Biochimica et Biophysica Acta, 469 (1977) 292—300

BBA 77789

ACTIVE TRANSPORT OF L-SORBOSE AND 2-DEOXY-D-GALACTOSE IN *SACCHAROMYCES FRAGILIS*

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(Received February 14th, 1977)

Summary

Sorbose and 2-deoxy-D-galactose are taken up in *Saccharomyces fragilis* by an active transport mechanism, as indicated by the energy requirement of the process and the accumulation of free sugar against the concentration gradient. There are no indications for transport-associated phosphorlyation as mechanism of energy coupling with these two sugars.

The measured sugar-proton cotransport and the influx inhibition by uncouplers suggest a chemiosmotic coupling mechanism. Thus there are at least two different active transport mechanisms operative in *Saccharomyces fragilis*: transport-associated phosphorylation in the case of 2-deoxy-D-glucose and chemiosmotic coupling in the case of sorbose and 2-deoxy-D-galactose. The differences between the two mechanisms are discussed.

Uncouplers do not stimulate downhill sorbose transport in energy-depleted cells and evoke an almost complete inhibition of efflux and of exchange transport.

The differences between this sugar-proton cotransport system and similar systems in bacteria and *Chlorella* are discussed.

Introduction

Both 2-deoxy-D-glucose (dGlc) and 2-deoxy-D-galactose (dGal) are taken up by *Saccharomyces fragilis* cells by an active mechanism, as described in previous papers [1,2]. With dGlc energy coupling appeared to occur via the mechanism of transport-associated phosphorylation [1], as described before in other yeast strains [3,4]. dGal, on the other hand, enters the cells as the free sugar, with subsequent intracellular phosphorylation [2]. Further studies were undertaken to elucidate the mechanism of energy coupling to dGal transport.

During these studies it was felt that the energy-requiring intracellular phosphorylation of this sugar was a complicating factor, obscuring the experimental

results to some extent. The huge accumulation of 2-deoxy-D-galactose-1-phosphate for example, is accompanied by a strong decrease of the polyphosphate fraction. Thus, a possible intermediate role of polyphosphate in energy coupling to the transport process per se would be difficult to evaluate, due to the secondary intracellular reactions. Therefore the transport mechanism of several other sugars was investigated, in order to find a sugar that is transported against a concentration gradient, but without subsequent intracellular phosphorylation.

It appeared that sorbose fulfilled these requirements. In most investigations described in the present communication the influence of experimental conditions was studied both on dGlc and on dGal and sorbose transport, to facilitate evaluation of the different mechanisms of energy coupling.

Methods

Saccharomyces fragilis was grown, harvested, washed and, when necessary, starved as described before [2]. Proton cotransport was measured on yeast cells washed and resuspended in 1 mM Tris/citrate buffer. The pH of the suspension was registered with a Corning-110 research pH-meter coupled to a 1 mV Vitatron recorder, adapted to give full-scale deflection of 0.1 pH units. The buffering capacity of the incubation mixture was calibrated later by the addition of known amounts of H_2SO_4. K^+ concentrations in the medium were measured with a flame photometer.

Transport studies, utilizing ^{14}C-labeled substrates, were conducted at pH 5.4 in 1 mM Tris/citrate buffer, unless otherwise stated. Uptake measurements and analysis of cellular extracts were performed as described before [2].

Analytical methods were: ATP, with the firefly tail luciferin-luciferase system, according to the method of Addanki et al. [5]; polyphosphates, according to Lohmann and Langen [6]; orthophosphate, by the method of Meyerhof and Oesper [7].

Anaerobic conditions were achieved by leading purified nitrogen through the suspension for 3 min. Subsequently the vessels were tightly closed. In control experiments no oxygen could be detected in the suspensions, utilizing a YSI oxygen probe.

Results

During incubation of *S. fragilis* with ^{14}C-labeled sorbose for periods up to 4 h the total amount of radioactivity in the incubation mixture remained constant. These experiments were done at a final yeast concentration of 10% (wet weight) and substrate concentrations of 0.1—1 mM. Analysis of cellular extracts was done via paper chromatography and autoradiography as described previously [1,2]. These methods allow to distinguish between various sugars and their phosphorylated derivatives. Only one spot, with the R_F value of sorbose was found. These facts indicate that no measurable conversion of sorbose takes place after transport of the free sugar into the cells.

Sorbose transport is linear with time for several minutes. From the initial rates of transport during the first 30 s Michaelis-Menten kinetics with a V of

0.30 ± 0.09 mmol/g per h and a K_m of 12.8 ± 3.1 mM (n = 5) could be derived, assuming the formation of a 1 : 1 sugar-carrier complex.

Transport of sorbose into fresh yeast cells is shown in Fig. 1. Apparently uptake proceeds against a concentration gradient. The ratio of the intracellular over the extracellular sorbose concentration (C_i/C_o) at stationary state was about 200 at an initial sugar concentration in the medium of 0.1 mM, decreasing to about 130 at 1 mM and 4 at 100 mM. This accumulation of free sugar inside the cells indicates active transport. The only other possible explanation for these results would be facilitated diffusion, combined with a strong binding of the sugar inside the cells, as discussed by Kotyk and Michaljaničová [8]. This remote possibility was ruled out by the effect of basic macromolecules like protamine and chitosan. After treatment with these macromolecules the cell membrane becomes permeable to small molecules like monosaccharides, whereas the intracellular organization is not affected [9—11]. As shown in Fig. 1, addition of chitosan to the cells preloaded with [^{14}C]sorbose leads to a very fast efflux of the accumulated sugar. Apparently the accumulated sorbose was present inside the cells in the free, unbound form.

Moreover, the active character of sorbose transport is also indicated by the energy requirement of the process. As shown in Fig. 2, anaerobic conditions and starvation, but especially a combination of these two conditions, lead to a substantial inhibition of transport, both with reference to the transport velocity and the accumulation capacity. After 2 h C_i/C_o ratios were reached of 130 for normal cells under aerobic conditions, 10 for normal cells under anaerobic conditions, 8 for starved cells under aerobic conditions and 1 for starved cells under anaerobic conditions.

During incubation with 100 mM sorbose there was no measurable change in

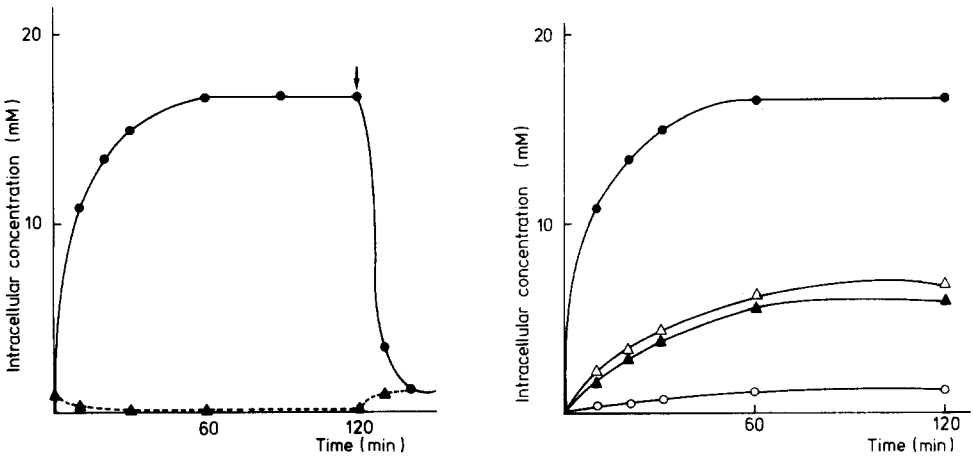


Fig. 1. Uptake of sorbose under aerobic conditions (●———●) and the effect of treatment with chitosan (250 γ/ml), added after 2 h (arrow). Initial concentration of sorbose: 1.08 mM. Yeast concentration: 10% (wet wt./v). The experiment was carried out in a 1 mM Tris/citrate buffer, pH 5.4. ▲- - - - -▲, Concentration of sorbose in the medium.

Fig. 2. Uptake of sorbose in normal cells under aerobic conditions (●———●); normal cells under anaerobic conditions (△———△); starved cells under aerobic conditions (▲———▲); starved cells under anaerobic conditions (○———○). Initial substrate concentration in the medium: 1.08 mM.

the intracellular ATP and polyphosphate concentrations. As shown previously the maximal ATP production under these experimental conditions would be about 0.5 mmol/g per h, assuming an ATP/O ratio of 2 [1]. As the sorbose uptake rate is of the same order of magnitude (0.3 mmol/g per h), these results make it unlikely that phosphorylated intermediates would play a direct role in energy coupling to the transport process.

A possible coupling of sugar influx to K^+ efflux was investigated by measuring the K^+ concentration in the medium, with dGlc, dGal and sorbose as substrates. In many experiments the efflux of K^+ with any of these sugars present in the medium actually exceeded the K^+ efflux in the absence of sugar. The results varied considerably, however, with different yeast batches. In about 30 experiments the number of K^+ leaving the cells as a consequence of sugar influx varied from 0 to 25% of the sugar molecules taken up. In this respect there were no differences between the three sugars studied. These results made it very unlikely that a direct coupling of sugar and K^+ fluxes would exist.

Further experiments were designed to investigate the possible role of a sugar-proton cotransport. In preliminary experiments it appeared that washing and resuspending of the yeast cells in 1 mM Tris/citrate buffer in the pH range 5.4—6.5 was necessary to obtain a reasonable constant baseline pH of the suspension. Without this pretreatment the pH of a suspension of *S. fragilis* cells exhibits irregular and rather large fluctuations. As shown in Fig. 3, uptake of dGlc, dGal and sorbose is associated with a concomitant rise in the medium pH, reflecting influx of protons.

As discussed previously, dGlc is taken up via transport-associated phosphorylation [1]. According to this theory the sugar is phosphorylated at the outside of the membrane with polyphosphate as phosphate donor [1,3,4]. In this concept cotransport of cations e.g. protons as compensation of the negative charge of the sugar-phosphate-carrier complex can be anticipated. The observed influx of protons during dGal and sorbose transport cannot be explained along these lines, however. Proton-substrate symport utilizing the

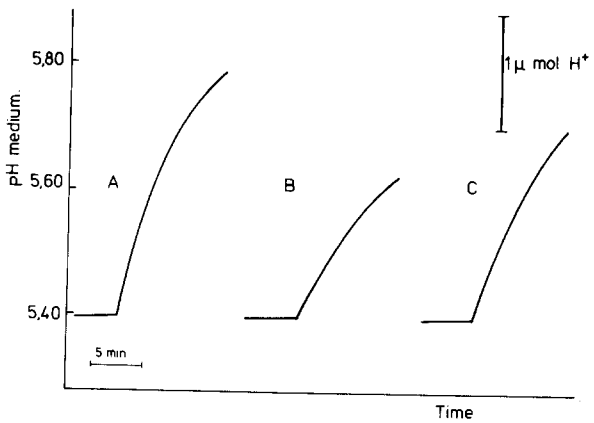


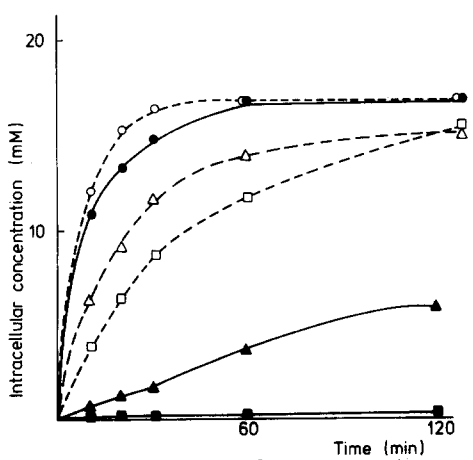
Fig. 3. Alkalinization of the medium with uptake of dGlc (A), sorbose (B) and dGal (C) in the course of time under anaerobic conditions in a 1 mM Tris/citrate buffer, pH 5.4. Initial substrate concentration: 10 mM. Yeast concentration: 10% (wet wt./v).

TABLE I

INFLUX OF SUGAR (10 mM) AND PROTONS UNDER ANAEROBIC CONDITIONS IN A 1 mM TRIS/CITRATE BUFFER, pH 5.4

Substrate	Incubation time (min)	Influx (μM/g)		H^+/sugar
		Sugar	H^+	
Sorbose	2	1.9	1.0 (n = 2)	0.53
	3	3.2	1.3 (n = 5)	0.41
	4	3.8	1.9 (n = 2)	0.50
dGal	2	1.5	1.4 (n = 2)	0.93
	3	1.8	1.8 (n = 4)	1.00
	4	2.2	2.3 (n = 2)	1.04
dGlc	2	3.3	1.5 (n = 2)	0.45
	3	3.8	2.1 (n = 4)	0.55
	4	4.4	2.4 (n = 2)	0.55

electrochemical gradient of H^+ across the membrane for uphill sugar transport seems conceivable. Data on proton-sugar symport in short-term experiments at pH 5.4 are summarized in Table I. At higher pH values, under anaerobic conditions, the stoichiometry of protons cotransported per sugar molecule decreased and became virtually zero at pH 8. At the same time increase of the medium pH from 5.4 to 9 progressively inhibited initial rates of sugar uptake with, in the case of sorbose, a concomitant decrease of the accumulation factor (Fig. 4). At pH 8 and higher the intracellular sorbose concentration never exceeded the medium concentration, even under aerobic conditions. The influence of pH



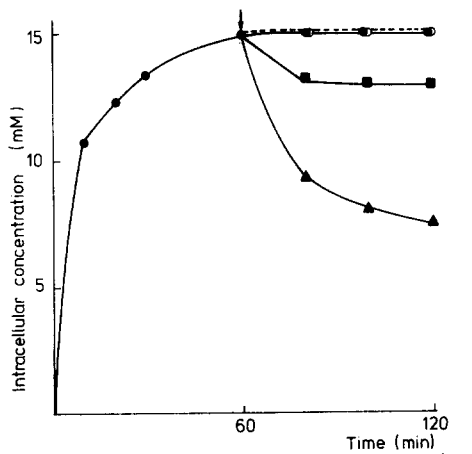


Fig. 4. Uptake of sorbose in a 200 mM Tris/citrate buffer at pH 5.4 (●———●), at pH 7 (▲———▲) and at pH 9 (■———■) and of dGlc at pH 5.4 (○- - - - -○), at pH 7 (△- - - - -△) and at pH 9 (□- - - - -□) under aerobic conditions. The uptake of dGal shows a similar sensitivity to pH-change as sorbose. It was necessary to use high buffer concentrations in order to maintain high pH-values in the medium.

Fig. 5. Uptake of sorbose under aerobic conditions at pH 5.4 (●———●) and the influence of a pH-change in the medium to 7 (▲———▲) or to 9 (■———■) after 60 min (arrow). ○- - - - -○, Intracellular concentration of sorbose after addition of 1 mM Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (arrow). Dinitrophenol (1 mM) and azide (40 mM) gave identical results.

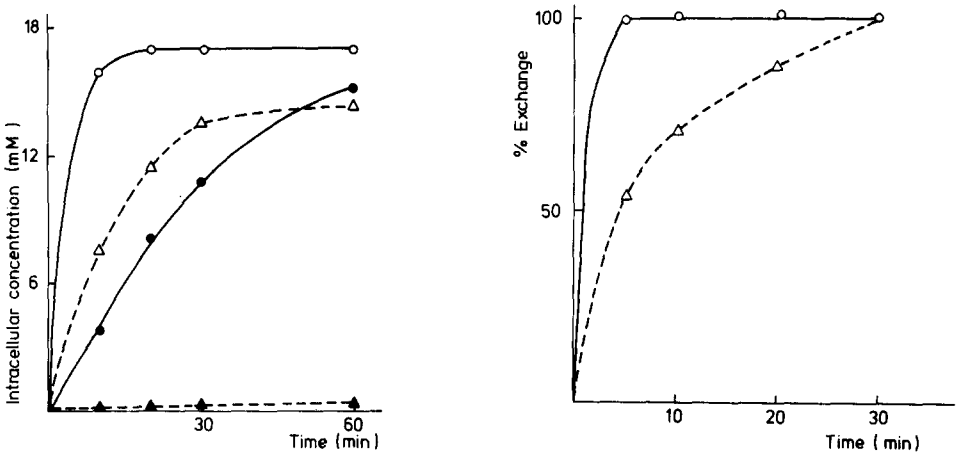


Fig. 6. Influence of dinitrophenol (1 mM) on the uptake of dGlc and sorbose under aerobic conditions at pH 5.4. ●———●, sorbose uptake, control; ▲- - - - -▲, sorbose uptake, dinitrophenol added; ○———○, dGlc uptake, control; △- - - - -△, dGlc uptake in the presence of dinitrophenol. The results with dGal were similar to those obtained with sorbose. CCCP (1 mM) and azide (40 mM) gave identical results.

Fig. 7. Influence of azide (40 mM) on dGlc-exchange under aerobic conditions at pH 5.4. ○———○, dGlc control; △- - - - -△, dGlc, azide added. CCCP (1 mM) and dinitrophenol (1 mM) gave identical results.

on the accumulation factor is also reflected by pH-dependent efflux from preloaded cells, as shown in Fig. 5. Changing the pH from 5.4 to either 7 or 9 caused efflux of accumulated sugar, although the velocity at pH 9 is extremely slow.

The influence of uncouplers on sugar uptake is depicted in Fig. 6. If added to cells preloaded with sugar, uncouplers did not induce sugar efflux (Fig. 5). In relation to these phenomena the influence of uncouplers on steady-state flux was studied. Cells were incubated with unlabeled sugar until a steady-state was reached. Then the uncoupler and subsequently a pulsing dose of the ^{14}C-labeled

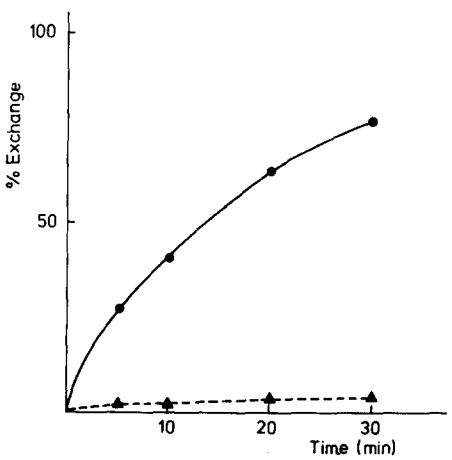


Fig. 8. Influence of azide (40 mM) on sorbose-exchange under aerobic conditions at pH 5.4. ●———●, sorbose, control; ▲- - - - -▲, sorbose, azide added. The results with dGal were comparable to those with sorbose. CCCP (1 mM) and dinitrophenol (1 mM) gave identical results.

sugar were added to the medium. As shown in Figs. 7 and 8, exchange transport of dGlc was slightly retarded, whereas exchange transport of dGal and sorbose was almost completely inhibited. Finally the influence of uncouplers on downhill transport was studied. Sorbose transport in starved cells under anaerobic conditions is essentially always a downhill process, with intracellular sugar concentrations never exceeding the medium concentration. Even this transport was strongly inhibited by uncouplers.

Discussion

Uptake of dGlc, dGal and sorbose in *S. fragilis* apparently takes place via active transport, as indicated by the C_i/C_o ratios during steady-state and the energy requirement of the transport processes (Figs. 1 and 2, refs. 1 and 2). Energy coupling to dGlc transport occurs via transport-associated phosphorylation as discussed amply in preceding papers [1—4]. In the case of dGal transport a different mode of energy coupling must take place, as previously shown [2]. This applies imperatively also to sorbose transport, as this sugar is not phosphorylated by *S. fragilis*. Consequently it could be anticipated that certain characteristic differences would be found between dGlc transport on the one hand and dGal and sorbose transport on the other. A crucial difference between dGlc and dGal transport could be deduced from pulse-labeling experiments and was discussed in a preceding paper [2].

As discussed before, transport-associated phosphorylation [1,3,4] implies the probability of H^+ cotransport (as found in the present studies for dGlc), not as the driving force but rather as a fortuitous consequence of the transport step. Some inhibition by uncoupling agents, as shown in Fig. 6, is also quite conceivable for a transport system driven by phosphate-bond energy, as pointed out, e.g. by Berger [12]. Thus the observations on dGlc transport as described in the present paper are well compatible with the mechanism of transport-associated phosphorylation.

The observed sugar-H^+ cotransport with dGal and sorbose, however, should be explained along different lines. The most obvious interpretation seems to be a symport system in the sense of the chemiosmotic theory [13,14]. The influence of uncouplers on sorbose transport in fresh yeast cells supports this concept (Fig. 6). Uncouplers permit leakage of protons across the membrane in the direction of the electrochemical gradient, thus dissipating the energized state of the membrane [14,15]. The fact that sorbose transport is much more sensitive to uncouplers than dGlc transport (which is not dependent on a protonmotive force) is in accordance with theoretical anticipations. The strong inhibition of sorbose influx, caused by increased pH in the medium, may also be explained in terms of the chemiosmotic theory. Increased extracellular pH values diminish the H^+ gradient across the membrane and thus the protonmotive force. Again, dGlc transport is much less influenced by changes in extracellular pH (Fig. 4).

In several bacterial systems various effects of valinomycin on active transport support the chemiosmotic theory. For instance, in membrane vesicles of *Escherichia coli* lacking substrate, an electrical potential can be created by valinomycin-induced K^+ efflux. This induces certain active transport processes [16].

During the present investigations attempts (not described in detail here) to produce similar effects in yeast were not successful. Valinomycin did not induce any increase of K^+ permeation across the yeast cell membrane, not even after pretreatment of the cells with Tris/EDTA, as described by Leive [17] and Pavlasova and Harold [18]. Similar negative results were obtained by Borst-Pauwels (personal communication).

The described kinetic studies indicate a one-to-one sorbose-carrier complex. Based on the chemiosmotic interpretation a proton-sorbose stoichiometry of at least 1 should thus be expected during transport. Experimentally a ratio of about 0.5 was found at pH 5.4 (Table I) and even lower at higher pH values. By extrapolation of these results a ratio closer to the theoretical expectations could be anticipated at low pH values. This could not be tested experimentally, as strongly buffered media (obscuring proton movements) were required to stabilize pH values below 5.0. Although a residual activity of a proton-pump mechanism under the employed anaerobic conditions (thus lowering the measured H^+/sorbose stoichiometry) could not be excluded, the shift of the H^+/sorbose ratio at higher pH levels seems to indicate a mixed transport mechanism, resembling the hexose-proton cotransport system of *Chlorella*. Sugar transport in *Chlorella* changes gradually from a high-affinity, uphill system via a protonated carrier at low pH, into a low-affinity, facilitated diffusion system via the same, unprotonated carrier at high pH values [19]. A similar pH-dependent shift of H^+/sorbose stoichiometry, initial transport velocity and accumulation ratio was observed in the present studies on *S. fragilis*. With dGal the proton/sugar stoichiometry was close to one at pH 5.4 (Table I), decreasing again at higher pH values. This indicates a similar transport mechanism as with sorbose.

In bacteria and bacterial membrane vesicles, uncouplers produce a rapid efflux of accumulated substrates [15,20,21]. Further, downhill transport of sugar in energy-depleted bacteria is stimulated by uncouplers, due to their shorting-out the reversed proton-motive force, created by the substrate-H^+ cotransport. Koch et al. even consider this phenomenon as "critical evidence in support of Mitchell's hypothesis" [22,23]. Both in the *Chlorella* system [19, 24—26] and in the present studies the results were quite different. Uncouplers do not induce efflux of accumulated substrate (Fig. 5) and downhill transport of sorbose in energy-depleted cells is inhibited. As pointed out by Tanner et al. for the *Chlorella* system [19,24—26], this suggests that translocation of the protonated carrier depends critically on the membrane potential. The complete inhibition of sorbose exchange transport by uncouplers (Fig. 8) is in accordance with this assumption. On this point the difference between the sorbose and the dGlc transport systems is salient and noteworthy: whereas uncouplers inhibit sorbose exchange transport almost completely, they have a much less pronounced effect on dGlc exchange (Figs. 7 and 8).

A pH shift to 7 after preloading the cells with sorbose at pH 5.4 causes a pronounced net efflux (Fig. 5). This should be expected, considering the much lower accumulation ratio achieved at pH 7, as compared to pH 5.4 (Fig. 4). A shift to pH 9, however, results in only a very limited efflux, despite the extremely low accumulation level at this pH during influx experiments (Fig. 5). This suggests that not only the absence of a membrane potential, but also the unprotonated state of the carrier impedes translocation.

Though not fully understood, these observations may appear of crucial importance to elucidate this active transport system in *S. fragilis* on the molecular level.

Acknowledgements

The authors wish to thank Miss Karmi Christianse for her expert technical assistance. This work was done under auspices of the Netherlands Foundation for Biophysics and with financial support from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

References

1 Jaspers, H.T.A. and Van Steveninck, J. (1975) Biochim. Biophys. Acta 406, 370—385
2 Jaspers, H.T.A. and Van Steveninck, J. (1976) Biochim. Biophys. Acta 443, 243—253
3 Van Steveninck, J. (1970) Biochim. Biophys. Acta 203, 376—384
4 Van Steveninck, J. (1969) Arch. Biochem. Biophys. 130, 244—252
5 Addanki, S., Sotos, J.F. and Rearick, P.D. (1966) Anal. Biochem. 14, 261—264
6 Lohmann, K. and Langen, P. (1957) Biochem. Zeitschr. 328, 1—11
7 Meyerhof, O. and Oesper, P. (1947) J. Biol. Chem. 170, 1—22
8 Kotyk, A. and Michaljaničová, D. (1968) Folia Microbiol. 13, 212—220
9 Jaspers, H.T.A., Christianse, K. and Van Steveninck, J. (1975) Biochem. Biophys. Res. Commun. 65, 1434—1439
10 Schlenk, F. and Zydek-Swick, C.R. (1970) Arch. Biochem. Biophys. 138, 220—225
11 Svihla, G., Dainko, J.L. and Schlenk, F. (1969) J. Bacteriol. 100, 498—504
12 Berger, E.A. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1514—1518
13 Mitchell, P. (1966) Biol. Rev. 41, 445—502
14 Harold, F.M. (1974) Ann. N.Y. Acad. Sci. 227, 297—311
15 Harold, F.M., Altendorf, K.H. and Hirata, H. (1974) Ann. N.Y. Acad. Sci. 235, 149—160
16 Hirata, H., Altendorf, K. and Harold, F.M. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1804—1808
17 Leive, L. (1967) J. Biol. Chem. 243, 2373—2380
18 Pavlasova, E. and Harold, F.M. (1969) J. Bacteriol. 98, 198—204
19 Komor, E. and Tanner, W. (1974) J. Gen. Physiol. 64, 568—581
20 Asghar, S., Levin, E. and Harold, F.M. (1973) J. Biol. Chem. 248, 5225—5233
21 Lombardi, F.J., Reeves, J.P., Short, S.A. and Kaback, H.R. (1974) Ann. N.Y. Acad. Sci. 227, 312—327
22 Cecchini, G. and Koch, A.L. (1975) J. Bacteriol. 123, 187—195
23 Purdy, D.R. and Koch, A.L. (1976) J. Bacteriol. 127, 1188—1196
24 Komor, E., Haass, D. and Tanner, W. (1972) Biochim. Biophys. Acta 266, 649—660
25 Komor, E., Loos, E. and Tanner, W. (1973) J. Mem. Biol. 12, 89—99
26 Grüneberg, A. and Komor, E. (1976) Biochim. Biophys. Acta 448, 133—142