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THE EFFECT OF INTRACELLULAR pH ON THE RATE OF HEXOSE UPTAKE IN *CHLORELLA*

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Summary

The rate of hexose uptake by *Chlorella* is reduced by uncouplers such as carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone or dinitrophenol even before concentration equilibrium is reached. The addition of uncouplers changes the membrane potential and the intracellular pH. The membrane potential does not influence the initial velocity of net sugar uptake, whereas manipulation of the cell pH by means of dimethyloxazolidinedione or by butyric acid uncovered a dramatic influence of cell pH on the rate of hexose uptake: at pH values of 7.5—6.8 maximal rate of uptake is observed but at more acid pH a strong inhibition takes place with virtually total blockage of uptake at pH 6.1. The decrease of cell pH to 6.1 in the presence of carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone could therefore account for the decrease in hexose transport rate. It was shown that the intracellular pH as such determines the rate of uptake and not the pH difference between inside and outside; the transport rate did not correlate with ΔpH.

Introduction

The transport of hexoses by the green alga *Chlorella vulgaris* is directly powered by the protonmotive potential difference. Metabolic inhibitors like the uncoupler FCCP abolish the accumulation of 6-deoxyglucose, a non-metabolized glucose analogue, but inhibitors also reduce the translocation velocity for example for 6-deoxyglucose. As a consequence the uptake rate for 6-deoxyglucose into cells is inhibited by FCCP even far below the concentra-

Abbreviations: DMO, dimethyloxazolidinedione; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

tion equilibrium. Or when FCCP is added to cells which had previously accumulated 6-deoxyglucose only a slow rate of efflux of sugar is observed despite the entire halt of sugar influx [1].

Apparently the catalyzed transfer of hexose into the cell and out of the cell is strongly affected by FCCP, a feature also observed for the yeast *Rhodotorula gracilis* [2], but in contrast to lactose transport in *Escherichia coli* [3] and a number of other transport systems [4].

The simplest explanation of the unusual behaviour of *Chlorella* would be to assume a direct reaction of FCCP with an essential sulfhydryl group of the transport-catalyzing protein [5]. However, this possibility is ruled out by the observation that not only FCCP but also the chemically quite different uncoupler dinitrophenol affects sugar transport in the same way [6]. In addition it has been observed that in the presence of the antibiotic nystatin *Chlorella* cells are able to catalyze facilitated diffusion even in the absence of energy. Under such conditions FCCP has no inhibitory effect neither on influx nor on efflux of hexose [7]. The addition of the sulfhydryl-reactive agent *N*-ethylmaleimide, however, completely stops this hexose transport in the presence of nystatin [8].

Since there exists strong evidence for a direct role of protons in energization of active sugar transport in *Chlorella* [9,10], it seemed likely that the inhibition of the rate of sugar transport by a proton conductor such as FCCP [11] is exerted via a reduction of ΔpH and/or the membrane potential. It will be shown, however, that this is not the case. The crucial factor rather seems to be the lowered intracellular pH value.

Materials and Methods

The unicellular green alga *C. vulgaris* was grown as described previously in mineral salt medium with light and air (enriched with 3% carbon dioxide) [1]. 6-Deoxyglucose and dimethyloxazolidinedione was obtained from Koch-Light, Colnbrook, U.K. 6-[^{3}H]Deoxyglucose was tritiated by and [^{14}C]dimethyloxazolidinedione purchased from The Radiochemical Centre Amersham, England; FCCP was a gift of Dr. P. Heytler.

Uptake of 6-deoxyglucose. Chlorella cells (25 μl packed cells/ml) were induced for hexose uptake by incubation in 25 mM sodium phosphate, pH 6.0, with 2.8 mg glucose/ml present. After 3 h incubation at 27°C the cells are fully induced and the glucose has been used up. Then the cells at a density of 9 μl packed cells/ml were shaken in the same buffer together with 1 mM 6-[^{3}H]-deoxyglucose (specific activity 10 nCi/μmol).

Samples were withdrawn at 30-s intervals, rapidly filtered, washed and extracted by boiling in 0.01 N HCl for 10 min. The radioactivity of the extract was measured by liquid scintillation counting.

Determination and manipulation of cell pH. The algae (9 μl packed cells/ml) were incubated in 10 μM [^{14}C]dimethyloxazolidinedione for 15 min and were then separated from the medium by centrifugation through a silicon oil layer. The radioactivity of cells and medium was determined after combustion by liquid scintillation counting. When a more rapid determination of the intracellular pH was necessary, the cells were equilibrated with radioactive dimethyl-

oxazolidinedione as above, then a sample was taken to determine internal radioactivity at equilibrium and then the cell suspension was diluted 100-fold into a medium with or without FCCP present. Samples were removed at 1 min intervals, filtered, and the radioactivity in the cells was determined as above. The rate of efflux of radioactivity from the cells was used as a measure of internal dimethyloxazolidinedione in the undissociated form. By this figure the internal pH could be calculated using the mass law.

For manipulation of internal pH the algae were shaken with dimethyloxazolidinedione or butyric acid of different concentrations (5—100 mM).

Results and Discussion

FCCP decreases the membrane potential of *Chlorella* from —130 to —90 mV [10]. But as has been shown previously [12] the magnitude of the membrane potential between —30 and —130 mV does not affect the velocity of net hexose uptake. It seemed likely, therefore, that the ΔpH regulates the transport rate. On the other hand, it also has to be considered possible that either the external or the internal pH value as such controls the rate of transport. Since in the usual experiment the external pH is maintained more or less constant at pH 6.0 by external buffer, it remains unaffected by the addition of uncoupler. The internal pH of *Chlorella* was previously measured by means of the distribution of the weak acid dimethyloxazolidinedione (DMO). Since equilibration of DMO takes about 10 min with this method faster pH transients cannot be sensed. The most prominent inhibition of sugar uptake by FCCP occurs, however, during the first few minutes; later on the cells apparently recover from the uncoupling action by a mechanism unknown so far. To meet the requirement for a fast determination of intracellular pH a slight modification of the common method was used. The cells were equilibrated with radioactive dimethyloxazolidinedione, then the cell suspension was diluted into a non-radioactive medium and the rapid efflux of dimethyloxazolidinedione was followed. The rate of efflux is directly dependent on the amount of the protonated acid within the cell which is determined by the cell pH. Thus, the rate of efflux measured in the first minutes can be used to calculate the cell pH. *Chlorella* cells were equilibrated with radioactive DMO and then diluted into a large volume of DMO-free medium with or without FCCP.

As documented in Fig. 1 the efflux of DMO in FCCP-treated cells is 4.1 times faster than in control cells. The internal pH of the control cells was 7.1 as measured by the conventional distribution technique [13]. Since in control cells the uncharged form of DMO amounts to 14%, it must occur to 4.1 times more, i.e. 58% in FCCP-poisoned cells; from this a cell pH of 6.15 can be calculated.

The question is whether this drastic change in the intracellular pH causes the observed inhibition of sugar by FCCP. To test this possibility the influence of cell pH on the rate of sugar uptake was measured. The manipulation of cell pH was achieved by high concentrations of permeable acids such as dimethyloxazolidinedione or butyric acid. These compounds diffuse into the cell as the protonated acid and, due to the alkaline interior, dissociate into anions and protons, which acidify the cell interior. As shown in Fig. 2 at an internal pH

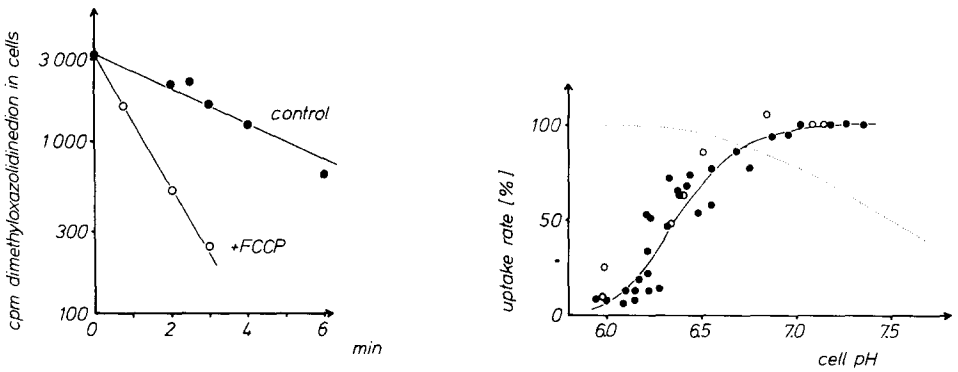


Fig. 1. Efflux of previously equilibrated dimethyloxazolidinedione out of cells by dilution into control or FCCP-containing medium. The cells (200 µl packed cells/ml) were incubated for 20 min in 0.3 µCi dimethyloxazolidinedione (specific activity 46 Ci/mol) and then diluted into 100-fold volume of 25 mM sodium phosphate (pH 6.0) and 1 mM 6-deoxyglucose in absence or presence of 50 µM FCCP. Samples of 50 ml were filtered and the radioactivity in the cells was measured. The internal pH at the beginning of the efflux exeriment was 7.09.

Fig. 2. 6-Deoxyglucose uptake rate at different cell pH values. The different cell pH values at constant outside pH of 6.0 were achieved by 15 min incubation in dimethyloxazolidinedione of 0—100 mM (closed symbols) or 5—50 mM butyric acid (open symbols). · · · · · ·, the transport activity at the corresponding outside pH values.

from 7.5 to 6.8 sugar uptake is optimal. At lower values a strong inhibition is seen and at pH 6.0 hardly any uptake activity can be measured. The same results were obtained independent of whether dimethyloxazolidinedione or butyric acid was used for cell pH manipulation. A direct inhibitory action of these compounds seems unlikely, therefore. At an inside pH value of 6.15, the condition obtained by treatment with FCCP, about 10% uptake activity is expected, a value which is in good agreement with experimental results. Thus the decrease of cell pH by FCCP most likely is the reason for the observed inhibition of sugar uptake rate. Since FCCP also strongly inhibits the efflux of accumulated sugar [1] it has to be assumed that low intracellular pH values negatively affect catalyzed transport steps. As indicated by the dotted line in Fig. 2, the dependence of the uptake rate on outside pH shows a considerably different characteristic.

In the experiments described the cell pH was decreased at a constant outside pH. Thus in fact also ΔpH was decreased and it might be argued, therefore, that the proton gradient is responsible for the changes observed in uptake velocity. To test this possibility the uptake of 6-deoxyglucose was determined at constant ΔpH, but different internal pH values as well as at different ΔpH but constant internal pH values. No correlation between ΔpH and the rate of sugar uptake has been observed (Table I). High transport velocities are observed at ΔpH values of 0.3 or 1.05, and, at low inside pH, low velocities at ΔpH values of 0.31 and 1.02. Again, however, a close correspondence between uptake rate and cell pH has been observed: full activity above pH 7.0 and a greatly decreased rate at pH 6.2 or below.

From the data above and from those published previously [12] it has to be

TABLE I

COMPARISON OF CELL pH AND ΔpH AND THEIR EFFECT ON 6-DEOXYGLUCOSE UPTAKE

As 100% was taken the rate of uptake achieved at an outside pH of 6.0. It amounted to 200 μmol/ml packed cells per hour.

Experimental condition	Intracellular pH	ΔpH	Rate of uptake (%)
Outside pH 6.0	7.05	1.05	100
Outside pH 5.07 plus 40 mM dimethyloxazolidinedione	6.09	1.02	6.2
Outside pH 7.0	7.30	0.30	85
Outside pH 5.84 plus 40 mM dimethyloxazolidinedione	6.15	0.31	7.8

concluded that of the different effects caused by FCCP such as a decrease in membrane potential, a decrease in the pH difference and a lowering of the intracellular pH, only the latter seems responsible for the significant inhibition of sugar transport velocity. It is expected, therefore, that FCCP does not inhibit the rate of 6-deoxyglucose transfer under conditions where the internal pH cannot fall below the critical value of 6.5, for instance at alkaline pH values of the medium. Indeed no inhibition by FCCP of for example 6-deoxyglucose efflux occurs at an outside pH of 8.0 (Fig. 3). A strong respiratory increase due to the uncoupling action of FCCP could be measured and shows that the uncoupler was active under these conditions.

There exist a number of possible explanations, how the intracellular pH might affect the activity of the sugar transport system. Thus, it is known for example that the protonated transport protein does not catalyze H^+ translocation in the absence of hexoses. Therefore, the addition of sugars to cells leads to a transient disappearance of protons from the medium [9]. Also the mere logics of H^+ cotransport, and for that matter of any ion cotransport, demand

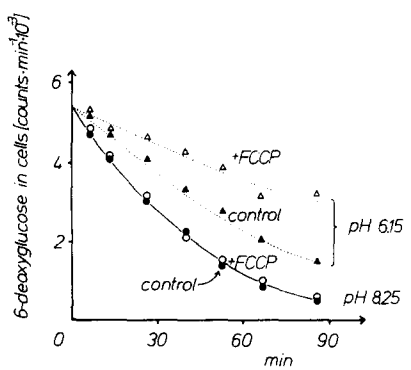
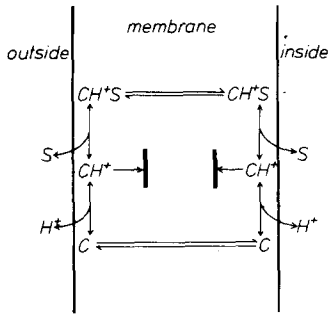


Fig. 3. Efflux of 6-deoxyglucose at acid and alkaline pH value in absence or presence of FCCP. The cells (3.5 μl packed cells/ml) were allowed to take up 2 mM labeled 6-deoxyglucose (specific activity 0.25 Ci/mol) till a plateau value was reached. Then the cells were diluted 230-fold into 25 mM sodium phosphate at pH 8.25 with or without 100 μM FCCP, or into buffer, pH 6.15, with or without 20 μM FCCP. These FCCP concentrations achieved the same respiration enhancement at the respective pH value.



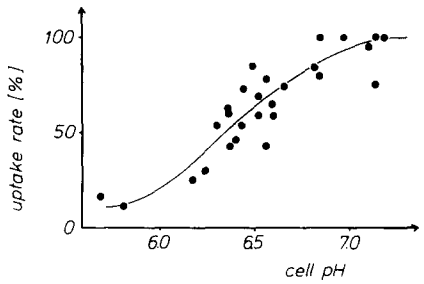


Fig. 4. Model for hexose proton cotransport in *Chlorella*. C, the transport protein; S, sugar. The arrows perpendicular to the membrane indicate that in some way the transport protein, except in the protonated form CH^+, is able to 'communicate' with both the bulk phases on either side of the membrane.

Fig. 5. Steady-state uptake of 6-deoxyglucose at different cell pH values. The cells had been preloaded with 10 mM 6-deoxyglucose for 4 h. Then the cells were washed and resuspended in 1 mM radioactive 6-deoxyglucose. Simultaneously the cell pH was manipulated by incubation with 0—100 mM dimethyloxazolidinedione.

that the transport substrate is required for the translocation step, since otherwise the transport system itself would act like an uncoupler and catalyze the breakdown of the corresponding ion gradients. Assuming a symmetric transport system would have the consequence that the protonated transport protein cannot 'enter' nor 'get to the outside again' without the sugar. Only the 'empty' transport protein (C) and the ternary complex (CH^+S) can 'traverse' the membrane (Fig. 4). If these assumptions were correct it would be expected that a low intracellular pH does not affect hexose exchange transport. When the cells were preloaded with non-radioactive 6-deoxyglucose and then the exchange transport with radioactive sugar was measured at different intracellular pH values the data of Fig. 5 were obtained. Surprisingly the identical pH dependence as for net uptake of sugars has been observed. It seems unlikely, therefore, that the assumptions made above (Fig. 4) about a symmetric behaviour of the ternary complex CH^+S are correct, since certainly the pH optimum for the 'entry' of CH^+S is pH 6 and below (dotted line in Fig. 2).

Although the results of Fig. 5 do not support the simplest possible explanation of the effect of intracellular pH as given above, they are in agreement with the previous report, that FCCP does severely inhibit even the exchange transport of hexoses in *Chlorella* [1]. Whether the intracellular low pH inhibits transport at all directly (e.g. by protonation of the transport protein) or indirectly, e.g. by more general effects on the cytoplasmic membrane or a conformation change of the transport protein, could only be a matter of speculation at the moment. It seems certain, however, that an asymmetric transport system has to be assumed, since such a drastic difference is observed depending on whether a pH value of 6 exists within the cell or in the medium surrounding the cell.

Another unexpected observation has been the steep slope of the pH dependence curve of Fig. 2. This curve does not follow a simple titration curve, but rather has the slope of a second or third-order reaction. This indicates either

the existence of several cooperative anionic groups of the same pH value or oligomers of the transport protein, or that the determination of the internal pH becomes increasingly more erroneous in the acid pH range by interference of cell organelles.

Inhibition of β-hydroxybutyrate uptake by acidification of cell pH was reported for rat thymocytes [7], and similarly for phosphate transport by yeasts [8] where, too, a slope of the titration curve is shown which corresponds to a third or fourth-order reaction.

The inactivation of proton cotransport processes by low internal pH may be seen as a protecting mechanism against further acidification of the protoplasma in situations, caused by poisons or other external factors, where internal pH is in danger to get entirely out of control.

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