J. Membrane Biol. 12, 89–99 (1973)

A Confirmation of the Proposed Model for the Hexose Uptake System of *Chlorella vulgaris*. Anaerobic Studies in the Light and in the Dark

E. Komor, E. Loos, and W. Tanner

Fachbereich Biologie der Universität Regensburg, Germany

Received 10 August 1972

Summary. Energy for accumulation of sugar analogues in *Chlorella vulgaris* can be supplied by respiration and by light. Under anaerobic conditions a quantum efficiency of close to one has been determined for light-dependent uptake of 6-deoxyglucose at 712 nm. Although the rate of uptake under anaerobic conditions in the dark is less than 15% of that in the light, the steady-state plateau of accumulation does not change when the light is turned off. This is explained by the observation that efflux of sugar is inhibited by lack of energy to the same extent as influx. This agrees with a model proposed earlier (Komor, Haass & Tanner, *Biochim. Biophys. Acta* **266**:649, 1972). Under aerobic conditions during the steady-state plateau in the dark, the rate of influx of 6-deoxyglucose is twice that of initial influx (positive transmembrane effect). By anaerobiosis the initial influx is almost completely inhibited, whereas the steady-state influx is affected only to 50%. This points to an energyless "homoexchange." However, this flux is completely inhibited by uncoupling agents. To explain these results, the existence of an energy-generating efflux has been assumed, which is affected by uncouplers. The efficiency of energy production by efflux and the re-use of this energy for influx is inversely related to the amount of energy supplied by other energy-generating processes in the cells; under anaerobic conditions in the dark, 90% of the efflux energy seems to be available for influx.

Autotrophically grown cells of the unicellular green alga *Chlorella vulgaris* are able to take up hexoses via an inducible transport system (Tanner, 1969). Nonmetabolizable hexose analogues like 6-deoxyglucose are accumulated against aconsiderable concentration gradient. The energy required for this concentration work can be supplied by respiration or, under anaerobic conditions, by light (Tanner, 1969; Komor & Tanner, 1971). Upon addition of sugar analogues to the cells in the dark an increase in respiration is observed which corresponds to the additional production of one ATP (or an equivalent) per sugar molecule transported (Decker & Tanner, 1972). The possibility has been excluded, however, that the energy is used to phosphorylate the sugar (Komor & Tanner, 1971).

The active transport system of *Chlorella* possesses several unusual features and a model has been proposed explaining all the results obtained so far when uptake was studied under aerobic conditions (Komor, Haass & Tanner, 1972). This model has been checked and confirmed now by measurements of uptake under anaerobic conditions in the presence and absence of light. The results of these investigations will be reported in this paper.

Materials and Methods

The strain of *Chlorella vulgaris* and the growth conditions were the same as described previously (Komor & Tanner, 1971). 6-Deoxyglucose was purchased from Roth, Karlsruhe, and was tritiated by the Radiochemical Centre, Amersham, England. Carbonylcyanide-*p*-trifluoro-methoxy-phenylhydrazone (FCCP) was a generous gift of Dr. P. Heytler.

Adaptation of algae: The algae were adapted by incubation with glucose as described previously (Komor & Tanner, 1971).

Net uptake in air: 60 µliters of packed cells (not corrected for intercellular water) were shaken in 6 ml 0.025 M sodium phosphate buffer, pH 6.5, in an Erlenmeyer flask. The experiment was started by the addition of ^{3}H-6-deoxyglucose and samples of 0.5 ml were withdrawn within the first 5 min.. In the first few seconds some radioactivity is unspecifically adsorbed by the cells; therefore, the various curves (e.g., Fig. 1) do not run through the zero point. Uptake rates were determined from the final slopes. The concentration of 6-deoxyglucose used was high enough to saturate the uptake system.

Net uptake in nitrogen: The cell suspension was put into a glass cuvette (10 × 26 × 120 mm) in a water bath and nitrogen was bubbled through a porous glass bottom of the cuvette. After a 10-min preincubation in the dark, 9 µC of ^{3}H-6-deoxyglucose (specific activity 0.1 µC/µmole) dissolved in 200 µliters were added and samples of 0.5 ml were withdrawn with an Eppendorf pipette within the first 8 min. For light-dependent uptake the cuvette was illuminated on the total front area. The light source was a slide projector in conjunction with a Schott interference filter of 712 nm peak wavelength, half bandwidth 17 nm. Light intensity was measured with a calibrated silicon-cell inside the cuvette. The absorption was 62% at 712 nm as measured with an integrating sphere.

Steady-state uptake: 270 µliters of packed cells were shaken in 27 ml 0.025 M sodium phosphate buffer with 270 µmoles of 6-deoxyglucose for 3 to 4 hr until the steady-state plateau of accumulation was reached. Next, 9 ml of suspension were put into a glass cuvette and preincubated in nitrogen for 10 min, or shaken further in an Erlenmeyer flask when aerobic conditions were required. Then 18 µC of ^{3}H-6-deoxyglucose (specific activity 100 µC/µmole) were added in a volume of 50 µliters and samples of 0.5 ml were withdrawn as above. All uptake experiments were carried out at 27 °C.

Extraction and determination of radioactivity: The algae were filtered through membrane filters (0.8 µm pore width; Sartorius, Göttingen) and extracted in 1 ml 0.01 N hydrochloric acid in boiling water for 10 min. All sugar was extracted in this way. After centrifugation, 0.5 ml of the supernatant was pipetted into scintillation vials with a dioxane-naphthalene-PPO mixture. The counting efficiency for tritium was about 50%.

Results

The Quantum Requirement for Light-Driven Active Uptake of 6-Deoxyglucose

Before the model recently published for sugar transport in *Chlorella* has been tested with experiments carried out anaerobically in the light, the quantum requirement for 6-deoxyglucose uptake under these conditions has been studied.

Fig. 1 shows the stimulation of 6-deoxyglucose uptake at two different light intensities. The aerobic uptake rate in the dark is considerably higher than the anaerobic one even at saturating light intensity. The light-stimulated uptake was completely unaffected by DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea; 2×10^{-6}M], an inhibitor of photosynthetic oxygen evolution. This shows that light is not merely generating oxygen to allow active uptake to proceed again under aerobic conditions. Obviously, a process of cyclic photophosphorylation (Arnon, 1967; Tanner, Loos, Klob & Kandler, 1968) supports transport under these conditions.

As can be seen from Fig. 2, the uptake is saturated at the rather low light intensity of 1,200 erg cm^{-2} sec^{-1}. Far-red light (712 nm) was used in these experiments, since it is especially suited for cyclic photophosphorylation. From the light dependence below the saturation point a quantum

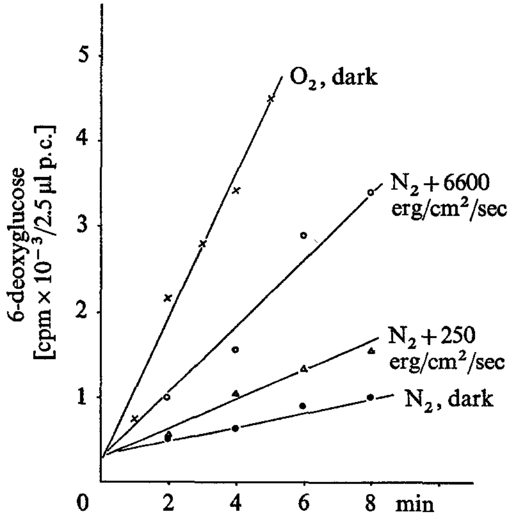

Fig. 1. Uptake of 6-deoxyglucose in nitrogen at various light intensities and in air in the dark. 90 µliters of packed cells (p.c.) were incubated in 9 ml 0.025 M sodium phosphate buffer, pH 6.5, with 90 µmoles of ^{3}H-6-deoxyglucose (9 µC). Under aerobic conditions the cells are using endogenous substrates

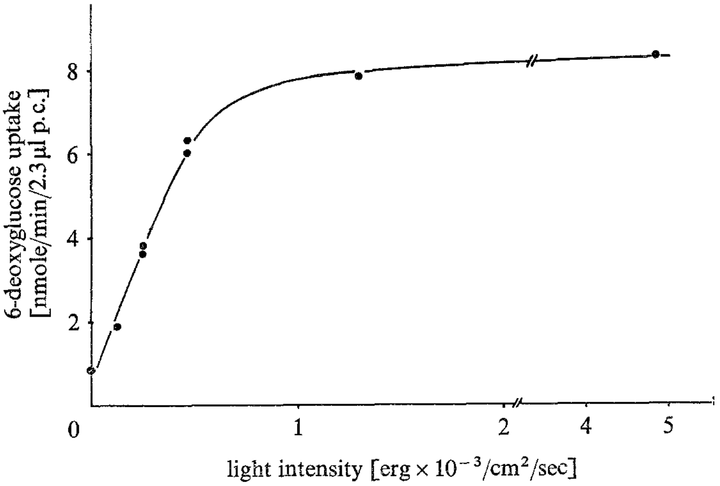

Fig. 2. Light dependence of 6-deoxyglucose uptake. Conditions as in Fig. 1

requirement for 6-deoxyglucose uptake of 1.26 ±0.51 (19 experiments) has been determined. A more detailed consideration of these data in relation to photophosphorylation will be published elsewhere.

The Efflux of 6-Deoxyglucose under Anaerobiosis in the Presence and Absence of Light

In a previous publication (Komor *et al.*, 1972) a model for active hexose-transport of *Chlorella* was presented, which explained among other things, the observation that uncoupling agents such as 2,4-dinitrophenol or FCCP did not cause any net efflux when applied during steady-state accumulation, whereas they completely inhibited both the net and the steady-state influx. These data have been obtained with 3-O-methylglucose but in the meantime the same has been shown to be true for 6-deoxyglucose (*unpublished results*).

For the model it has been assumed that efflux can proceed only via the carrier. Furthermore, carrier should move from the outside to the inside only when it is loaded with substrate and when the required form of energy is present. In the presence of uncouplers no carrier moves to the inner side of the membrane; consequently influx as well as efflux are inhibited (i.e., no net change of radioactivity in the cells can be seen during steady state).

The following question now arose: What happens to accumulated sugar during steady state under anaerobiosis in the light when the light is turned off? According to the model, when the energy for carrier translocation is

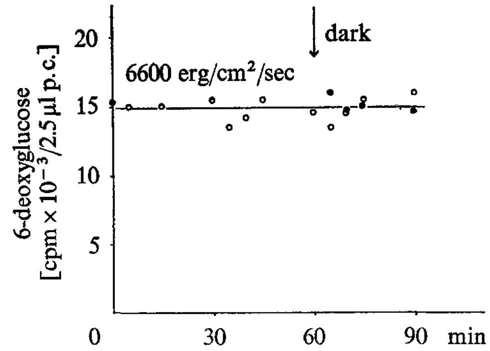

Fig. 3. Effect of a light-to-dark change on accumulation of 6-deoxyglucose. Algae were incubated aerobically as in Fig. 1 for 4 hr, then transferred in two parallel samples to a nitrogen atmosphere (time 0 min) and illuminated with light of 6,600 erg cm^{-2} sec^{-1}. At time 60 min in one sample, light was turned off (filled symbols)

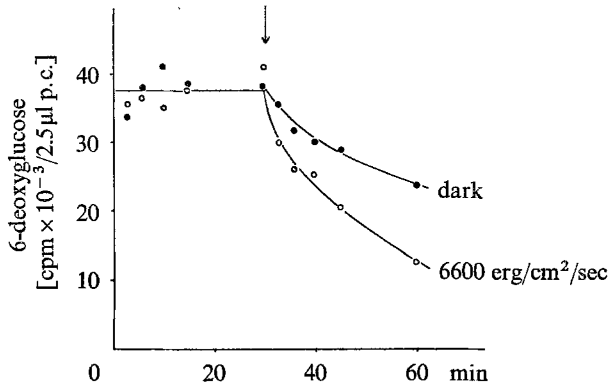

Fig. 4. Exchange of accumulated radioactive 6-deoxyglucose with unlabeled one in the presence and absence of light. Experimental procedure as in Fig. 3, except for the addition of 18 µC of ^3H-6-deoxyglucose (specific activity 0.2 µC/µmole); light in one sample was turned off at 0 min. At 30 min, unlabeled 6-deoxyglucose was added to the final concentration of 0.1 M

cut off, no efflux should be observed. Fig. 3 demonstrates that indeed no radioactivity is lost in the dark.

Efflux during steady state in *Chlorella* can be measured by substantial dilution of the outside specific radioactivity of 6-deoxyglucose. Under aerobic conditions this efflux is strongly inhibited by uncoupling agents. An analogous experiment was now carried out under anaerobic conditions in dark and light (Fig. 4). In accordance with previous results, the efflux is slower when the energy available is reduced; i.e., in the case when the light is turned off. Since in principle the same results are obtained by turning off the light as by adding uncouplers, the possibility of a nonspecific side effect of the poisons is excluded.

The observation that the efflux rate in the dark is still about half that in the light (Fig. 4) whereas it can be completely inhibited with uncouplers (Komor *et al.*, 1972) can be explained by the model previously proposed and will be discussed below (experiment of Fig. 5).

Effect of Anaerobiosis on Steady-State and Net Influx

As with β-galactoside uptake of *E. coli* (Kepes, 1957), in *Chlorella* under aerobic conditions an increased oxygen consumption is initiated by the addition of sugar analogues. This increase remains constant from the beginning through the steady state of accumulation (Decker & Tanner, 1972). The uptake rate, however, rises with increasing inside concentration of sugar. (A positive transmembrane effect of approximately 2 is generally observed with 6-deoxyglucose.) As a consequence, the following has to be assumed: the stoichiometry of energy required per sugar transported either changes from 1 ATP to 0.5 ATP or it stays the same all the time and half the influx during steady state does not require energy. In the latter case, part of the transport would resemble the postulated "homoexchange" of amino acids in Ehrlich cells described by Heinz and Walsh (1958). To test these assumptions, net and steady-state influx were measured under anaerobic conditions and compared with the corresponding aerobic fluxes.

Net influx in the dark is strictly dependent on respiratory energy (Fig. 5*A*; *see also* Fig. 1). During steady state, the rate of aerobic influx is about twice as high as that of the net influx (Fig. 5*B*). However, under anaerobic conditions in the dark, the aerobic steady-state influx is inhibited only to about 50%. This result excludes the possibility that the uptake in the steady state is solely driven by respiratory energy, which is being used with improved efficiency as compared to net influx.

On the other hand, as has already been pointed out previously (Decker & Tanner, 1972), the explanation of an energyless "homoexchange" is not in accordance with the experimental results either. It had been shown that uncouplers such as FCCP completely inhibit steady-state fluxes under aerobic conditions. Fig. 5*B* also demonstrates that the apparently energyless influx during steady state in N_2 does require some form of energy which is affected by uncoupling agents.

We, therefore, have postulated (Decker & Tanner, 1972; Komor *et al.*, 1972) that some form of energy is generated by efflux; the amount being greatest at high intracellular concentrations of sugar. It seems possible that

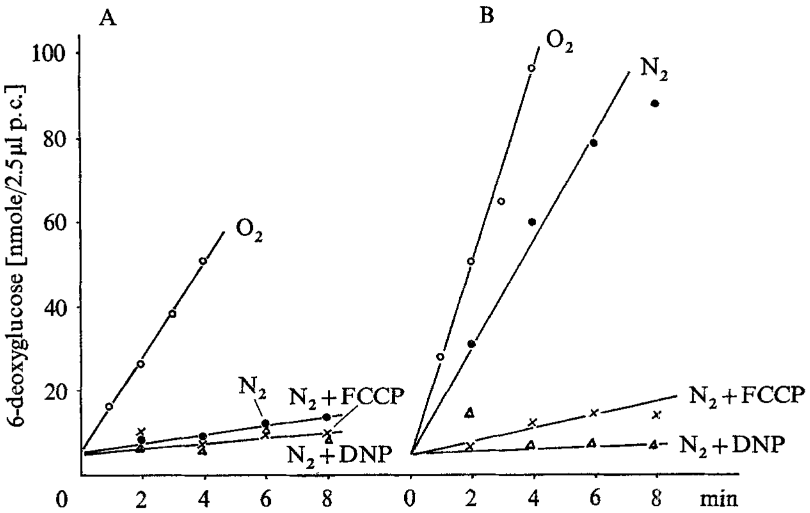

Fig. 5. Net and steady-state uptake of 6-deoxyglucose under aerobic conditions in the dark. Net uptake (*A*) was measured as in Fig. 1. For measuring steady-state uptake (*B*), cells were incubated as in Fig. 1, but with unlabeled 6-deoxyglucose, for 4 hr aerobically in the dark. Then parallel samples were transferred to nitrogen atmosphere or kept in air. Where indicated, uncouplers were added (DNP 4×10^{-4} M, FCCP 5×10^{-5} M). After a 10-min incubation, 9 µC of ^{3}H-6-deoxyglucose were added (specific activity 100 µC/µmole)

this simply happens by reversal of the energy-consuming influx reaction. For ion translocating systems, energy generation by reversal of active fluxes has been reported before (Lew, Glynn & Ellroy, 1970; Makinose & Hasselbach, 1971).

The observation that efflux measured during steady state by high dilution of external sugar with a nonradioactive one (Fig. 4) still occurs to some degree under anaerobiosis, but is completely blocked by FCCP (Komor *et al.*, 1972), can now simply be explained: anaerobiosis does not affect energy generation by efflux, whereas uncoupling agents inhibit both energy generation by efflux and by respiration (Fig. 5). Thus, under anaerobic conditions, even in the dark, the steady-state translocation of sugar molecules can be energized by that energy form, which is generated by the efflux.

Varying Amounts of Energy Generated by Efflux

Under aerobic conditions in the dark, the efficiency of energy generation by efflux and its re-use is about 50%, since during steady state the total efflux drives about half the influx, whereas the other half is driven by the increased respiration. Under anaerobic conditions in the dark, however,

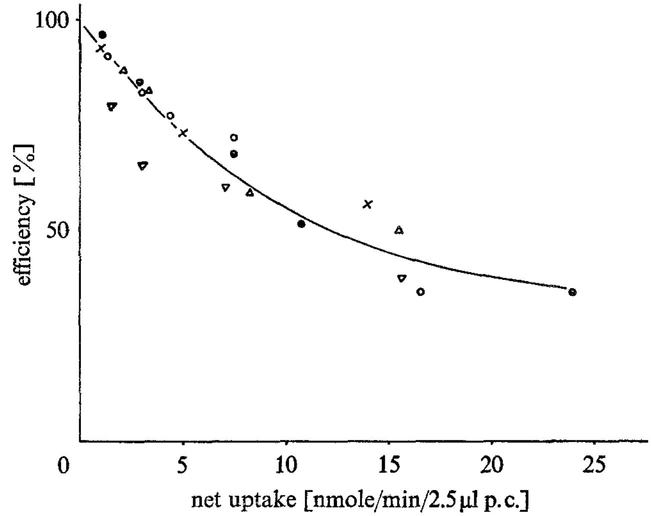

Fig. 6. Efficiency of generation and re-use of "efflux energy" in relation to the energy state of the algae. Experiments were performed as in Fig. 5. Experiments with uncouplers have not been considered. The rate of net uptake of 6-deoxyglucose is taken as measure for the energy state of the algae. Low "energy state" of the algae has been achieved by anaerobic conditions either in the dark or at low light intensities; high "energy state" by high light intensities or aerobic conditions. Equal symbols stand for experiments carried out on the same day with the same algal suspension

the efficiency must be considerably higher. The anaerobic steady-state concentration in the cells reached in the light is maintained almost constant after switching to the dark (Fig. 3) (a slow decrease might be concealed by the scattering of the points); this means that influx equals efflux and that the energy generated by efflux plus the low amount of energy derived from fermentation must drive the influx.

As efficiency for energy generation by efflux and the re-use of this energy for influx the following expression has been used:

$$\frac{\text{steady-state influx} - \text{net influx}}{\text{steady-state influx}},$$

whereby steady-state influx equals steady-state efflux.

From the data of Fig. 5, two extreme values in efficiency can be calculated. In oxygen, an efflux rate of 23 nmoles sugar/min per 2.5 µliters of packed cells (p.c.) generates energy for the additional influx rate of 11.5 nmoles sugar/min per 2.5 µliters p.c. This means an efficiency of 0.5 or 50%. In nitrogen an efflux of 13 nmoles sugar/min per 2.5 µliters p.c. generates energy for an additional influx of 11.5 nmoles sugar/min per 2.5 μliters p.c. yielding an efficiency of 0.89.

There seems to exist a steady relationship between the "energy content" of the algae and the efficiency of the energy generation by efflux. In Fig. 6, data for efficiency are depicted for varying "energy contents" of the algae caused under anaerobic conditions by illumination with different light intensities. The "energy content" is expressed as the rate of net uptake of 6-deoxyglucose, which should be directly dependent on energy until the transport system is energy saturated. The graph of Fig. 6 shows a continuous relationship between the "energy content" of the cells and the efficiency of efflux in generating energy. Under conditions where a large amount of energy is available from the light reactions (at high-light intensities) or by respiration, the efflux is least efficient and vice versa.

Discussion

As far as we know there have been no quantitative data published so far on light-driven active uptake of nonelectrolytes. The quantum requirement of 1.26 measured for 6-deoxyglucose uptake can be compared with a quantum requirement of 4 for glucose uptake (Tanner *et al.*, 1968). In the case of glucose, however, the energy is required not only for the transport step, but also for assimilation to oligo- and polysaccharides (Tanner, Loos & Kandler, 1965). From the increased respiration of *Chlorella* cells it has been calculated previously (Decker & Tanner, 1972) that 1.18 ATP are required for one 6-deoxyglucose to be taken up and 2.55 ATP for 1 glucose to be taken up *and* assimilated. A comparison of all these data shows that the quantum requirement determined for hexose transport is a reasonable value. The results obtained under anaerobic conditions with and without light fully confirm the transport model proposed earlier (Komor *et al.*, 1972). A main postulate has been that the carrier is translocated to the inside only as carrier-substrate-complex and only when energy is available. As a consequence, steady state or exchange efflux is affected to the same extent as influx by energy poisons (Decker & Tanner, 1972; Komor *et al.*, 1972). In analogy, turning off the light under anaerobic conditions should affect influx and efflux rates as well and this exactly has been shown (Figs. 1, 3 and 4).

Another important assumption has been the existence of an energy-generating efflux. The phenomena which led to this postulate for *Chlorella* shall be shortly summarized here:

(1) Although the rate of influx increases with increasing intracellular concentration becoming twice as high under steady-state conditions, the increased O_2-uptake stays constant from the beginning throughout the experiment (Decker & Tanner, 1972).

(2) Although the extra influx is also maintained under anaerobiosis and appears thus to proceed without energy, it can be fully inhibited by FCCP and DNP (Fig. 5).

(3) Finally, it has been observed that with decreasing "energy content" of the cells the efflux becomes more and more efficient in supporting the influx (Fig. 6). This is best explained by assuming that two forms of energy, derived from respiration (or photophosphorylation) and derived from efflux, are competing to drive sugar influx.

Although several features of hexose uptake by *Chlorella* are unusual, especially when compared to bacterial sugar uptake systems (Kepes, 1971), they nevertheless might not be restricted to *Chlorella*. Thus, all sugar transport data obtained with the yeast *Rhodotorula* (Kotyk & Höfer, 1965; Höfer, 1971*a*) can be explained by the *Chlorella* model as well as with the model designed for *Rhodotorula* (Höfer, 1971*b*). Also the model for amino acid transport in Ehrlich cells (Heinz & Walsh, 1958), which assumes that 80% of the steady-state flux is by energyless homoexchange, has failed to explain so far why DNP to a large extent inhibits this "energy-independent" flux (Heinz & Mariani, 1957). The assumption of an energy-generating efflux system would abolish this discrepancy. It seems possible, therefore, that the *Chlorella* transport model may be of general importance to explain active transport of nonelectrolytes by eucaryotic cells.

This work was supported by the Deutsche Forschungsgemeinschaft.

References

Arnon, D. I. 1967. Photosynthetic activity of isolated chloroplasts. *Physiol. Rev.* **47**:317.

Decker, M., Tanner, W. 1972. Respiratory increase and active hexose uptake of *Chlorella vulgaris*. *Biochim. Biophys. Acta* **266**:661.

Heinz, E., Mariani, H. A. 1957. Concentration work and energy dissipation in active transport of glycine into carcinoma cells. *J. Biol. Chem.* **228**:97.

Heinz, E., Walsh, P. M. 1958. Exchange diffusion, transport and intracellular level of amino acids in Ehrlich carcinoma cells. *J. Biol. Chem.* **233**:1488.

Höfer, M. 1971*a*. Transport of monosaccharides in *Rhodotorula gracilis* in the absence of metabolic energy. *Arch. Mikrobiol.* **80**:50.

Höfer, M. 1971*b*. A model of the monosaccharide uphill transporting cell membrane system in yeast. *J. Theoret. Biol.* **33**:599.

Kepes, A. 1957. Metabolisme oxidatif lié au fonctionnement de la galactoside perméase d'*Escherichia coli*. *Compt. Rend. Acad. Sci., Paris.* **244**:1550.

Kepes, A. 1971. The β-galactoside permease of *Escherichia coli*. *J. Membrane Biol.* **4**:87.

Komor, E., Haass, D., Tanner, W. 1972. Unusual features of the active hexose uptake system of *Chlorella vulgaris*. *Biochim. Biophys. Acta* **266**:649.

Komor, E., Tanner, W. 1971. Characterization of the active hexose transport system of *Chlorella vulgaris*. *Biochim. Biophys. Acta* **241**:170.

Kotyk, A., Höfer, M. 1965. Uphill transport of sugars in the yeast *Rhodotorula gracilis*. *Biochim. Biophys. Acta* **102**:410.

Lew, V. L., Glynn, D. M., Ellroy, J. C. 1970. Net synthesis of ATP by reversal of the sodium pump. *Nature* **225**:865.

Makinose, M., Hasselbach, W. 1971. ATP synthesis by the reversal of the sarcoplasmic calcium pump. F.E.B.S. **12**:271.

Tanner, W. 1969. Light-driven active uptake of 3-O-methylglucose via an inducible hexose uptake system of *Chlorella*. *Biochem. Biophys. Res. Commun.* **36**:278.

Tanner, W., Loos, E., Kandler, O. 1965. Photoassimilation of glucose by *Chlorella* in monochromatic light of 658 and 711 mμ. *In*: Currents in Photosynthesis. J. B. Thomas and J. C. Goedheer, editors. p. 243. Ad. Donker, Rotterdam.

Tanner, W., Loos, E., Klob, W., Kandler, O. 1968. The quantum requirement for light dependent anaerobic glucose assimilation by *Chlorella vulgaris*. *Z. Pflanzen Physiol.* **59**:301.