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THE INFLUENCE OF UNCOUPLERS ON PROTON-SUGAR SYMPORT IN *SACCHAROMYCES FRAGILIS*

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Uncouplers of oxidative phosphorylation inhibit proton-sugar symport in *Saccharomyces fragilis*. However, they do not induce efflux of accumulated sugar. It is shown that the effect cannot be explained by uncoupler-induced alterations in the transmembrane potential or transmembrane pH difference. It is also indicated that a decrease in intracellular pH is not involved in inhibition of sugar transport. It is argued that inhibition of transport by uncouplers is most likely caused by a direct interaction with the translocator.

Introduction

Active transport of solutes can be energized by a transmembrane electrochemical proton gradient, via so-called proton-solute symport. Thus, it should be expected that alterations of this H^+ gradient will affect these transport systems. For instance, uncouplers of oxidative phosphorylation should, through their protonophoric properties, influence H^+-solute symport, via dissipation of the electrochemical proton gradient.

For bacteria it was found that uncouplers indeed act in the way expected theoretically, changing the active transport system into an equilibrating one. Both amino acid [1,2] and carbohydrate transport [3] are sensitive to uncouplers, in the sense that uphill transport is inhibited, whereas downhill transport into energy-depleted bacteria and out of preloaded cells is stimulated [4].

In algae [5] and in yeast [6–8] sugar influx via H^+ symport is also inhibited by uncouplers. However, in *Rhodotorula gracilis* it was shown that 2,4-dinitrophenol reduced sugar influx to far below the equilibration level [9]. Moreover, uncouplers inhibited efflux of ^{14}C-labeled sugar from this yeast after addition of excess unlabeled sugar to the medium [9]. These phenomena cannot be explained via the protonophoric properties of uncouplers.

Also, in *Chlorella vulgaris* uncouplers do not induce any significant outflow of accumulated sugar from preloaded cells [5]. For this organism it was argued that this effect is not due to alterations in the proton-motive force, but rather to an uncoupler-induced decrease in the intracellular pH, resulting in inactivation of transport systems [10].

In the present paper it is shown that also in *Saccharomyces fragilis* the effect of uncouplers on sugar transport cannot be ascribed to their activity as protonophores. A direct interaction of uncouplers with the translocator is suggested.

Materials and Methods

S. fragilis was grown, harvested and washed as described before [11]. Glucose was used as carbon source when fucose transport was measured, whereas lactose was used in growing yeast for methyl-β-D-thiogalactoside transport measurements.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TCSA, 3,3′,4′,5-tetrachlorosalicylanilide.

Transport was measured under aerobic conditions at 25°C in 10% (w/v) yeast suspensions, in some experiments buffered with 0.2 M Tris-maleate, using the method described before [8].

Proton fluxes were measured under aerobic conditions as described before [12]. The intracellular pH was measured according to the method of Borst-Pauwels and Dobbelman [13].

The intracellular water space was measured as described previously [14]. A value of 0.45 ml/g yeast (wet weight) was found and this was used to calculate intracellular sugar concentration.

[^{14}C]Methyl-β-D-thiogalactoside was obtained from New England Nuclear and D-[^{3}H]fucose from Amersham International.

Results and Discussion

As shown in previous studies [8,15], fucose and methyl-β-D-thiogalactoside transport in *S. fragilis* proceeds via proton symport. Uncouplers inhibit this transport up to or even below diffusion equilibrium (Fig. 1). Maximal inhibition of the initial influx velocity is obtained with about 75 μM CCCP, 50 μM TCSA, 200 μM 2,4-dinitrophenol or 200 μM pentachlorophenol.

When the uncoupler was added after the cells had accumulated sugar, however, hardly any efflux could be observed, not even at extremely high (1 mM) concentrations of the uncouplers (Fig. 1). This lack of efflux cannot be explained by assuming that sugar uptake in this yeast is basically unidirectional. As shown previously, countertransport of methyl-β-D-thiogalactoside is readily evoked by lactose [15]. Further, as will be discussed below, depolarization of the membrane by tetraphenylphosphonium leads to a rapid efflux of accumulated sugar. Therefore, the observations depicted in Fig. 1 cannot be simply explained by the protonophoric properties of uncouplers and indicates that the effect of these drugs on symport in yeast is more complicated. The question also arises as to whether the inhibition of influx by uncouplers as shown in Fig. 1 is, in fact, caused by dissipation of the transmembrane electrochemical proton gradient. In this context, the effects of uncouplers on fucose and methyl-β-D-thiogalactoside transport in this yeast strain were studied in some detail.

As shown in Fig. 2 uncouplers act as protonophores in *S. fragilis*. The velocity of dissipation of the transmembrane pH difference strongly depends on the concentration of the uncoupler. At low concentrations complete dissipation takes at least 1 h, as can be deduced from the continuing increase in the medium pH. However, preincuba-

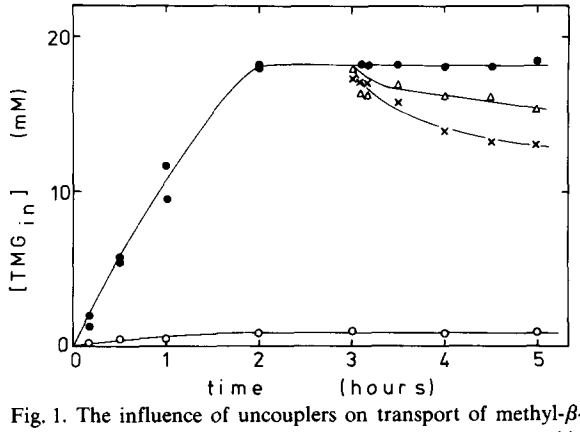

Fig. 1. The influence of uncouplers on transport of methyl-β-D-thiogalactoside (TMG) in *S. fragilis*. Uptake was measured in suspensions, buffered at pH 4.5. Initial methyl-β-D-thiogalactoside concentration: 1 mM. (●——●) Control, (○——○) 1 mM CCCP added 1 min before starting transport, (△——△) 1 mM CCCP added after 3 h of uptake, (x——x) 1 mM 2,4-dinitrophenol added 3 h after starting transport.

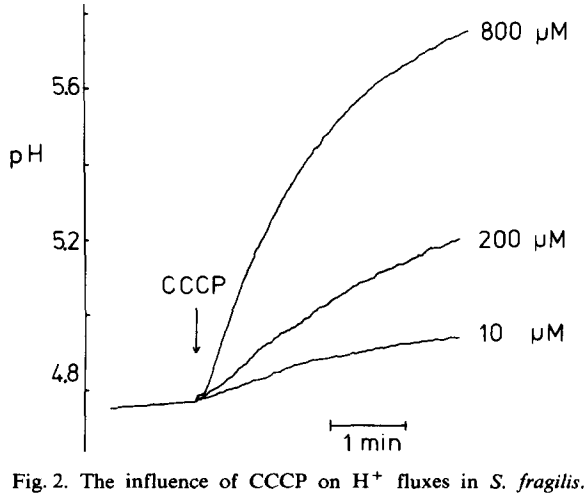

Fig. 2. The influence of CCCP on H^+ fluxes in *S. fragilis*. Yeast was washed three times and subsequently incubated anaerobically in 10% (w/v) suspension buffered with 1 mM Tris-maleate. After recording a baseline, CCCP was added (10, 200 or 800 μM).

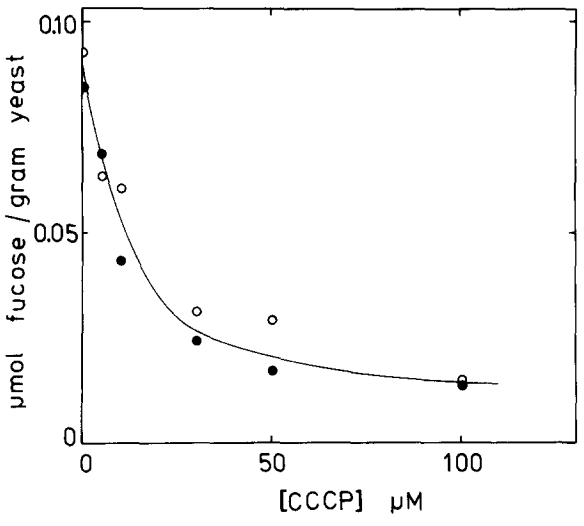

Fig. 3. The influence of the period of preincubation of CCCP with yeast on the fucose influx velocity. Yeast was incubated aerobically at pH 5 with CCCP. After 1 min (●———●) or 2 h (○———○) transport was started by adding 0.1 mM fucose. Uptake was measured 1 and 2 min after starting uptake. Identical results were obtained, using unbuffered suspensions.

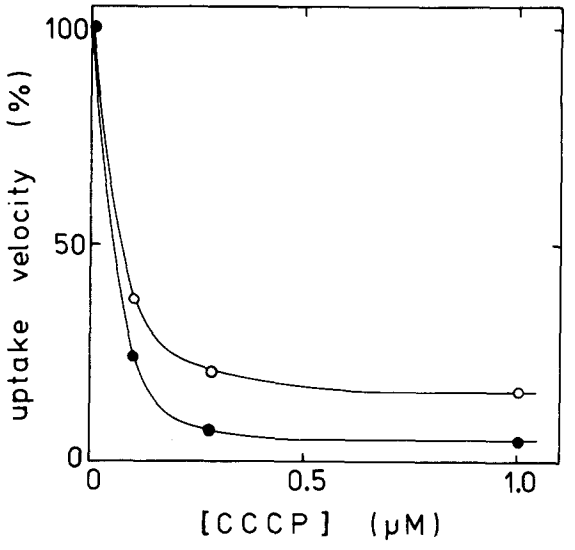

Fig. 4. The pH dependence of the CCCP inhibition of methyl-β-D-thiogalactoside (TMG) influx. Yeast was incubated at pH 4.5 (●———●) or 7.5 (○———○). 1 min before methyl-β-D-thiogalactoside (0.1 mM), CCCP was added to the suspension. The influx velocity was determined from measurements at 1, 2, 3 and 4 min after starting sugar transport.

tion of the cells with uncouplers for short (1 min) or longer times (2 h) did not result in a different sensitivity of sugar influx (Fig. 3). This lack of effect of the preincubation period, especially at low uncoupler concentrations, indicates that the uncoupler-induced inhibition of sugar uptake is not caused by dissipation of the proton-motive force, but rather by a different mechanism.

As a first possibility it was considered that a relatively fast acidification of the cytoplasm might occur, even at low uncoupler concentrations. Assuming that this might cause inactivation of the transport system in a similar way to that suggested for *C. vulgaris* [10], the results depicted in Figs. 1 and 3 could be explained along these lines. This was not borne out experimentally, however. As shown in Fig. 4 the inhibitory effect of CCCP is almost independent of the medium pH. The percent inhibition by this uncoupler is about the same at pH 4.5 and pH 7.5. (It should be noted that the initial sugar uptake velocity in the absence of uncoupler is about 15-times higher at pH 4.5 than at pH 7.5.) Direct measurements of the intracellular pH revealed that after short incubation periods with uncoupler no acidification took place (Table I). After longer incubation periods in the absence of uncoupler the intracellular pH was increased. This increase was lower in the presence of uncouplers (Table I). These observations demonstrate that acidification of the cytoplasm is not involved in inactivation of the transport system.

In the second place it was considered that uncouplers cause a rapid depolarization of the membrane, due to their protonophoric activity [8]. If

TABLE I

THE INFLUENCE OF CCCP ON THE INTRACELLULAR pH

Yeast was incubated aerobically at pH 4.72 or 7.23 for 2 min or 1 h, with or without 100 µM CCCP. The intracellular pH was determined after removing extracellular medium by rapid filtration and washing.

pH_{out}	pH_{in}			
	2 min incubation		1 h incubation	
	−CCCP	+CCCP	−CCCP	+CCCP
4.72	6.30	6.25	6.70	6.41
7.23	6.42	6.43	6.84	6.68

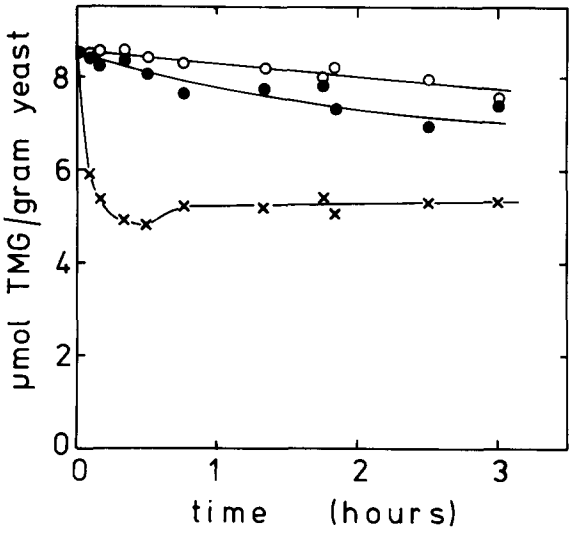

Fig. 5. The influence of CCCP and tetraphenylphosphonium on efflux of methyl-β-D-thiogalactoside (TMG). Yeast was incubated for $3\frac{1}{2}$ h with 1 mM [^{14}C]methyl-β-D-thiogalactoside (TMG) at pH 4.5. After centrifugation, the supernatant was removed. Efflux was started by mixing the yeast with fresh buffer (pH 4.5), without sugar. Extra additions in the efflux medium: 0.2 mM CCCP (O——O) or 10 mM tetraphenylphosphonium (x——x). (●——●) Control without extra additions.

depolarization were to lead to inactivation of the transport system, the effects of uncouplers could thus be explained. For the case of influx inhibition by uncouplers this seems a priori unlikely. As shown before [8], the membrane potential at a medium pH of 7.5 is much higher than at pH 4.5 and therefore it should be expected that the sensitivity of sugar influx to uncouplers would be pH dependent, if depolarization were an important determinant in this context. As shown in Fig. 4, this is not the case. Also, the lack of sugar efflux after addition of uncouplers to preloaded cells (Fig. 1) cannot be explained by inactivation of the transport system via depolarization. As shown previously [8,15], high concentrations of tetraphenylphosphonium cause depolarization of the membrane. As shown in Fig. 5 this leads to a rapid partial efflux of accumulated sugar at a medium pH of 4.5, whereas CCCP does not induce such an efflux. At pH 9.0 this tetraphenylphosphonium-induced efflux is complete, whereas CCCP rather inhibited efflux (Fig. 6). The fact that uncouplers inhibit efflux even at high external pH (in contrast to results obtained with *C. vulgaris* [10]) shows again that this inactivation is not caused by acidification of the cytoplasm. The difference between the tetraphenylphosphonium effects at pH 4.5 and pH 9.0 can be easily explained from previous observations. The accumulation ratio of sugars, taken up via proton-sugar symport, is governed by the proton-motive force [8,15]. As shown before, at low medium pH the transmembrane pH difference is the main component of the proton-motive force, whereas at high medium pH the proton-motive force is completely determined by the transmembrane potential [8].

The results presented indicate that neither the sugar influx inhibition by uncouplers nor the lack of sugar efflux after addition of uncouplers to preloaded cells is related to the protonophoric properties of these compounds. Therefore, the effects of uncouplers should be explained in an alternative way. Most likely uncouplers can associate with the translocator, thus creating an inactive transport system. Although it has been argued [10] that chemically unrelated uncouplers are not likely to interact with translocator proteins in comparable ways, a number of papers have focussed on

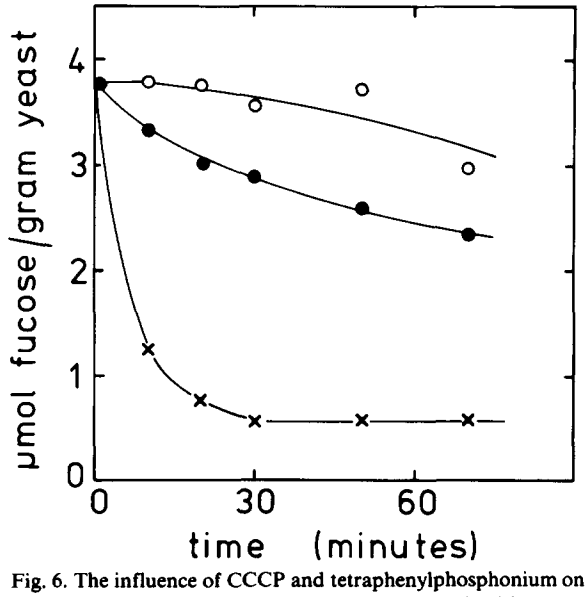

Fig. 6. The influence of CCCP and tetraphenylphosphonium on efflux of fucose. Yeast was incubated, unbuffered, with 1 mM [^{3}H]fucose. After 1 h the suspension was diluted 1:1 with buffer (final pH 9.0), without sugar. Extra additions after dilution: 0.2 mM CCCP (O——O) or 10 mM tetraphenylphosphonium. (●——●) Control without extra additions.

uncoupler binding to membrane proteins (see e.g. Refs. 16 and 17). It could be shown, for instance, that the mitochondrial ATPase can bind structurally unrelated uncouplers. On this basis a direct interaction of uncouplers with transport proteins seems conveivable.

In this context, it should be noted that uncouplers also inhibit energy-independent facilitated diffusion of sorbose in *Saccharomyces cerevisiae*. In this case, again a direct interaction of uncouplers with the translocator seemed to be the most probable explanation for the experimental observations [18]. Finally, inhibition of amino acid transport in *Bacillus subtilis* by uncouplers has also been explained by a direct interaction of the uncoupler with the transport system [19,20]. Further experiments will be necessary, however, to elucidate the exact mode of action of uncouplers in these systems.

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