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REGULATION OF CELLULAR METABOLISM BY INTRACELLULAR PHOSPHATE

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SUMMARY

1. Incubation of liver cell suspensions with 10 mM glycerol or 10 mM fructose caused a decrease in intracellular $[P_i]$ and a fall in the [ATP]/[ADP] while the respiratory rate, the redox state of the intramitochondrial NAD couple, and the [ATP]/[ADP] · $[P_i]$ remained essentially unaltered.

2. The concentration of intracellular phosphate in yeast *Candida utilis* was found to be dependent on phosphate concentration in the growth medium: it was 25–30 μmol/g wet weight in cells grown in the presence of 50 mM P_i, 10–12 μmol/g wet weight in cells grown in 5 mM P_i, and 3–4 μmol/g wet weight in cells grown with 1 mM P_i.

3. The [ATP]/[ADP] ratios were found to be highest in yeast cells grown in the presence of 50 mM P_i ($\geq$ 30) and lowest in cells grown with 1 mM P_i (8–9). The increase in [ATP]/[ADP] was due to an increase in [ATP].

4. The endogenous levels of glucose 6-phosphate in washed and aerated cells were lowest in cells grown in the presence of 50 mM P_i and highest in those grown in the presence of 1 mM P_i. A suggestion is made that the high intracellular $[P_i]$ stimulates phosphorolytic breakdown of glycogen and depletes the cellular stores of this substrate.

5. It is postulated that the cellular respiratory rate and the NADH generation rate are regulated by [ATP]/[ADP] · $[P_i]$ and not by the "energy charge" as defined by Atkinson (Biochemistry 7, 4030–4034, 1968).

6. The concentration of intracellular $[P_i]$ is implicated as an important contributor to the regulatory mechanisms of cellular metabolism.

INTRODUCTION

The mechanism of regulation of cellular respiration with its accompanying

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phosphorylation reactions is of particular physiological importance. Respiration is the principle source of cellular ATP and at the same time provides the major sink for NADH, ADP, and phosphate, the metabolites which are also reactants in most of the other metabolic pathways of the cell. Changes in the concentrations of these metabolites which occur in response to cellular metabolic requirements provide the simplest regulatory mechanism through which the activities of the various pathways including oxidative phosphorylation can be adjusted and maintained.

Previous experiments indicated that in suspensions of isolated pigeon heart mitochondria respiring in the presence of substrate and oxygen [1], in suspensions of isolated liver cells [2, 3], in ascites tumor cells [3], and in perfused liver [3] and heart [4], a near equilibrium exists in the multistep reaction:

$$\mathrm{NADH}+2\,c^{3+}+2\,\mathrm{ADP}+2\,\mathrm{P_i} \rightleftharpoons \mathrm{NAD^+}+2\,c^{2+}+2\,\mathrm{ATP} \tag{1}$$

The equilibrium constant for this reaction is defined by:

$$K_{eq}=\frac{[\mathrm{NAD^+}]}{[\mathrm{NADH}]}\cdot\frac{[c^{2+}]^2}{[c^{3+}]^2}\cdot\frac{[\mathrm{ATP}]^2}{[\mathrm{ADP}]^2[\mathrm{P_i}]^2} \tag{2}$$

where $[c^{2+}]$ and $[c^{3+}]$ are the concentrations of the reduced and oxidized forms of cytochrome *c*, respectively, $[\mathrm{NAD^+}]$ and [NADH] are the intramitochondrial concentrations of the free coenzymes and [ATP], [ADP], and $[\mathrm{P_i}]$ are the extramitochondrial concentrations of adenine nucleotides and $\mathrm{P_i}$. Since all three phosphorylation sites share common intermediates in the phosphorylation reactions, these experimental results provided a foundation for a proposed near equilibrium model of mitochondrial oxidative phosphorylation [2, 5] in which the respiration is dependent on the cytoplasmic $[\mathrm{ATP}]/[\mathrm{ADP}]\cdot[\mathrm{P_i}]$ and the mitochondrial $[\mathrm{NAD^+}]/[\mathrm{NADH}]$. If this model is correct, it then follows that the metabolic pathways are regulated toward providing ATP under conditions for which its free energy of hydrolysis is nearly constant and that the $[\mathrm{ATP}]/[\mathrm{ADP}]\cdot[\mathrm{P_i}]$ may be an important regulatory factor of cellular metabolism.

In 1966, Atkinson [6] suggested that because the adenine nucleotides participate in every sequence of metabolic reactions, the stabilization of the so-called energy charge, defined as $([\mathrm{ATP}]+1/2[\mathrm{ADP}])/([\mathrm{ATP}]+[\mathrm{ADP}]+[\mathrm{AMP}])$ [7] might be a necessary prerequisite to metabolic homeostasis. The energy charge, which is proposed to regulate each sequence of reactions, depends on balance among ATP-utilizing and ATP-producing reactions. In order to perform this regulatory function in the cell, the energy charge must be maintained through operation of the control responses at a value between 0.80 and 0.95 [7, 8].

The two postulated regulatory factors of cellular metabolism, the $[\mathrm{ATP}]/[\mathrm{ADP}]\cdot[\mathrm{P_i}]$ [5] and the energy charge [6–8], are fundamentally different. The former stresses that in addition to ATP and ADP, the concentration of cellular phosphate is of primary importance in the regulatory mechanisms. The latter, in contrast, ascribes no role to the phosphate concentration, but involves AMP as a primary regulator. (In regulation by $[\mathrm{ATP}]/[\mathrm{ADP}]\cdot[\mathrm{P_i}]$, the concentration of AMP enters only indirectly through the adenylate kinase reaction). Both the $[\mathrm{ATP}]/[\mathrm{ADP}]\cdot[\mathrm{P_i}]$ and the energy charge and their respective influences on metabolism can be tested experimentally and evaluated quantitatively.

The experimental material used in this study is either yeast, *Candida utilis*, or

suspension of cells isolated from rat liver. Yeast requires phosphate for its growth and fermentation [9]. The yeast cell assimilates phosphate as a monovalent anion ($H_2PO_4^-$) [10] from the medium against a concentration gradient [11] via a carrier mechanism which obeys a saturation behavior. The inorganic phosphate taken up by the cells is stored as metaphosphate [12] and can be rapidly and reversibly transformed into orthophosphate. The present contribution describes the metabolic characteristics of yeast cells grown in the presence of various concentrations of inorganic phosphate in the medium.

It is found that in cells grown in high phosphate medium, the concentration of the intracellular inorganic phosphate rises to 25–30 μmol/g wet weight and the [ATP]/[ADP] increases, due to an increase in [ATP], to a value of $\geqslant 30$, so that the cellular $[ATP]/[ADP] \cdot [P_i]$ has a value of $1 \cdot 10^3$–$2 \cdot 10^3\ M^{-1}$, characteristic of other types of cells [2–4]. As the concentration of intracellular P_i is lowered by growing cells in medium containing less phosphate, the [ATP]/[ADP] declines and thus the $[ATP]/[ADP] \cdot [P_i]$ is maintained at the value essentially the same as that found in cells grown in high phosphate medium. Moreover, the results presented in this work show that when the intracellular phosphate concentration in liver cells is decreased during the metabolism of fructose or glycerol, the cellular respiratory rate (ATP supply) and the NADH generation rate $[NAD^+]/[NADH]$ are regulated by $[ATP]/[ADP] \cdot [P_i]$ and not by the energy charge.

MATERIALS AND METHODS

Rats. 24-h starved female Sprague-Dawley rats were used for isolation of the liver cells.

Liver cells. The liver cells were prepared by the method of Berry and Friend [13] incorporating the modifications described by Cornell et al. [14] and Krebs et al. [15].

C. utilis yeast cells were grown in 2 % yeast extract, 2 % glucose, 0.05 % $MgSO_4$, pH 4.4, medium containing various concentrations of phosphate (50, 5, or 1 mM) at 22 °C, with rapid shaking. The cells were harvested by centrifugation in the early stationary phase of growth, washed three times with 0.9 % NaCl and once in 0.1 M NaCl, 0.02 M KCl, and 0.05 M morpholinopropane sulfonate at pH 7.0 medium. The cells were suspended in the same medium in the absence of any added substrate at a concentration of approx. 70 mg wet weight/ml and oxygenated with pure oxygen gas for about 20 min at 25 °C prior to the onset of the experiment; these were called "starved" cells. Ethanol or glucose was then added and the cells were incubated with constant oxygenation for the appropriate length of time, at 25 °C and finally quenched by addition of cold $HClO_4$ (4 % final concentration). When both ethanol and glucose were used as substrates, glucose was added after 1 min preincubation with ethanol. Incubations of the liver cells were carried out at 25 °C as described previously [2, 3]. At the end of the incubation period, the cells were either quenched with $HClO_4$ (3.5 % final concentration) or used for the determination of the redox state of cytochrome *c*.

The $HClO_4$ extracts were neutralized with KOH and aliquots of the extracts were used for assays of metabolites (see below). $[P_i]$ was measured in parallel experiments in which the cells were rapidly separated from the suspending medium by the method of Hems et al. [16]. The dry weight of the cell suspension and the medium was

determined for each preparation. A factor of 3.7 was used to convert dry weight into wet weight of cells [15].

Measurement of the redox state of the cytochromes in cell suspensions. A sample of the incubated cell suspension was added to a spectrophotometric cuvette in a Johnson Research Foundation dual wavelength spectrophotometer using the wavelength pair 550–540 nm. The half-bandwidth of the measuring light was 1.6 nm. In order to prevent sedimentation, the suspension of cells in the cuvette was continuously mixed with a vibrating stirrer. After the initial spectrophotometric reading had become stable, 5 μl of a 10 mg/ml solution of antimycin A was added to cause complete oxidation of cytochrome *c*. The observed decrease in the concentration of reduced cytochrome *c* was taken to correspond to 100 % oxidation of this cytochrome [2, 3]. The fully reduced state of cytochrome *c* was taken to be that after the addition of 2 mM cyanide. The calculation of the redox state of cytochrome *c* in liver cells is described by Wilson et al. [2].

Measurements of the mitochondrial [NAD$^+$]/[NADH] ratio. The mitochondrial [NAD$^+$]/[NADH] ratio in suspensions of liver cells was calculated according to Williamson et al. [17] from the 3-hydroxybutyrate and acetoacetate concentrations measured by the method of Williamson et al. [18]. The $E_{m\,7.0}$ of [3-hydroxybutyrate]/[acetoacetate] was taken to be −0.266 V [19] at 25 °C.

Oxygen uptake was measured at 25 °C with a Clark oxygen electrode.

Measurements of ATP, ADP, AMP, and P_i. ATP was determined by the method of Lamprecht and Trautschold [20], ADP and AMP by the method of Adam [21], and P_i by the method of Martin and Doty [22]. When calculating the [P_i] values, a correction was made for adhering fluid as described by Hems et al. [16].

Thermodynamic symbols and conventions are those used previously [2, 3, 5].

Reagents. Analytical grade reagents were obtained commercially.

Enzymes (collagenase, Type I; hyaluronidase; lactate dehydrogenase, Type VI; pyruvate kinase, Type II; hexokinase/glucose-6-*P* dehydrogenase; 3-hydroxybutyrate dehydrogenase, Type II; myokinase, Grade III) were all obtained from Sigma Chemical Co., St Louis, Mo.

RESULTS

The effect of varying the intracellular orthophosphate concentration of liver cells by fructose metabolism

Several metabolites are known to lower the intracellular phosphate concentration in the perfused liver as a result of an accumulation of phosphorylated intermediates (for summary see ref. 23). These include fructose [24–26], glycerol [27, 28], and 2-deoxyglucose [29]. In this work, suspensions of liver cells were used as the experimental material. The cell suspensions were preincubated for 10 min before the addition of fructose (10 mM). At the desired time intervals, samples were withdrawn for measurement of the oxygen uptake, the redox state of cytochrome *c*, the concentration of intracellular inorganic phosphate and the concentrations of substrates and metabolites. The results are summarized in Tables I and II. In agreement with the results of other investigators [24–26], a rapid fall in inorganic phosphate (P_i) and a decrease both in ATP and in total adenine nucleotide concentration were observed.

The redox state of the intramitochondrial [NAD$^+$]/[NADH] remained essen-

TABLE I

CONCENTRATIONS OF METABOLITES IN THE LIVER CELLS INCUBATED WITH FRUCTOSE

The liver cells were obtained and treated as described in the text. The cells were preincubated for 10 min prior to the addition of 10 mM fructose. Cells incubated for 25 min in the absence of fructose gave similar values to controls incubated for 10 min. ATP, ADP, AMP, and P_i are expressed as μmol/g wet weight of cells. 3-Hydroxybutyrate and acetoacetate are expressed as μmol/flask. The cell concentration was approx. 80 mg wet weight in 4 ml. Incubations were carried out at 25 °C. Values are means $\pm$S.D. for the number of experiments in parentheses.

Metabolite	Control	10 mM fructose		
		1 min	2 min	5 min
ATP	2.15±0.39 (4)	1.69±0.35 (4)	1.22±0.24 (4)	0.55±0.1 (4)
ADP	0.52±0.13 (4)	0.84±0.15 (4)	1.21±0.29 (4)	1.09±0.2 (4)
AMP	0.46±0.14 (4)	0.47±0.15 (4)	0.52±0.19 (4)	0.52±0.2 (4)
P_i	3.91±0.42 (3)	1.09±0.14 (3)	0.88±0.12 (3)	0.63±0.15 (3)
3-Hydroxybutyrate	0.41±0.05 (3)	0.38±0.04 (3)	0.36±0.05 (3)	0.47±0.08 (3)
Acetoacetate	0.31±0.09 (3)	0.31±0.05 (3)	0.34±0.01 (3)	0.38±0.04 (3)

TABLE II

THE REDOX STATE OF CYTOCHROME *c* AND OF THE MITOCHONDRIAL NAD COUPLE, THE [ATP]/[ADP] · [P_i] AND THE RESPIRATORY RATES OF LIVER CELL SUSPENSIONS INCUBATED WITH FRUCTOSE

The experimental values are those from Table IV. Concentration of cytochrome *c* in the liver cells was found to be approx. 0.02 μmol/g wet weight.

Metabolite ratio	Control	10 mM fructose		
		1 min	2 min	5 min
[ATP]/[ADP]	4.13±1.21 (4)	2.01±0.38 (4)	1.01±0.3 (4)	0.50±0.1 (4)
[ATP]/[ADP] · [P_i] (M^{-1})	1056±260 (3)	1844±177 (3)	1148±325 (3)	793±75 (3)
$\frac{ATP+1/2\ ADP}{ATP+ADP+AMP}$	0.77±0.04 (4)	0.70±0.05 (4)	0.63±0.05 (4)	0.52±0.06 (4)
$\frac{3\text{-Hydroxybutyrate}}{\text{Acetoacetate}}$	1.31±0.07 (3)	1.23±0.09 (3)	1.06±0.05 (3)	1.24±0.06 (3)
$\frac{\text{Cytochrome } c_{ox}}{\text{Cytochrome } c_{red}}$	4.16±0.6 (4)	4.03±0.38 (4)	4.48±0.39 (4)	4.78±0.66 (4)
O_2 uptake (μmol/min per g)	1.40±0.43 (4)	2.12±0.51 (4)	1.37±0.36 (4)	1.25±0.44 (4)
Turnover number (e^-/s per cytochrome *c*)	5.13±0.72 (4)	7.49±1.35 (4)	4.79±0.33 (4)	4.32±0.63 (4)

tially constant perhaps with a slight tendency to decline after 15 min incubation. The behavior of the respiratory rate followed the same pattern in all four experiments: there was a transient (up to 1 min) enhancement of respiration (not more than 70–80 %) after the addition of fructose which was followed by a decline to near the initial value. The respiratory rate then remained constant for the next 10–15 min and slowly declined (not by more than 30–40 %) during prolonged incubations.

The redox state of cytochrome *c* reflected the behavior of the respiratory rate. There was a transient, small increase in the extent of reduction of cytochrome *c* which returned almost to the original value (i.e. that before the addition of fructose) by 1 min after the additon of fructose. At longer incubation times (15 min and more) an oxidation of cytochrome *c* was observed. The most dramatic effects were observed in the [ATP]/[ADP] ratios which decreased more than 8-fold in the first 5 min, whereas, the [ATP]/[ADP] · [P_i] remained essentially constant. A transient 2-fold increase in the [ATP]/[ADP] · [P_i] observed after 1 min incubation is due to the fact that the decrease in the level of inorganic phosphate appears to occur faster than the decline in ATP level which is in agreement with the observations of the other authors [24–26]. In accordance with the general changes in the adenine nucleotide system, the energy charge declined from 0.77 to 0.52 in 5 min.

The [ATP]/[ADP] · [P_i] at various time intervals after the addition of fructose can also be expressed as ΔG for ATP hydrolysis. The calculation of the results of Table II shows that the values are: 11.7 kcal/mol (48.3 kJ/mol) at time 0, 12.03 kcal/mol (49.7 kJ/mol) at 1 min, 11.76 kcal/mol (48.6 kJ/mol) at 2 min, and 11.54 kcal/mol (47.7 kJ/mol) at 5 min incubation. Thus the changes are less than 5 %, within the accuracy of the experimental technique.

The effect of varying the intracellular orthophosphate concentration of liver cells by glycerol metabolism

Essentially the same, although less dramatic, effects were observed in the liver

TABLE III

CONCENTRATIONS OF METABOLITES IN THE LIVER CELLS INCUBATED WITH GLYCEROL

The liver cells were obtained and treated as described in the text. The cells were preincubated for 10 min prior to the addition of 10 mM glycerol. Cells incubated for 25 min in the absence of glycerol gave similar values to controls incubated for 10 min. ATP, ADP, AMP, and P_i are expressed as μmol/g wet weight in 4 ml. Incubations were carried out at 25 °C. Values are means ±S.D. for four independent experiments.

Metabolite	Control	10 mM glycerol		
		2 min	5 min	15 min
ATP	2.02±0.27	1.66±0.33	1.29±0.35	0.95±0.42
ADP	0.48±0.15	0.76±0.30	0.79±0.27	0.75±0.33
AMP	0.45±0.07	0.47±0.07	0.42±0.11	0.42±0.06
P_i	3.38±0.04	1.39±0.13	1.01±0.03	0.80±0.10
3-Hydroxybutyrate	0.29±0.09	0.28±0.09	0.33±0.06	0.38±0.12
Acetoacetate	0.22±0.08	0.24±0.09	0.26±0.10	0.26±0.08
Total adenine nucleotides	2.95±0.48	2.85±0.48	2.44±0.33	2.01±0.43

TABLE IV

THE REDOX STATE OF CYTOCHROME *c* AND OF THE MITOCHONDRIAL NAD COUPLE, THE [ATP]/[ADP] · [P_i] AND THE RESPIRATORY RATES OF LIVER CELL SUSPENSIONS INCUBATED WITH GLYCEROL

The experimental values are those from Table VI. Concentration of cytochrome *c* in the liver cells was found to be approx. 0.02 μmol/g wet weight.

Metabolite ratio	Control	10 mM glycerol		
		2 min	5 min	15 min
[ATP]/[ADP]	4.21±1.5	2.6±0.15	1.99±0.14	1.57±0.25
[ATP]/[ADP] · [P_i] (M^{-1})	1346±329	1209±165	1380±204	1277±381
$\frac{ATP+1/2\ ADP}{ATP+ADP+AMP}$	0.76±0.01	0.68±0.01	0.65±0.04	0.58±0.02
$\frac{3\text{-Hydroxybutyrate}}{\text{Acetoacetate}}$	1.41±0.4	1.40±0.35	1.45±0.52	1.62±0.48
$\frac{\text{Cytochrome } c_{ox}}{\text{Cytochrome } c_{red}}$	5.12±2.0	4.74±2.0	4.50±2.0	4.46±1.6
O_2 uptake (μmol/min per g)	1.57±0.4	1.64±0.56	1.41±0.47	1.20±0.43
Turnover number (e^-/s per cytochrome *c*)	5.8±1.3	6.0±1.9	5.2±1.6	4.4±1.6

cells incubated with 10 mM glycerol (Tables III and IV). During 15 min incubation, the [ATP]/[ADP] decreased from 4.2 to 1.6 and the energy charge declined from 0.76 to 0.58, while the redox state of the mitochondrial NAD couple and of cytochrome *c*, the respiratory rate, and the [ATP]/[ADP] · [P_i] remained essentially unaltered (Table IV).

The effect of concentration of inorganic phosphate in the growth medium on the ATP, ADP, and P_i levels in C. utilis cells

The effect of varying the concentration of phosphate in the growth medium on the levels of ATP, ADP, and P_i in *C. utilis* cells is shown in Tables V–VII. The levels of the adenine nucleotides and [P_i] were determined in the "starved" cells (i.e. those in the absence of added substrate after a preliminary 20 min oxygenation), in cells supplied with either glucose or ethanol or with both. As seen from the tables, the most pronounced differences among cells grown on various [P_i] were in the levels of intracellular ATP and P_i. In cells grown in the presence of 50 mM P_i, the concentration of the intracellular phosphate rose to above 25 μmol/g wet weight while it was only about 10–13 μmol/g wet weight in cells grown on 5 mM P_i, and 3–4 μmol/g wet weight in cells grown on 1 mM P_i. It should be mentioned here that cells once separated from the growth medium were subsequently maintained in a medium containing no added phosphate. However, repeated washings and incubations as described in the method section did not induce any leakage of [P_i] into the environment (unpublished experiments). Moreover, the observation that the concentration of the intracellular P_i is higher than in the suspending medium (as in cells grown on 5 or on 1 mM P_i) is in

TABLE V

METABOLITE CONCENTRATIONS OR METABOLITES RATIO IN *C. UTILIS* YEAST GROWN IN 50 mM PHOSPHATE MEDIUM

C. utilis yeast was grown in the medium described in the method section containing 50 mM phosphate. The yeast cells were treated as described and the metabolites were assayed on the quenched samples. Concentrations of ATP, ADP and P_i are expressed in μmol/g wet weight. Values are mean $\pm$S.E. for three different experiments: $\approx$ 70 mg wet weight cells/ml.

Condition	ATP	ADP	P_i	ATP/ADP	ATP/ADP · P_i
"Starved"	2.78±0.05	0.28±0.03	25.1±2.8	10.1±1.2	413±21
25 mM ethanol	4.17±0.07	0.12±0.004	25.9±1.9	33.8±1.4	1322±151
50 mM glucose					
0.5 min	3.89±0.09	0.14±0.02	23.6±2.2	28.8±5.1	1254±328
1 min	3.84±0.09	0.16±0.02	24.6±1.1	23.9±2.6	966±98
2 min	3.69±0.15	0.18±0.02	19.9±1.8	20.9±2.9	1066±212
5 min	2.99±0.13	0.23±0.004	15.7±0.24	13.3±0.82	846±40
10 min	2.47	0.27	10.5	9.1	871
50 mM glucose +25 mM ethanol					
0.5 min	4.20±0.06	0.13±0.02	23.6±2.2	35.2±7.9	1520±419
1 min	3.98±0.12	0.17±0.004	22.9±2.5	22.9±0.8	1016±81
2 min	3.93±0.12	0.17±0.008	20.4±0.9	22.8±1.8	1120±88
5 min	3.18±0.07	0.2 ±0.008	16.6±0.2	15.9±0.33	962±31
10 min	2.66	0.22	12.0	12.1	1001

TABLE VI

METABOLITE CONCENTRATIONS OR METABOLITES RATIO IN *C. UTILIS* YEAST CELLS GROWN IN 5 mM PHOSPHATE MEDIUM

The yeast cells were grown in the medium containing 5 mM P_i as described in the methods section. The yeast cells were suspended in the salt medium and aerated for approx. 20 min prior to the onset of the experiment. Concentrations of ATP, ADP and P_i are expressed in μmol/g wet weight. Values are mean $\pm$S.E. for three experiments: $\approx$ 70 mg wet weight cells/ml.

Condition	ATP	ADP	P_i	ATP/ADP	ATP/ADP · P_i
"Starved"	1.23±0.008	0.16±0.008	12.9±1.7	7.7±0.3	619±109
25 mM ethanol	1.32±0.04	0.12±0.004	11.6±1.8	11.5±0.7	1109±4
50 mM glucose					
0.5 min	1.36±0.008	0.12±0.01	8.3±0.29	12.1±1.4	1454±113
1 min	1.41±0.05	0.12±0.01	7.1±0.2	12.6±1.8	1472±200
2 min	1.39±0.09	0.13±0.02	6.8±0.5	11.8±2.7	1710±259
5 min	1.18±0.016	0.15±0.016	5.5±0.04	8.0±0.7	1465±129
50 mM glucose +25 mM ethanol					
0.5 min	1.29±0.03	0.13±0.000	7.8±0.08	9.9±0.3	1268±42
1 min	1.36±0.02	0.14±0.01	7.5±0.53	10.2±1.1	1362±47
2 min	1.41±0.05	0.16±0.02	6.9±0.41	9.3±0.9	1340±52
5 min	1.31±0.03	0.21±0.05	6.2±0.65	7.1±1.7	1111±151

TABLE VII

METABOLITE CONCENTRATIONS OR METABOLITES RATIO IN *C. UTILIS* YEAST CELLS GROWN IN THE PRESENCE OF 1 mM PHOSPHATE

The yeast cells were grown in the medium containing 1 mM P_i as described in the methods section. The cells were suspended in 0.1 M NaCl, 0.02 M KCl, 0.05 M morpholinopropane sulfonate medium at pH 7.0 and oxygenated for approx. 20 min prior to the addition of substrates. Concentrations of ATP, ADP, and P_i are expressed in μmol/g wet weight. Values are mean $\pm$S.E. for three independent experiments; $\approx$ 70 mg wet weight cells/ml.

Condition	ATP	ADP	P_i	ATP/ADP	ATP/ADP · P_i
"Starved"	1.31±0.12	0.15±0.02	3.7±0.3	8.9±0.5	2439±254
25 mM ethanol	1.22±0.07	0.15±0.02	2.9±0.7	8.3±0.7	3140±602
50 mM glucose					
0.5 min	1.56±0.07	0.18±0.02	1.4±0.2	9.0±0.7	6620±849
1.0 min	1.57±0.14	0.19±0.01	1.3±0.3	8.4±0.3	7539±2118
2.0 min	1.72±0.11	0.18±0.01	1.5±0.3	9.4±0.5	8591±605
5.0 min	1.57±0.19	0.17±0.02	2.3±0.3	9.5±0.3	4215±596
25 mM ethanol + 50 mM glucose					
0.5 min	1.46±0.03	1.14±0.01	2.1±0.2	10.4±0.3	4952±385
1.0 min	1.62±0.17	0.19±0.01	1.3±0.1	8.6±0.5	6984±415
2.0 min	1.82±0.19	0.21±0.03	1.4±0.3	8.9±0.2	7059±519
5.0 min	1.79±0.17	0.18±0.02	1.8±0.4	10.0±0.1	6250±619

accord with the postulate of Goodman and Rothstein [11] that the uptake of phosphate occurs against a concentration gradient.

The increase in the intracellular $[P_i]$ was paralleled by an increase in the level of ATP, which rose from 1.2 to 1.6 μmol/g wet weight in cells grown on either 5 or 1 mM P_i to about 4 μmol/g wet weight in cells grown in medium containing 50 mM phosphate. The concentrations of ADP were 0.1–0.25 μmol/g wet weight, irrespective of whether the cells were grown in the high or in the low phosphate medium. The concentrations of AMP were below 0.02 μmol/g wet weight and could not be measured with sufficient accuracy to provide meaningful results. These low values of AMP are consistent with the adenylate kinase reaction being at near equilibrium. Since the ATP levels were much higher in cells grown in 50 mM phosphate medium as compared to those grown in media containing less phosphate while the ADP concentrations were almost the same, the [ATP]/[ADP] ratios were significantly higher in cells containing high intracellular levels of P_i and values of $\geqslant$ 30 were commonly observed. In spite of these high [ATP]/[ADP] ratios found in cells grown in medium containing 50 mM P_i, the [ATP]/[ADP] · $[P_i]$ ratios were the same in cells grown on either 50 or 5 mM P_i because the increase in the intracellular $[P_i]$ in the former case compensated for the increase in the [ATP]/[ADP]. The numerical values for the [ATP]/[ADP] · $[P_i]$ were between $1 \cdot 10^3$ and $2 \cdot 10^3$ M^{-1}, similar to those found in other types of cells (see e.g. refs. 2 and 3).

A detailed examination of Table V–VII allows us to evaluate the effect of substrate addition on the [ATP]/[ADP] and [ATP]/[ADP] · $[P_i]$ ratios in cells grown on the same $[P_i]$. Generally "starved" cells showed a tendency to have lower [ATP]/

TABLE VIII

THE EFFECT OF PREINCUBATION IN EXTRACELLULAR [P_i] ON THE METABOLITE CONCENTRATIONS IN *C. UTILIS* CELLS GROWN IN THE PRESENCE OF 1 mM P_i

C. utilis cells were grown in the medium containing 1 mM P_i, washed and suspended in the salt medium supplemented with 20 mM P_i. (For the control experiment, the inorganic phosphate was omitted.) Cells were oxygenated for 20 min prior to the addition of 25 mM ethanol. Concentrations of ATP, ADP and P_i are expressed in μmol/g wet weight. Values are means $\pm$S.E. for three experiments.

Conditions	ATP	ADP	P_i	ATP/ADP	ATP/ADP · P_i
Control	1.14±0.03	0.13±0.003	3.3±0.05	8.8±0.1	2666±122
Incubated in 20 mM P_i	1.91±0.05	0.16±0.003	15.0±0.2	11.9±0.2	793±125

[ADP] and [ATP]/[ADP] · [P_i] ratios. This was especially pronounced in cells grown in the presence of 50 mM P_i in which the concentration of endogenous substrate was usually very low (see below). There was no significant difference in the [ATP]/[ADP] · [P_i] ratios among cells supplied with either ethanol or glucose or with both. When the concentration of intracellular P_i was decreased during the metabolism of glucose, the [ATP]/[ADP] declined so that the [ATP]/[ADP] · [P_i] was maintained constant at a value between $1 \cdot 10^3$ and $2 \cdot 10^3$ M^{-1}.

An interesting situation which demands a separate comment was found in cells grown in the presence of 1 mM P_i and later incubated in the phosphate-free medium. The concentration of the intracellular P_i was approx. 3–4 μmol/g wet weight in the "starved" or ethanol-supplemented cells. The [ATP]/[ADP] ratios were slightly lower [7–9] than in cells grown with 5 mM P_i while the [ATP]/[ADP] · [P_i] ratios were somewhat higher ($2 \cdot 10^3$–$3 \cdot 10^3$ M^{-1}). The addition of glucose induced an abrupt fall in the intracellular [P_i] which was, however, not compensated by a decline in the [ATP]/[ADP] which, on the contrary, even showed a tendency to rise. Consequently, the [ATP]/[ADP] · [P_i] rose to values between $6 \cdot 10^3$ and $9 \cdot 10^3$ M^{-1}.

When these cells were incubated for 20 min in the medium containing 20 mM P_i, the concentration of intracellular P_i rose from 3.2 μmol/g wet weight to 14.8 μmol/g wet weight concomitantly with a small increase in the ATP concentration (Table VIII). The [ATP]/[ADP] ratio increased while the [ADP]/[ADP] · [P_i] decreased to the value observed in cells grown in either 50 or 5 mM P_i.

The respiratory activity measured at 22 °C with 25 mM ethanol (+ 50 mM glucose) and expressed as turnover number for cytochrome *c* (electrons/s) was: 52±5 for cells grown in 50 mM P_i, 50±3 for cells grown in 5 mM P_i and 27±0.3 for cells grown in 1 mM P_i. Preincubation of cells grown in the presence of 1 mM P_i with 20 mM phosphate (Table VIII) increased the respiratory activity to 48±0.2 e^-/s per cytochrome *c*. The significance of the low respiratory rates with ethanol and glucose of cells grown in 1 mM P_i and of the high concomitant [ATP]/[ADP] · [P_i] will be discussed elsewhere.

The effect of intracellular [P_i] on carbohydrate metabolism in C. utilis cells

The endogenous substrate which accumulates in yeast during growth is mostly glycogen and trehalose [30]. Estimates of the level of the endogenous substrate in cells

TABLE IX

GLUCOSE 6-PHOSPHATE LEVELS IN *C. UTILIS* CELLS GROWN IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF PHOSPHATE

The yeast cells were grown in the indicated concentration of P_i and incubated as described in Materials and Methods. Glucose 6-phosphate concentrations are expressed in μmol/g wet weight. Values are mean $\pm$S.E. for three independent experiments.

Conditions	50 mM P_i	5 mM P_i	1 mM P_i
"Starved"	0.12±0.008	0.40±0.05	1.18±0.07
Ethanol (25 mM)	0.67±0.1	0.90±0.13	1.21±0.04
Glucose (50 mM)			
0.5 min	3.89±0.18	2.92±0.29	2.74±0.15
1.0 min	4.37±0.27	2.85±0.22	2.67±0.11
2.0 min	4.79±0.42	3.05±0.27	2.73±0.2
5 min	4.09±0.09	3.22±0.43	3.02±0.38
Ethanol (25 mM)+ glucose (50 mM)			
0.5 min	3.67±0.24	2.31±0.19	2.20±0.12
1 min	3.86±0.23	2.35±0.25	2.30±0.26
2 min	4.32±0.29	2.96±0.24	2.75±0.29
5 min	4.16±0.31	3.50±0.29	3.50±0.42

grown under various conditions can be made indirectly by measuring the concentration of glucose 6-phosphate. Table IX shows the impact of the concentration of P_i in the growth medium on the level of glucose 6-phosphate. It is seen that after 20 min of oxygenation the cells grown in the medium containing 50 mM P_i had only about 0.12 μmol of this metabolite per g wet weight, while the cells grown on 5 mM P_i contained 0.4 μmol/g wet weight and those on 1 mM P_i, 1.2 μmol/g wet weight, values 4- and 10-fold higher, respectively. This suggests that less glycogen accumulates in cells grown in media containing high [P_i] and that high intracellular [P_i] stimulates the breakdown of glycogen which is a phosphorolytic reaction. (Preliminary results do indeed show that the concentration of glycogen is lower in cells grown in 50 mM P_i, unpublished data.)

The amount of glucose 6-phosphate formed upon the addition of glucose or glucose plus ethanol during the time course of the experiment was, however, higher in cells which contained high intracellular [P_i]. Since, in these cells, the concentration of ATP was higher than in cells grown in low phosphate media, the availability of this metabolite for the hexokinase reaction must have stimulated the phosphorylation of glucose.

DISCUSSION

The most important contribution of the present work lies in providing an insight into the mechanism of regulation of cellular homeostatis. The experiments utilizing the trapping of phosphate by the addition of fructose or glycerol demonstrate that under conditions when the [ATP]/[ADP] decreases 8-fold and the energy charge decreases from 0.77 to 0.52, the respiratory activity, the redox state of cytochrome *c*,

and the mitochondrial [NAD^+]/[NADH] remain essentially unaltered. Moreover, in *C. utilis* grown in the presence of various concentrations of phosphate, the intracellular [P_i] and the [ATP]/[ADP] ratios vary over a range of values while the respiratory activity remains essentially the same. Thus, neither the [ATP]/[ADP] nor the energy charge per se can play a crucial role in regulation of oxidative phosphorylation and other regulatory factors must be responsible for stabilization of metabolism. The most suitable candidate for this regulatory function is the [ATP]/[ADP] · [P_i] which changes little under the experimental conditions investigated.

It has been reported recently by Davis and Lumeng [31] and Küster et al. [32] that at constant [P_i] the respiratory rate of isolated mitochondria was dependent on the [ATP]/[ADP]. Although this is certainly true for their particular experimental conditions, only by systematic variations in [P_i], both in isolated mitochondria and in intact cells, can one distinguish whether respiration is dependent on the [ATP]/[ADP] or on the [ATP]/[ADP] · [P_i]. Experiments on tightly coupled mitochondria isolated from various tissues [33–35] in which the [P_i] as well as [ATP]/[ADP] were systematically varied, demonstrated that the respiratory rate was dependent on the extramitochondrial [ATP]/[ADP] · [P_i] and not on the [ATP]/[ADP] ratio. This leads to yet another important conclusion that the transport of adenine nucleotides across the mitochondrial membrane (the adenine translocase reaction) which is reported to be phosphate independent [36] cannot be the rate-limiting step in respiration in vivo.

If the [ATP]/[ADP] · [P_i] is indeed regulated under most metabolic conditions, it follows then that the concentration of inorganic phosphate is an important contributor to the regulatory mechanism. In line with this reasoning is the observation that the transport of inorganic phosphate into most cells is a strictly controlled phenomenon. In the liver cells and in *C. utilis*, the [P_i] inside the cell is higher than in the extracellular fluid, thus the entry occurs against an apparent concentration gradient. In addition, the entry of inorganic phosphate into the cell must be rate limiting at least under some experimental conditions. This is observed in the liver cells in the presence of added fructose or glycerol when a rapid intracellular utilization of inorganic phosphate is not compensated by sufficiently high rates of transport from the extracellular medium into the cell so that the intracellular P_i concentration falls to a value much lower than that of the extracellular fluid.

The ability of the cell to regulate the uptake of phosphate means that under most metabolic conditions the concentration of P_i inside the cell remains essentially constant. It follows then that if the intracellular phosphate does not vary and adenylate kinase is near equilibrium (as supported by the wealth of information summarized recently by Beis and Newsholme [37]), the [ATP]/[ADP] · [P_i] will behave essentially the same as does the energy charge; both will remain constant under most metabolic conditions. On the other hand, if the concentration of intracellular phosphate changes, the behavior of the energy charge will deviate from that of the [ATP]/[ADP] · [P_i].

To better illustrate this point, a plot of calculated values of the energy charge against the [ATP]/[ADP] · [P_i] is presented in Fig. 1. The [ATP]/[ADP] · [P_i] is expressed as ΔG of hydrolysis of ATP, the total adenine nucleotides are chosen to be 4 mM (calculated values as plotted are independent of the total adenine nucleotide concentration) and adenylate kinase is assumed to be near equilibrium ($K = 0.8$ [38]). Since the free energy of hydrolysis of ATP depends on the actual phosphate concentration, the plots are effectively three dimensional and represent the relations between the

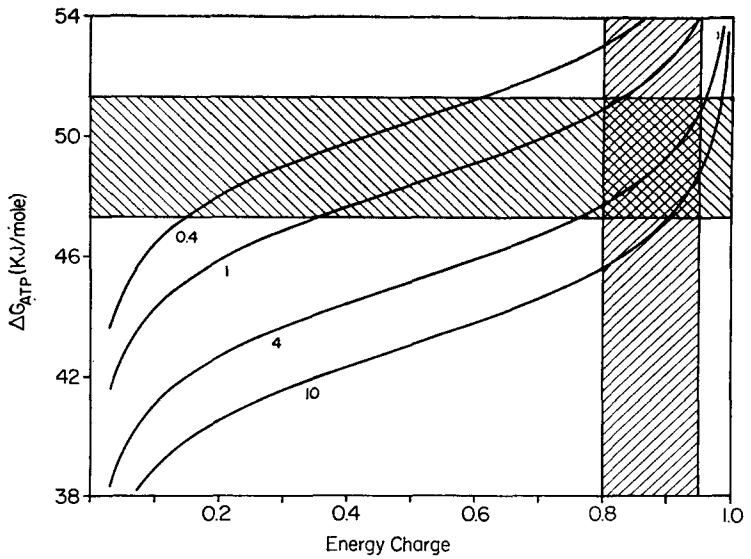

Fig. 1. Theoretical plots of the calculated energy charge against the calculated free energy of hydrolysis of ATP at four different concentrations of P_i. The vertical shaded area encompasses the values of the energy charge which are encountered in most cells in vivo (0.8–0.95). The horizontal shaded area delineates a similar physiological region for the free energy of hydrolysis of ATP (from 47.6 kJ/mol ($[ATP]/[ADP] \cdot [P_i] = 6 \cdot 10^2\ M^{-1}$) to 51.5 kJ/mole ($[ATP]/[ADP] \cdot [P_i] = 3 \cdot 10^3\ M^{-1}$)).

two parameters at four different P_i concentrations, specified in the figure (0.4, 1.0, 4.0, and 10 mM).

The vertical shaded area indicates the values for energy charge which are found in most cells in vivo (0.8–0.95), while the horizontal shaded area encompasses the physiological range for the free energy of hydrolysis of ATP (from 47.6 to 51.5 kJ/mol which corresponds to the $[ATP]/[ADP] \cdot [P_i]$ between $6 \cdot 10^2$ and $3 \cdot 10^3\ M^{-1}$). (Note that as the energy charge changes from 0 to 1, the ΔG of ATP hydrolysis changes from minus infinity to positive infinity.) It is seen that under most metabolic conditions, when the intracellular $[P_i]$ remains constant at the value characteristic for each type of cell, the values for the energy charge and the free energy of hydrolysis of ATP fall within a very narrow range common to both (cross-hatched region in Fig. 1).

The independence of the value for the energy charge from $[P_i]$ means that a change in the latter does not involve any readjustments in the adenine nucleotide pool. On the other hand, in order to maintain the $[ATP]/[ADP] \cdot [P_i]$ constant if the $[P_i]$ varies, the [ATP]/[ADP] must change. Moreover, the theoretical curves for the free energy of hydrolysis of ATP at four different phosphate concentrations allow us to predict that in cells in which the intracellular $[P_i]$ is higher, higher [ATP]/[ADP] are to be expected in order to maintain the $[ATP]/[ADP] \cdot [P_i]$ constant. The experimental results from this work and those of Woods et al. [26] and Raivio et al. [25], obtained at different intracellular $[P_i]$ (Table X and Fig. 2), show that as the intracellular phosphate is varied the $[ATP]/[ADP] \cdot [P_i]$ remains constant through compensating changes in the [ATP]/[ADP] (as illustrated by the movements of the experimental points to the left, but within the shaded area of the physiological values which is proposed to be maintained in functionally competent cells).

TABLE X

THE [ATP]/[ADP] · [P_i], THE ENERGY AND THE P_i CONCENTRATION IN THE LIVER CELLS OR FREEZE-CLAMPED LIVER INCUBATED OR PERFUSED WITH FRUCTOSE OR GLYCEROL

Substrate	Material	Time of incubation (min)	[ATP]/[ADP] · [P_i] (M^{-1})	Energy charge	[P_i] mM	Refs.
Fructose	Freeze-clamped liver	0	670	0.80	4.25	[26]
		10	463	0.61	1.67	[26]
		40	970	0.76	3.97	[26]
Fructose	Freeze-clamped liver	0	1980	0.87	3.86	[25]
		1	2036	0.79	1.89	[25]
		2	1946	0.69	1.28	[25]
		5	2140	0.72	1.29	[25]
		30	1006	0.88	5.42	[25]
Fructose	Liver cells	0	1056	0.77	3.91	This work
		1	1844	0.70	1.09	This work
		2	1148	0.63	0.88	This work
		5	793	0.52	0.63	This work
Glycerol	Liver cells	0	1346	0.76	3.38	This work
		2	1209	0.68	1.39	This work
		5	1380	0.65	1.01	This work
		15	1277	0.58	0.80	This work

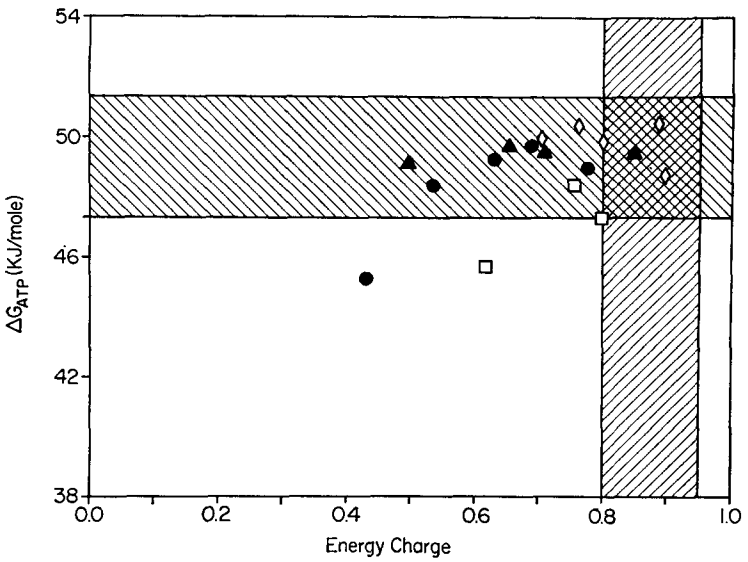

Fig. 2. Plots of the energy charge vs. the free energy of hydrolysis of ATP for the liver cells or perfused liver incubated with fructose or glycerol. The numerical values are summarized in Table VIII. □, Data of Woods et al. [26]; ◇, data of Raivio et al. [25]; ●, liver cells incubated with 10 mM fructose (this work); ▲, liver cells incubated with 10 mM glycerol (this work).

The metabolic importance of the intracellular phosphate so clearly observed in this work has not been adequately recognized in most studies, in part because of difficulties in measuring its concentration. Discussions of cellular energetics and metabolic regulation are not very fruitful, however, without this information particularly in cells (i.e. free living micro-organisms) and tissues (i.e. muscle) in which large changes in intracellular phosphate concentrations can occur. It should be pointed out here that in tissues in which maintaining a nearly constant level of ATP is required from metabolic reasons, the stimulation of respiration can occur effectively through the changes in $[P_i]$ (i.e. hydrolysis of phosphocreatine to creatine and phosphate) as those would tend to decrease the $[ATP]/[ADP] \cdot [P_i]$.

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REFERENCES

1 Erecińska, M., Veech, R. L. and Wilson, D. F. (1974) Arch. Biochem. Biophys. 160, 412–421
2 Wilson, D. F., Stubbs, M., Veech, R. L., Erecińska, M. and Krebs, H. A. (1974) Biochem. J. 140, 57–64
3 Wilson, D. F., Stubbs, M., Oshino, N. and Erecińska, M. (1974) Biochemistry 13, 5305–5311
4 Hassinen, I. E. and Hiltunen, K. (1975) Biochim. Biophys. Acta 408, 319–330
5 Wilson, D. F., Erecińska, M. and Dutton, P. L. (1974) Ann. Rev. Biophys. Bioeng. 3, 203–230
6 Atkinson, D. E. (1966) Annu. Rev. Biochem. 35, 85–124

7 Atkinson, D. E. (1968) Biochemistry 7, 4030–4034
8 Atkinson, D. E. (1971) in Metabolic Pathways (Vogel, H. J., ed.), Vol. V, pp. 1–21, Academic Press, New York
9 Suomalainen, H. and Oura, E. (1971) in The Yeast (Rose, A. H. and Harrison, J. S., eds.), Vol. 2, pp. 3–74, Academic Press, New York
10 Rothstein, A. (1963) J. Gen. Physiol. 40, 915–923
11 Goodman, J. and Rothstein, A. (1957) J. Gen Physiol. 40, 915–923
12 Wiame, J. M. (1947) Biochim. Biophys. Acta 1, 234–255
13 Berry, M. N. and Friend, D. S. (1969) J. Cell Biol. 43, 506–520
14 Cornell, N. W., Lund, P., Hems, R. and Krebs, H. A. (1973) Biochem. J. 134, 671–672
15 Krebs, H. A., Cornell, N. W., Lund, P. and Hems, R. (1974) in Alfred Benzon Symposium VI, pp. 718–743, Munksgaard, Copenhagen
16 Hems, R., Lund, P. and Krebs, H. A. (1975) Biochem. J. 150, 47–50
17 Williamson, D. H., Lund, P. and Krebs, H. A. (1967) Biochem. J. 103, 514–527
18 Williamson, D. H., Mellanby, J. and Krebs, H. A. (1962) Biochem. J. 82, 90–96
19 Krebs, H. A., Mellanby, J. and Williamson, D. H. (1962) Biochem. J. 82, 96–98
20 Lamprecht, W. and Trautschold, I. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 543–551, Academic Press, New York
21 Adam, H. (1963) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), pp. 573–577, Academic Press, New York
22 Martin, J. B. and Doty, D. M. (1949) Anal. Chem. 21, 965–967
23 Farber, E. (1973) Fed. Proc. 32, 1534–1539
24 Maënpää, P. H., Raivio, K. O. and Kekomaki, M. P. (1968) Science 161, 1253
25 Raivio, K. O., Kekomäki, M. P. and Maënpää, P. H. (1969) Biochem. Pharmacol. 18, 2615–2624
26 Woods, H. F., Eggleston, L. V. and Krebs, H. A. (1970) Biochem. J. 119, 501–510
27 Burch, H. B., Lowry, O. H., Meinhardt, L., Max, P. and Chyu, K. (1970) J. Biol. Chem. 245, 2032–2102
28 Woods, H. F. and Krebs, H. A. (1973) Biochem. J. 132, 55–60
29 Yushok, W. D. (1971) J. Biol. Chem. 246, 1607–1617
30 Manners, D. J. (1971) in The Yeast (Rose, A. H. and Harrison, J. S., eds.) Vol. 2, pp. 419–439, Academic Press, New York
31 Davis, J. E. and Lumeng, L. (1975) J. Biol. Chem. 250, 2275–2282
32 Küster, U., Bohnensack, R. and Kunz, W. (1976) Biochim. Biophys. Acta 440, 391–402
33 Klingenberg, M. (1969) in The Energy Level and Metabolic Control in Mitochondria (Papa, S., Tager, J. M., Quagliariello, E. and Slater, E. C., eds.), pp. 189–193, Adriatica Editrice Bari
34 Wilson, D. F., Owen, C. S., Mela, L. and Weiner, L. (1973) Biochem. Biophys. Res. Commun. 53, 326–333
35 Holian, A., Wilson, D. F. and Owen, C. S. (1977) Arch. Biochem. Biophys., 181, 164–171
36 Dúee, E. D. and Vignais, P. V. (1969) J. Biol. Chem. 244, 3920–3931
37 Beis, I. and Newsholme, E. A. (1975) Biochem. J. 152, 23–32
38 Markland, F. S. and Wadkins, C. L. (1966) J. Biol. Chem. 241, 4136–4145