The Hexose-Proton Cotransport System of *Chlorella*

pH-Dependent Change in K_m Values and Translocation Constants of the Uptake System

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ABSTRACT   The proton concentration in the medium affects the maximal velocity of sugar uptake with a K_m of 0.3 mM (high affinity uptake). By decreasing the proton concentration a decrease in high affinity sugar uptake is observed, in parallel the activity of a low affinity uptake system (K_m of 50 mM) rises. Both systems add up to 100 %. The existence of the carrier in two conformational states (protonated and unprotonated) has been proposed therefore, the protonated form with high affinity to 6-deoxyglucose, the unprotonated form with low affinity. A plot of extrapolated V_{max} values at low substrate concentration versus proton concentration results in a K_m for protons of 0.14 μM, i.e. half-maximal protonation of the carrier is achieved at pH 6.85. The stoichiometry of protons cotransported per 6-deoxyglucose is close to 1 at pH 6.0–6.5. At higher pH values the stoichiometry continuously decreases; at pH 8.0 only one proton is cotransported per four molecules of sugar. Whereas the translocation of the protonated carrier is strictly dependent on sugar, this coupling is less strict for the unprotonated form. Therefore at alkaline pH a considerable net efflux of accumulated sugar can occur. The dependence of sugar accumulation on pH has been measured. The decrease in accumulation with higher pH values can quantitatively be explained by the decrease in the amount of protonated carrier. The properties of the unprotonated carrier resemble strikingly the properties of carrier at the inner side of the membrane. The inside pH of *Chlorella* was measured with the weak acid 5,5-dimethyl-2,4-oxazolidinedion (DMO). At an outside pH of 6.5 the internal pH was found to be 7.2. To explain the extent of sugar accumulation it has to be assumed that the membrane potential also contributes to active sugar transport in this alga.

INTRODUCTION

The green alga *Chlorella vulgaris* possesses an inducible uptake system for hexoses, which is able to accumulate nonmetabolizable sugars like 6-deoxyglucose by a factor of more than 1,000 (15, 17). This uphill transport can be

energized by respiratory or photosynthetic energy supply (6, 16). It has been found that the uptake of 6-deoxyglucose as well as of other sugars is accompanied by a net uptake of protons in a 1:1 stoichiometry (13). This would be in accordance with Mitchell's theory of a nonelectrolyte-proton symport carrier for active nonelctrolyte transport (20). Similar results for sugar transport have been obtained with *Escherichia coli* and yeast (23, 27).

For *Chlorella* it is reasonably well understood which kinetic parameters, like affinity for transported sugar and maximal flux velocities, are responsible for the uphill sugar transport capacity (15). It has been studied now which of these parameters is affected by altering the proton concentration in the medium and how the accumulation capacity for sugar is changed with varying pH values. The kinetic features can be best explained if the existence of protonated and deprotonated carrier molecules is assumed. It was found that predominantly the affinity to sugar is decreased at alkaline pH values and that most likely the mobility of the empty carrier is also affected indirectly.

MATERIALS AND METHODS

The alga *Chlorella vulgaris* was grown in an inorganic medium as described previously (25). 6-Deoxyglucose was purchased from Koch-Light Laboratories, Colnbrook, England, and tritiated by the Labelling Service, Amersham, England. 5,5-Dimethyl-2,4-oxazolidinedione (DMO) was from Merck, Darmstadt, Germany, the [^{14}C]compound was from Radiochemical Centre, Amersham.

Sugar Uptake Measurements

Algae, which had been induced for the active sugar transport system (17, 24) were incubated in 0.025 M buffer. The following buffers have been used: for pH 1.0–3.0 sodium citrate/hydrochloric acid buffer; for pH 3.0–6.0 sodium citrate buffer; for pH 5.7–8.2 sodium phosphate buffer; for pH 8.0–9.0 Tris buffer; and for pH 9.0–11.0 sodium glycine buffer. In overlapping experiments no difference of more than 10% has been observed for sugar uptake by the different buffers, and no alteration of kinetic features has been seen. The cell concentration normally was 9 μl packed cells (packed cells, without correction for intercellular water) per milliliter suspension. The cell suspension was shaken at 27°C under aerobic conditions in the dark and the uptake experiment was started by addition of labeled sugar (6-deoxyglucose). Samples were withdrawn and filtered on membrane filters (0.8-μm pore size). For sugar influx measurements samples were withdrawn at intervals of 30 s. At low sugar concentrations the influx is linear for about 90 s at saturating concentrations for several minutes. The algae were extracted by boiling in 0.01 N HCl for 10 min and the radioactivity of the extract was determined by scintillation counting in a dioxane-PPO-naphthalene fluid.

Determination of Proton/Sugar Stoichiometry

The proton flux was determined using the pH-measuring equipment described previously (13, 18). The system has been calibrated in the presence of algae by addition of small amounts of acid. After a short preincubation time (5 min) and adjustment of

the algal suspension (100 μl packed cells/ml) to the pH desired by sodium hydroxide solution, radioactive 6-deoxyglucose (50 mM) was added to the algae and the initial pH change was recorded. Then samples were withdrawn from the reaction vessel, filtered, and handled as described before. The sampling times were 30, 60, 90, and 120 s after the addition of 6-deoxyglucose.

Measurement of 5,5-Dimethyl-2,4-Oxazolidinedion (DMO) Distribution

A thick suspension of algae (400 μl packed cells/ml) in 25 mM buffer was shaken vigorously, then [^{14}C]DMO in a small volume was added, and samples of 0.4 ml were rapidly centrifuged (total centrifugation time 20 s). 100 μl of the supernatant were counted directly in dioxane scintillation fluid.

Reproducibility of Results

The number of experiments performed and in some cases mean values of results are given in the legends to the figures. Typical results are presented in the figures.

RESULTS

pH-Dependance of Sugar Uptake and Accumulation

The uptake of 6-deoxyglucose measured as influx velocity is pH dependent with a broad optimum from pH 4.5 to pH 6.5 and with steep declines beyond these points (Fig. 1). Also the accumulation plateau reached depends on the pH of the medium: it decreases with decreasing H$^+$ concentration (Fig. 2), and at pH 9.5 only concentration equilibrium is reached. Both the decrease in influx velocity as well as in accumulation ratio with increasing pH could be due either to a change of the K_m or of the V_{max} values of the uptake system.
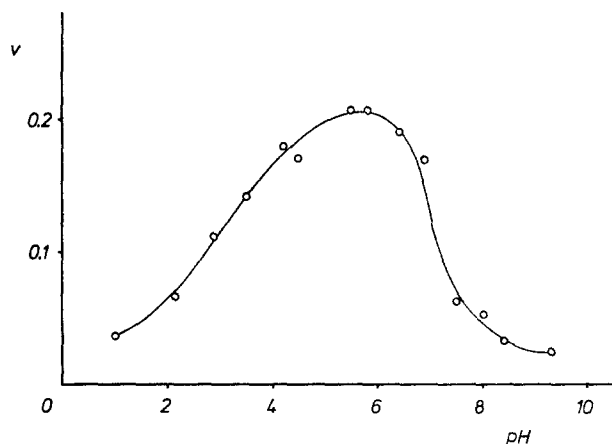

FIGURE 1.   pH-profile of sugar uptake. The uptake of 1 × 10^{-3} M 6-deoxyglucose was followed in 0.025 M buffer (v is expressed as millimoles per hour per milliliter packed cells), (five experiments).
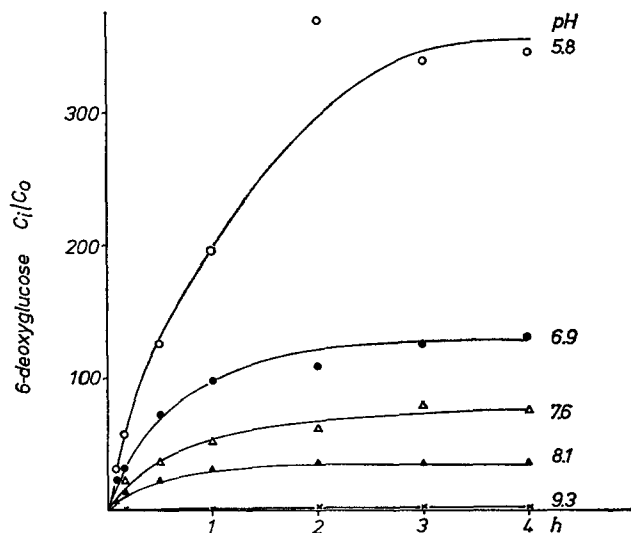
Figure 2. Uptake of 6-deoxyglucose at neutral and alkaline pH values. The algae were incubated in sodium phosphate buffer with 5×10^{-4} M 6-deoxyglucose (sp act 0.5 μCi/μmol). C_i = inside, C_o = outside concentration of sugar; (six experiments).

K_m for 6-Deoxyglucose Uptake at Different pH Values

The velocity of 6-deoxyglucose uptake was determined at different external pH values with different 6-deoxyglucose concentrations, yielding the values for K_m and V_{max}. From Fig. 3 it is evident that at all pH values the apparent K_m measured at low sugar concentration is about 3×10^{-4} M, whereas the intercept at the $1/v$ axis is quite different for each pH. The apparent maximal velocity V_{max} for sugar influx thus is decreased with increasing pH values. But at relatively high sugar concentrations a deviation from the straight lines in the Lineweaver-Burk plots has been observed at the more alkaline pH values. When the sugar concentration dependence of influx velocity is measured with hundred to thousand times higher 6-deoxyglucose concentrations an additional saturation function with a large K_m value (90 mM in Fig. 4, in other experiments 20–50 mM) is observed at alkaline pH (here 9.0). This saturable component is missing however at acid pH like 6.3 (Fig. 4). Since the maximal uptake velocity at pH 9.0 is at least half that at pH 6.3, it should be detectable if present at pH 6.3. (In six experiments the V_{max} values at alkaline pH had been 46–100% of those at acidic pH values; mean value 65 ± 23%.)

At acid pH values and at very alkaline pH values the uptake of 6-deoxyglucose corresponds to simple Michaelis-Menten kinetics at all substrate concentrations, whereas at slightly alkaline pH values the observed kinetics are similar to those expected for the simultaneous action of two uptake systems with largely differing K_m values for 6-deoxyglucose. When the sugar uptake
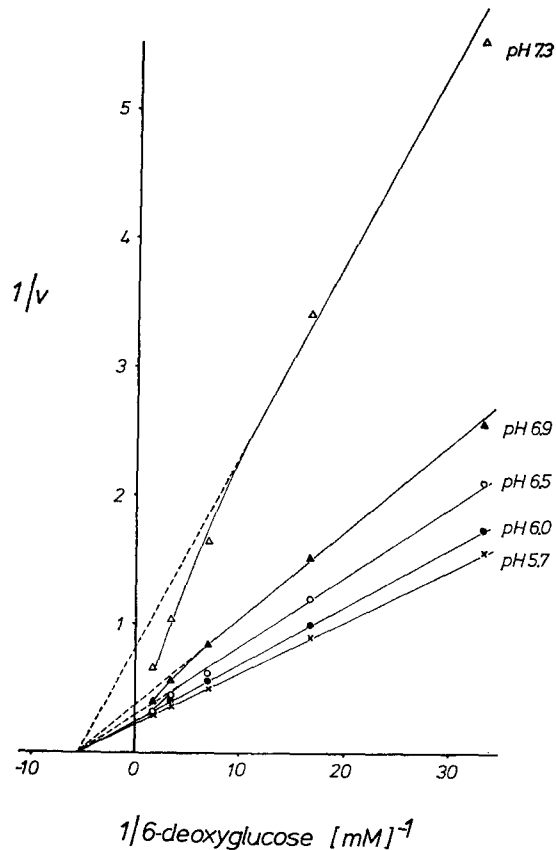
FIGURE 3.   Lineweaver-Burk plots of 6-deoxyglucose uptake velocity in dependence of 6-deoxyglucose concentration at various pH values (v is expressed as micromoles per minute per milliliter packed cells). The cells were incubated in sodium phosphate buffer at the pH values indicated; (five experiments; the K_m value has been found to be 0.23 ± 0.03 mM).

activity is plotted dependent on sugar concentration the two uptake systems are clearly separated due to the two orders of magnitude difference in K_m values. Therefore, it was tried to estimate the uptake activity of each individual uptake system in the following way. At about 0.1 mM 6-deoxyglucose the high affinity system is operating almost exclusively with only half a percent of the low affinity system being active. Thus the uptake velocity under this condition is a relative measure of the uptake activity of the high affinity system, as well as are the extrapolated V_{max} values from these uptake velocities. On the other hand, the increase of sugar uptake activity, obtained by an increase of the 6-deoxyglucose concentration from 10–200 mM, can be attributed to the uptake activity of the low affinity system, the high affinity uptake activity should increase less than 3%.

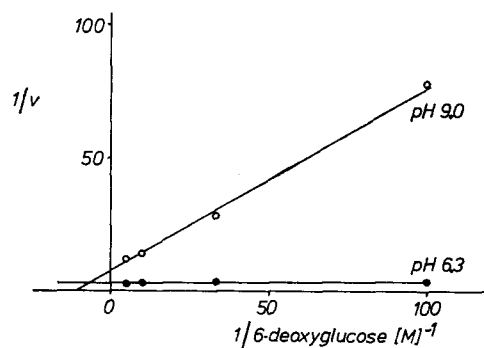Both uptake systems are strictly pH dependent whereby a surprising correla-

FIGURE 4. Uptake velocity for 6-deoxyglucose in dependence of high 6-deoxyglucose concentration at pH 6.5 and 9.0. Lineweaver-Burk plot (v is expressed as millimoles per hour per milliliter packed cells). (six experiments; range of K_m values at alkaline pH 22–90 mM; 43 ± 23 mM).

tion exists: the high affinity system decreases in the same way as the low affinity system increases, so that at pH 6.9 half of each is present. Both uptake activities, expressed in percent of maximal uptake activity at optimal conditions, add up to 100% at all pH values tested (Fig. 5). Algae, which had not been induced for the hexose uptake system by incubation with glucose, showed none of the above uptake systems.

A coincidence of two independent uptake systems with exactly the same pH dependence in opposite directions seems unlikely. In addition so far no evidence for two uptake systems for hexoses has been obtained from coinduction and from specificity experiments (17). We interpret the data above, therefore, in the following way: since protons are cotransported together with sugar (13, 18) the carrier molecules most likely bind protons with a distinct affinity, and an increase in pH should decrease the protonation of the carrier. It is suggested, therefore, that the high affinity uptake system is due to the activity of protonated carrier molecules, and that the low affinity system is due to the activity of the unprotonated carrier molecules. To test this concept, the stoichiometry of proton cotransport together with 6-deoxyglucose was determined at different pH values at high 6-deoxyglucose concentration (50 mM). At pH 6.5 the stoichiometry has been found to be approximately one (18). According to the conclusions drawn above, this value was expected to decrease at more alkaline pH values, since part of the sugar should be transported by the low affinity system, assumed to be identical with the activity of unprotonated carrier molecules. The experimental results, shown in Fig. 6, agree well with this prediction. At the high sugar concentration used three out of four sugar molecules are translocated without protons at pH 8.0.

Affinity of the Sugar Transport to Protons

The proton concentration in the medium (i.e. the pH value) would determine how many of the carrier molecules are protonated and how many remain
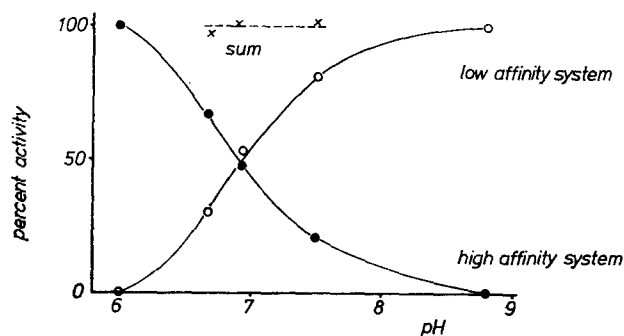
FIGURE 5   FIGURE 6

FIGURE 5.   Relative activity of high affinity and low affinity carrier at different pH values. As activity of high affinity carrier was taken the extrapolated maximal velocity of 6-deoxyglucose uptake at low sugar concentration as in Fig. 2. As activity of low affinity carrier was taken the difference in influx velocity, which was produced by elevating the 6-deoxyglucose concentration from 10 to 200 mM. The uptake velocity of the high affinity carrier should not be enhanced significantly thereby (less than 3%); (four experiments).
FIGURE 6.   The stoichiometry of proton-6-deoxyglucose cotransport at different pH values. The stoichiometry was determined as described in Materials and Methods and reference 18; (three experiments).
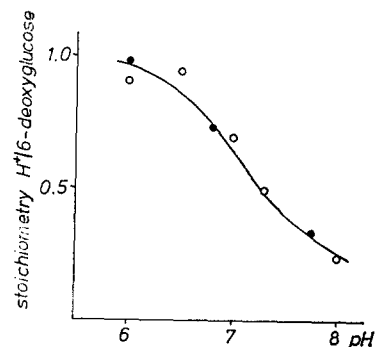
unprotonated. At extreme pH values like 5.7 or 9.0 only one population of carrier molecules would exist (the protonated or the unprotonated ones, respectively); at pH values in between, the relative amounts of the two possible populations would be shifted according to the pH values and the affinity of carrier to protons. The sugar uptake activity at very low 6-deoxyglucose concentrations (10–100 μM) which is proportional to the extrapolated V_{max} values in Fig. 3, may indicate the relative "amount" of high affinity carrier (i.e. protonated carrier). For example when assuming that at pH 5.7 all carrier is protonated, at pH 6.9 only half of it should be protonated. When the proton concentration is plotted against the extrapolated V_{max} values, a one-phasic Michaelis-Menten kinetic is obtained (at least in this pH range) with a K_m value for protons of 0.14 μM i.e. pH 6.85 (Fig. 7).

Protonation of Carrier and Its Relationship to Sugar Accumulation

6-Deoxyglucose is accumulated by *Chlorella* at low outside sugar concentration more than 1,000-fold (15). Evidence had been presented that this accumulation is partly caused by a different K_s value, i.e. the true carrier substrate dissociation constant, at the inside as compared to that at the outside and partly by a considerable difference of the rate of flux of the carrier without sugar, when the flux towards the outside is compared to that towards the inside (15). It has to be assumed that at low substrate concentration, where the largest accumulation ratios can be observed, only the protonated carrier
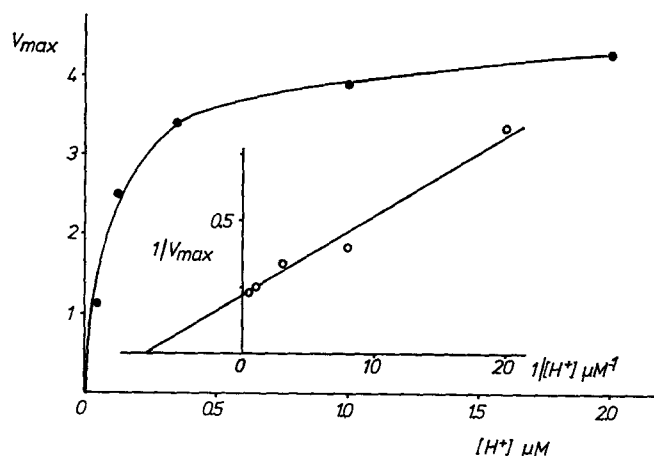
FIGURE 7. Maximal velocity of 6-deoxyglucose uptake of high affinity in relation to proton concentration. Insert: Lineweaver-Burk plot (V_{max} is expressed as micromoles per minute per milliliter packed cells). K_m for protons has been found to be $1.2 \times 10^{-7} \pm 0.2 \times 10^{-7}$ M; (six experiments).

is working. In addition the unprotonated form of the carrier has the same affinity to the substrate at the inside as at the outside and also for that reason should not be an important factor contributing to accumulation. The accumulation capacity would, therefore, be expected to correlate with the amount of protonated carrier, which should be proportional to the V_{max} values of the high affinity system. Such a correlation has indeed been observed (Table I).

Change in Accumulation Plateau by a Stepwise Change in pH

Indications have also been obtained that the motility of the carrier without sugar is different for the protonated and the deprotonated form. This can be shown by net efflux measurements since under this condition the movement of the empty carrier is the rate limiting reaction (14). When the pH value of the external medium is increased the accumulation plateau for 6-deoxyglucose decreases (Fig. 2). Therefore, it is expected that a change in pH when the cells are already in the steady-state plateau, would induce an alteration in the accumulation plateau until a new plateau is reached. When the pH is changed for example from 6.3 to 8.3, a net efflux of sugar should occur. With cells kept at pH 6.3, the rate of net efflux induced by high dilution with buffer is extremely slow, as has been shown previously: at an inside concentration of 0.11 M 7 h are needed to lose half the accumulated 6-deoxyglucose, and net efflux is hardly observable, therefore, within short times (14, 15). Fig. 8 demonstrates that the alkalization of algae in the plateau level induces a rather rapid loss of sugar and within about 4 h the new plateau level about three times lower than the previous one is reached. When alkaline algae were treated with acid

TABLE I

PH-DEPENDENCE OF MAXIMAL 6-DEOXYGLUCOSE INFLUX VELOCITY AND
6-DEOXYGLUCOSE ACCUMULATION

All values are expressed relative to the values at pH 5.8. The maximal velocity was obtained from
extrapolated values of Lineweaver-Burk plots as in Fig. 3. The accumulation was measured like
in Fig. 2 with 5×10^{-4} M 6-deoxyglucose.

pH	5.8	6.45	6.9	7.3	7.55
Maximal influx velocity	1 (i.e. 195 μmol/h/ml packed cells)	0.94	0.52	0.34	0.17
Accumulation factor	1 (i.e. 350 times)	0.97	0.52	0.36	0.20

to lower the pH value again, the steady-state plateau was elevated to a new
level typical for the new pH value. This shows that no irreversible changes
occur by prolonged incubation of the cells at alkaline pH values.

The relatively rapid net efflux at alkaline pH values can be explained in
two ways: (a) the membrane is made leaky, resulting in a highly increased
diffusional part of sugar efflux; (b) the net efflux is still carrier mediated, but
the normally limiting reaction, i.e. the translocation of free carrier from the
outside to the inside of the membrane (14) is largely increased at high pH val-
ues. The following experiments were designed to distinguish between these
two possibilities.

*Passive Diffusion and 6-Deoxyglucose Uptake at Alkaline pH Values below
Concentration Equilibrium*

The pentose D-ribose does not have any measurable affinity to the hexose
transport system, thus it was chosen as substrate for measurement of a possibly
passive diffusion of sugar. A thick suspension of *Chlorella* cells (about 50% of
volume) was incubated with labeled D-ribose and the disappearance of radio-
activity in the supernatant was followed. Thus any possible washout effects of
the filtering method were circumvented. There is evidently no increase in
ribose permeability at alkaline pH values, rather a slight decrease is observ-
able (Fig. 9).

Additional evidence that net sugar flux at alkaline pH is carrier mediated
was obtained by following the uptake of 6-deoxyglucose below concentration
equilibration and its competitive inhibition by glucose. The same technique as
above was used. Glucose totally prevents the uptake of 6-deoxyglucose at pH
8.5 as it does at the "normal" pH of 6.5 (Fig. 10). Furthermore the experi-
ment shows that even below concentration equilibrium the uptake velocity at
an alkaline pH value is considerably lower than at an acidic pH value. These
data are strong evidence for possibility (b) mentioned above, i.e. the motility
of free carrier into the cell is significantly increased, when no proton is bound
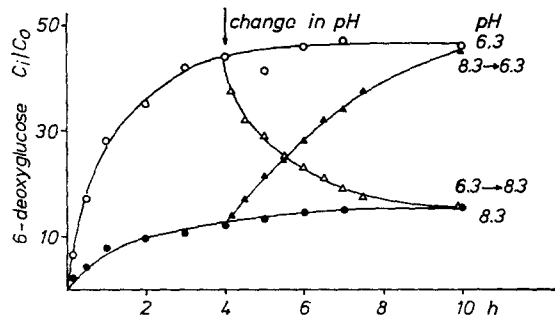to the carrier molecule.

FIGURE 8   FIGURE 9

FIGURE 8.   Change of the accumulation plateau by a change in pH. 4.7 mM 6-deoxy-glucose (sp act 0.26 μCi/μmol) was accumulated by the cells in sodium phosphate buffer. In the plateau level the pH was rapidly altered by addition of sodium hydroxide or hydrochloric acid; (five experiments).
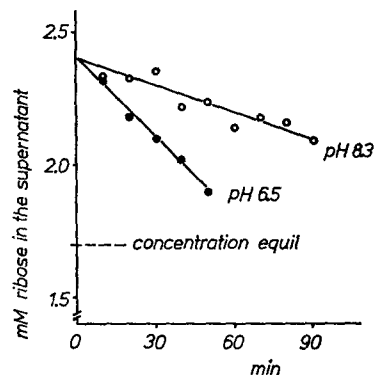
FIGURE 9.   Uptake of D-ribose at pH 6.5 and 8.9. Algae (430 μl packed cells/ml) were incubated in sodium phosphate buffer with 2.4 mM D-ribose in the external medium. Samples of the suspension were removed, rapidly centrifuged and the radioactivity of an aliquot of the supernatant was determined; (two experiments).
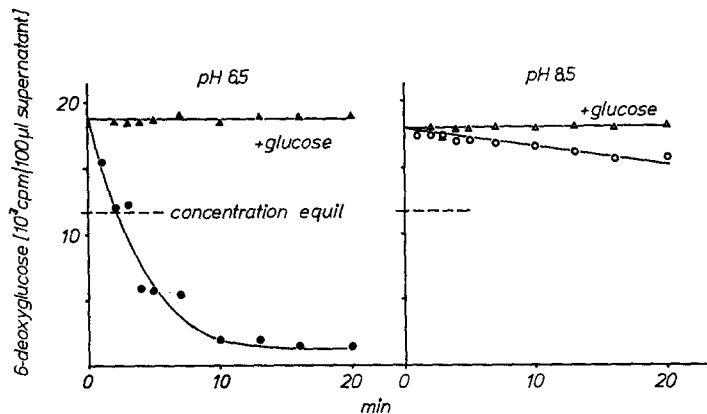

FIGURE 10.   Uptake of 6-deoxyglucose at pH 6.5 and 8.5. Algae (540 μl packed cells/ml) had been incubated as described in Fig. 8 with 10 mM 6-deoxyglucose (sp act 10.5 μCi/μmol) in the external medium, in the presence or absence of 10 mM glucose; (four experiments).

Estimation of Cytoplasmic pH by Dimethyloxazolidinedion (DMO) Distribution

The distribution of the weak acid DMO, a metabolically inert compound, has been used as method to measure the internal pH of the cells. With animal cells, mitochondria, and chloroplasts (1, 3, 9) this method has given reliable results, when compared with other methods (9, 19, 21). In *Chlorella* DMO also

shows the necessary prerequisites: there is no indication of DMO metabolism by the cells, and the velocity of DMO movement into the cells is proportional to the DMO concentration, so is most likely passive. The steady state of DMO distribution is reached in 5–10 min (Fig. 11). The cytoplasmic pH, indicated by DMO, is dependent on the external pH value, showing a Δ pH of about 0.7 U at an outside pH of 6.5 (Fig. 12).
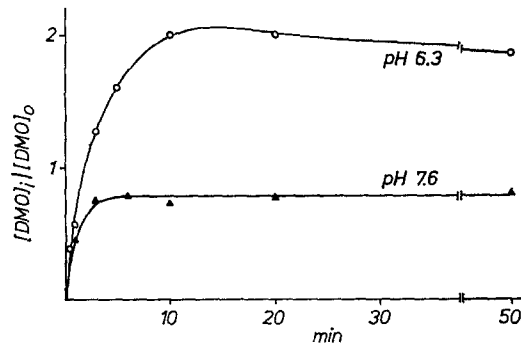

FIGURE 11.   The time-course of DMO distribution. The experiment was performed as described in Material and Methods; (four experiments).
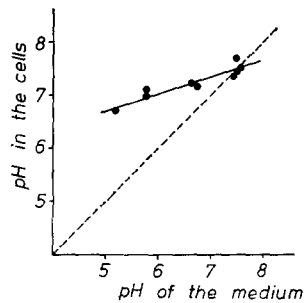

FIGURE 12   FIGURE 13

FIGURE 12.   Intracellular pH values determined by DMO distribution at different external pH values. Broken line would correspond to an internal pH identical to the external one. The points originate from four different experiments.

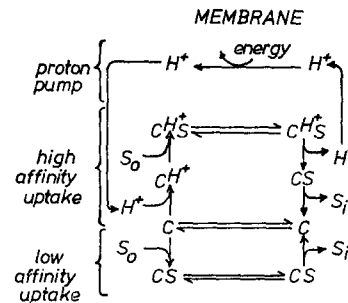FIGURE 13.   Proposed model for hexose uptake by *Chlorella vulgaris*.

Although the estimation of cytoplasmic pH in a eucaryotic cell can be subject to errors due to the various organelles with individual pH values, it seems rather unlikely that the pH gradient across the cytoplasmic membrane in *Chlorella* can account for the high accumulation ratio of 6-deoxyglucose. It has to be assumed, therefore, that the membrane potential also plays an important role in sugar transport by *Chlorella*.

DISCUSSION

There exists evidence that β-galactoside uptake by *Escherichia coli* and maltose uptake by *Saccharomyces carlsbergensis* are accompanied by simultaneous proton

uptake (23, 27), however the role of protons in these sugar transport systems has not been elaborated so far. There exist, however, similarities between the *Chlorella* system and the role of sodium ions for sugar and amino acid transport in animal systems (22), where sodium ions are affecting the affinity (4, 5, 26), the maximal velocity (7, 8, 12), or both (10, 28).

The data described here and previously (6, 13–18, 24) would best be fitted by the model in Fig. 13. The carrier molecules can exist in two different states ready for binding sugar: either in the unprotonated one with a low affinity for sugar (K_m of 50 mM), or in the protonated one with a high affinity for sugar (K_m of 0.3 mM). The protonation of the carrier molecules occurs with an apparent K_m for protons of 0.14 μM. This property explains the peculiar pH dependence of the affinity for 6-deoxyglucose. It also explains the apparent affinity of "inside" carrier (K_m of 30 mM), measured previously (15), since a cellular pH of 7.3 would be high enough to deprotonate about 80% of the inside carrier molecules. Due to the protonation-deprotonation reaction the cyclic flow of carrier should cause flow of protons into the cell at acid pH outside (less than 6.3) with a constant stoichiometry of 1, and at more alkaline pH values with a decreasing stoichiometry of protons per carrier and thus per sugar translocated. The protonated carrier molecule without sugar does not seem to be capable of passing the membrane to a significant extent, because in acid medium (pH 6.5) there is a very slow rate of net efflux of sugar. This property would also assure that energy, used for maintenance of alkaline cell interior, is not wasted. In contrast the unprotonated carrier also in the absence of sugar traverses the membrane more easily, as shown by net efflux at alkaline pH outside. This is in agreement with the observation that at acidic pH unprotonated carrier moves outwardly much faster under conditions of net influx than under net efflux (14, 15).

The velocity of carrier-sugar complex movement seems not to be strongly affected by protonation of the carrier molecule, since under very alkaline conditions about 65% of the uptake velocity is reached (only at much higher sugar concentrations) as at acid pH. The inhibition of net efflux, of net influx below and above concentration equilibrium, and of all steady-state fluxes of sugar by uncouplers as found previously (14–16), cannot be explained readily, since uncouplers should not change the protonation of carrier molecules outside to any significant extent, and so far no evidence for an unspecific inhibition by carbonylcyanide-p-trifluoromethoxy phenylhydrazone(FCCP), e.g. of diffusive processes through *Chlorella* membranes, has been obtained. It is assumed, therefore, that the membrane potential, which should collapse in the presence of uncouplers (11, 21), is influencing strongly the translocation rates of protonated carrier. Since the membrane potential is negative inside (2), and the carrier-proton complex might carry a net positive charge, its movement to the inside could be enhanced. Together with the pH gradient of

about 1 U, the membrane potential needed to account for the high accumulation ratio for 6-deoxyglucose should be at least 120 mV.

The involvement of the membrane potential in sugar transport is also indicated by the inhibition of sugar influx by the addition of high concentrations of potassium or sodium salts (0.2 M). The inhibition was especially strong when the salt was added just before the sugar addition; these alkali ions did not, however, change the proton-sugar stoichiometry (18).

It is rather certain that the carrier, once at the inner side of the membrane, becomes deprotonated, since a net uptake of protons together with sugar is observed (13). The fact then, that the carrier at the outer side of the membrane in its deprotonated form exerts important properties of the normal inside carrier, strongly indicates that protonation/deprotonation of the sugar carrier is the main cause for sugar accumulation by *Chlorella*. The protonation is thought to change the conformation of the carrier and thus increases the affinity to sugar, and in addition the charged sugar-carrier complex becomes susceptible to the driving force of membrane potential. Energy for this active transport system might be largely supplied by a proton pump transporting protons back to the outside against an electrochemical gradient. (11, 20)

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