Biochimica et Biophysica Acta 861 (1986) 413–419
Elsevier

BBA 73316

A study of the mechanism by which inhibitors of the plasmamembrane ATPase enhance uptake of divalent cations in yeast

G.W.F.H. Borst-Pauwels, A.W. Boxman, A.P.R. Theuvenet, P.H.J. Peters and J. Dobbelmann

Laboratory of Chemical Cytology, R.C. University, Toernooiveld, 6525 ED Nijmegen (The Netherlands)

(Received 6 June 1986)

Key words: Membrane potential; Divalent cation uptake; ATPase inhibitors; (*S. cerevisiae*)

The enhancement of divalent cation uptake in yeast provoked by the membrane ATPase inhibitors trifluoperazine, miconazole, compound 48/80, ethidium, DIO-9 and calmidazolium should be ascribed to an increase in cation permeability of the yeast rather than to hyperpolarisation of the yeast cell membrane. For trifluoperazine and miconazole it is unequivocally shown that the cells are hyperpolarized though for miconazole only transiently. Whether the other drugs also hyperpolarize the yeast cells is uncertain. The apparent hyperpolarisation caused by trifluoperazine and miconazole may be attributed to a specific increase in the K^+ permeability of the yeast plasmamembrane evoked by these compounds.

Introduction

A great number of inhibitors of yeast plasmamembrane ATPase stimulate divalent cation uptake in yeast with concomitant release of K^+. This has been found for DIO-9 [1], ethidium [2–5], miconazole [5], diethylstilbestrol [6] and for the calmodulin antagonists trifluoperazine [5–8], compound 48/80 [5] and calmidazolium [5] which are inhibitors of plasmamembrane ATPase as well [5]. The stimulation of Sr^{2+} uptake by DES [6] and the enhancement of Ca^{2+} uptake by trifluoperazine [7,8] are accompanied by an increase in the equilibrium distribution of the lipophilic cation tetraphenylphosphonium (TPP) which may indicate that the increase in divalent cation influx is due to hyperpolarisation of the yeast cells by these compounds. It has been hypothesized that the enhancement of Ca^{2+} uptake into yeast by DIO-9 [1] and ethidium [3] is due to hyperpolarisation of the cells as well. This hyperpolarisation may be caused by an increase in K^+ permeability of the plasmamembranes induced by these drugs.

We have now examined whether DIO-9 and ethidium cause an increase in uptake of TPP. Besides the effect of DIO-9 and ethidium we also studied the effects of miconazole, trifluoperazine, compound 48/80 and calmidazolium upon both Sr^{2+} and TPP uptake and the effects of ethidium, trifluoperazine and chitosan upon both Ca^{2+} and TPP uptake.

Methods

TPP, Sr^{2+} and Ca^{2+} uptake. Yeast cells, *Saccharomyces cerevisiae*, were resuspended in distilled water to 4% (w/v) and aerated for one night in order to exhaust the cells for internal substrate. The cells were resuspended at 2% (w/v) in 45 mM Tris-succinate (pH 4.5) provided with 3% (w/v) glucose at 25°C. N_2 was bubbled through the

Abbreviation: TPP, tetraphenylphosphonium.

Correspondence address: Prof. Dr. G.W.F.H. Borst-Pauwels, Laboratory of Chemical Cytology, R.C. University, Toernooiveld, 6525ED Nijmegen, The Netherlands.

suspension. Unless otherwise stated the cells were preincubated for one h before adding the drugs. Carrier-free ^{14}C-labelled TPP (final concentration 0.36 μM) or ^{89}Sr was added either together with the drugs or 30 min after the addition of the drugs, ^{45}Ca was added only together with the drugs and was diluted by 1 mM nonradioactive Ca^{2+}. 1.8-ml samples were taken at appropriate times and filtrated under suction after rapidly mixing the yeast samples with 20 ml of ice-cold 20 mM $MgCl_2$ solution in case of TPP uptake or 50 mM NaEDTA (pH 8.5) in case of Sr^{2+} or Ca^{2+} uptake. Subsequently the cells were washed with 1.5 ml ice-cold water and in case of ^{89}Sr uptake also with 2 ml of acetone in order to dry the sample rapidly. By this procedure externally bound TPP, Ca^{2+} or Sr^{2+} were removed almost quantitatively. The radioactivity of ^{14}C-labelled TPP or ^{45}Ca was determined by means of liquid scintillation and that of ^{89}Sr was determined by means of an end window Geiger Mueller tube. For studying efflux of ^{14}C-labelled TPP from yeast, the cells were loaded during 4 h with radioactive TPP. Then the various drugs were added. The efflux of TPP was determined after taking yeast samples at appropriate times and determining the radioactivity of the yeast samples as described above for TPP uptake.

Chemicals, DIO-9 and calmidazolium were added as alcoholic solutions, miconazole was dissolved in dimethyl sulfoxyde. The other compounds were dissolved in distilled water. The ultimate concentrations of alcohol or dimethyl sulfoxyde were at maximum 1% (v/v). ^{14}C-labelled TPP, ^{89}Sr and ^{45}Ca were obtained from Amersham International (U.K.). Trifluoperazine, compound 48/80 and chitosan were from Sigma Chemie, Taufkirchen (F.R.G.), calmidazolium from Janssen Life Sciences Products, Beerse (Belgium), miconazole was a gift from Dr. A. Goffeau, Louvain la Neuve (Belgium) and DIO-9 was a gift from Gist-Brocades, Delft (The Netherlands). 1 mg/ml DIO-9 gave an absorbance of 2.1 at 303 nm after correction for the absorbance at 400 nm.

Results

Fig. 1 shows that trifluoperazine greatly increased the TPP content of cells which were preloaded with carrier-free TPP for 4 h, at which time distribution equilibrium had been established. Ater 20–40 min maximum uptake of TPP was reached whereafter TPP was extruded again. The time at which maximum uptake was reached decreased with increasing trifluoperazine concentrations. The maximum height of TPP uptake increased with increasing trifluoperazine concentrations up to 0.0 mM. With 40 μg/ml miconazole a transient uptake of TPP was found which was followed by a rapid decrease in cellular TPP content to amounts far below the TPP content of control cells. At 100 μg/ml miconazole no significant uptake was found anymore and the TPP release started almost immediately (data not shown). 15 μg/ml calmidazolium did not affect the cellular TPP content significantly (data not shown) and DIO-9, ethidium and compound 48/80 led only to release of previously accumulated TPP.

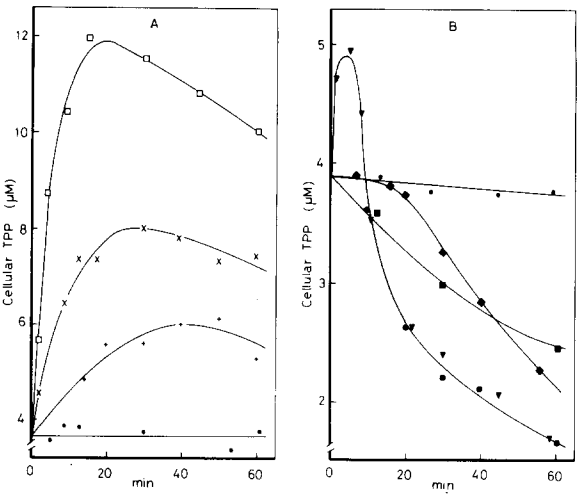

Fig. 1. Effect of various drugs upon the TPP content of metabolizing yeast cells, which are preloaded with ^{14}C-TPP for 4 h. (A) Effect of trifluoperazine: (+) 20 μM, (×) 40 μM, (□) 80 μM. (B) Effect of compound 48/80: (◆) 20 μg/ml; DIO-9: (■) 200 μg/ml; ethidium; (●) 2 mM; miconazole: (▼) 40 μg/ml; (●) control.

Finding a decrease in the TPP content of the cells on adding the drugs is no unequivocal proof that the cells are depolarized. All the drugs added to the yeast suspension are cations. If these cations are accumulated into the cells they might be able to compete with TPP for intracellularly located negatively charged binding sites which may lead to a decrease in the total TPP content of the

cells even when the cells are not depolarized. Therefore an eventual hyperpolarisation of the cells by the drugs may be masked due to displacement of TPP from internally located binding sites.

Whereas the equilibrium distribution ratios of TPP may be affected by changes in the binding capacity of the cells, the initial rates of uptake of TPP in the cells are not expected to be effected by the extent of binding of TPP inside the cells. Fig. 2 shows that all compounds examined increased the influx rate of TPP into the cells. With ethidium the net uptake rate of TPP decreased very rapidly. The uptake became already soon below that of the control. The decrease in net uptake rate developed less rapidly with DIO-9 and even still slower with compound 48/80. Addition of miconazole led to a great increase in the influx rate of TPP (data not shown). At relatively high miconazole concentrations the influx was followed by an efflux of TPP from the cells. This was also found at high trifluoperazine concentrations (data not shown). Calmidazolium gave rise to an almost immediate uptake of TPP followed by a slow gradual uptake.

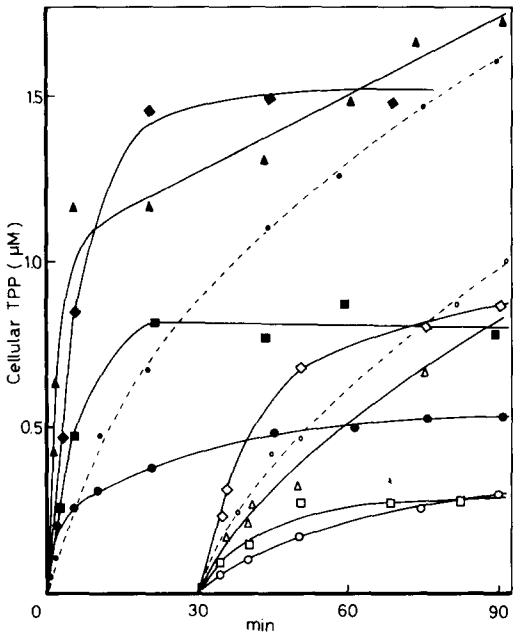

Fig. 2. Effect of the drugs upon the initial rate of TPP influx into metabolizing yeast cells. The yeast cells were preincubated for 1 h in the presence of 3% (w/v) glucose before addition of the drugs. ^{14}C-TPP was added either together with the drugs (closed symbols) or after 30 min (open symbols). Same symbols as in Fig. 1B; (▲), calmidazolium.

It appeared that the influx rates of TPP in cells incubated with the various drugs greatly decreased during this incubation, see Fig. 2. With cells preincubated with ethidium, miconazole (data not shown) and DIO-9 the influx rate observed after 30 min incubation were even lower than that of the control cells without added drugs. With compound 48/80 and calmidazolium the influx rates of TPP though being greatly decreased, were still greater than that of the control.

We also determined the effect of the drugs upon uptake of radioactive Sr^{2+} under similar conditions as under which the uptake of TPP was studied. For a proper comparison of the effects of the various drugs upon TPP and Sr^{2+} uptake we calculated relative rates of influx by dividing the influx rates observed in the presence of the drugs by the rate of influx found in the absence of added drugs. Fig. 3 shows that DIO-9 had a far greater effect upon the relative influx rate of Sr^{2+} than upon the relative influx rate of TPP. This

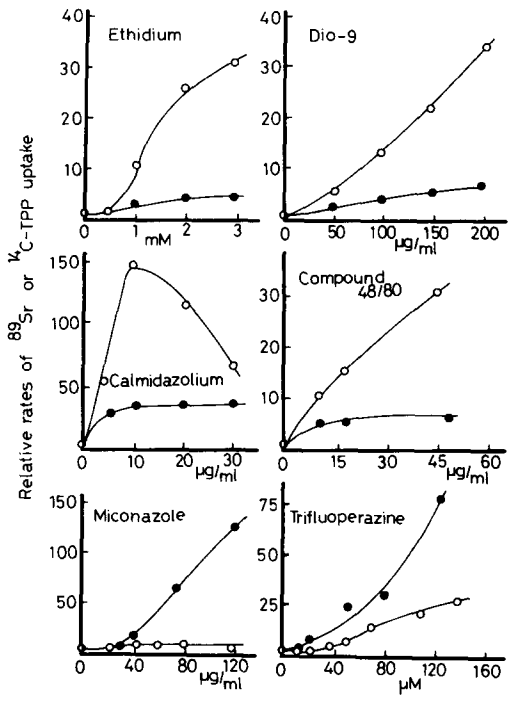

Fig. 3. Dependence of the relative rates of ^{14}C-TPP and ^{89}Sr influx upon the concentrations of the various drugs. The relative rates of influx are equal to the quotient of the influx rates found in the presence of the drugs and the influx rates found in the absence of the drugs. Open symbols relative rate of Sr^{2+} influx; closed symbols relative rate of TPP influx.

also applies to the effects of ethidium, calmidazolium and compound 48/80. The relative influx rates found for TPP uptake induced by ethidium, DIO-9 or compound 48/80 did not exceed a value of 6. On the other hand with miconazole and trifluoperazine relative rates of TPP influx of the order of magnitude of 100 could be obtained. The effect of these two compounds upon the relative rate of TPP influx was far more greater than upon the relative Sr^{2+} uptake contradictory to what had been found with the other drugs. It was also found that the influx rate of radioactive Sr^{2+} decreased during the incubation of the cells with the various drugs just as has been found for TPP uptake (data not shown).

The drugs applied also give rise to release of K^+ from the cells to the medium [1–5,7–9]. Fig. 4 shows that by approximation a single correlation existed between the amount of Sr^{2+} being absorbed within one min and the amount of K^+ being released within the same period of time after the addition of ethidium, compound 48/80 and DIO-9. With trifluoperazine the datapoints lie only slightly below those found for the other drugs.

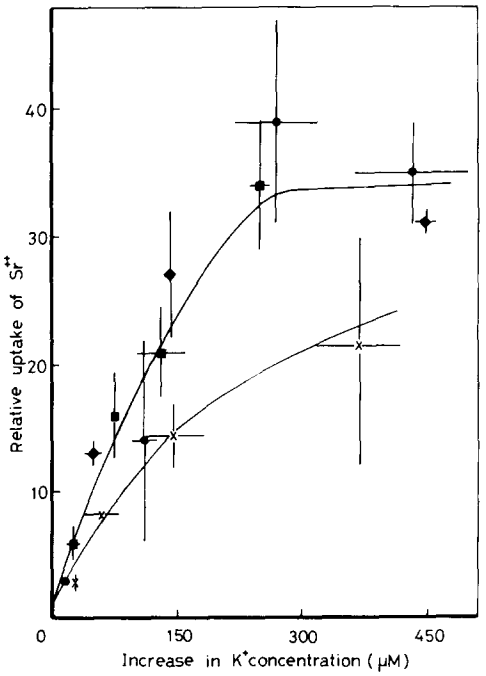

Fig. 4. Relation between the amount of ^{89}Sr accumulated into the yeast cells and the amount of K^+ released within one min after the addition of the drugs. The uptake of ^{89}Sr is expressed in relative units obtained by dividing the amount of ^{89}Sr accumulated in the presence of the drugs and the ^{89}Sr accumulated in the absence of the drugs. The amount of K^+ released is expressed as the increase in K^+ concentration of the medium one min after the addition of the drugs to 2% (w/v) yeast. Same symbols as in Fig. 1B; (×) trifluoperazine.

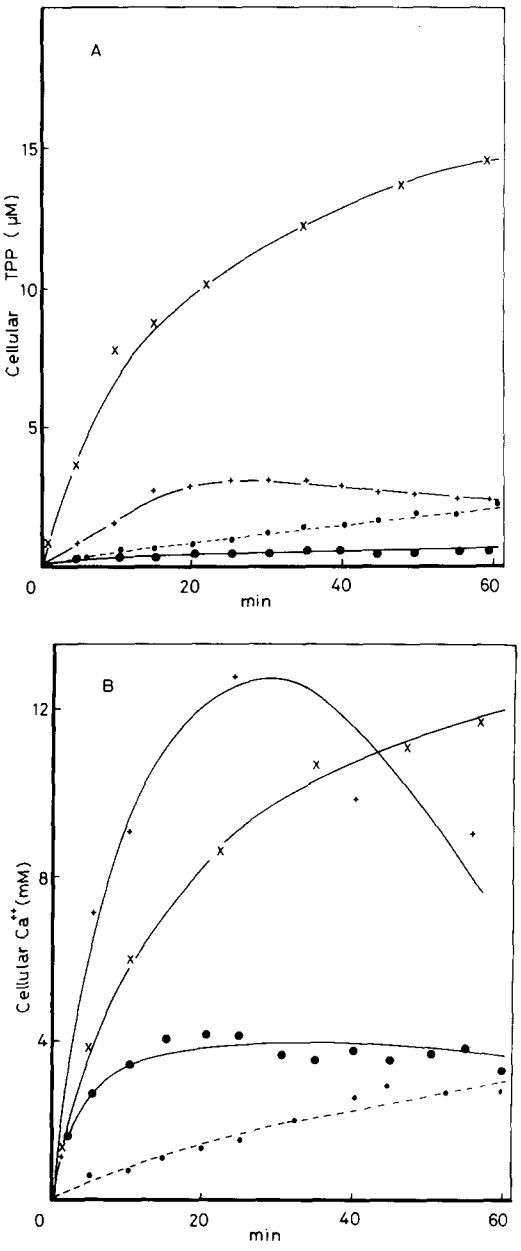

Fig. 5. A comparison of the effect of chitosan, ethidium and trifluoperazine upon uptake of TPP (A) and 1 mM Ca^{2+} (B). (+) 50 μg/ml chitosan; (×) 0.1 mM trifluoperazine; (●) 3 mM ethidium; (•) control.

We finally examined whether chitosan an agent which specifically permeabilizes the yeast plasmamembrane [10] enhances divalent cation uptake as well. In order to minimalize a possible decrease in divalent cation influx due to release of cellular Ca^{2+} from the cells after permabilizing the plasmalemma, we added 1 mM Ca^{2+} to the medium. Furthermore instead of ^{89}Sr uptake ^{45}Ca uptake was studied. Fig. 5 shows that chitosan increased the uptake of ^{45}Ca greatly. The enhancement of ^{45}Ca uptake was accompanied by an increased influx of TPP. We also examined whether under these experimental conditions ethidium and trifluoperazine enhanced ^{45}Ca uptake. This appeared to be true. Furthermore the enhancement of ^{45}Ca uptake by trifluoperazine was accompanied by a relatively great increase in TPP uptake and that being provoked by ethidium did not show even a significant increase in TPP uptake.

Discussion

Eilam [8] has observed that trifluoperazine increases the distribution of TPP between yeast cells and medium greatly at pH 6. On the other hand the effect of trifluoperazine at pH 4.5 was much smaller [11]. Our results show that also at low pH a relatively great increase in TPP uptake can occur and that trifluoperazine hyperpolarizes the cells. The uptake of TPP, however, is reversible showing a maximum at about 5 to 30 min depending upon the trifluoperazine concentration and upon the batch of yeast. Apparently miconazole transiently hyperpolarizes the yeast cells. Whether the other drugs also lead to a hyperpolarisation of the cells is less certain. The fact that the equilibrium distribution of TPP between cells and medium is decreased by compounds like ethidium and DIO-9 and also by compound 48/80 and calmidazolium does not necessarily mean that the cells are depolarized by these compounds. Part of the TPP accumulated into the cells is bound to intracellular constituents [12]. This bound TPP may be displaced by the cationic drugs after their uptake into the cell causing a reduction in the partition ratio of TPP. Secondly the cells shrink during loss of K^+ [13,14]. This also causes a decrease in the cellular TPP content. A third factor which may contribute to the apparent depolarisation is that part of the cells are completely permeabilized allowing the entry of relatively large molecules, whereas the remainder of the cells are still intact [13]. All these factors will lead to an underestimation of the true membrane potential when calculating the membrane potential from the equilibrium distribution of TPP between cells and medium.

Theoretically the initial influx rate of TPP into the cells does not depend upon the extent of binding of TPP inside the cells. Furthermore both shrinkage of the cells and complete permeabilization of the cells will be initially minimal. In that respect initial influx rates may be a better relative measure for the membrane potential than the equilibrium distribution ratios of TPP. However, we cannot exclude the possibility that the drugs have increased the permeability of the plasmamembrane for TPP. Therefore the increases in TPP influx rate found with the various drugs are no ultimate proof that the cells are hyperpolarized by these drugs. This view is supported by the fact that after permabilizing the cells with chitosan also an increase in the TPP influx rate is found.

Foury et al. [1] and Peña et al. [3] have hypothesized that the increase in Ca^{2+} uptake into yeast cells provoked by either DIO-9 or ethidium is due to hyperpolarisation of the cells caused by an electrogenic exit of K^+ from the cells. Our results do not support this view. It can be argued that even if DIO-9, ethidium, compound 48/80 and calmidazolium hyperpolarize the yeast cells, it is still unlikely that the enhancement of Sr^{2+} uptake by these compounds is due to this hyperpolarisation. Fig. 6 shows that the great increase in the influx rate of TPP provoked by trifluoperazine is not accompanied by a comparable increase in the rate of Sr^{2+} influx. This also applies to the effect of miconazole. This shows that Sr^{2+} influx is rather insensitive to variations in the magnitude of the membrane potential. This makes it very unlikely that the great increase in Sr^{2+} influx observed with DIO-9, ethidium and calmidazolium, which is only accompanied by a relatively small increase in the TPP influx rate, is due to hyperpolarisation of the cell membranes. Furthermore as shown in Fig. 3, on increasing the concentration of compound 48/80 from 10 μg/ml to 50 μg/ml the rate of Sr^{2+} influx still increases

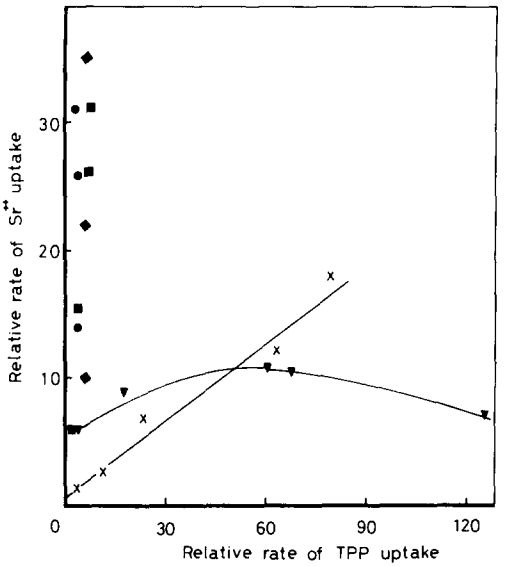

Fig. 6. Relation between the influx rate of ^{89}Sr and the influx rate of ^{14}C-TPP found with the various drugs. Data of Fig. 3. Same symbols as in Fig. 1B and Fig. 4.

though the influx rate of TPP is virtually constant.

A more likely explanation for the enhancement of Sr^{2+} influx evoked by DIO-9, ethidium, compound 48/80 and calmidazolium is that the permeability of the cell membrane for small cations is increased in a nonselective way by these drugs. The correlation found between Sr^{2+} uptake and K^+-efflux provoked by ethidium, DIO-9 and compound 48/80 is in accordance with this view. Furthermore compounds which selectively permeabilize the yeast cell membrane, like DEAE-dextran [4,15] and as shown in this publication chitosan, also enhance the uptake of Ca^{2+}. Probably the enhancement of Sr^{2+} influx by trifluoperazine also is mainly due to an increase in cation permeability of the cell membrane instead of to the hyperpolarisation of the cells. This is indicated by the fact that almost the same relation is found between the uptake of Sr^{2+} and the efflux of K^+ provoked by trifluoperazine as is found when DIO-9, ethidium or compound 48/80 are added to the cells.

It may be expected that in cells of which the plasmamembrane has become permeable to small cations the accumulation of divalent cations will occur predominantly into the vacuoles [15]. Accordingly the selectivity between Sr^{2+} and Mn^{2+} accumulation being found in intact cells [16] and which resides in the plasmalemma [17] has been disappeared completely in the presence of the permeabilizing agent DEAE-dextran [4]. Ethidium has a similar effect like DEAE-dextran supporting our view that ethidium and probably also the other drugs primarily act as a permeabilizing agent making the cytosol accessible to small cations.

In order to explain the great hyperpolarisation caused by both trifluoperazine and miconazole under conditions that divalent cation uptake is only slightly stimulated we have to assume, that these two compounds increases predominantly the K^+ permeability of the yeast cells and to a far less extent the permeability for other ions like Ca^{2+} and Sr^{2+}.

Acknowledgement

The provision of yeast by Gist-Brocades at Delft is gratefully acknowledged.

References

1 Foury, F., Boutry, M. and Goffeau, A. (1977) J. Biol. Chem. 252, 4577–4583
2 Peña, A. and Ramirez, G. (1976) J. Membrane Biol. 22, 369–384
3 Peña, A. (1978) J. Membrane Biol. 42, 199–213
4 Theuvenet, A.P.R., Nieuwenhuis, B.J.W.M., Van de Mortel, J. and Borst-Pauwels, G.W.F.H. (1986) Biochim. Biophys. Acta 855, 383–390
5 Borst-Pauwels, G.W.F.H., Theuvenet, A.P.R., Boxman, A.W., Peters, P.H.J. and Dobbelmann, J. (1986) J. Bioelectrochem. Bioenerg., in the press
6 Borst-Pauwels, G.W.F.H., Boxman, A.W. and Theuvenet, A.P.R. (1984) in Environmental Regulation of Microbial Metabolism (Kulaev, I.S., Dawes, E.A. and Tempest, D.W., eds.), pp. 369–376, Academic Press, New York
7 Eilam, Y. (1983) Biochim. Biophys. Acta 733, 242–248
8 Eilam, Y. (1984) Biochim. Biophys. Acta 769, 601–610
9 Boutry, M., Foury, F. and Goffeau, A. (1980) Biochim. Biophys. Acta 464, 602–612
10 Jaspers, H.T.A., Christianse, K. and Van Steveninck, J. (1975) Biochem. Biophys. Res. Commun. 65, 1434–1439
11 Eilam, Y., Lavi, H. and Grossowicz, N. (1985) J. Gen. Microbiol. 131, 2555–2564
12 Boxman, A.W., Dobbelmann, J. and Borst-Pauwels, G.W.F.H. (1984) Biochim. Biophys. Acta 772, 51–57
13 Borst-Pauwels, G.W.F.H., Theuvenet, A.P.R. and Stols, A.L.H. (1983) Biochim. Biophys. Acta 732, 186–192
14 Borst-Pauwels, G.W.F.H. and Theuvenet, A.P.R. (1985) FEMS Microbiol. Lett. 29, 221–224

15 Eilam, Y., Lavi, H. and Grossowicz, N. (1985) J. Gen. Microbiol. 131, 623–629
16 Rothstein, A. and Hayes, A., Jennings, D. and Hooper, D. (1958) J. Gen. Physiol. 41, 585–594
17 Nieuwenhuis, B.J.W.M., Weijers, C.A.G.M. and Borst-Pauwels, G.W.F.H. (1981) Biochim. Biophys. Acta 649, 83–88