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DIFFERENT PROTON-SUGAR STOICHIOMETRIES FOR THE UPTAKE OF GLUCOSE ANALOGUES BY *CHLORELLA VULGARIS*

EVIDENCE FOR SUGAR-DEPENDENT PROTON UPTAKE WITHOUT CONCOMITANT SUGAR UPTAKE BY THE PROTON-SUGAR SYMPORT SYSTEM

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SUMMARY

The uptake of hexoses by *Chlorella vulgaris* is accompanied by the uptake of protons. For 6-deoxyglucose a stoichiometry of one proton taken up per sugar molecule has been measured, whereas for 1-deoxyglucose approximately two protons are taken up per sugar molecule.

It was found that in the presence of 1-deoxyglucose a considerable proportion of "carrier" catalyzes the transport of protons without the concomitant transport of sugar. Presumably the binding of sugar initiates the translocation of the carrier-proton-sugar complex, but whereas 1-deoxyglucose can still dissociate from the complex at the external side of the cytoplasmic membrane, the translocation of the carrier-proton complex continues.

This conclusion was reached since (a) the composition of the translocated carrier-proton-sugar complex is the same for both sugar. Its formation is a first order reaction with respect to protons. (b) When 6-deoxyglucose, present inside cells, is exchanged for external sugar, the exchange ratio is two to one when the external sugar is 1-deoxyglucose, two molecules of 6-deoxyglucose are lost for each molecule of 1-deoxyglucose entering. This result indicates that during uptake of 1-deoxyglucose statistically only each second carrier molecule appearing at the internal side of the cytoplasmic membrane is carrying sugar.

INTRODUCTION

In the unicellular green alga *Chlorella vulgaris* the synthesis of a hexose uptake system can be induced, which enables the organism to accumulate non-metabolizable analogues of glucose [1, 2]. The net transport of glucose and its analogues is accompanied by a net transport of protons in a strictly stoichiometric ratio [3, 4]. Thus the uptake of sugar by *Chlorella* cells appears to work as a proton symport system, in accordance with the universal mechanism for metabolism coupled

transport processes proposed by Mitchell [5–8]. The stoichiometry of hexoses taken up to protons taken up has been determined for several hexoses, all of which are transported via the same uptake system. Different values have been obtained however, for different hexoses, for example a value of one for glucose and 6-deoxyglucose, but a value of two for 1-deoxyglucose* [4]. It seems difficult at first sight to reconcile this result with the detailed model derived from previous experiments with 6-deoxyglucose [9]. The experiments described herein have been carried out to explain why different stoichiometries are obtained for different hexoses taken up by the same uptake system. A similar problem has been studied by Christensen [11] with animal cells, in which sodium and amino acids are undergoing exchange.

Apparently different sodium to amino acid stoichiometries, depending on the chemical structure of the amino acid, have been observed. It was found that the composition of the transported complex was always the same whereas the exchange probability of sodium and amino acid at the two surfaces of the membrane was different. In this way net transport of labeled sodium can take place without net transport of labeled amino acid and vice versa [10, 11]. For *Chlorella* evidence has been obtained that the stoichiometry of proton per carrier** is the same for all glucose analogues. But some analogues initiate the translocation of carrier-proton complex without themselves becoming translocated, so that protons are transported in excess of the sugar transported.

MATERIALS AND METHODS

The green alga *Cl. vulgaris* was grown in inorganic medium as described previously [12] harvested and induced for the hexose uptake system by incubation with glucose [1]. The glucose analogue 6-deoxyglucose was obtained from Koch and Light, Colnbrook, 3-*O*-methylglucose from Calbiochem, San Diego, and 1-deoxy-glucose was prepared according to Ness and Fletcher [13]. The 3-*O*-methyl-[^{14}C] glucose was purchased from Radiochemical Centre, Amersham, and [^{3}H] labeled 6-deoxyglucose and 1-deoxy-glucose were tritiated by the Labeling Service of the Radiochemical Centre, Amersham.

The filtration of algae was performed on membrane filters of 0.8 μm pore size from Sartorius, Göttingen, with help of a filtration assembly from Hölzel, Dorfen (G.F.R.), or a syringe filtration set from Sartorius, Göttingen. All experimental values are expressed $\pm$ their average deviation.

Measurement of sugar uptake

Algae, which had induced for sugar uptake by prior incubation with glucose, were shaken in 25 mM sodium phosphate buffer (pH 6.0) at a final cell density of 20 μl packed cells/ml in a water bath at 27 °C. The experiment was started by addition of sugar to a final concentration of 10 mM for 6-deoxyglucose, 1-deoxyglucose or 3-*O*-methylglucose and a specific activity of 50 mCi/mol. Samples were taken within the first 2 min and filtered onto membrane filters. The extraction and determination of radioactivity was accomplished as described previously [2].

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* 1,5-Anhydro-D-glucitol.

** The transport mediating catalyst in the cell membrane is indiscriminately called carrier for the sake of brevity.

Determination of the energy requirement for sugar uptake

The stoichiometry of protons taken up per sugar molecule had been determined previously [4]. The ratio of extra oxygen respired per sugar molecule taken up was measured either by manometry analogous to Decker and Tanner [14] or with an oxygen electrode (Gilson oxygraph) in the same medium as above, but with an algal density of 3 μl packed cells/ml. Non-radioactive sugar was added to give the same final concentration as for the uptake measurements.

Determination of the reaction order with respect to protons of the uptake system

Adapted algae were incubated in 25 mM sodium phosphate buffer at a cell density of 20 μl packed cells/ml at different pH values and sugar was added to a final concentration of 0.2 mM for 6-deoxyglucose, or 0.4 mM for 1-deoxyglucose or 1 mM for 3-*O*-methylglucose at a specific activity of 50 mCi/mol. The sampling and preparation of the algae was as described above.

Determination of the stoichiometry, carrier translocated per sugar molecule transported

To measure the stoichiometry of a carrier that is "translocated" to the inside, the transstimulated efflux was followed. It is assumed that for each molecule of sugar that comes out of preloaded cells (radioactive sugar inside) due to the addition of non-radioactive sugar to the outside, one carrier molecule had to contact the inside. Since on the other hand the actual amount of sugar entering the cells can be measured in a parallel experiment (cells preloaded with non-radioactive sugar and radioactive sugar added to the outside), the stoichiometry of carrier translocated per sugar entering the cells can be determined. This stoichiometry thus is expressed as the ratio of efflux of 6-deoxyglucose to influx of the external sugar.

Induced algae at a cell density of 20 μl packed cells/ml were preloaded with 5 mM 6-deoxyglucose in 25 mM sodium phosphate buffer (pH 6.0) for 4 h. Then the algae were separated from the medium by centrifugation and washed with cold buffer. For the influx test these algae were rapidly resuspended in 25 mM sodium phosphate buffer (pH 6.0) to a final cell density of 10 μl packed cells/ml in the presence of labeled sugar (1 mM for 6-deoxyglucose, 3 mM for 1-deoxyglucose or 10 mM for 3-*O*-methylglucose) at the specific activity 50 mCi/mol. Samples have been taken and filtered on membrane filters and the radioactivity in the cells was extracted and determined as described previously [2].

For the efflux test algae were preloaded with labeled 6-deoxyglucose of a specific activity of 50 mCi/mol. These algae, when separated from the medium and washed after the preloading period as described above, were resuspended in the same medium as for the influx test, but the sugar now present (in concentrations as above) was in the unlabeled form. Samples were withdrawn by a syringe with a filtering unit at the top which retained the cells. An aliquot of the filtrate in the syringe was taken for determination of radioactivity.

In both experimental sets the sampling was accomplished within 2 min during which the inflowing sugar contributed only negligibly to the total internal sugar, and vice versa.

RESULTS

Energy requirement for sugar uptake

The uptake of sugar by *Cl. vulgaris* is so intimately linked to the metabolism of the cell that metabolic energy is even necessary for "downhill" sugar uptake. Since there are protons taken up together with the sugar molecules, it is assumed that the translocation of the carrier-sugar complex is only possible if a simultaneous binding and translocation of a proton is occurring (at least at acid pH values in the outside medium). The electrochemical potential difference of protons across the cytoplasmic membrane would act as the metabolic energy reservoir for sugar uptake in *Chlorella*. During proton-coupled uptake of sugar this potential difference would vanish unless some metabolic mechanism, such as a proton-translocating ATPase, is recycling the protons. In *Chlorella* an immediate increase in respiration is observed after addition of transportable sugar [14], reflecting the increased activity of a proton-translocating device to maintain the protonmotive potential difference. A distinct stoichiometry of protons taken up, per sugar molecule taken up was determined previously for different sugars [4]. This was found to be one for 6-deoxyglucose, but two for 1-deoxyglucose. The same relationship was established when the stoichiometry of extra oxygen respired per sugar molecule taken up was determined, again the "energy" requirement was twice that for 1-deoxyglucose as compared with 6-deoxyglucose (Table I).

Three alternative models to explain different stoichiometries

There are three basic possibilities (depicted in Fig. 1) to explain these results.

I. One might postulate that the carrier molecule has at least two proton-binding sites, which have access to both sides of the cytoplasmic membrane, so that two protons are translocated for each carrier cycle. The structure of the transported sugar molecule determines the number of protons which have to be bound for translocation to be achieved: one for 6-deoxyglucose, two for 1-deoxyglucose. Though this explanation might be the simplest one, it is hard to understand why one sugar would be translocated with two extra protons whereas another one would be translocated by the same carrier molecule with only one proton.

II. The carrier molecule does bind one proton and one sugar molecule and therefore one proton only is translocated per carrier cycle, but at the inner side of the cytoplasmic membrane only the proton is released leaving the carrier-sugar complex to be translocated out again. For some glucose-analogues this failure to dissociate from the carrier at the inner side of the membrane may be negligible, and for others, considerable. The stoichiometry of net uptake of protons per net uptake of sugar

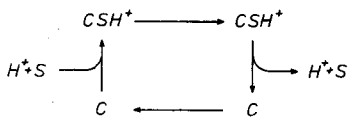
TABLE I

STOICHIOMETRIES OF ENERGY REQUIREMENT FOR 6-DEOXYGLUCOSE AND 1-DEOXYGLUCOSE UPTAKE

The stoichiometries have been determined as described in Materials and Methods.

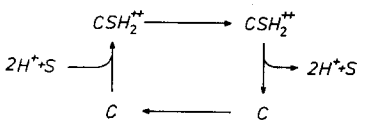
	6-Deoxyglucose	1-Deoxyglucose
Protons taken up/sugar taken up	0.96±0.13	1.95±0.64
Extra oxygen respired/sugar taken up	0.236±0.038	0.386±0.051

Uptake of 6-deoxy glucose:

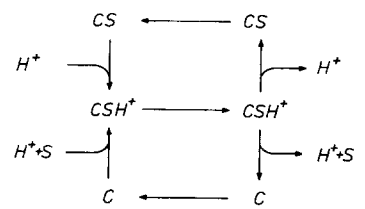


Uptake of 1-deoxy glucose according to:

Model I:



Model II:



Model III:

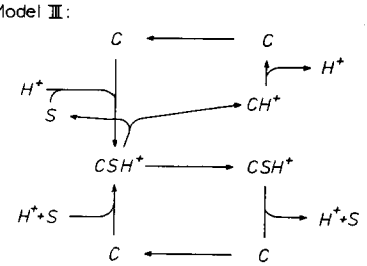


Fig. 1. Three alternative models for the explanation of different stoichiometries for different sugars. (C stands for carrier, S for sugar and H^+ for proton).

would thus become more than one. This model is similar to the one offered by Wheeler and Christensen [10, 11] to explain the different exchange stoichiometries of sodium and various amino acids in animal cells.

III. The carrier molecule has only one proton binding site with access to both sides of the cytoplasmic membrane and therefore only one proton is translocated per carrier cycle. Normally the protonated carrier molecule is locked at the external side of the membrane and the translocation must be initiated by the binding of sugar. It is possible though, that with some sugars the translocation of the carrier-proton-sugar complex is initiated but the sugar molecule itself still dissociates with a certain probability at the external side and the translocation of the protonated carrier alone takes place (Fig. 1, III). For 6-deoxyglucose this side reaction would have to be negligible

and, therefore, each translocated carrier-proton complex would also carry 6-deoxyglucose. This would result in a stoichiometry of one proton per 6-deoxyglucose. For 1-deoxyglucose the probability of "jumping-off" would have to be 50 % and only half of the translocated carrier-proton complexes would also carry 1-deoxyglucose. The overall stoichiometry in this case would be two protons per 1-deoxyglucose.

In summary, all models would result in about twice as much energy dissipated per sugar molecule transported for 1-deoxyglucose as for 6-deoxyglucose. To decide whether model I, II or III is the correct one two kinds of experiment have been performed. In the first type of experiment the stoichiometric composition of the transported carrier-sugar-proton complex was deduced from measurements of the reaction order with respect to protons. In the second kind of experiment an attempt was made to measure the stoichiometry of carrier translocated per sugar molecule transported.

Determination of the reaction order with respect to protons

Two alternatives described above can be distinguished by the composition of the translocated carrier-sugar-proton complex: in model I for 6-deoxyglucose the composition is one proton per sugar and carrier molecule, for 1-deoxyglucose it is two protons per sugar and carrier molecule; in models II and III the stoichiometry is, for both sugars, one proton per sugar and carrier molecule. Thus the formation of the complex proton-sugar-carrier should be a first order reaction for 6-deoxyglucose throughout with respect to protons, but a second order reaction for 1-deoxyglucose if model I is correct and a first order reaction if model II or III is correct. Therefore, an attempt was made to titrate the carrier molecules with protons in the presence of the respective sugars and to determine the reaction order by graphic analysis of the titration curve. Since it was not possible to measure the formation of the carrier-sugar-proton complex directly, the rate of net sugar uptake into cells without sugar inside was measured under conditions, where the rate of sugar uptake should be

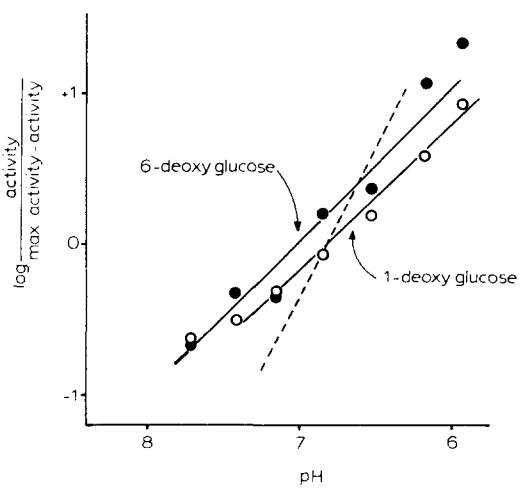


Fig. 2. Titration of 6-deoxyglucose and 1-deoxyglucose uptake with protons. The experiments have been performed as described in Materials and Methods. The broken line corresponds to a slope of two, leading through the half-saturation point of 1-deoxyglucose uptake (6-deoxyglucose, closed symbols; 1-deoxyglucose, open symbols).

directly proportional to the amount of the carrier-sugar-proton complex formed at the external side of the membrane. It was necessary to prevent the translocation of the non-protonated complex, however which dominates at high pH. This was accomplished by chosing a sugar concentration equal to the K_m for uptake via the protonated carrier, a concentration which is 0.01–0.02 times the K_m values of the uptake via the deprotonated carrier [9].

The titration curve was brought to linear form by transformation of the uptake rate to the expression: logarithm of measured activity divided by the difference of maximal activity to measured activity (or simply: logarithm of active carrier/inactive carrier), where the highest rate obtained (normally at pH 6.0) was taken as maximal activity. The most accurate part of the curve experimentally is near the half-saturation point (zero on the ordinate), whereas at the two ends small experimental errors produce great deviations from the theoretical line. Hence the numerical value of the slope, which is the reaction order, was determined near the half-saturation point of the curve. The titration curve is shown for 6-deoxyglucose and 1-deoxyglucose in Fig. 1. It is evident that the reaction is first order for both sugars; the reaction order for 6-deoxyglucose is 1.11 ± 0.16 and for 1-deoxyglucose 1.00 ± 0.05 in the average of four experiments. These results are incompatible with model I (Fig. 1), but expected for models II and III.

Appearance of free carrier inside per sugar molecule transported

Another clear distinction between model I, II and III is the number of free carrier molecules appearing at the inner side of the cytoplasmic membrane per one molecule of 1-deoxyglucose transported into the cell: one for model I and II, but about two for model III. Each carrier appearing at the inner side of the cytoplasmic membrane should be able to bind internally accumulated sugar, such as 6-deoxyglucose, and to transport it out again. This reaction commonly takes place during steady-state conditions. The rate of 6-deoxyglucose exit will be directly dependent on the amount of free carrier at the inner side of the membrane. The ratio of efflux of internal 6-deoxyglucose to influx of the respective external sugar, 6-deoxyglucose or 1-deoxyglucose was determined. For 6-deoxyglucose a ratio of one is expected since each carrier molecule "coming in" brings one sugar molecule and takes out one internal 6-deoxyglucose. The same is true for 1-deoxyglucose according to models I and II, but according to model III approximately each second carrier molecule coming in would carry a molecule of 1-deoxyglucose, but each of course would carry out one molecule of internal 6-deoxyglucose, resulting in an efflux to influx ratio of two.

The experiments were performed with cells which had been preloaded with 6-deoxyglucose at an internal concentration saturating for efflux [15]. After removal of external sugar the cells were incubated with 6-deoxyglucose or 1-deoxyglucose, respectively, and the influx of the respective sugar as well as the efflux of preloaded 6-deoxyglucose was monitored over a short time period to avoid dilution of internal 6-deoxyglucose with the incoming sugar. The net efflux of internal 6-deoxyglucose via diffusion is negligible; practically the entire efflux is carrier-mediated and in fact initiated by the addition of external sugar.

The results, summarized in Table II show that the efflux to influx ratio is close to one for 6-deoxyglucose but it is about two when 1-deoxyglucose is at the outside. This result is incompatible with models I and II, but postulated for model III.

TABLE II

CARRIER-MEDIATED EFFLUX OF INTERNAL 6-DEOXYGLUCOSE COMPARED DURING STEADY-STATE CONDITIONS WITH CARRIER-MEDIATED INFLUX OF EXTERNAL 6-DEOXYGLUCOSE OR EXTERNAL 1-DEOXYGLUCOSE

The experiments have been performed as described in Materials and Methods.

Conditions	Ratio	Number of experiments
6-Deoxyglucose outside:		
Efflux of 6-deoxyglucose/influx of 6-deoxyglucose	1.03 ± 0.22	(8)
1-Deoxyglucose outside:		
Efflux of 6-deoxyglucose/influx of 1-deoxyglucose	2.22 ± 0.61	(7)

DISCUSSION

Both types of experiments designed to distinguish between the three possible models of variable proton-sugar stoichiometry have brought results (summarized in Table III) incompatible with model I (carrier-two proton complex) and model II (carrier-one proton complex, but only partial dissociation of sugar from the carrier-proton-sugar complex). But they are compatible with model III (carrier-one proton complex) which assumes in addition to the translocation of carrier-proton-sugar complex a sugar-induced translocation of carrier-proton complex alone. Thus the titration of carrier with protons clearly established a first order reaction with respect to protons for both 6-deoxyglucose and 1-deoxyglucose, and no second order reaction for 1-deoxyglucose as expected according model I. It should be remembered, of course, that due to the complicated reaction sequence of carrier-mediated sugar transport the slope of the proton titration curve can be smaller than two, even in the

TABLE III

SUMMARY OF THE EXPERIMENTAL RESULTS AND THEIR COMPARISON WITH THE PROPOSED MODELS

The values obtained for 1-deoxyglucose (Tables I and II) are divided by the values for 6-deoxyglucose (Tables I and II) for the various kinds of experiment performed.

	Experimental result	Compatible with model		
		I	II	III
Energy requirement				
$\frac{\text{Protons/1-deoxyglucose}}{\text{Protons/6-deoxyglucose}}$	2.04	−	+	+
$\frac{\text{Extra oxygen/1-deoxyglucose}}{\text{Extra oxygen/6-deoxyglucose}}$	1.64			
Reaction order for protons				
1-deoxyglucose/6-deoxyglucose	0.91	−	+	+
Free carrier appearing inside				
1-deoxyglucose/6-deoxyglucose	2.15	−	−	+

case of a second order reaction [16], but the slope actually obtained for 1-deoxyglucose seems to fit exactly to a first order reaction, as does the one for 6-deoxyglucose. Whereas, this result reveals a one-to-one proton-sugar stoichiometry of the translocated carrier complex for both glucose analogues, the experiments for determination of the efflux to influx ratio indicate a different stoichiometry of "empty" carrier appearing at the inner side of the membrane per sugar molecule transported into the cell, so that 1-deoxyglucose initiates twice as much efflux of preloaded sugar as influx of 1-deoxyglucose, in contrast to 6-deoxyglucose, an expected result only according model III.

The key feature of model III is the occurrence of a reaction sequence, which with certain sugars like 6-deoxyglucose is negligible [17], that is the translocation of the carrier-proton complex without sugar. Conceivably the living cell may have evolved so as to avoid this wasteful reaction, since it would constitute a permanent leak of protons into the cell interior, i.e. an energy dissipating reaction without achievement of useful work. Thus only the binding of sugar to the carrier-proton complex initiates the translocational reaction step, possibly by an "unlocking" conformational change of the carrier molecule. But apparently 1-deoxyglucose is able, due to its chemical structure, to dissociate from this complex to a considerable degree prior to translocation, whereas the "relocking" conformational change might be slower than the translocational step, thus resulting in net transport of proton alone. This would explain a sugar-induced translocation of carrier-proton complex alone. But this explanation implies that there is at least one surface reaction (for example the hypothetical conformational change) with a slower velocity constant than the velocity constant of at least one transmembrane reaction. A similar implication had to be made by Wheeler and Christensen [10, 11], when explaining the amino acid-dependent stoichiometry of Na^+ and amino acid exchange in animal cells by only partial exchange of Na^+ or amino acid, respectively, at the outer or inner membrane surface. Similarly, the sugar transport versus pH kinetic data obtained with *Chlorella* cells, are most easily explained by the assumption of some limiting surface reaction [9].

TABLE IV

STOICHIOMETRY OF ENERGY REQUIREMENT, REACTION ORDER RELATIVE TO PROTONS AND RATIO OF EFFLUX OF INTERNAL 6-DEOXYGLUCOSE/INFLUX OF EXTERNAL SUGAR FOR 3-*O*-METHYLGLUCOSE

In the last column the figures obtained for 3-*O*-methylglucose are compared with the respective figures for 6-deoxyglucose (Tables I and II).

	3-*O*-Methylglucose	3-*O*-Methylglucose/ 6-deoxyglucose
Energy requirement		
Protons/sugar	1.41 ±0.24	1.47
Extra oxygen/sugar	0.294±0.029*	1.25
Relative reaction order to protons	0.91 ±0.12	0.82
Efflux of 6-deoxyglucose/influx of 3-*O*-methylglucose	1.40 ±0.12	1.36

* From Decker, M., unpublished results.

The explanation for variable stoichiometry, given above, is also valid for fractional stoichiometries since they would not reflect the stoichiometric composition of the translocated complex, but would depend on the probability of the sugar-induced translocation of carrier-proton complex. So for the hexose transport system of *Cl. vulgaris* 1-deoxyglucose is not the only glucose-analogue behaving extraordinarily since 3-*O*-methylglucose also exhibits a higher fractional stoichiometry of 1.5 proton per sugar transported, which would not reasonably be explained by a mechanism like model I. When experiments are performed with 3-*O*-methylglucose analogous to those with 1-deoxyglucose, the results are compatible with the predictions according to model III (Table IV). In this case the translocation of carrier-proton complex seems to occur at a probablity of about 30 %. It would be interesting to know, if similar phenomena occur in other uptake systems of other organisms and if perhaps a similar explanation would be valid.

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