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RESPIRATORY INCREASE AND ACTIVE HEXOSE UPTAKE OF *CHLORELLA VULGARIS*

M. DECKER AND W. TANNER

Fachbereich Biologie der Universität Regensburg, Regensburg (Germany)

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SUMMARY

1. When non-metabolizable glucose analogues like 3-*O*-methylglucose and 6-deoxyglucose are added to *Chlorella vulgaris* cells an increase in the rate of respiration is observed.

2. This respiratory increase is strictly correlated with sugar transport since (a) non-induced cells do not respond to the addition of sugars; (b) only transportable sugars cause the respiratory increase; (c) the extent of the increase in O_2 uptake depends on the amount of sugar added, whereby the different K_m values for uptake of various sugars correspond closely to the "K_m values" for the increased O_2 uptake.

3. Each extra O_2 brings about the uptake of 5.1 molecules of 6-deoxyglucose, which corresponds to 1.18 "ATP" per sugar. For glucose, which is rapidly metabolized in the cells, this ratio is 2.5 ATP per glucose taken up. The extra ATP covers exactly the energy required for the conversion to sucrose and starch of most of the glucose taken up.

4. In the presence of 6-deoxyglucose the increased respiration is the same during steady state (net influx being zero) as at the onset of sugar uptake. Since the influx during steady state is about twice the initial influx (positive transmembrane effect) the ratio "ATP" required per 6-deoxyglucose taken up decreases during uptake and reaches 0.5 to 0.6 during steady state.

5. Steady-state influx is completely energy dependent; it can be fully inhibited by uncoupling agents. It is considerably less sensitive, however, to sodium azide than net influx. These and the above observations (see 4) can be explained by assuming that during steady-state part of the transport energy required is supplied by respiration and part by a direct reversal of the influx reaction; uncouplers inhibit both kinds of energy generation.

6. All data reported are compatible with the model presented in the preceding publication[1].

INTRODUCTION

In the preceding paper[1] evidence for a detailed model for hexose transport and accumulation by *Chlorella* cells has been presented. In the following paper the

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

relation of sugar transport to respiratory metabolism of these cells in the dark has been investigated.

Increased respiratory rates following the addition of salts[2,3] or organic molecules[4,5] to various organisms as well as stoichiometric relations of transport and respiration have repeatedly been reported[2-7]. The possible fallacies of such experiments and the conclusions drawn from them are generally realized[2,3,8]. Nevertheless it seems to be a valid approach for estimating the energy requirement of active uptake processes as long as a strict correlation between uptake and the increased respiration exists.

Kepes[5] has found that *Escherichia coli* show increased respiration rate when exposed to β-galactoside analogues like thiomethyl-β-D-galactoside. From the data it was deduced that 1 ATP is required for one β-galactoside transported. It was also observed that the increased respiratory rate remained constant even when net influx became zero during steady state.

Similar findings have now been made for hexose transport of *Chlorella*. However, the respiratory rate stays constant in spite of the fact that the influx rate during steady state is twice that found at the beginning of uptake (positive transmembrane effect). This is explained by an energy-producing efflux, an energy which partly drives the uptake. The steady-state influx is completely inhibitable by uncoupling agents.

MATERIALS AND METHODS

6-Deoxyglucose from Koch–Light Laboratories, Colnbrook, England, was tritiated by the Radiochemical Centre, Amersham and purified subsequently by paper chromatography (butanol–pyridine–water–acetic acid = 60:70:30:3, by vol.); its specific activity was 100 μCi/μmole.

All other chemicals used and the strain of *Chlorella vulgaris* were the same as in the preceding paper[1].

Uptake measurements

Uptake of radioactive sugars was measured by the membrane filter technique as described previously[10]. D-[^{14}C]Glucose uptake was followed by measuring the disappearance of radioactivity from the medium (1 μCi D-glucose with a final concentration of $2 \cdot 10^{-2}$ M was present initially). Aliquots were taken from after a few minutes up to 3 h, centrifuged and the radioactivity was determined by pipetting an aliquot of the supernatant directly into Dioxan/PPO scintillation fluid. The disappearance of radioactivity from the medium was linear with time. The exact incubation conditions were identical to those used during respiration measurements. The stoichiometry of sugar and O_2 uptake was measured in parallel experiments carried out at the same time and with the same batch of algae. All data are based on packed cell volume without correction for extracellular water (about 33%).

Measurement of respiration

O_2 uptake was measured by conventional manometric technique. Induced algae (60 μl packed cells) were incubated in 2 ml 0.025 M sodium phosphate buffer, pH 6.5. After the rate of respiration was followed for 30 min 0.5 ml of the corresponding sugar was added from the side arm; the final concentration was $1 \cdot 10^{-2}$ M

in the case of 6-deoxyglucose and $2 \cdot 10^{-2}$ M in the case of glucose. To two controls 0.5 ml of mannitol and water respectively was added.

For the experiments of Fig. 1 and Table I respiration was measured polarographically with a Clark electrode and a Gilson Oxygraph; 5–10 times more dilute cell suspension could be used than with manometric techniques.

RESULTS AND DISCUSSION

Correlation between transport and increased respiratory rate

Chl. vulgaris cells possess an inducible active hexose uptake system[9,10]. The addition of D-glucose or 3-*O*-methylglucose to glucose induced *Chl. vulgaris* cells results in an increased respiration. Non-induced cells do not show this effect (Fig. 1a);

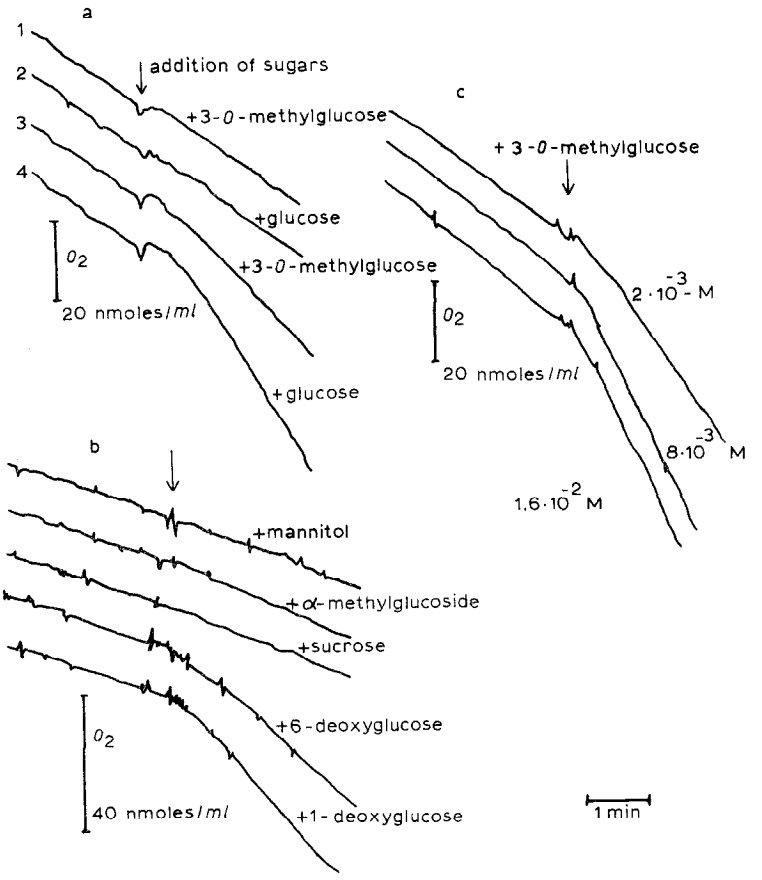

Fig. 1. Polarographic determination of increased O_2 consumption of *Chlorella* cells due to the addition of sugars. (a) In a total volume of 1.8 ml algal suspension (12 μl packed cells) in 0.04 M sodium phosphate buffer (pH 6.5) the decrease in O_2 content was followed before and after the addition of the corresponding sugars ($5 \cdot 10^{-3}$ M final concn). Traces 1 and 2 were obtained with non-induced, Traces 3 and 4 with induced cells. Temperature 26 °C. (b) Experimental conditions as in (a). All traces were obtained with induced cells. All sugar concentrations were $1.1 \cdot 10^{-2}$ M except for 6-deoxyglucose, where it was $5.5 \cdot 10^{-3}$ M. Temperature 20 °C. (c) Concentrations of 3-*O*-methylglucose as given in the figure; other conditions as in (a). Temperature 26 °C.

with these cells a slight continuous increase can be observed and a rate comparable to the increased rate of induced cells is only reached after more than 30 min.

Since 3-*O*-methylglucose is not metabolized in *Chlorella* at all[10] it seemed reasonable to assume that in the case of sugar analogues the increased respiration directly reflects the energy required for transport work. Furthermore this is supported by the observation that only transportable sugars cause this respiratory increase (Fig. 1b). Disaccharides and sugar alcohols which have been found previously[11] not to inhibit the uptake of 3-*O*-methylglucose also did not affect respiration of these cells. This correlation has been found to hold for sucrose and mannitol (Fig. 1b) but also for the non-transportable sugars trehalose, lactose, rhamnose, ribose and α-methylglucoside. In addition to the sugars shown in Fig. 1, an increased respiration was observed with D-xylose and 2-deoxyglucose, which again agrees with previous data[11].

The increase in the rate of respiration depends on the concentration of the sugar added and is saturable (Fig. 1c). A strong argument in favour for a direct relation of this increased respiration to transport arises from the fact that the K_m values for uptake of various sugars, although differing among each other by a factor of 100, show fairly close agreement to the sugar concentration causing half maximal respiratory increase (Table I).

TABLE I

K_m VALUES FOR SUGAR UPTAKE AND FOR INCREASED OXYGEN CONSUMPTION

The K_m values for sugar uptake except that for 6-deoxyglucose are taken from a previous publication[11]. The K_m values for increased O_2 consumption were determined polarographically (see Fig. 1). The rates of extra O_2 taken up were plotted against concentration of sugar present.

Sugars	*K_m for uptake (M)*	*K_m for increased O_2 consumption (M)*
3-*O*-Methylglucose	$1 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$
6-Deoxyglucose	$3.2 \cdot 10^{-4}$	$2 \cdot 10^{-4}$
2-Deoxyglucose	$1–2 \cdot 10^{-5}$	$2–4 \cdot 10^{-5}$
D-Glucose	$1–2 \cdot 10^{-5}$	$2–3 \cdot 10^{-5}$

Since more than 80% of the glucose taken up by these cells are assimilated to oligo- and polysaccharides[12] the increased respiration also reflects the draw upon energy for assimilation. In spite of this, the K_m values for glucose uptake and extra O_2 consumption also correspond to each other (Table I), which suggests that the uptake of glucose is the rate-limiting step for glucose metabolism of these cells. This is supported by the observation that less than 1% of the glucose taken up is present as free glucose in these cells[12].

Stoichiometry of sugar uptake and increased rate of respiration

The stoichiometric relation between sugar taken up and extra oxygen consumed was studied in detail for 6-deoxyglucose. The data are summarized in Table II. The mean values of five experiments show that slightly more than 5 molecules of 6-deoxyglucose are taken up for each additional O_2. Assuming a P/O ratio of 3,

Kandler[13] has obtained a value of 2.7 from *in vivo* experiments, these data could mean that slightly more than 1 ATP (or an energetic equivalent) are required per sugar molecule transported from the outside to the inside of the cell. The ratio "ATP" required per 6-deoxyglucose has been found to remain fairly constant in various experiments, although the absolute rates of sugar uptake vary by more than a factor of 2 (Table II).

Under the conditions used for these experiments, *i.e.* a 6-deoxyglucose concentration of 10^{-2} M, an accumulation ratio $(\text{sugar})_{\text{inside}}/(\text{sugar})_{\text{outside}}$ of close to 10 is observed. Thus 1 mole "ATP" would easily suffice to bring about the osmotic work (1.3 kcal/mole) required; even for the highest accumulation of 350-fold so far observed[10] for 6-deoxyglucose at an initial outside concentration of $3.3 \cdot 10^{-4}$ M the osmotic work only amounts to 3.5 kcal/mole.

TABLE II

STOICHIOMETRY OF 6-DEOXYGLUCOSE UPTAKE AND INCREASED RESPIRATION

Uptake of 6-[^{3}H]deoxyglucose was measured in a total volume of 7.5 ml 0.025 M sodium phosphate buffer (pH 6.5) which contained 180 μl *Chlorella* cells (packed cells) and 2.5 μCi 6-[^{3}H]deoxyglucose (0.01 M). Under these conditions the rate of uptake stayed constant for almost 2 min. For more details and the measurement of respiration see Materials and Methods.

Expt	*Initial influx* (*μmoles/ml packed cells per h*)	*μmoles O_2/ml packed cells per h*			*Sugar taken up per O_2*	*"ATP" required per sugar transported**
		Endogenous respiration	*Respiration in the presence of 6-deoxyglucose*	*Respiration increase*		
1	150	42.5	78.5	36	4.20	1.44
2	210	38	71.5	33.5	6.27	0.96
3	370	28	106	78	4.75	1.26
4	320	37.5	95.5	58	5.51	1.08
5	350	67	134	67	5.22	1.14
Average	280	42	97	55	5.10	1.18

* The values are obtained by dividing 6 by the value of the previous column.

TABLE III

STOICHIOMETRY OF D-GLUCOSE UPTAKE AND INCREASED RESPIRATION

For experimental conditions see Materials and Methods.

Expt	*Initial influx* (*μmoles/ml packed cells per h*)	*μmoles O_2/ml packed cells per h*			*Sugar taken up per O_2*	*"ATP" required per sugar transported**
		Endogenous respiration	*Respiration in the presence of glucose*	*Respiration increase*		
1	340	42.5	182.5	140	2.43	2.46
2	260	28	140	112	2.32	2.58
3	290	43	162	119	2.44	2.46
4	240	54	158	104	2.36	2.54
5	350	47.5	195.5	148	2.36	2.54
Average	296	43	167	124	2.38	2.51

* The values are obtained by dividing 6 by the value of the previous column.

It has been reasoned above that in the case of D-glucose uptake the increased respiration should be responsible for the uptake work as well as for the energy required to assimilate part of the sugar taken up. If this were so the stoichiometry of glucose taken up per extra O_2 should be lower. That this is indeed the case can be seen in Table III. The mean values of 5 experiments show a requirement of 2.5 ATP per glucose taken up.

It is known[12] that more than 80% of the glucose taken up is assimilated: 50% is found in sucrose (3 ATP are required to convert 2 molecules of glucose to sucrose) and 30% in starch (2 ATP are required per glucose). If it is assumed that the residual 20% need at least 1 ATP in the hexokinase reaction before they are further metabolized, then a value of close to 1.5 ATP can be calculated as a fair approximation of the amount of energy required for glucose assimilation. Therefore, the experimental value of 2.5 for uptake *plus* assimilation fits ideally.

Increased respiration, steady-state uptake and positive transmembrane effect for influx

The data of Table II have all been obtained from initial uptake measurements. However, it has been shown that the rate of influx into empty cells also in *Chlorella* is smaller than that into preloaded cells[1,10]. For 3-*O*-methylglucose the ratio of the two rates was found to be 1.2–1.5; for 6-deoxyglucose an even higher ratio of 2 has been observed (Table IV). These latter data were obtained by incubating 2 samples with 10^{-2} M 6-deoxyglucose, of which one sample also contained radioactive 6-deoxyglucose. When this sample had reached its steady state of uptake, normally after 3 h at an inside concentration of around 10^{-1} M, radioactive 6-deoxyglucose was added to the second sample. After correcting for the higher specific radioactivity of the second sample the steady-state influx rates of Table IV were obtained.

When the O_2 uptake was followed during several hours it was observed that in the case of 6-deoxyglucose the increased O_2 consumption stayed completely constant, *i.e.* O_2 uptake was identical during steady state where net influx is zero to that in the very beginning of sugar uptake. The same has been observed by Kepes[5] for β-galactoside uptake of *E. coli*. However, due to the much faster steady-state

TABLE IV

TRANSMEMBRANE EFFECT FOR 6-DEOXYGLUCOSE INFLUX

Experimental conditions as given in Table II and in Materials and Methods. Steady-state influx was measured in a parallel sample which contained only 10^{-2} M nonradioactive 6-deoxyglucose. 2.5 μCi 6-[^{3}H]deoxyglucose (spec. act. 100) were added to this sample after the first sample (*i.e.* initial influx sample) was in the steady state.

Expt	*μmoles/ml packed cells per h*		*Ratio*
	Initial influx	*Steady state influx*	
1	340	600	1.76
2	200	400	2.0
3	240	580	2.4
4	350	730	2.1
Average	282	577	2.0

TABLE V

STOICHIOMETRY OF 6-DEOXYGLUCOSE UPTAKE AND INCREASED RESPIRATION DURING INITIAL UPTAKE AND STEADY-STATE UPTAKE

Experimental conditions as given in Table IV and in Materials and Methods.

	Initial uptake	*Steady state*
6-Deoxyglucose uptake (μmoles/ml packed cells per min)	5.9	12.5
Respiration increase (μmoles O_2/ml packed cells per min)	1.12	1.12
Sugar taken up per O_2	5.3	11.3
"ATP" required per sugar transported	1.13	0.53

influx the uptake seems to be more efficient. The ratio "ATP" required per 6-deoxyglucose taken up drops to 0.5–0.6, which is shown for a typical experiment in Table V.

Theoretically, of course, the above ratio could even drop much more, since steady-state influx could be running almost without an energy requirement. Thus Heinz and Mariani[14] have suggested that the amino acid transport during steady state in Ehrlich cells proceeds largely by homoexchange without energy requirement and only about 20 % show true active transport. However, this explanation does not hold for *Chlorella*, as has already been shown in the preceding paper[1], since steady-state influx can be completely inhibited by FCCP. It is proposed, therefore, that steady-state influx has the same energy demand of roughly 1 "ATP" per sugar as net influx. But, whereas during net influx energy is solely supplied by respiration, it is suggested here that energy derived from respiration *plus* energy generated by efflux during steady state supplies steady-state influx. The latter could be brought about by a simple reversal of the energy-requiring uptake reaction (see model in Fig. 7 of the preceding paper). In the case of 6-deoxyglucose the efficiency of the energy-producing efflux *plus* the re-use of this energy would have to run at about

TABLE VI

INHIBITION OF INITIAL INFLUX AND STEADY-STATE INFLUX OF 6-DEOXYGLUCOSE BY FCCP AND SODIUM AZIDE

Experimental conditions as given in Table IV and in Materials and Methods. The poisons were added to the initial influx sample 5 min before the radioactive and nonradioactive sugar were added and in the case of the steady-state influx sample 5 min before the radioactive 6-deoxyglucose was put in. However, basically the same results were obtained, when the poisons were added in each case with the total sugar and the 6-[^{3}H]deoxyglucose, respectively.

Expt		*Concn of poison used (M)*	*Initial influx (μmoles/ml packed cells per h)*		*% of control*	*Steady-state influx (μmoles/ml packed cells per h)*		*% of control*
			Control	*+Poison*		*Control*	*+Poison*	
1	FCCP	$5 \cdot 10^{-5}$	230	7.2	3	410	58	14
2		$2.5 \cdot 10^{-5}$	210	17	8	320	53	17
3		$2.5 \cdot 10^{-5}$	200	20	10	400	133	33
1	Sodium azide	10^{-2}	200	30	15	500	200	40
2		10^{-2}	240	34	14	580	210	36

50%, since only half of the steady-state influx would have to be brought about by this efflux energy.

The fact that the respiratory rate stays constant with 6-deoxyglucose from the beginning to the steady state might then be a mere accidental reflection of the size of the positive transmembrane influx effect of 2 and the efficiency of 50% for the re-use of the energy generated by efflux. That this might be indeed so is indicated by the preliminary observation that with 3-*O*-methylglucose, which shows a lower transstimulated influx, the increased O_2 consumption slightly drops with time.

If the assumption were correct that part of the energy for uptake is generated by efflux it would be expected that some inhibitors affect steady state and net influx differently. This was indeed observed: FCCP as well as sodium azide inhibited uptake less during steady state, although the effect was only small in the case of FCCP (Table VI). However, the same data do show that the extra influx during steady state as compared to net influx is inhibited by both poisons. Thus energy is certainly also necessary for this additional uptake. The observation that steady-state uptake is less inhibitable than net influx has also been made for other uptake systems[15,16].

CONCLUSIONS

The unusual features of hexose uptake of *Chlorella* made it necessary to propose a rather involved model[1]. Evidence for the postulated direct reversal of the influx reaction (preceding paper, Fig. 7), whereby energy is regenerated, is presented in this paper. Thus the observation that the efficiency of the system increases during uptake is most easily explained in this way. On the other hand, there is no part of the steady-state influx which is energy independent (Table VI and Fig. 1 of the preceding paper). The observation that steady-state influx is less sensitive to FCCP and sodium azide is best explained by the assumption that in the steady state an additional process besides respiration supplies the energy required for transport and that this additional process is less sensitive to the poisons.

The transmembrane effect for influx, which has been observed for 3-*O*-methylglucose[1,11] and 6-deoxyglucose (Table IV) can be explained according to Heinz and Walsh[17]. During steady state the reaction 2

is mainly responsible for bringing the carrier to the outside again, whereas it is reaction 1 $C_{in} \rightarrow C_{out}$ during initial uptake. When the rate of reaction 2 is greater than the rate of reaction 1 a transmembrane effect for influx will be observed.

A number of conclusions drawn in this and the preceding paper will be checked in the future under different experimental conditions. *Chlorella* cells can bring about active uptake of sugars either using respiration or under anaerobic conditions using light as energy source[9]. Quantitative experiments on the light-driven active sugar uptake of *Chlorella* are in progress.

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