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OXIDATIVE PHOSPHORYLATION IN YEAST

V. PHOSPHORYLATION EFFICIENCIES IN GROWING CELLS DETERMINED FROM MOLAR GROWTH YIELDS

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SUMMARY

1. Growth yields of *Saccharomyces cerevisiae* grown in complex media with glucose, ethanol, D- and L-lactates, pyruvate or acetate, respectively, as the energy source were measured. The cell mass formed per mole of ATP which would have been produced from the substrates according to theoretical stoichiometry, was determined. Assuming that on the average 10.5 g dry wt. of cells were formed per mole ATP (the Elsden constant), the amount of ATP furnished by fermentation and oxidation of glucose and by the oxidation of the other substrates by growing cells was calculated.

2. Phosphorylation efficiencies in growing respiration-deficient and oxidative phosphorylation-deficient mutants were evaluated from growth yield measurements.

3. The effects of antimycin A, 2,4-dinitrophenol, oligomycin and azide on phosphorylation efficiencies in growing cells were examined.

4. The results suggest that in yeast growing in a complex medium on glucose and lactate, energy-producing and energy-consuming reactions are effectively coupled. The lack of the first oxidative phosphorylation site in yeast is corroborated. Functioning of the antimycin-binding site and equilibration of cellular ATP with mitochondrial high-energy state do not seem to be obligatory for anaerobically growing yeast.

INTRODUCTION

The efficiency of energy transformations in the living cell is one of the essential problems of cell biology. Various procedures have been elaborated to measure phosphorylation efficiencies within intact cells[1–15], but the validity of some of them has been questioned[9,13].

A method for the determination of the efficiency of energy transformations in growing cells has been devised by BAUCHOP AND ELSDEN[16]. They showed that different microorganisms synthesized the same amount of cell material per mole of ATP produced by catabolism of the energy source. The average value found by these authors, which was later corroborated and generalized by others[17–24], was 10.5 g dry wt. cells formed per mole ATP. From this value, having measured the amount of cells formed per mole of the energy source consumed, either the amount of ATP furnished by catabolism can be estimated, or knowing the theoretical yield of ATP, the efficiency of energy transformation *in vivo* can be evaluated.

This method has been used in the present work to study the phosphorylation efficiencies in wild-type and mutant yeast cells growing under various conditions. Effects of several compounds affecting energy transformations *in vivo* have also been examined.

EXPERIMENTAL

Chemicals

Antimycin A was purchased from Sigma; casein hydrolysate from Difco; D- and L-lactates were obtained from Calbiochem; oligomycin was a gift from Upjohn; and other chemicals were obtained from Lachema.

Microorganisms

The following strains of *Saccharomyces cerevisiae* were used: DT XII (wild type), DT XII A (respiratory-deficient mutant, prepared from DT XII by acriflavin treatment[25]), DH 1 (oxidative phosphorylation-deficient mutant[26]). In some experiments a laboratory strain of *Candida utilis* was also used.

Culture technique

The complex medium containing minerals, vitamins and casein hydrolysate was the same as that used by BAUCHOP AND ELSDEN[16] for *S. cerevisiae*, but the pH was kept constant at pH 5.0 by buffering with 0.05 M phthalate buffer. The medium contained varying concentrations of glucose or other energy sources. The medium for anaerobic culture in Thunberg tubes was supplemented with Tween 80 and ergosterol[27]. The adenine-requiring strain DH 1 was cultured in the presence of 0.004 % adenine.

The yeasts were stored on nutrient agar slants at 4°. To prepare an inoculum, a loopful of cells was transferred into 10 ml of a complex medium containing 2 % glucose or other energy sources in a 100-ml erlenmeyer flask and cultured on a shaker for 20 h at 30°. The cultures were centrifuged, and the cells washed with distilled water and suspended in water. Experimental flasks were inoculated with this suspension, so that they contained 10^4 cells/ml at the beginning of the cultivation. The 100-ml erlenmeyer flasks, fitted with cotton plugs, each contained 10 ml of culture medium. For aerobic growth, the flasks were agitated at 30° on a reciprocal shaker (5 cm amplitude, 110 oscillations/min). To measure anaerobic growth yields, each flask was given 2.5 μg antimycin A (dissolved in ethanol which was evaporated after adding to the flask) prior to addition of the medium. Some anaerobic growth experiments were carried out using Thunberg tubes which, after inoculation, were twice filled with purified nitrogen and reevacuated.

In each experiment, a series of flasks with a range of concentrations of the energy source was used. Cultivation proceeded until growth in the flask containing the highest concentration of the energy source had ceased. This was estimated from growth curves measured for each experimental condition.

Other methods

The amounts of cells were determined either by gravimetric or turbidometric measurements. Separate calibration curves relating turbidity to dry wt. of cells were constructed for each strain and each energy source. Dry weight was estimated after drying the cells at 105° overnight.

Radioactivity was measured with an end-window Geiger–Müller counter.

Terms used and method of calculation

For each experiment, a curve was constructed showing the relation between cell yields and concentrations of the substrate used as energy source in the medium. The slope of the curve gives the molar yield coefficient for the particular substrate $Y_{\text{substrate}}$ (g dry wt. cells per mole of substrate) (see refs. 16 and 17). The ATP coefficient, Y_{ATP}, expresses g dry wt. of cells formed per mole ATP. The latter value was calculated from molar yield coefficients assuming the following stoichiometries for ATP formation:

Anaerobically:

$$\text{Glucose} + 2\ \text{ADP} + 2\ \text{P}_i = 2\ \text{ethanol} + 2\ \text{CO}_2 + 2\ \text{ATP} \quad (1)$$

Aerobically:

$$\text{Glucose} + 6\ \text{O}_2 + 28\ \text{ADP} + 28\ \text{P}_i = 6\ \text{H}_2\text{O} + 6\ \text{CO}_2 + 28\ \text{ATP} \quad (2)$$

$$\text{Lactate} + 3\ \text{O}_2 + 12\ \text{ADP} + 12\ \text{P}_i = 3\ \text{H}_2\text{O} + 3\ \text{CO}_2 + 12\ \text{ATP} \quad (3)$$

$$\text{Ethanol} + 3\ \text{O}_2 + 12\ \text{ADP} + 12\ \text{P}_i = 3\ \text{H}_2\text{O} + 2\ \text{CO}_2 + 12\ \text{ATP} \quad (4)$$

$$\text{Pyruvate} + 2.5\ \text{O}_2 + 11\ \text{ADP} + 11\ \text{P}_i = 2\ \text{H}_2\text{O} + 3\ \text{CO}_2 + 11\ \text{ATP} \quad (5)$$

$$\text{Acetate} + 3\ \text{O}_2 + 8\ \text{ADP} + 8\ \text{P}_i = 2\ \text{H}_2\text{O} + 2\ \text{CO}_2 + 8\ \text{ATP} \quad (6)$$

In deducing these equations it has been assumed that the oxidative phosphorylation site corresponding to Site I of animal mitochondria is lacking in Saccharomyces mitochondria[28–30].

The average ATP coefficient compiled from numerous data obtained from widely different organisms, $Y_{\text{ATP}} = 10.5$ (see ref. 17), will be called the Elsden constant in this paper. The ratio of the molar yield coefficient on a given substrate to the Elsden constant will be called the phosphorylation coefficient and will be assumed to represent moles ATP formed from 1 mole of the substrate under the conditions given.

In Figs. 1–4, the standard deviations from the mean are represented by the heights of the vertical bars.

RESULTS

Assimilation of glucose by cells growing in a complex medium

In accordance with previous reports[16,17], it has been assumed that when cells grow in a complex medium containing casein hydrolysate, glucose is used essentially as a source of energy and not as a source of carbon, the latter being supplied by casein hydrolysate. However, cellular carbohydrates may be derived directly from glucose rather than from components of casein hydrolysate.

In order to estimate the fraction of glucose assimilated, cells of strain DT XII were grown in the presence of uniformly ^{14}C-labeled glucose, and the radioactivity incorporated into the cells was estimated. The results are summarized in Table I.

TABLE I

ASSIMILATION OF GLUCOSE BY *S. cerevisiae* DT XII GROWING IN A COMPLEX MEDIUM

The cells were grown on a range of concentrations of uniformly ^{14}C-labeled glucose. Anaerobiosis was attained by cultivation in the presence of 0.25 µg/ml antimycin A. When growth ceased, the cells were centrifuged, washed twice with water, 3 times with ice-cold 5 % trichloroacetic acid, then suspended in water, filtered, and washed on the filter with water and with acetone. Radioactivity of the washed cells was counted and compared with the original radioactivity of the medium. The values are means (± S.E.) from 3 experiments.

Conditions	*Glucose incorporated by cells (%)*
Aerobic	10.7 ± 2.9
Anaerobic	1.5 ± 0.1

Only 1 % of the added glucose was assimilated by cells which only ferment it and 10 % was assimilated by respiring cells. The correction of molar yield coefficients for this assimilated part will be inserted in some calculations.

Similarly, a fraction of other substrates used as energy sources could be assimilated. However, these fractions were not measured and the corrections were not applied.

Since almost no growth of *S. cerevisiae* occurred when glucose was omitted from the medium, casein hydrolysate alone was not used as a source of energy. On the other hand, cells of *C. utilis* were able to grow considerably in the complex medium in the absence of glucose.

Growth yields of fermenting cells and mutants

Fig. 1 shows the relation between cell mass formed by fermenting cells and glucose present in the complex growth medium. The curve marked 0 corresponds to cells of wild-type strain DT XII grown in the presence of antimycin A. The inhibitor was used in a sufficiently high concentration to stop respiration, thus the total amount

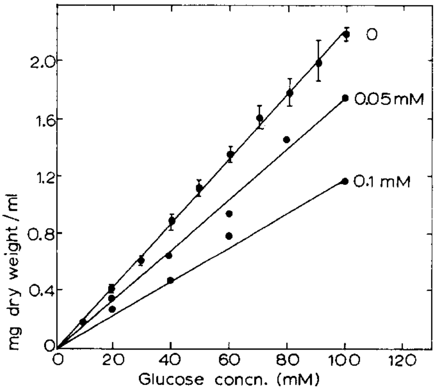


Fig. 1. Growth yield of cells fermenting glucose in a complex growth medium. Curve 0 corresponds to wild-type strain DT XII grown in the presence of 0.25 µg/ml antimycin A which arrested respiration; the values were obtained in 6 independent experiments. The other curves correspond to respiration-deficient mutant DT XII A grown in the presence of 2,4-dinitrophenol in the concentrations indicated on the curves.

of glucose was catabolized only by fermentation. Exactly the same curve was obtained when cells were cultured anaerobically in Thunberg tubes in the absence of antimycin A. The same relation was also found with cells of the respiratory-deficient mutant DT XII A, grown both in the presence and absence of antimycin A, and similarly with cells of the oxidative phosphorylation-deficient mutant DH 1.

As can also be seen from Fig. 1, 2,4-dinitrophenol lowered the growth yield of fermenting cells. It acted similarly on the three strains examined.

Growth yield of respiring cells

The relation between the cell mass formed and the amount of glucose present in the complex growth medium for cells DT XII, grown aerobically, was linear only at low glucose concentrations (Fig. 2). This was not due to a lowering of pH during growth at higher glucose concentrations since the pH of the buffered medium remained unchanged, even after growth on 0.1 M glucose. As may be seen from Fig. 3, neither casein hydrolysate nor aeration as used in standard experiments limited growth at higher glucose concentrations. Supplementation of the complex medium with tryptophan, which might be lacking from the casein hydrolysate, was also without effect.

Since higher glucose concentrations are known to repress the synthesis of several enzymes of oxidative metabolism in yeast[31,32], the essentially biphasic growth yield curves can be accounted for by assuming that, at higher concentrations, glucose was utilized aerobically in 2 steps[31–34]: first transformed to ethanol and acetate and followed by oxidation of two products. It should be noted, however, that direct oxidation of glucose to CO_2 and H_2O furnished more useful energy than the 2-step transformation *via* ethanol and acetate. This is evident from Table II and the data presented below.

As can be seen from Fig. 2, the aerobic growth yields on glucose were lower in the presence of 2,4-dinitrophenol. Fig. 4 shows the effect of oligomycin and the combined effects of antimycin A, dinitrophenol and oligomycin on growth yields. The

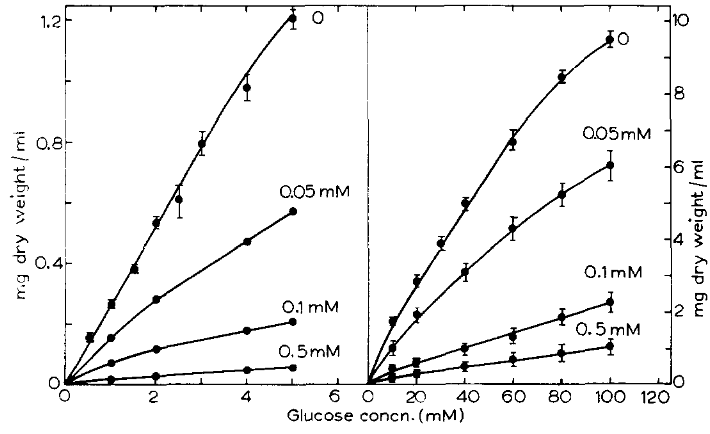


Fig. 2. Growth yield of wild-type strain DT XII utilising glucose aerobically in a complex growth medium. Values of Curve 0 were obtained in 15 experiments. Numbers on curves indicate concentrations of 2,4-dinitrophenol present.

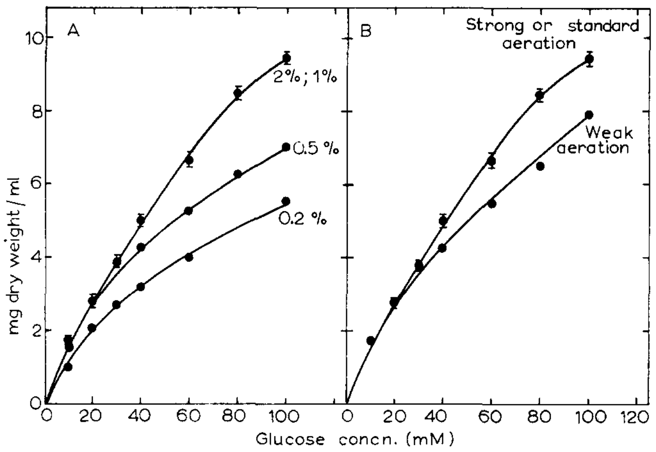


Fig. 3. Aerobic growth yield curves of strain DT XII at different concentrations of casein hydrolysate (A) and under different aeration conditions (B). Yeast cells were grown in a complex medium containing either standard concentrations of casein hydrolysate (1 %, in B) or the concentrations indicated on the curves (in A). In A, standard aeration conditions were provided by shaking 10 ml of culture in 100-ml erlenmeyer flasks. In B, the culture was weakly aerated by shaking 40 ml of the culture in a 100-ml erlenmeyer flask and strongly aerated by shaking 20 ml of culture in a 500-ml erlenmeyer flask.

TABLE II

MOLAR YIELD COEFFICIENTS, ATP COEFFICIENTS AND PHOSPHORYLATION EFFICIENCIES OF *S. cerevisiae* DT XII GROWN UNDER VARIOUS CONDITIONS

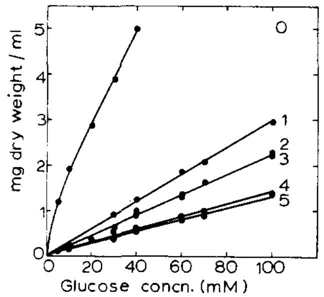
The data from growth yields in the complex medium (Figs. 1 to 5) were used for calculation. DNP, 2,4-dinitrophenol. $Y_{\text{substrate}}$: molar yield coefficient, g dry wt. cells formed per mole substrate catabolized. Y_{ATP}: ATP coefficient, g dry wt. cells formed per mole ATP. The values are calculated from molar yield coefficients assuming theoretical stoichiometry of Eqns. 1–6. Phosphorylation efficiency (theoretical): moles ATP formed per mole substrate catabolized, expressed by Eqns. 1–6. Phosphorylation efficiency (experimental): ratio of $Y_{\text{substrate}}$ to the Elsden constant, assumed to represent moles ATP formed per mole substrate catabolized under the given conditions.

Growth conditions	*Energy source*	*Inhibitor (concn.)*	$Y_{substrate}$	Y_{ATP}	*Phosphorylation efficiency*	
					Theoretical	*Experimental*
Anaerobic	Glucose	—	22.1	11.0	2	2.1
	Glucose	—	22.3*	11.1*	2	2.1*
	Glucose	DNP (0.05 mM)	16.7	8.3	—	1.6
	Glucose	DNP (0.1 mM)	12.0	6.0	—	1.1
Aerobic	Glucose (< 3 mM)	—	262.0	9.4	28	25.0
	Glucose (< 3 mM)	—	289.0*	10.3*	28	27.5*
	Glucose (> 20 mM)	—	123.0	4.4	28	11.7
	Glucose	Antimycin A (0.25 µg/ml)	22.1	11.0	—	2.1
	Glucose	Oligomycin (10 µg/ml)	30.0	1.1	—	2.9
	Glucose	DNP (0.05 mM)	150.0	5.4	—	14.3
	Glucose	DNP (0.1 mM)	70.0	2.5	—	6.7
	Glucose	DNP (0.5 mM)	20.0	0.7	—	1.9
	Lactate	—	129.2	10.8	12	12.3
	Ethanol	—	70.0	5.8	12	6.7
	Pyruvate	—	58.4	5.3	11	5.6
	Acetate	—	48.3	6.0	8	4.6

* Values corrected for glucose assimilated by cells.

growth rate was not affected by these compounds. Oligomycin lowered the aerobic growth yield but had no effect on the growth yield of cells whose respiration was arrested by antimycin A. Dinitrophenol affected the two yields and potentiated the aerobic effect of oligomycin. In order to test the effect of azide, which is volatile at acid pH, the cells of the respiration-deficient strain DT XII A were incubated with the inhibitor in tightly stoppered flasks until growth had ceased. The growth yield of these fermenting cells decreased in the presence of azide; with 0.02 mM azide the yield was only 0.5 of that of the control.

Fig. 5 presents growth yield curves for the wild-type strain grown aerobically on ethanol, pyruvate, acetate and DL-lactate. The molar growth yield on the last substrate was higher than those on the other substrates. The molar growth yields on D- and on L-lactate were exactly the same as that on racemic DL-lactate.



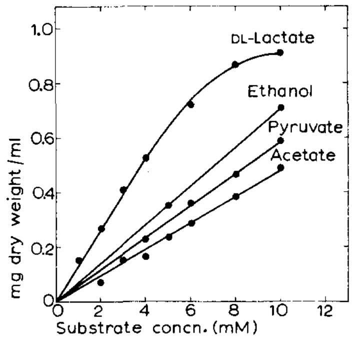


Fig. 4. Growth yield of wild-type strain DT XII grown aerobically on glucose in a complex medium in the presence of inhibitors. The inhibitors were used in the following concentrations: Antimycin A, 0.25 μg/ml; 2,4-dinitrophenol, 0.1 mM; oligomycin, 10 μg/ml (a concentration of 20 μg/ml had the same effect). Curve 0 corresponds to the control without inhibitor. Curve 1, oligomycin; 2, antimycin A + oligomycin; 3, antimycin A; 4, oligomycin + 2,4-dinitrophenol; 5, antimycin A + 2,4-dinitrophenol.

Fig. 5. Growth yield of wild-type strain DT XII, grown aerobically on different energy sources.

Calculation of molar yield coefficients, ATP coefficients and phosphorylation efficiencies

The data obtained were used for calculations which are summarized in Table II. Their significance will be discussed in the next section.

DISCUSSION

The validity of the phosphorylation efficiency values obtained by the method used in this work depends on several assumptions. Some of these assumptions have been extensively discussed by previous investigators[16,17,19,35–37], while others have been verified experimentally in the present study. Providing that the theoretical basis of the method holds, the following considerations can be drawn from the data obtained:

(a) The aerobic phosphorylation coefficient with low glucose concentrations, representing the amount of ATP produced and appropriately corrected for glucose assimilated by the cells, is 13 times higher than the anaerobic one. This is in accord with the stoichiometry of Eqns. 1 and 2 and supports the *in vitro* findings[28–30] that the first phosphorylation site of the oxidative phosphorylation system is missing in Saccharomyces cells.

(b) The ATP coefficients from anaerobic growth on glucose and aerobic growth on glucose and lactate, based on the theoretical stoichiometries of Eqns. 1 to 3, are very close to the Elsden constant. Assuming that the universality of the Elsden constant can be interpreted as an indication of an effective coupling between energy-producing and energy-consuming reactions in growing microbial cells[19], the yeast cells growing on glucose and lactate in a complex medium may also be considered as being effectively coupled. It has been suggested that a loose coupling between respiration and phosphorylation exists in nongrowing yeast[38], and P/O ratios as low as 1 have been proposed[1]. On the other hand, low phosphorylation coefficients on ethanol, pyruvate and acetate suggest that in cells growing on these substrates, catabolism and anabolism may be partially uncoupled, or that catabolism of these substrates may not conform to Eqns. 4–6.

(c) The observation that the phosphorylation coefficient of the oxidative phosphorylation-deficient mutant DH 1, grown aerobically on glucose, was substantially lower than that of the wild-type and essentially equal to the phosphorylation coefficient of anaerobically growing yeast, proves that the oxidative phosphorylation system, which was found to be substantially damaged in isolated mutant mitochondria[26], is also essentially inoperative in intact growing cells.

(d) Since antimycin A lowered the aerobic growth yield on glucose to the anaerobic level, but did not affect the anaerobic growth yield, operation of the respiratory chain site which binds antimycin A (*e.g.* in reversed electron flow through phosphorylation site II of mitochondria) is not obligatory for the anaerobic growth of yeast.

(e) Lowering of the anaerobic growth yield by the uncoupler 2,4-dinitrophenol indicates that it also exerts its uncoupling action under anaerobic conditions when ATP is supplied only by glycolytic phosphorylation. The mechanism of its anaerobic action has been previously discussed[39,40].

(f) The fact that the aerobic phosphorylation coefficient on glucose decreased in the presence of oligomycin, but remained higher than the anaerobic one, suggests that substrate-level phosphorylation coupled to α-ketoglutarate oxidation was not eliminated *in vivo* by oligomycin. As the anaerobic growth yield was not affected by oligomycin, equilibration of cellular ATP with mitochondrial high-energy states or intermediates, which should be prevented by the inhibitor, does not seem to be obligatory for growing cells.

On the other hand, the fact that the anaerobic growth yield was lowered by azide indicates that azide cannot be presumed to act only as an oligomycin-like inhibitor[41,42], and its uncoupling action[43–45] should also be taken into consideration.

Although pointing out an effective energy coupling in growing yeast, the results of the present work cannot provide a numerical value for the P/O ratio, the calculation of which would be independent of the empirical Elsden constant. From measurements of the growth yields and metabolized gases as a function of the specific growth rate in aerobic continuous culture, VON MEYENBURG[46] recently calculated the effective P/O ratio in growing Saccharomyces cells as 1.1. Since, however, the author's assumption that the P/O ratio is equally high in cells under different degrees of catabolic repression cannot be taken for granted, and lower P/O ratios in repressed cells have not been excluded[47], the value may be underestimated.

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