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CHARACTERIZATION OF THE ACTIVE HEXOSE TRANSPORT SYSTEM OF *CHLORELLA VULGARIS*

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SUMMARY

1. The green alga *Chlorella vulgaris* possesses an inducible active uptake system for hexoses. Uptake of 3-*O*-methylglucose by *Chlorella* is strongly inhibited by the uncoupler carboxylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). The inhibition is also observed below concentration equilibration.

2. The sugar accumulation of *Chlorella* can be described as a pump and leak system. The apparent K_m of $1 \cdot 10^{-3}$ M for the steady-state accumulation of 3-*O*-methylglucose corresponds closely to the K_m for its uptake.

3. The K_m values both for D-glucose and 2-deoxyglucose uptake were found to be $1 \cdot 10^{-5}$ to $2 \cdot 10^{-5}$ M.

4. 6-Deoxyglucose as well as 1-deoxyglucose get accumulated several 100-fold within the cells. This shows that phosphorylation of the sugar does not have to take place for the transport of the sugar.

5. Cells induced with glucose take up 1-deoxyglucose and 6-deoxyglucose without a lag. These analogues on the other hand are also able to induce a linear uptake of glucose.

INTRODUCTION

It has been shown previously that autotrophically grown *Chlorella vulgaris* cells do not take up glucose as well as other hexoses until a rather specific hexose transport system is induced[1-3]. Induced cells are able to accumulate sugar analogues such as 3-*O*-methylglucose more than a 100-fold[2]. The energy necessary for the accumulation can be supplied *via* respiration. However, light energy can also be utilized for this sugar concentration work[2]. The latter feature especially seems to justify a detailed study of this uptake system, since additional experimental approaches should be possible with *Chlorella*, which might help our understanding of the links between energy supply and active transport.

In recent years evidence has accumulated that sugar transport in bacteria is either brought about by the phosphoenolpyruvate-phosphotransferase system[4-6] or, as in the case of β-galactosides in *Escherichia coli*, by a transport mechanism which leaves the transported sugar unphosphorylated[7,8]. In yeasts this question does not seem to be settled[9,10] whereas in other fungi evidence is available that the transported

Abbreviation: FCCP, carboxylcyanide *p*-trifluoromethoxyphenylhydrazone.

sugars tested remain unphosphorylated[11,12]. It is also clear that hexose transport of the intestine is brought about by a non-phosphorylating uptake system[13].

In this paper the sugar transport into *Chlorella* will be characterized further. It will be shown that hexoses are accumulated in *Chlorella* until a steady state is reached (pump and leak) and evidence will be presented that hexose accumulation does not require phosphorylation of the sugar.

MATERIALS AND METHODS

The strain of *Chlorella vulgaris* used and the conditions of culture were the same as previously described[1]. 3-*O*-Methylglucose was obtained from Calbiochem; 3-*O*-methyl [^{14}C]glucose and 2-deoxy [^{14}C]glucose from NEN, Boston; 2-deoxyglucose was obtained from Roth, Karlsruhe; D-[^{14}C]glucose from the Radiochemical Centre, Amersham; 6-deoxyglucose was prepared by acid hydrolysis of 6-deoxy-β-methylglucoside from Pierce Chemical Co., Rockford; 1-deoxyglucose (1,5-anhydro-D-glucitol) was synthesized according the method of NESS *et al.*[14]. Carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) was a generous gift of Dr. P. Heytler, Dupont de Nemours; membrane filters of 0.8 μm pore size were purchased from Sartorius GmbH, Göttingen.

Adaption of Chlorella cells

Approximately 360 μl packed cells* were incubated in 10 ml 0.032 M sodium phosphate buffer (pH 6.5) with 14 mg D-glucose present and shaken in an erlenmeyer flask. The glucose was used up by the algae after 2–3 h. The cells remained induced thereafter for at least 10 h (ref. 3).

Uptake experiments

The incubations with sugars were carried out in 0.025 M sodium phosphate buffer (pH 6.5) and the experiment was started by addition of adapted algae. The reaction mixture was shaken in an erlenmeyer flask in the dark at room temperature (22°). Samples were withdrawn at the times indicated and filtered rapidly through membrane filters. The filters were washed with 2 ml 0.08 M ice-cold sodium phosphate buffer (pH 6.5) and extracted with 5 ml aqueous solution of 80% ethanol at 65° for 1 h. The concentrated extracts were chromatographed on Whatman 1 chromatographic paper ascending in toluene for 20 min to remove the pigments. The origins with the radioactive sugar were cut out and the radioactivity determined directly on paper in toluene–PPO with the liquid scintillation spectrometer Beckmann LS 100. The counting efficiency was 70%. After it had been established that at least 95% of 3-*O*-methylglucose can be extracted from the cells as free sugar (see Fig. 1) another extraction procedure was employed for this sugar. The cells were extracted by boiling the filter with the cells in 1 ml 0.1 M HCl for 10 min. This method was advantageous because no pigments were extracted. Thus the extract could be measured directly by pipetting an aliquot into the scintillation fluid (dioxane–naphthalene–PPO).

When non-radioactive sugars taken up by the cells had to be assayed, the ethanol extracts of the cells were chromatographed in Solvent A, *i.e.* butanol–pyridine–

* All cell volumes are given as packed cell volume without any correction for extracellular water, except for the experiment of Fig. 2.

water–acetic acid (60:70:30:3, by vol.) to separate the sugars from other cell constituents. The sugars were subsequently eluted and the amounts determined colorimetrically: 6-deoxyglucose according the method of DISCHE AND SHETTLES[15]; 1-deoxyglucose by periodate cleavage[16] and measurement of periodate with the method of AVIGAD[17].

RESULTS

(1) *Inhibition of 3-O-methylglucose uptake by FCCP*

It has been shown before[1] that the green alga *Chlorella* is able to take up 3-*O*-methylglucose against an inside concentration more than 100 times the concentration in the surrounding medium. The sugar taken up can be extracted again by warm 80 % ethanol, and on chromatography the radioactivity proves to be due solely to 3-*O*-methylglucose (see Fig. 1). Even after prolonged incubations of 20 h and more, the sugar in the cell is present as free 3-*O*-methylglucose to at least 95 %. The uptake of the sugar is strongly inhibited by poisons like azide or the uncoupling agent FCCP. Fig. 2 shows this inhibition by $5 \cdot 10^{-5}$ M FCCP. Although this FCCP concentration is fairly high, it is the concentration showing optimal uncoupling action as judged by the increased respiratory rate of the cells (see insert of Fig. 2). It should be pointed out that FCCP not only inhibits the accumulation of the sugar but also the uptake into the cells before concentration equilibration is reached (Fig. 2).

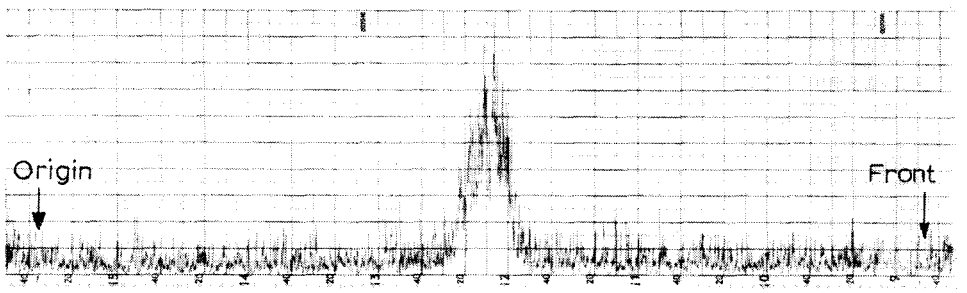

Fig. 1. Scan of a paper-chromatographic separation of the radioactive products extracted from *Chlorella* after 3-*O*-methyl[^{14}C]glucose uptake. In a total volume of 12 ml 145 μl of packed cells were incubated together with 60 μmoles of labelled 3-*O*-methylglucose (specific activity 0.017 μC/μmole). After 3 h 1.5 ml were filtered and the cells extracted with ethanol (see MATERIALS AND METHODS). Half of the extract was chromatographed on Whatman 1 in Solvent A (see MATERIALS AND METHODS).

(2) *The steady state of 3-O-methylglucose uptake*

It has been shown for active uptake systems that the net influx decreases with time due to an increased efflux. However, RING *et al.*[18], VALLÉE AND JEANJEAN[19], CRABEEL AND GRENSON[20] have shown that exceptions to this pattern exist, *i.e.* the net uptake into cells can also decrease with time due to a decrease in the rate of influx. The experiment of Fig. 3 shows that 3-*O*-methylglucose uptake into *Chlorella* follows the pattern of a pump-and-leak system. The steady-state concentration in the cells is reached between 3 and 6 h and then remains constant for more than 20 h. There is a slight but significant increase in the rate of 3-*O*-methylglucose uptake in the

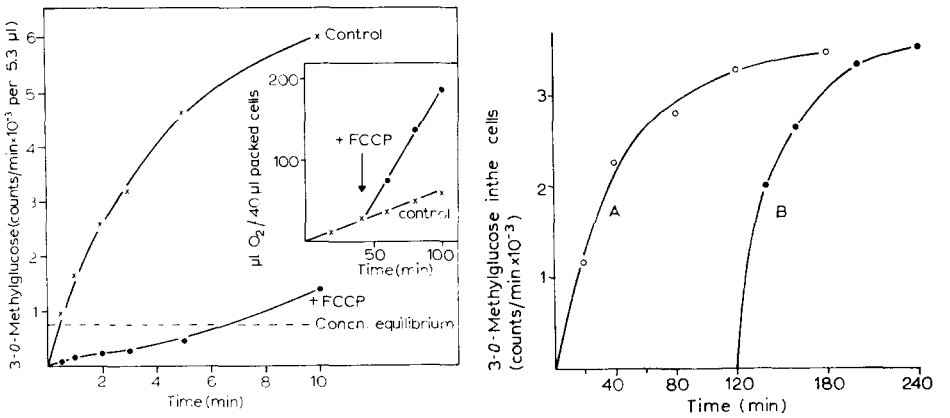

Fig. 2. Inhibition of 3-*O*-methylglucose uptake by (FCCP ($5 \cdot 10^{-5}$ M). Insert: Effect of (FCCP ($5 \cdot 10^{-5}$ M) on respiration. In a total volume of 4 ml 28 μl of cells (i.e. packed cell volume corrected by 33% of extracellular water) were incubated together with 0.44 μmole of labelled 3-*O*-methylglucose (specific activity 0.91 μC/μmole). At the times indicated 0.5 ml were filtered and the cells extracted as described in MATERIALS AND METHODS. Insert: in a total volume of 2 ml 0.04 M sodium phosphate buffer (pH 6.5) and 40 μl of packed cells were shaken in Warburg vessels.

Fig. 3. Steady-state uptake of 3-*O*-methylglucose into *Chlorella*. In a total volume of 6 ml 37 μl packed cells, were incubated in the presence of $1.5 \cdot 10^{-4}$ M 3-*O*-methylglucose. To A 3-*O*-methylglucose (0.2 μC, specific activity 10 μC/μmole) were added together with the non-radioactive sugar, to B the same radioactivity was added after 2 h incubation with the non-radioactive sugar. Aliquots of 0.2 ml were counted.

steady state compared to the initial influx into non-preloaded cells. Thus the initial uptake at $1.5 \cdot 10^{-4}$ M 3-*O*-methylglucose is 5.7 μmoles/ml packed cells per h whereas it is 7.4 μmoles/ml packed cells per h after 120 min, although the outside concentration has dropped slightly ($1.15 \cdot 10^{-4}$ M). This possibly reflects the trans-membrane effect observed in facilitated diffusion as well as in active uptake systems[21,22].

The steady-state concentration reached in the cell is dependent on the outside concentration: it increases with increasing concentrations in the medium and the

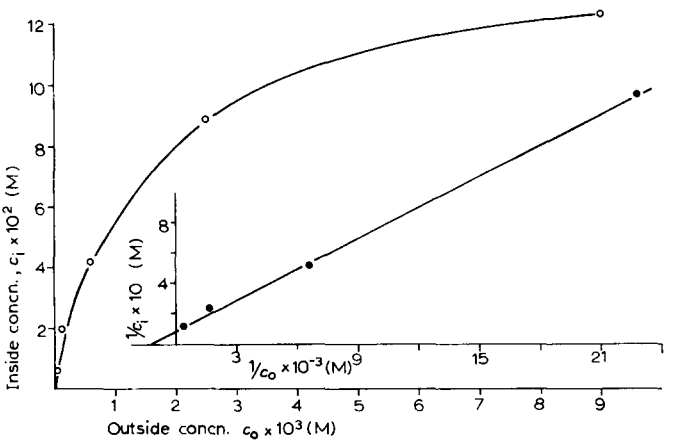

Fig. 4. The steady-state concentration of 3-*O*-methylglucose in the cells at varying outside concentrations. The time course of uptake was followed at each individual concentration; the steady-state plateau was reached between 4 and 6 h.

accumulation ratio ($c_{inside}/c_{outside}$) decreases. When the steady-state concentrations in the cells are plotted against the corresponding concentration in the medium a saturation curve is obtained with an apparent K_m of approximately $1 \cdot 10^{-3}$ M (Fig. 4) which is identical to the K_m of 3-*O*-methylglucose uptake (Fig. 5). Thus the same phenomenon applies here as in the β-galactoside system of *E. coli*[23,24]. The K_m value for 3-*O*-methylglucose uptake is very high, but D-glucose, one of the natural substrates of the uptake system, has an apparent affinity approximately 50 to 100 times higher than 3-*O*-methylglucose (Fig. 6). The same low K_m of $1 \cdot 10^{-5}$ to $2 \cdot 10^{-5}$ M has been observed for 2-deoxyglucose, a sugar which, however, does get phosphorylated to some extent in the cells and, therefore, does not serve well as a transport analogue.

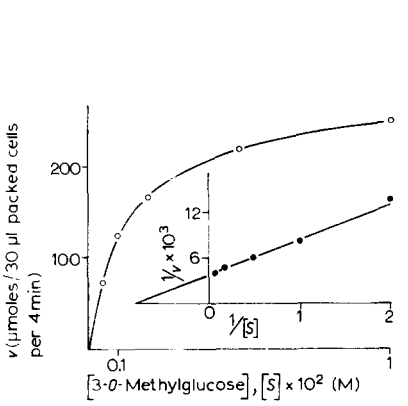

Fig. 5. Concentration dependence of the initial rate of 3-*O*-methylglucose uptake.

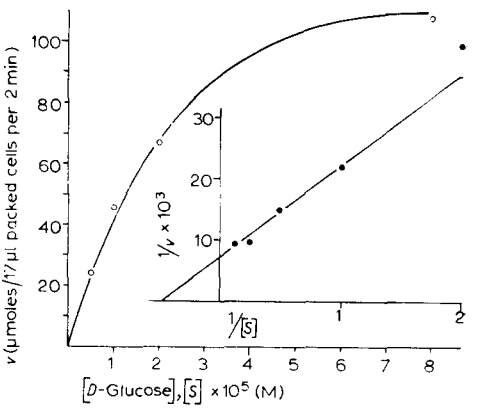

Fig. 6. Concentration dependence of the initial rate of D-glucose uptake.

(3) Evidence that no phosphorylation of the sugar is involved in active uptake by Chlorella

It had been shown before[1] that induced cells as compared to non-induced cells did not contain any increased content of soluble hexokinase. These investigations have been extended to the membrane fraction and the same negative results have been obtained. In addition an intensive search for a phosphoenolpyruvate-phosphotransferase activity also failed to give positive results.

It was thought to be of interest, therefore, to see whether 6-deoxyglucose and 1-deoxyglucose can be actively accumulated by *Chlorella* cells since this would exclude a possible phosphorylation at these two positions. The previous observation[3] that 6-deoxy-6-fluorogalactose inhibited the uptake of 3-*O*-methylglucose did not really prove that phosphorylation of the 6-position is not required for active uptake, since the inhibitor might just have interfered on the outside without being transported itself. Fig. 7 shows, however, that 6-deoxyglucose is taken up by *Chlorella* and is accumulated about 350-fold under the experimental conditions. 6-Deoxyglucose is not at all metabolized in the cells since the sum of the 6-deoxysugar found in the cells and on the outside stays constant during the experimental time. Essentially the same result has been obtained with 1-deoxyglucose (Fig. 8), which not only shows that a sugar without a hydroxyl group in the 1-position gets accumulated, but also that it must be the pyranose form of glucose which passes through the membrane.

The accumulation in this case was 160-fold. These results clearly show that phosphorylation neither at the 1- nor the 6-position is required for hexose accumulation in *Chlorella*. It remains possible, however, that various hexose uptake systems exist in *Chlorella*; D-glucose and 2-deoxyglucose, for example could still be taken up by a system phosphorylating in the 6-position, whereas 6-deoxyglucose enters the cell by a different but non-phosphorylating mechanism. The following results, however, strongly suggest that the various hexoses tested are all transported by one and the same non-phosphorylating uptake system.

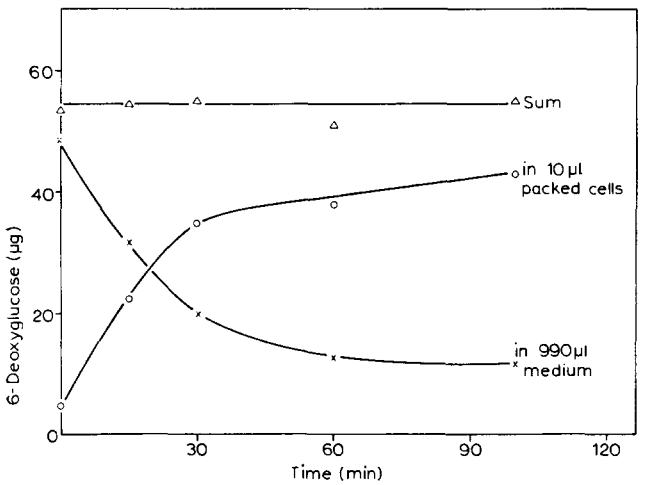

Fig. 7. Uptake of 6-deoxyglucose. In a total volume of 6 ml 60 µl of packed cells were incubated together with 2 µmoles of 6-deoxyglucose. At the times indicated 1 ml of the suspension was centrifuged rapidly; the supernatant and the cells were treated as described in MATERIALS AND METHODS.

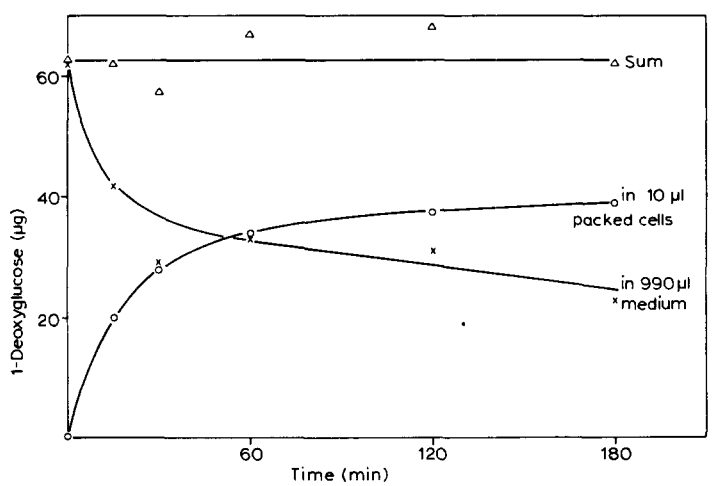

Fig. 8. Uptake of 1-deoxyglucose. Conditions as in Fig. 7 except that 2.4 µmoles of 1-deoxyglucose have been added.

Fig. 9 shows Lineweaver–Burk plots of the 6- and 1-deoxyglucose inhibition of 2-deoxyglucose uptake. The two sets of data were obtained in two individual experiments. It can be seen that the v_{max} for 2-deoxyglucose uptake differs somewhat, and this was observed generally from experiment to experiment; the K_m value, however, always remained quite constant. In the presence of the inhibitors the K_m values increase but the v_{max} also is affected; it decreases. Mixed-type inhibitions have been observed, however, between all sugars tested, *e.g.* also between D-glucose, 3-*O*-methylglucose and 2-deoxyglucose.

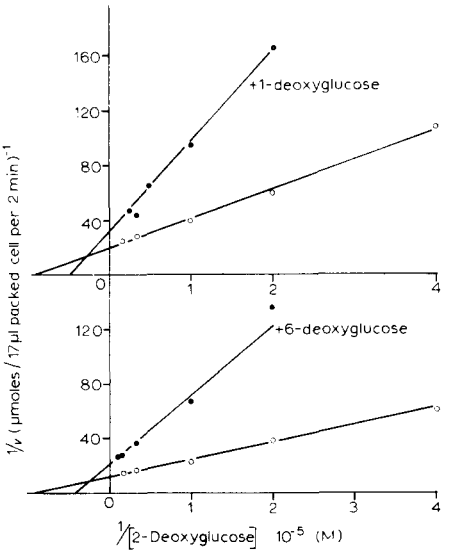

Fig. 9. Lineweaver–Burk plots of the uptake of 2-deoxyglucose and its inhibition by 6-deoxyglucose ($2.5 \cdot 10^{-4}$ M) and 1-deoxyglucose ($7.5 \cdot 10^{-4}$ M).

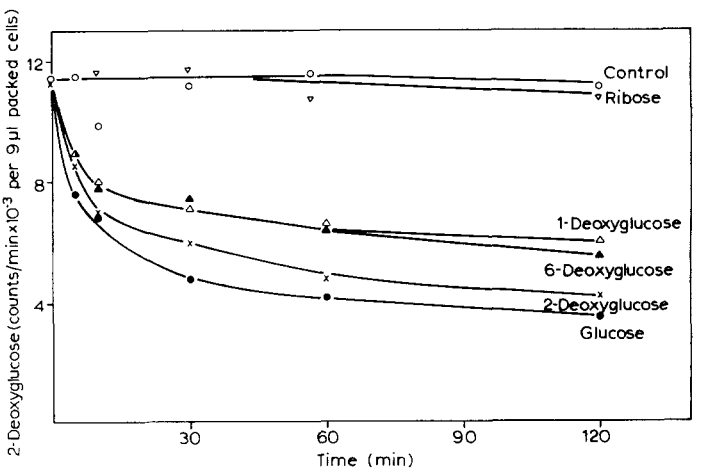

Fig. 10. Exchange of accumulated 2-deoxy[^{14}C]glucose. In a total volume of 18 ml 162 μl of algae (packed cells) were shaken together with 18 μmoles of labelled 2-deoxyglucose (specific activity 0.033 μC/μmole). After steady state had been reached, 3 ml of this suspension were added rapidly to 0.2 ml of a glucose analogue solution ($3.2 \cdot 10^{-1}$ M) or water, respectively. Aliquots of 0.5 ml were filtered and the radioactivity in the cells determined.

A second piece of evidence that 6-deoxy-, 1-deoxyglucose and the various other hexoses are taken up by the same uptake system comes from exchange transport experiments. When these sugars are added to cells that are in the steady state of taking up, for example, 2-deoxy[^{14}C]glucose outflow of radioactivity can be seen (Fig. 10). The addition of D-ribose, which is not transported by this system[3], does not result in an outflow of 2-deoxy[^{14}C]glucose.

Finally a co-induction between the sugars in question also has been observed. When the hexose uptake system has been induced by preincubating the cells with glucose the uptake of 6-deoxyglucose subsequently proceeds linearly whereas hardly any uptake is observed for at least 1 h with non-induced cells (Fig. 11). Identical results results were obtained with 1-deoxyglucose. On the other hand, 1-deoxy-, 6-deoxy-glucose as well as 3-*O*-methylglucose are also able to induce the uptake system, since after pretreatment with these sugars glucose is taken up without a lag (Fig. 12).

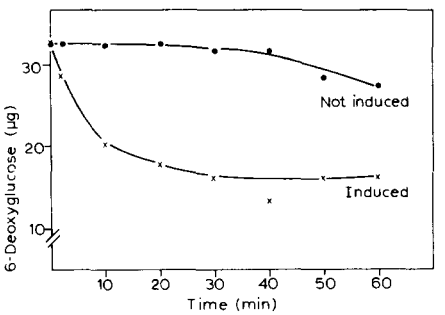

Fig. 11. Induction of 6-deoxyglucose uptake by D-glucose. The cells were induced by glucose as described in MATERIALS AND METHODS and a parallel sample was shaken in buffer alone for the same time. In a total volume of 2 ml 110 µl algae (packed cells) were incubated then together with 2.4 µmoles 6-deoxyglucose. The disappearance of 6-deoxyglucose from the medium was followed.

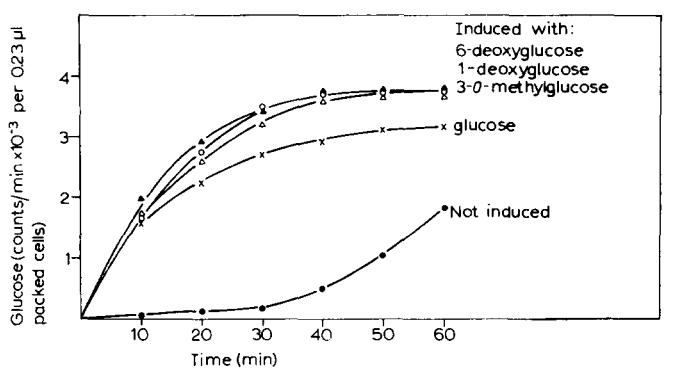

Fig. 12. Induction of D-glucose uptake by various D-glucose analogues. The cells were induced as described in MATERIALS AND METHODS using various glucose analogues in a final concentration of 7.8 mM or shaken in 0.032 M sodium phosphate buffer alone without a sugar, respectively. After 3 h the external sugar was removed by centrifugation and 2 µl algae (packed cells) were incubated in a total volume of 4 ml together with 0.04 µmole labelled D-glucose (specific activity 0.5 µC/µmole). At the times indicated aliquots of 0.5 ml were filtered and counted directly in scintillation vials.

DISCUSSION

The results of the co-induction experiments as well as the partly competitive inhibition between the various sugars tested strongly suggest that these substances are transported and accumulated by one and the same hexose transport system. The fact that 6- and 1-deoxyglucose are accumulated clearly shows that the hexoses are not phosphorylated at either of these two positions during transport. The phosphoenolpyruvate-phosphotransferase system, therefore, cannot be involved. Since 2-deoxy- and 3-*O*-methylglucose are also transported *via* the same uptake system only the 4-position remains as a possibility for a chemical modification (phosphorylation, oxidation) of the transported substrate. This latter possibility can be considered as rather unlikely for the moment. Therefore, a transport model has to be suggested similar to the one for the β-galactoside transport in *E. coli*[25] where not the substrate but some transport protein is modified through an energy dissipating process. The observation, however, that a decreased energy supply brought about either by inhibition with FCCP (Fig. 2) or by anaerobiosis[2] leads to a strong inhibition of the uptake velocity even before concentration equilibrium is reached in *Chlorella* cannot easily be reconciled with β-galactoside uptake models[25] for *E. coli*. Since the K_m of 3-*O*-methylglucose uptake in the presence of FCCP is not different from the K_m of unpoisoned cells (unpublished results) it could be assumed that energy is necessary for the actual translocation process. MANNO AND SCHACHTER[26] have interpreted in a similar manner results obtained for the thiomethyl-β-galactoside uptake of *E. coli* and KOTYK AND HÖFER[27] results obtained for the L-xylose uptake of *Rhodotorula gracilis*.

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REFERENCES

1 W. TANNER AND O. KANDLER, *Z. Pflanzenphysiol.*, 58 (1967) 24.
2 W. TANNER, *Biochem. Biophys. Res. Commun.*, 36 (1969) 278.
3 W. TANNER, R. GRÜNES AND O. KANDLER, *Z. Pflanzenphysiol.*, 62 (1970) 376.
4 W. KUNDIG, F. D. KUNDIG, B. ANDERSON AND S. ROSEMAN, *J. Biol. Chem.*, 241 (1966) 3243.
5 H. R. KABACK, *J. Biol. Chem.*, 243 (1968) 3711.
6 W. HENGSTENBERG, J. B. EGAN AND M. L. MORSE, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 274.
7 E. R. KASHKET AND T. H. WILSON, *Biochim. Biophys. Acta*, 193 (1969) 294.
8 E. M. BARNES AND H. R. KABACK, *Proc. Natl. Acad. Sci. U.S.*, 66 (1970) 1190.
9 S.-C. KOU, M. S. CHRISTENSEN AND V. P. CIRILLO, *J. Bacteriol.*, 103 (1970) 679.
10 J. VAN STEVENINCK, *Biochim. Biophys. Acta*, 163 (1968) 386.
11 C. E. BROWN AND A. H. ROMANO, *J. Bacteriol.*, 100 (1969) 1198.
12 W. KLINGMÜLLER, *Z. Naturforsch.*, 22b (1967) 188.
13 R. K. CRANE, *Physiol. Rev.*, 40 (1960) 389.
14 R. K. NESS, H. G. FLETSCHER AND C. S. HUDSON, *J. Am. Chem. Soc.*, 72 (1950) 4547.
15 Z. DISCHE AND L. B. SHETTLES, *J. Biol. Chem.*, 175 (1948) 595.
16 J. M. BOBBIT, *Advan. Carbohydrate Chem.*, 11 (1956) 1.
17 G. AVIGAD, *Carbohydrate Res.*, 11 (1969) 119.
18 K. RING, W. GROSS AND E. HEINZ, *Arch. Biochem. Biophys.*, 137 (1970) 243.
19 M. VALLÉE AND R. JEANJEAN, *Biochim. Biophys. Acta*, 150 (1968) 599.

20 M. CRABEEL AND M. GRENSON, *European J. Biochem.*, 14 (1970) 197.
21 M. LEVINE AND W. D. STEIN, *Biochim. Biophys. Acta*, 127 (1966) 179.
22 J. P. ROBBIE AND T. H. WILSON, *Biochim. Biophys. Acta*, 173 (1969) 234.
23 G. N. COHEN AND J. MONOD, *Bacteriol. Rev.*, 21 (1957) 169.
24 A. KEPES, in C. D. TOSTESON, *The Molecular Basis of Membrane Function*. Prentice Hall, Englewood Cliffs, 1969, p. 353.
25 E. P. KENNEDY, in N. O. KAPLAN AND E. P. KENNEDY, *Current Aspects of Biochemical Energetics*, Academic Press, New York, 1966, p. 433.
26 J. A. MANNO AND D. SCHACHTER, *J. Biol. Chem.*, 245 (1970) 1217.
27 A. KOTYK AND M. HÖFER, *Biochim. Biophys. Acta*, 102 (1965) 410.