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Are proton symports in yeast directly linked to H^+-ATPase acidification?

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Transport of amino acids in *Saccharomyces cerevisiae* is an H^+-driven secondary active transport. Inhibitors of the plasma membrane H^+-ATPase, particularly heavy water, diethylstilbestrol and suloctidil, were shown to affect the H^+-extruding ATPase activity as well as the ATP-hydrolyzing activity, to a similar degree as they inhibited the transport of amino acids. The inhibitors had virtually no effect on the membrane electric potential or on the ΔpH which constitute the thermodynamically relevant source of energy for these transports. Transport of acidic amino acids was affected much more than that of the neutral and especially of the basic ones. The effects were greater with higher amino acid concentrations. All this is taken as evidence that the amino acid carriers respond kinetically to the presence of protons directly at the membrane site where they are extruded by the H^+-ATPase, rather than to the overall protonmotive force.

Introduction

Most transports of nutrients in various yeast species are now known to proceed in symport with H^+ ions, driven by the electrochemical potential difference of these ions [1–5]. This difference apparently arises through concerted action of several mechanisms, such as active pumping by the plasma membrane H^+-ATPase [6], release of CO_2 caused by a burst of endogenous metabolism with subsequent hydration to H_2CO_3 and dissociation to $H^+ + HCO_3^-$ [7], and a passive K^+ diffusion potential (the measured membrane potential is strongly reduced in the presence of extracellular K^+).

The resulting electrochemical potential difference $\Delta\tilde{\mu}_{H^+}$ or, after dividing with F, the protonmotive force, is thermodynamically competent to maintain the observed accumulation ratios of proton-driven solutes [8] even on assuming that only a single proton is bound to the carrier according to

$$-RT \ln(c_S''/c_S') = n[\Delta\psi F + RT \ln(c_H''/c_H')]$$

or, at 30°C,

$$c_S''/c_S' = \exp(-38.32\, n\Delta\psi - 2.3\ \mathrm{pH})$$

where c_S'' and c_S' are the solute concentrations (in mol per litre) intra- and extracellularly, n is the number of protons symported per one solute molecule, F is the Faraday constant (96485 C mol^{-1}), R is the gas constant (8.314 J mol^{-1} K^{-1}), T is the absolute temperature in Kelvin and $\Delta\psi$ the membrane potential in volt.

However, it was indicated several times before [9,10] that far from equilibrium, which is the common situation in a living cell and hence also during the transport process, the $\Delta\tilde{\mu}_{H^+}$ or the protonmotive force is not directly related either to the capacity or to the half-saturation constant of the transport system. Specifically, it was shown by using heavy water [11] that many H^+-associated transports in yeast are generally depressed by it quite substantially while neither the membrane potential nor the pH difference are appreciably affected. It is shown here that there is a much clearer link of these transports to the function of the membrane H^+-ATPase.

Materials and Methods

Strains and cultivation. The following strains of *Saccharomyces cerevisiae* were used: *S. cerevisiae* K (CCY

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21-4-60), *S. cerevisiae* Σ1278b and its *gap* mutant 2512c, lacking the general amino acid permease (the two isogenic strains were a kind gift from Prof. Marcelle Grenson of the Université libre in Brussels), and *S. cerevisiae* Y55 and its *pma1*-105 mutant, with defective plasma membrane H^+-ATPase (these were a kind gift from Prof. James F. Haber of the Rosenstiel Basic Sciences Research Center in Waltham, MA, USA). Also a strain of *Lodderomyces elongisporus* (CCY 65-1-1, ATCC 11503) was used (a kind gift from Prof. Ervin Novák of the Institute of Hygiene in Budapest).

The strains were grown aerobically at 30°C in a yeast extract-glucose medium with mineral salts as described by Kotyk [12]. After harvesting, the cells were washed in distilled water, aerated for 60 min on a magnetic stirrer, incubated for 60 min with 50 mM glucose at 30°C, washed and used immediately for the experiment. As the transport systems synthesized during incubation with glucose [13] tend to be degraded with half-times of tens of minutes [14–16] utmost standardization and speed were an essential requirement for comparability of results.

Uptake measurements. Transport of labelled solutes was estimated in 25-ml Erlenmeyer flasks agitated at 1.2 Hz in a Dubnoff reciprocal water bath, using 0.1 M triethanolamine-phthalic acid buffer of pH 5.5, aerobically at 30°C. The advantages of this buffer and the particular pH value are described in Ref. 17. The details of the procedure were described in Ref. 12.

pH determination. The extracellular pH was monitored with a Radiometer glass electrode (less than 5 mm diameter) submerged in 5 ml of suspension in a jacketed constant-temperature vessel, connected to a WGW pH-meter and a conventional flatbed recorder. The intracellular pH was estimated using the dual-wavelength excitation method with fluorescein (diacetate) as the fluorophore [18].

Membrane potential estimation. This was done by equilibrating cells at 30°C with a 75 nM solution of ^{14}C-labelled tetraphenylphosphonium chloride (see Ref. 19). The equilibrium was always achieved within less than 20 min. The potential value was calculated from the Nernst equation.

ATP hydrolysis. The ATP-hydrolyzing activity was determined in membrane fragments as described in [20] but 20 mM KNO_3 was added to block any vacuolar ATPase activity present.

Reagents. All the amino acids used were from Lachema (Czechoslovakia) and were of the highest purity available. Heavy water (D_2O) was from Merck (FRG) and was 99.9% pure. Diethylstilbestrol, miconazol, erythrosin B, suloctidil and dicyclohexylcarbodiimide were from Sigma (FRG), fusicoccin was a kind gift of Dr. Ullrich-Eberius of the Technische Hochschule in Darmstadt. All other chemicals were from Lachema (Czechoslovakia).

The ^{14}C-labelled amino acids were from the Institute for Research, Production and Uses of Radioisotopes (Czechoslovakia), with the exception of L-ornithine and 2-aminoisobutyric acid which were from Amersham International (UK). The same firm supplied ^{14}C-labelled tetraphenylphosphonium chloride.

Results and Discussion

ATPase activity

Two types of reaction can be followed in this respect, the hydrolysis of ATP by an ATPase-containing membrane preparation [20,21] in vitro and acidification of external medium upon addition of glucose [22,23]. While the former reaction appears to be a straightforward reflection of the enzyme activity, the latter is a complex process, apparently involving exchanges of cations (results prepared for publication) as well as extrusion of organic acids [24]. Hence, even the most powerful inhibitors of the ATPase reaction (such as diethylstilbestrol, suloctidil or D_2O) diminish the glucose-induced acidification only partially (Fig. 1).

The various reported inhibitors of the H^+-ATPase [25,26] do not display identical effects on ATP hydrolysis and on glucose-induced acidification (Table I) but, clearly, D_2O, diethylstilbestrol and suloctidil inhibit both processes very powerfully.

The different effects of inhibitors during the initial phase of acidification and during a later period only support the view that the acidification process is a

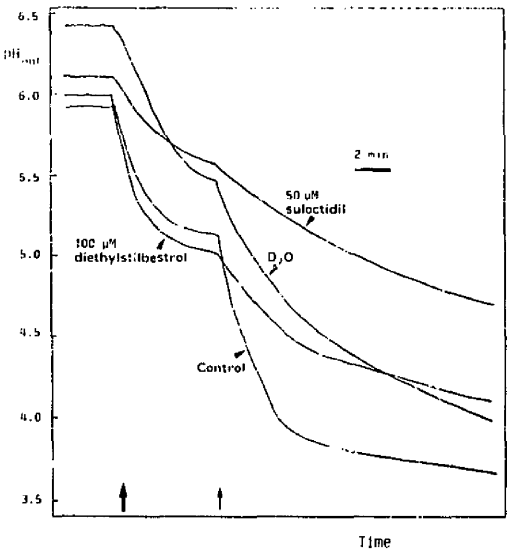

Fig. 1. Traces of extracellular pH of a suspension of *S. cerevisiae* K at 30°C, aerobically, with 5.5–6.1 mg dry wt. per ml. Cells were added at heavy arrow, 1% glucose at light arrow.

TABLE I

Effects of inhibitors on H^+-ATPase activity of S. cerevisiae K plasma membranes

Ranges of at least four experiments are shown.

Inhibitor added	Concn. (μM)	ATP hydrolysis[a]	Acidification[b] A	Acidification[c] B
None	–	100	100	100
D_2O	–[d]	37–58	18–31[e]	75–98[e]
Diethylstilbestrol[f]	100	10–31	30–45	89–120
Erythrosin B[f]	50	15–22	68–75	95–100
Miconazol[f]	50	32–56	98–120	18–28
Suloctidil[f]	50	5–8	12–26	12–28
Sodium vanadate	50	45–70	75–85	69–92
Cupric sulfate	10	12–22	52–68	69–130[g]
Dicyclohexylcarbodiimide	100	48–60	89–95	96–102
Fusicoccin[f]	100	85–92	100–105	98–108

[a] In per cent of the control; 100% = 0.19 ± 0.05 μmol P_i split off per min per mg membrane protein.

[b] In per cent of the control; 100% = 7.0 ± 2.3 nmol H^+ per min per mg dry wt., for the period between 30 and 90 s after addition of glucose.

[c] In per cent of the control; 100% = 4.3 ± 1.8 nmol H^+ per min per mg dry wt., for the period between 5 and 10 min after addition of glucose.

[d] Not less than 97%.

[e] The measured pH (or pD) values were by 0.4 units higher than would correspond to reality [27].

[f] Corrections for the effect of ethanol as solvent were always made.

[g] The behaviour here was anomalous.

combination of several H^+-releasing reactions [22]. The ATPase proper appears to be most active during the first period after adding glucose.

Protonmotive force

The effects of inhibitors on the protonmotive force at pH 5.5 (the optimum pH for transport studies in yeast, see Ref. 17) were quite different from those on the ATPase activity (Table II) which indicates, among other things, that the protonmotive force is not primarily the result of ATPase activity. Two of the most powerful inhibitors of ATPase activity, D_2O and diethylstilbestrol, in fact increase the membrane potential and the protonmotive force, in case of diethylstilbestrol highly significantly. This refutes the notion that this substance may diminish the barrier properties of the plasma membrane [28].

Uptake of amino acids

Amino acids in yeasts are taken up by a secondary active transport involving protons [29,30], even if there is virtually no efflux of once accumulated amino acids which are present intracellularly as freely dissolved molecules [31,32]. The demonstration that terminated the debate of whether protons or other 'sources of energy' are involved in the uptake was the one using heavy water [11]. Now the pronounced effect on the ATPase as well as on all secondary transports studied suggested the possibility that the inhibition of the H^+ symports of nutrients is due to a primary inhibition of the plasma membrane ATPase. Such a mechanism would be justifiable if one realizes that the uptake of amino acids (with protons) causes an immediate acidification of the cell interior, one that is compensated by the activity of the plasma membrane ATPase. Because it is highly probable that the protons used for this type of transport are utilized in the immediate vicinity of the membrane [33] and because the pH optima of the symports coincide with or tend toward the pH optimum of the ATPase reaction (6.0–6.5; [10]) it is clear that a failure of the ATPase to provide these protons could be reflected in the activity of the secondary (symport) active uptake of any solute that is transported along with protons.

From a total of 13 amino acids tested glutamic acid was selected in the most extensive inhibitor screening (Table III). The inhibition percentages are seen to agree quite well with the effect of the various substances on glucose-induced acidification and fairly well with the ATP-hydrolyzing activity, although there the effects of most of the inhibitors tend to be greater than on acidification. There is poor agreement with the values of either the membrane potential or the total protonmotive force.

The effect of the two most powerful inhibitors, D_2O and diethylstilbestrol, was then examined with all the 13 amino acids and a clear dependence of the effect on the isoionic point of the amino acid was established (Table IV). While the two acidic amino acids are inhibited on the average by 77% in D_2O and 88% in 100 μM diethylstilbestrol, the corresponding figures

TABLE II

Membrane potential $\Delta\psi$ and the protonmotive force pmf of the S. cerevisiae K plasma membranes

Means from 3–6 experiments are shown.

Inhibitor	Concn. (μM)	$\Delta\psi$ (mV)	pmf (mV)
None	–	55	86
D_2O	(> 97%)	57	90
Diethylstilbestrol	100	70	117
Erythrosin B	50	57	75
Suloctidil	50	46	63
Sodium vanadate	50	47	72
Cupric sulfate	10	49	79
Dicyclohexylcarbodiimide	100	41	70
Fusicoccin	100	54	88
Ethanol	(0.7%)	51	78
Ethanol	(3.5%)	16	45

TABLE III

The initial rate of uptake of 10 μM L-glutamic acid by S. cerevisiae K in the presence of inhibitors, at pH 5.5, aerobically, at 30°C

Ranges of values from at 4–6 experiments are shown.

Inhibitor	Concn. (μM)	Uptake rate[a]
None	–	100
D_2O	(>97%)	18– 32
Diethylstilbestrol	100	9– 12
Erythrosin B	50	61– 74
Suloctidil	50	25– 31
Sodium vanadate	50	70– 85
Cupric sulfate	50	60– 71
Dicyclohexylcarbodiimide	100	91–109
Fusicoccin	100	79–105
Ethanol	(0.7%)	100–115

[a] 100% = 87 ± 16 nmol glutamic acid per ml cell water per min.

are 48% and 71% for the neutral ones and 17% and 24% for the basic ones, respectively. The simplest explanation for this grading would be that at pH 5.5 the acidic amino acids carry a net negative charge of nearly one (0.92–0.96), the neutral ones have no net electric charge and the basic ones one full positive charge. Thus, the basic amino acids could in principle enter cells without an additional proton (cf. the arguments for Na^+-stimulated uptake of amine acids in tumour cells [34]). In contrast, the neutral amino acids may need one and the acidic ones perhaps two protons. That this is not a matter of a particular transport system but a more general property is evidenced by the use of the mutant lacking the general amino acid permease where the effects are similar to those in the wild strain (Fig. 2).

These observations are in agreement with those of Eddy's group (summarized in Ref. 35) as far as the special position of glutamate goes but in the gap mutant both leucine and proline (not shown in the figure) are much more affected by D_2O than is lysine although in Eddy's work identical stoichiometries of 1 : 1 were found. Moreover, it appears here that basic amino acids display lower accumulation ratios than either the neutral or the acidic ones, this being an argument against multiple H^+ ions being involved in their uptake [8,36].

The direct link between ATPase pumping of protons and the transport of amino acids as proposed here is supported by several auxiliary observations.

(1) As mentioned before [11], at higher concentrations of the transported solute the effect of D_2O is more pronounced; thus the initial rate of glutamate transport is reduced in the presence of D_2O by 19% at 50 nM Glu, by 55% at 2.5 μM Glu, by 73% at 125 μM Glu and by 78% at 3.1 mM Glu. A reverse sequence would be expected if the transport rate depended on the 'source of energy', such as the protonmotive force, where the accumulation ratios are higher at lower than at high solute concentrations (e.g. Refs. 37 and 38).

(2) In *pma* mutants with an impaired function of the plasma membrane H^+-ATPase [39], the uptake rate of amino acids is substantially lower than in the parent strain, the ratio being 0.30 for L-proline, 0.48 for L-leucine, 0.62 for L-arginine and 0.28 for L-aspartic acid (very poorly transported both in the parent and in the mutant).

TABLE IV

Initial rates of uptake of 10 μM amino acids in the presence of D_2O (column A) and 100 μM diethylstilbestrol (column B), in S. cerevisiae K, at pH 5.5, aerobically, at 30°C

Expressed in nmol amino acid taken up per ml cell water per min, means from at least five experiments.

Group	Amino acid	pI	Control	Rate of uptake			
				A	%	B	%
I	L-Aspartic acid	2.95	52.0	9.4	18	6.2	12
	L-Glutamic acid	3.09	83.2	23.1	28	9.0	11
II	L-Phenylalanine	5.67	16.4	7.5	46	5.1	31
	L-Methionine	5.71	51.4	34.2	66	n.m.	–
	L-Tryptophan	5.94	34.3	21.5	63	8.9	26
	L-Leucine	6.04	97.0	46.7	48	25.2	26
	Glycine	6.07	52.4	21.3	41	n.m.	–
	L-Alanine	6.11	51.6	18.4	36	11.7	23
	2-Aminoisobutyric acid	6.29	9.3	4.2	45	3.9	42
	L-Proline	6.30	61.2	44.8	73	14.5	24
III	L-Ornithine	9.73	70.2	62.4	89	57.1	81
	L-Lysine	9.90	31.1	22.1	71	23.6	76
	L-Arginine	10.74	19.2	17.1	89	13.7	71

(3) In *Lodderomyces elongisporus*, where the effect of D_2O on the glucose-induced acidification is much lower than in *Saccharomyces cerevisiae*, its depression of tryptophan, leucine and glutamic acid transport is also much lower (not shown here quantitatively).

(4) As mentioned before [11] the effects of heavy water are generally smaller at low pH values than at high ones, the working hypothesis being that at low external pH values the protons (or deuterons) for the secondary transports can be supplied from the extracellular medium. At high external pH, the protons must be supplied by the ATPase and hence its inhibition is felt more strongly.

(5) At higher suspension densities the transport capacity of virtually all systems is reduced [12,40] by a noncompetitive effect of CO_2 dissolved at the cells, quite probably on the H^+-ATPase. At a density of 0.25 mg dry wt. of *S. cerevisiae* K cells per ml the initial rate of uptake of 1 mM L-tryptophan at 30°C was 450 nmol per ml intracellular water per min, while at a density of 12.5 mg dry wt. per ml the rate was only 130 nmol per ml per min. The lower-density rate was decreased in D_2O by 55%, the high-density rate by 19%.

Conclusions

Taken together, these data suggest that inhibitors or conditions (genetic or physiological) that affect the function of the plasma membrane H^+-ATPase depress in a similar fashion the transports of amino acids in yeast. The rates of transport have very little to do with either the membrane potential or the protonmotive force, contrary to predictions from various kinetic models [41,42] where the components of the protonmotive force measured macroscopically are included and their coupling with the transport system is assumed. The dependence of the rate of glutamic acid uptake on ATP-hydrolyzing activity, on medium-acidifying activity and on protonmotive force is summarized in Fig. 3. Clearly, considering the complicated system used, there is a fine agreement between the rate of uptake and the acidification capacity (correlation coefficient 0.925) but a poor agreement between the rate of uptake and the

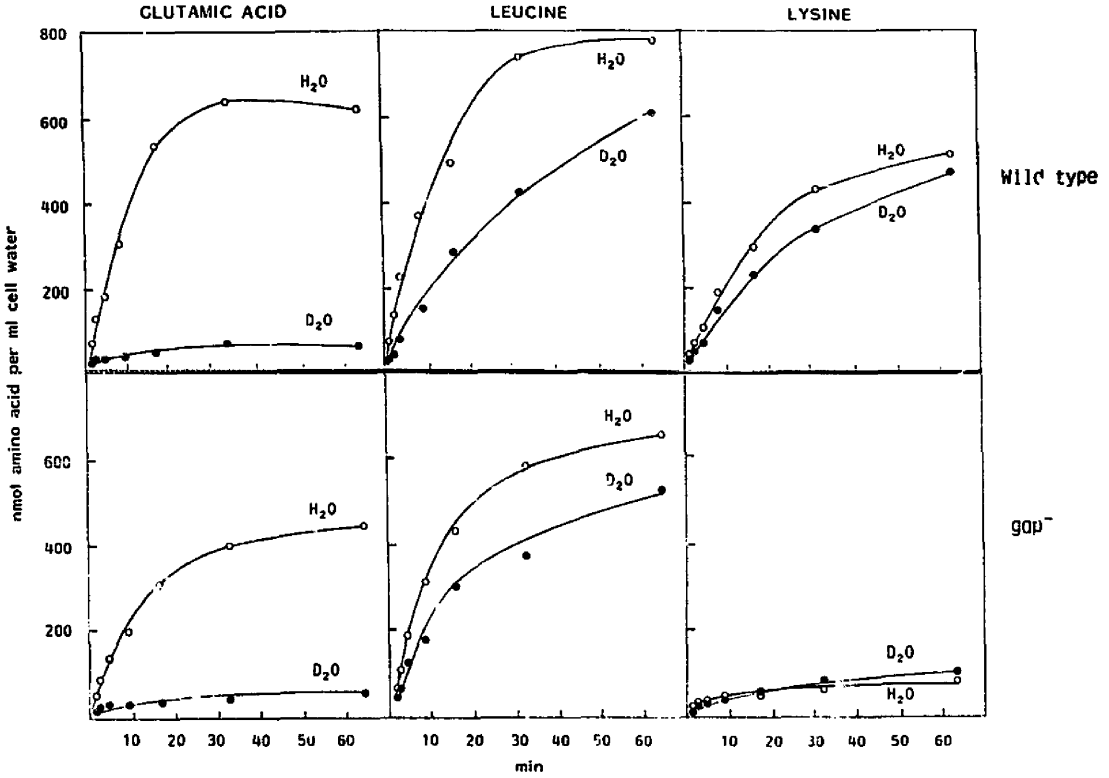

Fig. 2. Effect of heavy water on the uptake of 10 μM amino acids by the wild-type strain *S. cerevisiae* Σ1278b and by its *gap* mutant, deficient in the general amino acid permease.

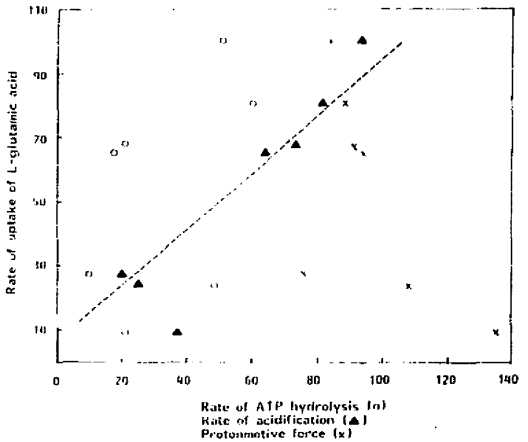

Fig. 3. Dependence of the rate of uptake of 10 μM L-glutamic acid by the wild-type strain of *S. cerevisiae* on the rate of ATP hydrolysis by the plasma membrane H^+-ATPase in vitro, on the medium acidification by this ATPase (the first segment of the pH curve corresponding to column A of Table I) and on the protonmotive force. The median values of the range of values were always taken. The dashed line was computed by the least-squares method for the dependence on the rate of acidification.

ATP hydrolysis (correlation coefficient 0.487) and absolutely no relationship between the uptake rate and the protonmotive force (the correlation coefficient is negative).

It is likely that what the transport systems see in the membrane is the local concentration of H^+ ions as is generated, in the case described here, apparently mainly by the plasma membrane H^+-ATPase.

References

1 Misra, P.C. and Hofer, M. (1975) FEBS Lett. 52, 95–99.
2 Serrano, R. (1977) Eur. J. Biochem. 80, 97–102.
3 Eddy, A.A. (1982) Adv. Microbiol. Physiol. 23, 11–76.
4 Reichert, U. and Forêt, M. (1977) FEBS Lett. 83, 325–328.
5 Roomans, G.M., Kuypers, G.A.J., Theuvenet, A.P.R. and Borst-Pauwels, G.W.F.W. (1979) Biochim. Biophys. Acta 551, 197–206.
6 Vara, F. and Serrano, R. (1982) J. Biol. Chem. 257, 12826–12830.
7 Opekarová, M. and Sigler, K. (1985) Cell Mol. Biol. 31, 195–200.
8 Kotyk, A. (1983) J. Bioenerget. Biomembr. 15, 307–320.
9 Kotyk, A. (1980) in Frontiers of Bioorganic Chemistry and Molecular Biology (Ananchenko, S.N., ed.), pp. 417–425, Pergamon Press, Oxford.
10 Kotyk, A. and Horák, J. (1985) in Water and Ions in Biological Systems (Vasilescu, V., Pullman, B., Packer, L. and Leahu, L., eds.), pp. 343–353, Plenum Press, New York.
11 Kotyk, A., Koryta, J. and Dvořáková, M. (1990) FEBS Lett. 264, 203–205.
12 Kotyk, A. (1987) Yeast 3, 263–270.
13 Kotyk, A., Horák, J. and Knotková, A. (1982) Biochim. Biophys. Acta 698, 243–251.
14 Alonso, A. and Kotyk, A. (1978) Folia Microbiol. 23, 118–125.
15 Kotyk, A. and Michaljaničová, D. (1979) J. Gen. Microbiol. 110, 323–332.
16 Horák, J., Sychrová, H. and Kotyk, A. (1990) Biochim. Biophys. Acta 1023, 380–382.
17 Kotyk, A. and Georghiou, G. (1991) Biochem. Int. 24, 641–647.
18 Slavík, J. (1982) FEBS Lett. 140, 22–26.
19 Hauer, R. and Höfer, M. (1978) J. Membr. Biol. 43, 335–349.
20 Serrano, R. (1983) FEBS Lett. 140, 22–26.
21 Sychrová, H. and Kotyk, A. (1985) FEBS Lett. 183, 21–24.
22 Sigler, K., Knotková, A. and Kotyk, A. (1981) Biochim. Biophys. Acta 643, 572–582.
23 Sigler, K., Kotyk, A., Knotková, A. and Opekarová, M. (1981) Biochim. Biophys. Acta 643, 583–592.
24 Sigler, K., Knotková, A., Páca, J. and Wurst, M. (1980) Folia Microbiol. 25, 311–317.
25 Serrano, R. (1990) in The Plant Plasma Membrane (Larsson, C. and Møller, I.M., eds.), pp. 127–153, Springer-Verlag, Berlin-Heidelberg.
26 Anraku, Y., Umemoto N., Hirata, R. and Wada, Y. (1989) J. Bioenerget. Biomembr. 21, 588–603.
27 Glasoe, P.K. and Long, F.A. (1960) J. Phys. Chem. 64, 188–190.
28 Borst-Pauwels, G.W.F.H., Theuvenet, A.P.R. and Stols, A.L.H. (1983) Biochim. Biophys. Acta 732, 186–192.
29 Seaston, A., Carr, G. and Eddy, A.A. (1976) Biochem. J. 154, 669–676.
30 Kotyk, A. and Metlička, R. (1986) Stud. Biophys. 110, 205–210.
31 Kotyk, A. and Říhová, L. (1972) Biochim. Biophys. Acta 288, 380–389.
32 Horák, J. (1986) Biochim. Biophys. Acta 864, 223–256.
33 Slavík, J. and Kotyk, A. (1984) Biochim. Biophys. Acta 766, 679–684.
34 Christensen, H.N., Handlogten, M.E. and Thomas, E.L. (1969) Proc. Natl. Acad. Sci. USA 63, 948–955.
35 Eddy, A.A. (1982) in Membranes and Transport (Martonosi, A.N., ed.), Vol. 2, pp. 47–53, Plenum Press, New York.
36 Rottenberg, H. (1979) Methods Enzymol. 55 F, 547–569.
37 Horák, J. and Kotyk, A. (1986) Biochim. Biophys. Acta 857, 173–179.
38 Kotyk, A. and Stružinský, R. (1977) Biochim. Biophys. Acta 470, 484–491.
39 McCusker, J.H., Perlin, D.S. and Haber, J.E. (1987) Mol. Cell. Biol. 7, 4082–4088.
40 Kotyk, A. and Michaljaničová, D. (1987) Folia Microbiol. 32, 459–464.
41 Kotyk, A. (1985) in Structure and Properties of Cell Membranes (Bengo, Gh., ed.), Vol. 2, pp. 11–17, CRC Press, Boca Raton.
42 Sanders, D., Hansen, U.-P., Gradmann, D. and Slayman, C.L. (1984) J. Membr. Biol. 77, 123–152.