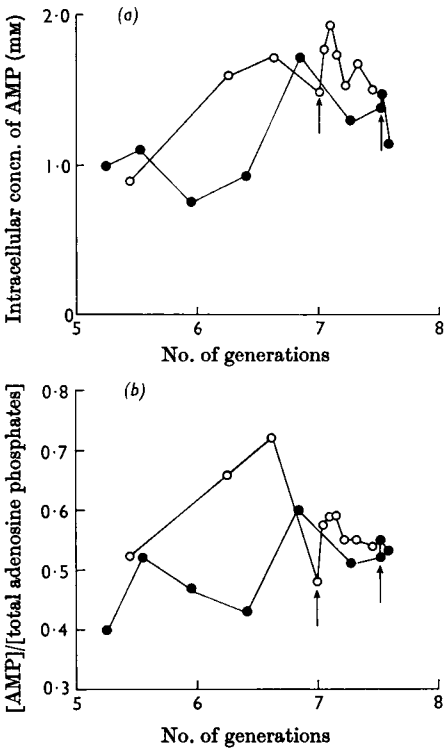


Fig. 5. Changes in the intracellular concentration of AMP (*a*) and in the [AMP]/[total adenosine phosphates] ratio (*b*) during the aerobic growth of yeast cells on 0·9% sugar in a synthetic medium: ○, glucose-grown cells; ●, galactose-grown cells. Glucose and galactose had been consumed at the points indicated by the arrows.

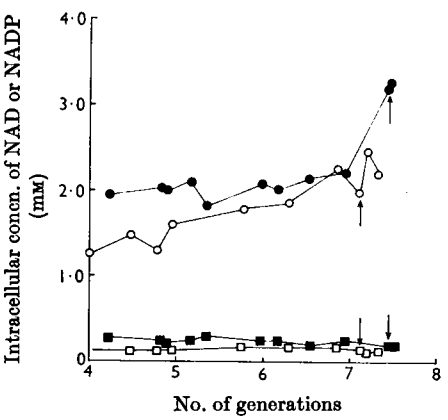


Fig. 6. Changes in the total intracellular concentrations of NAD and NADP during the aerobic growth of yeast cells on 0·9% sugar in a synthetic medium. NAD: ○, glucose-grown cells; ●, galactose-grown cells. NADP: □, glucose-grown cells; ■, galactose-grown cells. Glucose and galactose had been consumed at the points indicated by the arrows.

0·8mM. By the end of growth on glucose, the concentration of ADP rose to that found in the galactose-grown cells. The differences in the concentrations of ADP observed in cells grown on glucose and galactose resulted in great differences in the [ATP]/[ADP] ratio (Fig. 10), especially in the first stages of growth.

Changes in the concentration of AMP (*Fig.* 5). In general, the concentration of AMP was higher in glucose than in galactose-grown cells. During growth on glucose the concentration of AMP increased continuously in parallel with the concentration of total adenosine phosphates. In galactose-grown cells the AMP concentration increased only during the last generation.

Changes in the concentrations of reduced and oxidized nicotinamide nucleotides during the aerobic growth of yeast on different carbon sources

In the description of the results and the discussion below, NAD refers to the sum NAD^+ plus NADH and NADP to the sum $NADP^+$ plus NADPH.

Changes in the concentrations of NAD and NADP (*Fig.* 6). At all stages of growth the concentrations of both NAD and NADP were higher in galactose-grown cells. Until the sugar was exhausted, NADP concentration remained approximately constant; subsequently it declined slightly. NAD concentration remained constant (approx. 2mM) in galactose-grown cells and as long as galactose was present in the growth medium. After this it increased by 60%. By contrast, a continuous increase in the concentration of NAD in glucose-grown cells was noted: 1·25mM at 4 generations and approx. 2mM at 7 generations.

Changes in the intracellular concentration of NAD^+ (*Fig.* 7). While the sugars were still present in the growth medium, the concentration of NAD^+ remained substantially constant at 1·6mM in galactose-grown cells and 1·2mM in glucose-grown cells. After this the NAD^+ concentration rose by about 80% in galactose-grown cells and by 45% in glucose-grown cells.

Changes in the intracellular concentration of NADH (*Fig.* 7). After about 5 generations the NADH concentration started increasing. In glucose-grown cells the increase continued until glucose was exhausted from the growth medium. The highest concentration recorded was 0·8mM. In galactose-grown cells the increase was transitory and corresponded to the decrease in the concentration of NAD^+ (Fig. 7). When the sugars had been consumed the concentration of NADH fell.

Changes in the intracellular concentration of $NADP^+$ (*Fig.* 8). The concentration of $NADP^+$ was always higher in galactose-grown cells than in glucose-grown cells (0·13–0·16mM). A continuous

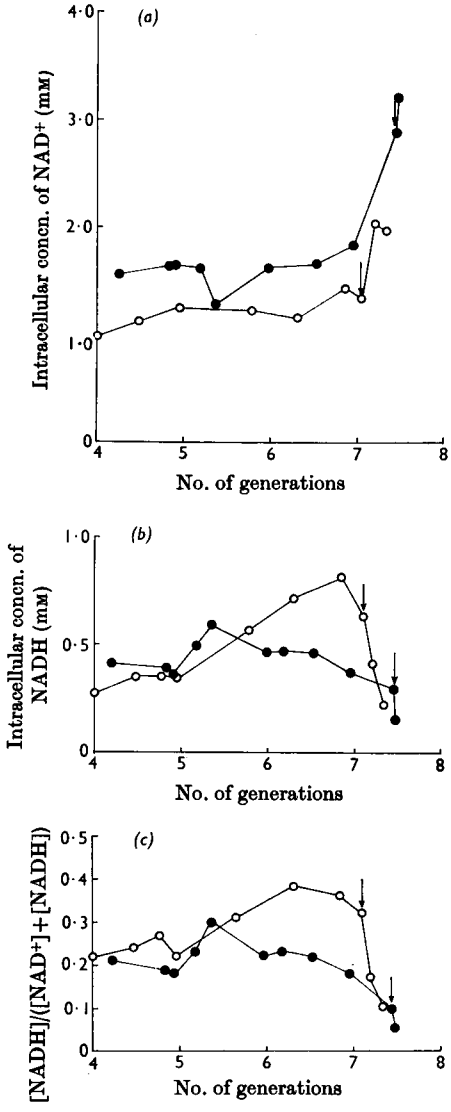


Fig. 7. Changes in the intracellular concentration of NAD+ (*a*), in the intracellular concentration of NADH (*b*) and in the [NADH]/([NAD+]+[NADH]) ratio (*c*) during the aerobic growth of yeast cells on 0·9% sugar in a synthetic medium: ○, glucose-grown cells; ●, galactose-grown cells. Glucose and galactose had been consumed at the points indicated by the arrows.

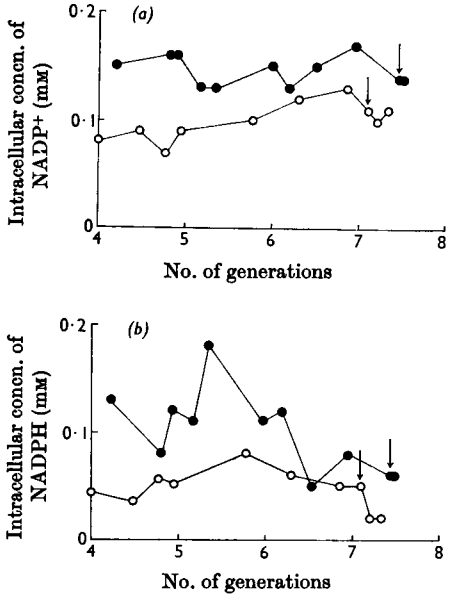


Fig. 8. Changes in the intracellular concentration of NADP+ (*a*) and in the intracellular concentration of NADPH (*b*) during the aerobic growth of yeast cells on 0·9% sugar in a synthetic medium: ○, glucose-grown cells; ●, galactose-grown cells. Glucose and galactose had been consumed at the points indicated by the arrows.

increase in the concentration of NADP+ was observed in glucose-grown cells, reaching a maximum of 0·13mM immediately before the disappearance of glucose.

Changes in the intracellular concentrations of NADPH (*Fig.* 8). At any stage of growth the concentration of NADPH was higher in galactose-grown cells than in glucose-grown cells. The lowest concentrations were observed during the growth of cells on C_2 compounds.

DISCUSSION

In the discussion below, 'compartmentation' within the cell has not been taken into consideration.

During the growth of yeast on sugars the rate of growth remained constant in spite of the changes in the concentration of cofactors. Thus either the contribution of each of the main pathways of metabolism of the cell (glycolysis and oxidative metabolism) to the total energy production changed, or the changes in the concentrations of cofactors were counterbalanced by appropriate changes in the other parameters controlling the rate of the steps of a pathway, or both. Both of these could happen in galactose-grown cells. Changes in the parameters controlling the rate of the step of glycolysis only would be expected in glucose-grown cells, since, as long as the sugar is still present in the medium, glycolysis seems to be the only pathway of energy production (Polakis & Bartley, 1965; Polakis *et al.* 1965; Swanson & Clifton, 1948; Lemoigne, Aubert & Millet, 1954). The parameters controlling the rate of a step are:

(*a*) the concentrations of reactants and products; (*b*) the concentrations of activators and inhibitors; (*c*) the rate of formation of the appropriate enzyme.

Glycolytic pathway. Galactose is consumed more slowly than glucose (Fig. 1). Consequently the rate of glycolysis would be expected to be lower in galactose-grown cells. The concentration of glucose 6-phosphate was not lower in galactose-grown cells than in glucose-grown cells (Fig. 9). All this means that the glycolytic enzymes would most probably be less active in galactose-grown cells.

Five steps of glycolysis will be directly influenced by the intracellular concentration of cofactors measured in this work. Of these, the properties of the yeast enzymes glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase have not been studied.

According to the data of Fromm, Silverstein & Boyer (1964) and Fromm & Zewe (1962), at any time the intracellular concentration of ATP was insufficient to saturate the yeast hexokinase, and the concentration of ADP was high enough to compete with ATP. Consequently at any time the rate of the hexokinase reaction will depend on both the concentration of ATP and the [ATP]/[ADP] ratio.

Yeast phosphofructokinase has been studied at pH 7·5 (Viñuela, Salas & Sols, 1963; Ramaiah, Hathaway & Atkinson, 1964; Salas, Viñuela, Salas & Sols, 1965; Sols, De La Fuente, Viñuela & Heredia, 1963). However, the pH of the yeast cell is about 6·5. At pH 7·5 the intracellular concentrations of ATP (Fig. 3) would be high enough to inhibit the enzyme at the fructose 6-phosphate concentrations likely to be found inside the cell (Fig. 9). At these fructose 6-phosphate concentrations the enzyme is activated by AMP (Ramaiah *et al.* 1964). Our data suggest an activation of this step *in vivo* by AMP. Thus increases in [AMP]/[ATP] ratio (Fig. 10) corresponded to decreases in glucose 6-phosphate concentration (Fig. 9). In glucose-grown cells the increase in [AMP]/[ATP] ratio was accompanied by an increase in NADH concentration (Fig. 7*b*).

According to the data of Bücher (1955) and Reynard, Haas, Jacobsen & Boyer (1961) the rate of the phosphoglycerate kinase in yeast will depend on both the ADP and ATP concentrations. In galactose-grown cells the concentration of ADP may not limit the rate of the reaction. In glucose-grown cells both the concentration of ADP (Fig. 4*a*) and the [ATP]/[ADP] ratio (Fig. 10) are not favourable to this reaction. The higher NADH concentration in glucose-grown cells (Fig. 7*b*) might be due to control of this reaction or of the pyruvate-kinase step by lack of ADP. Alternatively, the high NADH concentration of glucose-grown cells might be a reflection of low alcohol-dehydrogenase activity (this is unlikely; see Polakis & Bartley, 1965) or of the low NADH-oxidase activity of these cells (Polakis *et al.* 1965).

According to the data of Alvarado (1960), the ATP concentration in galactose-grown cells is high enough to saturate the galactokinase.

Pentose phosphate pathway. This is used in yeast mainly for the production of intermediates. Only a small proportion of glucose breakdown can be accounted for by this pathway (for example, see Kovachevich & Guzman-Barron, 1964).

According to the kinetic properties of yeast glucose 6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase from *Candida utilis* (Rose, 1961; Noltmann & Kuby, 1963) the intracellular concentration of $NADP^+$ (Fig. 8*a*) was always high enough to saturate both enzymes. During the metabolism of sugars the concentration of NADPH was also high enough to cause a substantial inhibition. Both $NADP^+$ and NADPH concentrations were higher in galactose-grown cells, but no explanation seems possible with the present

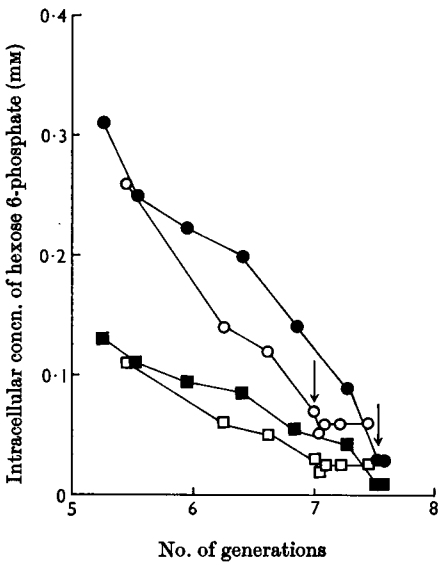
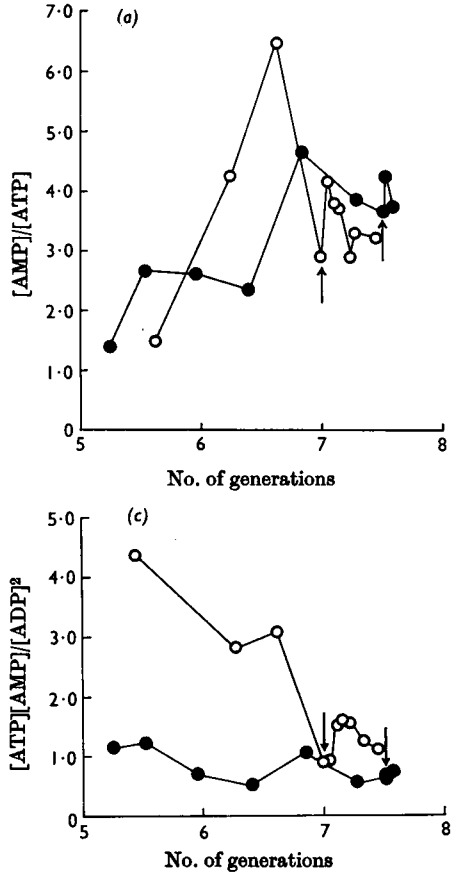


Fig. 9. Changes in the intracellular concentration of glucose 6-phosphate during the aerobic growth of yeast cells on 0·9% sugar in a synthetic medium. The intracellular concentrations of fructose 6-phosphate, calculated on the assumption that the phosphoglucose-isomerase reaction is at equilibrium at any time during growth, are also presented. Glucose 6-phosphate: ○, glucose-grown cells; ●, galactose-grown cells. Fructose 6-phosphate: □, glucose-grown cells; ■, galactose-grown cells. Glucose and galactose had been consumed at the points indicated by the arrows.



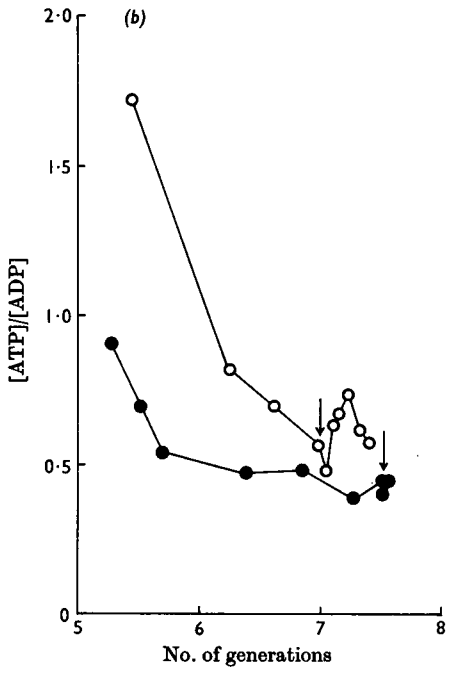


Fig. 10. Changes in the [AMP]/[ATP] ratio (*a*), in the [ATP]/[ADP] ratio (*b*) and in the expression [ATP][AMP]/[ADP]² during the aerobic growth of yeast cells on 0·9% sugar in a synthetic medium: ○, glucose-grown cells; ●, galactose-grown cells. Glucose and galactose had been consumed at the points indicated by the arrows.

data. During the growth of yeast on sugars the concentration of glucose 6-phosphate was also high enough to saturate its dehydrogenase. Thus at this stage of growth the two dehydrogenases of the pentose phosphate pathway will most probably function at almost maximal rate. During growth on C_2 compounds the intracellular concentration of glucose 6-phosphate fell to suboptimum levels for the functioning of the dehydrogenase (Fig. 9). At the same time the concentration of NADPH decreased (Fig. 8*b*) in spite of the decrease in the rate of growth and the increase in the activity of $NADP^+$-linked isocitrate dehydrogenase (Polakis & Bartley, 1965). These changes will influence the anabolic reactions in the cell, especially the syntheses of amino acids, lipids and nucleic acids.

Metabolism of pyruvate. The concentration of NAD^+ found inside the cell gave maximal activities in the assay of pyruvate oxidase (Polakis & Bartley, 1965).

From the kinetic properties of yeast alcohol dehydrogenase (Racker, 1955; Sund & Theorell, 1963), of K^+-activated acetaldehyde dehydrogenase of yeast (Black, 1955; Milstein & Stoppani, 1958) and of $NADP^+$-linked acetaldehyde dehydrogenase of yeast (Seegmiller, 1955) it may be concluded that the concentration of nicotinamide nucleotides found in yeast was high enough to saturate all of these enzymes. Thus at any time the rate of NAD^+-linked enzymes will depend on the product inhibition. The high [NADH]/[NAD^+] ratio during growth on glucose will favour the formation of ethanol, whereas the low [NADH]/[NAD^+] ratio during growth on galactose will be more favourable to the aldehyde-dehydrogenase reaction.

Tricarboxylic acid cycle. The concentration of NAD^+ found in the cells gave maximal activity in the assay of NAD^+-linked isocitrate dehydrogenase (Polakis & Bartley, 1965). Owing to the very complex kinetics of this enzyme (Smith, Hathaway & Atkinson, 1965) and to lack of knowledge about

'compartmentation', the stimulatory effect of AMP, which is also pH-dependent (Kornberg & Pricer, 1951; Hathaway & Atkinson, 1963), cannot be assessed.

The concentration of NAD^+ was also sufficient to saturate the yeast α-oxoglutarate-oxidase system (see Holzer, Hierholzer & Witt, 1963).

Adenylate-kinase reaction (*Fig.* 10c). The equilibrium constant of the adenylate-kinase reaction depends on the concentration of Mg^{2+} (Bowen & Kerwin, 1956).

In galactose-grown cells and with the correct concentration of Mg^{2+} the values of the expression $[ATP][AMP]/[ADP]^2$ might reflect equilibrium conditions. During the growth of cells on glucose the adenylate-kinase reaction was definitely out of equilibrium.

The rate of growth of cells should be directly proportional to the rate of utilization of ATP. ATP will be converted either into ADP (syntheses of polysaccharides, of some amino acids and of ribose 5-phosphate, and carboxylation of acetyl-CoA) or into AMP (activation of acetate and amino acids, and synthesis of nucleic acids). Calculations on the basis of the cell constituents (see Polakis & Bartley, 1966) as well as the values of the relationship $[ATP][AMP]/[ADP]^2$ in glucose-grown cells indicate that during growth the rate of consumption of ATP to give AMP exceeds the rate of consumption of ATP that yields ADP. The values of the expression $[ATP][AMP]/[ADP]^2$ are higher during growth on glucose than during growth on galactose. This could be due to differences in the activity of adenylate kinase, but it is at least partly due to the faster rate of growth of cells on glucose.

Patterns of metabolism in yeast during the aerobic growth on glucose or galactose. The patterns presented here, although not proven, could explain all the known changes that take place during the aerobic growth of yeast on either sugar.

The intracellular concentration of glucose 6-phosphate is of importance for the function of the pentose phosphate pathway, which provides ribose 5-phosphate and NADPH. A fall in the intracellular concentration of glucose 6-phosphate will result in a lower rate of supply of these intermediates, and consequently in a lower rate of growth. Thus for maximal rate of growth the cells will have to keep the glucose 6-phosphate concentration optimum for the reaction of its dehydrogenase and at the same time must adjust the energy production to the maximal capacities of the biosynthetic pathways.

During the growth of cells on glucose, glycolysis seems to be the only pathway of energy production (Swanson & Clifton, 1948; Lemoigne *et al.* 1954; Polakis *et al.* 1965). In this case the phosphorylation of glucose by the hexokinase reaction should be adjusted to keep the glucose 6-phosphate concentration optimum for the dehydrogenase reaction, without any increase in the rate of glycolysis, which would provide more energy than required for maximal rate of growth. This might be achieved by balancing the reactions of energy production to the reactions of energy consumption by adjusting the concentrations of different cofactors. Hence the importance of the adenylate-kinase reaction. Evidence that the production of energy by glycolysis is regulated by the concentration of ADP is presented in this paper. In a previous paper (Polakis *et al.* 1965) evidence was presented that the repression by glucose of the mitochondrial enzymes and structure was possibly brought about by a compound involved in the transformation of energy inside the cell. The results of the present paper point to ADP as the most probable candidate (Fig. 4). Its effect should be inducible and could be brought about by a mechanism similar to the one described by Jacob & Monod (1961), assuming that the repressor molecules, formed by the regulator genes, which govern the formation of mitochondrial enzymes and structural protein, are deactivated by the binding of ADP, which ideally should follow sigmoid-curve kinetics. By the end of the growth on glucose the concentration of glucose 6-phosphate (Fig. 9) fell to suboptimum levels for the reaction of its dehydrogenase. This must retard the rate of energy consumption and the rate of growth. Thus for a short time the ATP concentration should increase and the AMP concentration and the [AMP]/[ATP] ratio should decrease. These changes happened (Figs. 3*a*, 5*a* and 10*a*). They will lead to inhibition of phosphofructokinase and of glycolysis and will facilitate the formation of reserve carbohydrate from ATP and glucose 6-phosphate (see Polakis & Bartley, 1966). The deceleration of AMP production from ATP by the low rate of supply of ribose 5-phosphate and NADPH (which in turn slows down the rate of formation of amino acids and lipids) will allow the adenylate-kinase reaction to increase the concentration of ADP. This will induce the formation of the mitochondrial enzymes and structure (see Polakis, Bartley & Meek, 1964, 1965; Polakis & Bartley, 1965). The aerobic adaptation is brought about at the expense of the reserve carbohydrate of cells (see Polakis & Bartley, 1966). The low intracellular concentration of glucose 6-phosphate during the growth of cells on C_2 compounds could also be the reason for the low rate of growth of these cells and for the decreases in the protein/dry wt. and RNA/dry wt. ratios, and for the increase in the carbohydrate/dry wt. ratio (see Polakis & Bartley, 1966). These changes could be interpreted as a preferential allotment of energy, produced by the oxidation of C_2 compounds, to carbohydrate

rather than protein and RNA synthesis. This in turn could suggest a requirement for intermediates rather than energy for the faster growth of cells in this stage.

The production of energy by glycolysis during the growth of cells on galactose is not enough to satisfy the need of cells for maximal rate of growth. It is reasonable to consider the production of glucose 6-phosphate from galactose the limiting factor. In this case any increase in the activities of the glycolytic enzymes (i.e. the enzymes catalysing the formation of pyruvate from glucose 6-phosphate) will lower the intracellular concentration of glucose 6-phosphate and the rate of growth without increasing the rate of either pyruvate formation or energy production. For maximal rate of growth on galactose, the intracellular concentration of glucose 6-phosphate must be optimum for the dehydrogenase reaction. Indications that this is accomplished by regulation of phosphofructokinase have been presented. [Decreases in the concentrations of glucose 6-phosphate after about 5·3 and 6·5 generations (Fig. 9) were accompanied by corresponding increases in the [AMP]/[ATP] ratio (Fig. 10); the NADH concentration in glucose-grown cells was higher (Fig. 7*b*) whereas the ADP concentration (Fig. 4*a*) was lower than in galactose-grown cells.] With a limiting supply of glucose 6-phosphate, the energy obtained by the glycolytic pathway alone will not be enough to satisfy the needs of cells for the highest possible rate of growth, and thus other supplementary energy-producing pathways are used by the cell (namely the citric acid cycle and the respiratory chain).

In this context the regulation of glycolysis at the level of phosphofructokinase in galactose-grown cells presents some advantages to these cells: it allows high intracellular concentrations of ADP and low concentrations of NADH, thus facilitating the reactions of acetaldehyde dehydrogenase, of the tricarboxylic acid-cycle enzymes and of the respiratory chain. By contrast, control at the level of phosphoglycerate or pyruvate kinase will result in high NADH concentrations and will require low ADP concentrations, thus facilitating the alcohol-dehydrogenase reaction and inhibiting the function of the tricarboxylic acid cycle and the respiratory chain.

Thus in general the changes in the concentrations of cofactors found during growth on sugars may be explained in terms of the requirements of cells for optimum growth. Consideration of mechanisms in terms of changes of enzyme activities and of quantities of glycolytic intermediates gives rise to a logical pattern of control that adjusts the metabolism of the cell to the most economical usage of the substrate available to it.

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