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PROCESSES INVOLVED IN THE CREATION OF BUFFERING CAPACITY AND IN SUBSTRATE-INDUCED PROTON EXTRUSION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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Summary

The high pH-maintaining capacity of yeast suspension after glucose-induced acidification, measured as its ability to neutralize added alkali, was found to be due mainly to actively extruded acidity (H^+). The buffering action of passively excreted metabolites (CO_2, organic acids) and cell surface polyelectrolytes contributed only 15—40% to the overall pH-maintaining capacity which was 10 mmol NaOH/l per pH unit between pH 3 and 4 and 3.5 mmol NaOH/l per pH unit between pH 4 and 7. The buffering capacity of yeast cell-free extract was still higher (up to 4.5-times) than that of glucose-supplied cell suspension; addition of glucose to the extract thus produced considerable titratable acidity but negligible net acidity. The glucose-induced acidification of yeast suspension was stimulated by univalent cations in the sequence $K^+ > Rb^+ >> Li^+ \simeq Cs^+ \simeq Na^+$. The processes participating in the acidification and probably also in the creation of extracellular buffering capacity include excretion of CO_2 and organic acids, net extrusion of H^+ and K^+ (in K^+-free media; in K^+-containing media this is preceded by an initial rapid K^+ uptake), and movements of some anions (phosphate, chlorides). The overall process appears to be electrically silent.

Introduction

Our preceding study [1] was concerned with the basic features of substrate-induced proton extrusion in *Saccharomyces cerevisiae*: the probable source of protons, acidification kinetics, multiplicity of proton transport systems, factors governing the total extent and rate of acidification, control of proton efflux by external pH and probable modes of this control. No attempt was made to char-

acterize in more detail the individual systems involved in proton extrusion. In the present study, examination and analysis of extra- and intracellular buffering action of the cells and of the relationship between the net fluxes of H^+, K^+, and other ions across the plasma membrane were used to obtain information on these features.

Materials and Methods

The experiments were performed with an aneuploid strain *Saccharomyces cereivisae* K derived from distillery yeast. The maintenance and cultivation of the strain, as well as the basic experimental technique, have been described previously [1].

The buffering capacity of yeast suspensions, suspension filtrates and cell-free extracts was determined by titration with 0.1 M NaOH; after each alkali addition the pH was allowed to return to a constant level.

Extracellular glycolytic metabolites were determined by gas chromatography as described elsewhere [2].

Cell-free extracts were prepared according to [3] using a Soniprobe 1130A sonicator (Dawe Instruments, London). Their metabolic activity was measured by the direct Warburg method. Titratable acidity produced by glucose fermentation was determined by incubating 1.8 ml cell-free extract with 0.2 ml 1 M D-glucose for 10 min under continuous pH monitoring, adding 1 mM *N*-ethylmaleimide to stop fermentation and titrating with 0.1 M NaOH to pH 7.0.

Extracellular level of K^+ was assayed by flame photometry on an EEL instrument (Evans Electroselenium Ltd., Halstead, U.K.) or by a K^+-selective electrode with membrane made of polymer-embedded AY-10 crown ether.

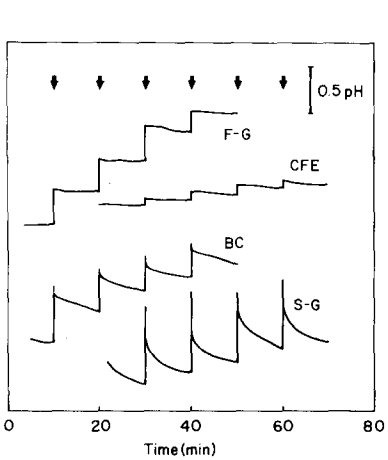
Suspensions of dead cells were obtained by 5-min boiling on a water bath. The leakiness of their plasma membranes was tested by checking the amount of substances absorbing at 280 nm in the medium.

Results

Titration of suspension components with alkali

Fig. 1 illustrates the time course of titration of whole yeast suspension and some of its components with 0.1 M NaOH. The time course of titration of suspension filtrate or cell-free extract has a simple 'staircase' character. However, on adding alkali to a suspension of dead cells the pH is seen to rise and then return slowly to a final, lower value. This effect is still much more conspicuous in a suspension of intact cells and has the form of an initial pH 'overshoot' and a subsequent rapid pH recovery. This recovery indicates the participation of a process which is absent in a cell-free extract and suspension filtrates but still retained to some degree in a suspension of boiled cells, e.g. delayed buffering by cell components.

Fig. 2 shows the titration curves of suspension components obtained by plotting the final pH values after each alkali addition against alkali concentration. The difference between the titration curve for distilled water and the theoretical curve calculated on the assumption of zero buffering capacity of water shows the considerable effect of dissolved CO_2 on the pH of water. A



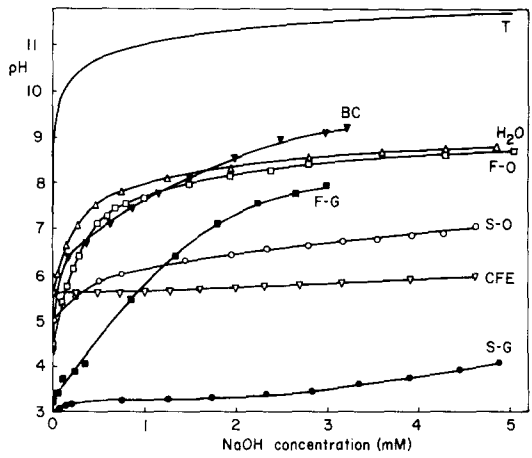


Fig. 1. Typical courses of titration of yeast suspension filtrate after flucose addition (F-G), cell-free extract (CFE), suspension of boiled cells (BC) and intact suspension after glucose addition (S-G) by 0.1 M NaOH. Suspension dry weight 10 mg/ml, cell-free extract adjusted accordingly. Alkali additions (about 0.2 mmol/l) indicated by arrows.

Fig. 2. Titration curves of yeast suspension components. Suspension dry weight 26 mg/ml, cell-free extract adjusted accordingly, 50 mM D-glucose. T, theoretical curve for unbuffered medium; H_2O, water; F-O, suspension filtrate before glucose addition; F-G, suspension filtrate after acidification; S-O, whole suspension without glucose; S-G, whole suspension after acidification; BC, suspension of boiled cells; CFE, cell-free extract.

titration of 10 ml water stripped of CO_2 by 2 h vigorous stirring in a closed manometric vessel, the side-arm of which contained 1 ml of 2 M NaOH, produced a curve essentially identical with the theoretical one. The buffering capacity of suspension filtrate in the absence of glucose is only slightly higher than that of water; after acidification due to glucose addition the buffering capacity increases considerably. Cell-free extracts have an extremely high buffering capacity; the implications of this finding will be discussed below.

In a preliminary communication [4] the titration of whole yeast suspension before and after acidification was found to indicate a high, constant buffering capacity, the titration curves being in both cases approximated by straight lines. A more careful analysis showed, however, that the titration of cell suspension before acidification to pH about 6 yielded a conventional titration curve, albeit with high pH-maintaining capacity. In contrast, the titration curve of the suspension after complete glucose-induced acidification exhibited an initial linear segment of very high buffering capacity; at pH about 4 the line bent upwards and its following part was also approximately linear in a considerable pH range. It should be noted that at pH 4 changes occur also in other characteristics of the proton extrusion system [1].

Proton fluxes and acidification extents during titration

The shape of the suspension titration curves was found to depend on the mode of alkali addition, i.e. on the magnitude of individual NaOH doses added. With small doses the pH-maintaining capacity was sufficient to restore the sus-

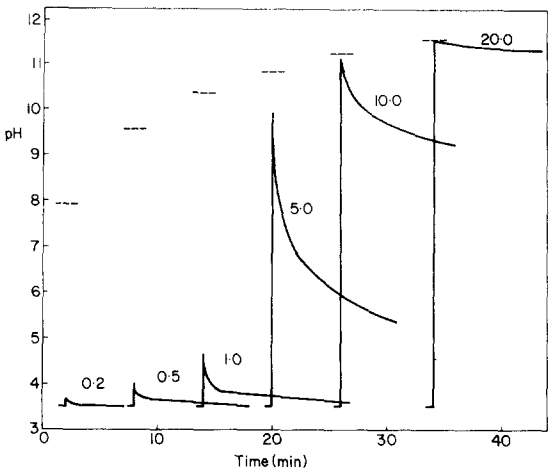


Fig. 3. Ability of yeast suspension supplied with glucose to neutralize different doses of alkai. 10 ml suspension (dry weight 9 mg/ml) was acidified by adding 40 mM D-glucose. When the pH reached 3.5 the suspension was supplied with a single dose of 0.1 M NaOH to reach the NaOH concentration (mmol · l^{-1}) indicated by the number at the appropriate curve. Dashed lines indicate pH values obtained by adding the same dose of alkali to 10 ml water previously acidified to pH 3.5 by HCl.

pension pH; if the doses were too large, the pH-restoring ability of the cells failed and the pH was irreversibly increased (Fig. 3).

In a preceding study [1] the substrate-induced acidification was studied under two sets of conditions: (1) 'physiological', i.e. under full control by external pH, and (2) 'pH-stat', when the pH_{out}-control was abolished by maintaining external pH artificially constant. During the titration by discrete NaOH doses used here the cells were subjected to still another set of conditions; the pH_{out}-control was not permanently lifted but the cells were challenged by a large dose of alkali which increased the pH above commonly found values. The 'titration' proton efflux, J_t, observed under these conditions, was considerably higher than the 'physiological' flux J_a though not as high as the 'pH-stat' flux J_s (Table I). It should be noted that a higher flux does not automatically imply a higher total extent of acidification. This is caused by the different time course

TABLE I

PROTON EFFLUX AND TOTAL EXTENT OF ACIDIFICATION IN GLUCOSE-SUPPLIED *SACCHAROMYCES CEREVISIAE* UNDER DIFFERENT CONDITIONS

Suspension dry weight 15 mg/ml, 50 mM D-glucose; pH-stat measurements and titrations performed with 0.1 M NaOH.

Conditions	Specific net proton efflux at pH 6 (pmol · s^{-1} · cm^{-2})		Total acidity produced for pH change 3.5 ↔ 6.0 (mmol H^+ · l^{-1}) *	
Physiological	J_a	1.0	A_a	0.32
pH-stat	J_s	51.6	A_s	2.58
Titration	J_t	9.8	A_t	6.05

* For explanation see text.

of acidification curves, pH-stat curves and titration curves. Also, values A_a, A_s and A_t in Table I have been calculated in a different way. While A_a represents a simple difference between proton concentrations at the beginning and at the end of acidification (pH 6.0 → 3.5), A_s is calculated as the amount of NaOH consumed in pH-stat experiment at pH 6.0 during the interval necessary for a pH drop of 6.0 → 3.5 in a parallel acidification experiment, and A_t is the amount of NaOH necessary in titration to produce a permanent rise in suspension pH from 3.5 to 6.0.

Buffering systems

The process of acidification and the attendant proton extrusion from cells includes the action of several systems exerting a buffering or pH-maintaining effect. These include:

A. Water with its content of dissolved CO_2 and possibly other substances.

B. CO_2 extruded from cells during glycolysis plus bicarbonate formed by its hydration at pH above 4.

C. Acidic metabolites (succinic, malic, lactic and acetic acids) extruded into the medium [5,6]; their pK_a values range from 3.5 to 5.2. Table II lists the levels of some of these acids in yeast suspension at various times after glucose addition.

D. Polyelectrolytes fixed on the cell surface [7].

E. Active membrane processes maintaining constant pH_{out} in the absence of glucose, energized by endogenous sources.

F. Active membrane processes functional in the presence of glucose and energized by its dissimilation (processes E and F need not necessarily be identical).

The quantitative role of all these systems was assessed in a series of titration experiments (Table III). The values of buffering capacities of suspension components, measured as slopes of titration curves, were determined at two pH values corresponding to the two components of the titration curve of the whole suspension after acidification. At $pH_{initial}$ (cf. Table III) factors A—E are seen to play an insignificant role, accounting together for a mere 15—17% of the total buffering capacity of the suspension. At pH 6, corresponding to the low-capacity part of titration curve S-G, these factors amount to about 40% of total

TABLE II

CONCENTRATIONS OF MAIN EXTRUDED ORGANIC ACIDS IN YEAST SUSPENSION IN INITIAL PHASES OF GLUCOSE-INDUCED ACIDIFICATION

Suspension dry weight 120 mg/ml, 200 mM D-glucose, detection of acids performed by gas chromatography [2]. No significant amounts of other acids (acetic, glyceric, etc.) were found in the medium during the experimental interval.

Time after glucose addition (min)	Concentration of extruded acid (mmol · l^{-1})		
	Succinic	Malic	Lactic
1	—	—	0.20
2	—	0.26	0.08
5	0.33	0.18	0.20
10	0.72	0.98	0.51

TABLE III

BUFFERING CAPACITIES β OF CELL SUSPENSION COMPONENTS MEASURED BY TITRATION WITH NaOH

Suspension dry weight 25—30 mg/ml. 100 mM D-glucose, titration with 0.1 M NaOH (cf. Fig. 2); β determined as tangent to titration curve at given pH. All values are means of six measurements. $pH_{initial}$ is the pH level at the beginning of titration.

Variant	$pH_{initial}$	β at $pH_{initial}$		β at pH 6.0	
		Absolute ($\beta_{(A)}$) (mmol NaOH/l per pH unit)	Relative to $\beta_{(A)}$ (%)	Absolute ($\beta_{(A)}$) (mmol NaOH/l per pH unit)	Relative to $\beta_{(A)}$ (%)
Suspension after glucose addition (A)	3.0	9.6	100	3.5	100
Suspension without glucose (B)	5.1	1.0	10	1.1	30
Filtrate of (A)	3.5	0.4	4	0.5	15
Filtrate of (B)	5.0	0.2	3	0.2	6
Suspension of dead cells	4.3	0.2	2	0.3	8
Water	5.5	0.03	1	0.03	1
Cell-free extract	6.1	11.4	119	15.7	445

buffering capacity. In any case, the majority of the yeast's buffering or pH-maintaining power is seen to be due to active membrane processes supported by exogenous sources (factor F).

Table III and Figs. 1 and 2 show also the results obtained with cell-free extracts. The extracts have a considerable glycolytic activity which is increased 7- to 8-times on glucose addition (Table IV). Their buffering capacity is high and nearly constant. The titration showed that the titratable acidity produced by 10-min glycolysis of 200 μmol D-glucose by 1.8 ml cell-free extract (90 μg protein/ml) was about 4 μmol H^+, i.e. about 2% of glucose added (assuming proton/glucose stoichiometry of 1 : 1). This glucose-produced acidity increased the total acidity of the extract by about 8%. This rise was not further increased

TABLE IV

GLYCOLYTIC, ACIDITY-PRODUCING AND BUFFERING CAPACITY OF CELL-FREE EXTRACT FROM *SACCHAROMYCE CEREVISIAE*

Cell-free extract (90 μg protein per ml) was prepared as described in Materials and Methods; 100 mM D-glucose, 1 mM ATP, 1 mM NADH, 1 mM $MgCl_2$.

Variant	pH	CO_2 production * (μl/100 mg protein)	NaOH consumption	
			(μmol/100 mg protein)	%
Extract	5.68	4.4	27.2	100
Extract + glucose	5.61	41.5	29.5	109
Extract + glucose + ATP + NADH + $MgCl_2$	5.68	32.7	29.6	109

* Determined 60 min after glucose addition.

on adding, besides glucose, 1 mM ATP, 1 mM $MgCl_2$ and 1 mM NADH; this addition caused merely a transient rise in CO_2 production followed by a drop below the level observed with glucose alone (Table IV).

Despite the acidity produced, the pH of the extract remained constant throughout the glucose fermentation.

Effect of univalent cations

Table V illustrates the well-known stimulating effect of alkali metal ions on the extent of proton extrusion. K^+ and Rb^+ caused a strong stimulation while Li^+, Na^+ and Cs^+ had only slight effect even at high concentrations. K^+ are also known to stimulate metabolic processes via K^+-dependent enzymes such as acetaldehyde dehydrogenase and acetyl-CoA synthetase [8]. Stimulation by K^+ and Rb^+ is observed whether the ion is added before or after acidification. If added before, the stimulation seems to be due to prolongation of component C_2 of the acidification curve [1]. As seen from Table V, the addition of about 15 mM ion caused an increase in the acidification extent and thus a drop of the final pH_{out} attained by the addition of glucose alone, to a new lower value. The stimulation of acidification due to the ions is sometimes 1—1.5 pH units, sometimes zero. Thus some yeast batches supplied with glucose reach the ultimate lowest pH_{out} spontaneously even in the absence of stimulating ions while others have to be supplied with K^+ or Rb^+ in order to reach it. The reasons for this difference will be the subject of further investigation.

H^+ versus K^+ movements during acidification

The strong stimulation of proton extrusion caused by K^+ induced us to study the interaction of fluxes of the two ions in some detail. Fig. 4 shows extracellu-

TABLE V

EFFECT OF UNIVALENT CATIONS ON THE EXTENT OF GLUCOSE-INDUCED ACIDIFICATION IN *SACCHAROMYCES CEREVISIAE*

Suspension dry weight 10 mg/ml, 24 mM D-glucose.

Ion present	Concentration (mM)	Total acidification extent (% of the control)
No ions	—	100
Li^+	7	93
	14	111
	100	135
Na^+	7	104
	14	118
	100	120
K^+	7	252
	14	313
	100	312
Rb^+	7	170
	14	200
	100	231
Cs^+	7	93
	14	120
	100	135

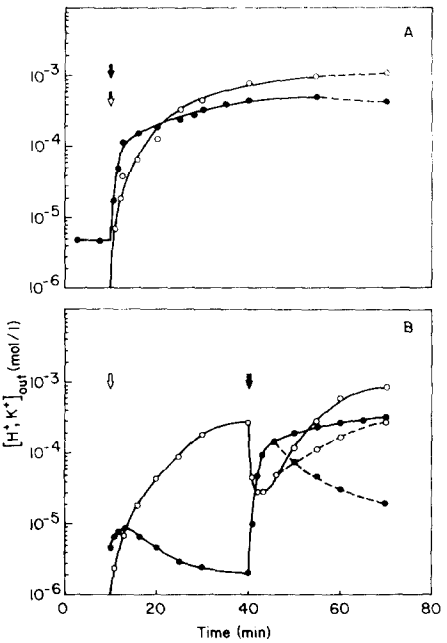


Fig. 4. Extracellular concentration of H^+ and K^+ during glucose-induced acidification of yeast suspension. Suspension dry weight 15 mg/ml, glucose concentration (A) 60 mM, (B) 60 mM (———) or 4 mM (- - - - -). ●———●, H^+; ○———○, K^+. A, glucose added at the moment of resuspension of cells; B, glucose added 30 min after resuspension. Empty arrow, cell resuspension; full arrow, glucose addition. Dashed lines in both panels reflect the situation after added glucose has been exhaused (in panel B this refers only to the case of 4 mM glucose).

lar concentrations of H^+ and K^+ measured during acidification. If glucose was added immediately after resuspension of cells in water, both H^+ and K^+ were extruded into the medium. If glucose was added 30 min after resuspension (Fig. 4B), the extracellular K^+ level increased spontaneously, reaching about 0.4 mM K^+ at the moment of glucose addition. The initial rate of proton extrusion in this case was somewhat higher while the extracellular K^+ was taken up by the cells. This apparent K^+/H^+ exchange continued until the extracellular concentrations of K^+ and H^+ were about the same, then the direction of the net K^+ flow reversed and both H^+ and K^+ were extruded from the cells. This apparent K^+,H^+ symport suggests a strongly electrogenic K^+,H^+-pump or a rapid massive outflow of anion(s). The sum of K^+ and H^+ efflux is 2—4 pmol · s^{-1} · cm^{-2}, the maximum total extracellular sum of their concentrations is about 1.6 mM. The anions considered as counterions include mainly HCO_3^- and organic anions (cf. Table II). The production of CO_2 by glycolyzing cells is reported to be 240—320 pmol · s^{-1} · cm^{-2} [9,10]; the concentration of the sum of organic acids at an early stage of acidification is about 2 mM, reaching 8—10 mM by the end of the process [5]. Glucose additions was found also to cause an efflux of phosphate and influx of chlorides (to be published). These movements of anions seem to add to the total electric balance of the acidification process. With respect to quantity, the anions would thus more than suffice to maintain the acidification electrically silent.

If glucose is exhaused from the medium during acidification, proton influx sets in immediately (Fig. 4, dashed lines). On the other hand, K^+ continues to be extruded from the cells, though at a lower rate, about equal to that observed after resuspending the cells in water without glucose.

The above results indicate an independent nature of the H^+ and K^+ fluxes during acidification.

Discussion

As shown by our present and previous [1,5] results, as well as by the data of other authors [6,10,12,13] the acidification of yeast suspension caused by glucose and other glycolytic substrates is accompanied by the extrusion of CO_2 and organic acids. It also involves the movements of ions, mostly K^+ and H^+, in a nonobligate antiport or apparent symport. The roles of these processes in different phases of the acidification are difficult to assess directly since the transport systems involved are located at the intracellular face of the plasma membrane which forms a permeability barrier for various inhibitors, probes and the like (vanadate, dicyclohexylcarbodiimide, lipophilic cations, etc.).

There are two basic processes to be considered as the major mechanisms of acidification: extrusion of a neutral substance which dissociates in the medium to yield protons, and extrusion of charged species, especially protons, which may be more or less electrically balanced by concurrently extruded anions. As shown by Janáček and Rybová [14] and Rybová et al. [15], the exponential time course of pH change [1] and the constant buffering capacity indicate a process governed by the gradient of chemical, rather than electrochemical, potential of the transported species, i.e. the transport of a neutral substance.

Moreover, the electrically silent extrusion of H^+ (and K^+) during acidification requires the presence of intracellular diffusible anions not only in sufficient quantity (as is seen to be the case) but also of sufficiently high permeability to prevent charge separation and creation of membrane potential. Although very likely [11,12], this condition has not yet been experimentally verified.

On the other hand, the transport of charged species is indicated above all by the presence of cation-activated ATPase (e.g. Ref. 11) in the yeast plasma membrane. Although its properties have been studied in purified preparations and no clear connection has been established between its in vitro properties and in vivo transport processes, it is a likely candidate for the system extruding protons (plus or versus other ions). Also, the acidification extent and time course are the same aerobically and anaerobically despite the much higher extracellular content of organic acids observed in the latter case. However, this observation can also be explained by assuming that higher extracellular level of buffering substances elicits a higher proton efflux due to a partial lifting of the pH_{out} control [1].

The measurements of buffering capacity by titration with alkali indicate also a minor role of extruded organic acids, CO_2 and cell-surface polyelectrolytes in alkali-induced acidification and a similar situation may be expected in the case of the substrate-induced process. Also, the extruded acidity cannot be due solely to organic acids and bicarbonate since the final pH_{out} is usually much lower than that attained in saturated CO_2 solutions (pH about 3.9) and also

lower than the pK_a values of the major extruded organic acids. The pH_{out} control also points to protons as the major extruded species.

The high buffering capacity of cell-free extract indicates that the acidification does not probably serve for the mere elimination of excess acidity from the cells. It rather appears that the generation of a low external pH is a process providing some important advantages for the yeast cell and, perhaps, controlling to some extent cell processes [16]. The obvious and well publicized aspect is the use of external protons (in concert with their electrochemical potential gradient) for the symport of other ions and, in particular, nonelectrolytes into the cells. To our mind, the very multiplicity of causes leading to the low external pH discussed above indicates that it is the objective (high external $[H^+]$) rather than the sources that is of survival value to the cell. This is in agreement with what is being found in various other microbial species where quite unrelated biochemical processes give rise to external acidity (cf. Ref. 17). It remains to be elucidated in what way the energy stored in the electrochemical potential of protons is used (for a caution tale see Ref. 18) and what role the attendant features of proton extrusion (such as the pH-maintaining capacity) may play in keeping the cell in its optimum state.

References

1 Sigler, K., Knotková, A. and Kotyk, A. (1981) Biochim. Biophys. Acta 643, 572—582
2 Wurst, M., Sigler, K. and Knotková, A. (1980) Folia Microbiol. 25, 306—310
3 Pye, E.K. (1969) Can. J. Bot. 47, 271—285
4 Sigler, K., Knotková, A. and Janáček, K. (1980) Bioelectrochem. Bioenerg. 7, 371—375
5 Sigler, K., Knotková, A., Páca, J. and Wurst, M. (1980) Folia Microbiol. 25, 311—317
6 Wilkinson, J.F. and Rose, A.H. (1963) in Biochemistry of Industrial Micro-organisms (Rainbow, C. and Rose, A.H., eds.), p. 393, Academic Press, London and New York
7 Katchalsky, A. (1971) Pure Appl. Chem. 26, 327—373
8 Rink, K. and Hoffmann, H. (1972) Z. Naturforsch. 27, 729—730
9 Lagunas, R. (1979) Mol. Cell. Biochem. 27, 139—146
10 Barford, J.P. and Hall, R.J. (1979) J. Gen. Microbiol. 114, 267—275
11 Serrano, R. (1980) Eur. J. Biochem. 105, 419—424
12 Conway, E.J. and Brady, T.G. (1950) Biochem. J. 47, 360—369
13 Oura, E. (1977) Process Biochem. 3, 19—21
14 Janáček, K. and Rybová, R. (1979) Studia Biophys. 73, 127—131
15 Rybová, R., Janáček, K. and Kleinová-Slavíková, M. (1980) Z. Pflanzenphysiol. 97, 5—12
16 Páca, J. (1981) Eur. J. Appl. Microbiol. Biotechnol., in the press
17 Haddock, B.A. and Hamilton, W.A. (eds.) (1977) Microbial Energetics, Cambridge University Press, Cambridge
18 Kotyk, A. (1980) in Frontiers of Bioorganic Chemistry and Molecular Biology (Ananchenko, S.N., ed.), pp. 417—426, Pergamon Press, Oxford