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Transformation of a Strictly Coupled Active Transport System into a Facilitated Diffusion System by Nystatin

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Summary. The active hexose transport system of *Chlorella vulgaris* is obligatorily coupled to metabolic energy. No facilitated diffusion component, as in the β-galactoside transport of *Escherichia coli*, for example, is observed with *Chlorella*. In the presence of nystatin, however, facilitated diffusion of sugar analogues occurs. Thus, only under this condition can the classical overshoot experiment be successfully carried out. The net efflux of sugars induced by nystatin does not take place through holes. It can be explained by the assumption that the mobility of the unloaded carrier is less restricted in the presence of nystatin. Nystatin also changes the K_m for influx, whereas the K_m for efflux is not significantly affected. Possible roles of sterols in this eucaryotic sugar transport system are discussed.

Polyene antibiotics like nystatin and filipin have been shown to severely affect membrane permeabilities for various substrates [7, 19, 21]. They are only active, however, when the membranes exposed to them contain sterols. Thus, only eucariotic cells [12, 19] and *Mycoplasma laidlawii* grown in the presence of sterols [5, 27] show high rates of efflux of ions and nonelectrolytes when polyenes are added. They bind to sterols [4, 20] and at least under certain conditions are thought to produce holes in membranes [12].

In *Chlorella vulgaris* cells an active, nonphosphorylating hexose transport system can be induced [16, 26]. Recently, evidence has been obtained that this transport of sugars, which can be driven by respiration or light, is a proton symport [13]. The following data will show that although nystatin greatly increases net efflux of sugar analogues accumulated by *Chlorella*, the efflux does not proceed through holes. The results obtained can be explained by assuming that the influx of carrier without substrate, previously shown to be the rate-limiting reaction for net efflux [14, 15], is greatly increased by nystatin. Sterols possibly constitute an important factor, therefore, responsible for the normal transport characteristics of these cells.

Materials and Methods

6-Deoxyglucose was obtained from Koch-Light Laboratories, Colnbrook, England, and tritiated by the Radiochemical Centre, Amersham. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was generously provided by Dr. P. Heytler, Dupont de Nemours. Nystatin (mycostatin) was obtained from Serva, Heidelberg. Stock solutions of 10 mg nystatin/ml dimethylsulfoxide were prepared, stored at −20 °C in the dark and used up within a week. The addition of the same amount of dimethylsulfoxide without nystatin was without effect on the algae. All experiments were performed with the green alga *Chlorella vulgaris* in air in the dark at 27 °C.

Growth of the algae and induction of the hexose uptake system was carried out as described previously [26]. The cells were incubated in 0.025 M sodiumphosphate buffer, pH 6.5. Samples were filtered, extracted and the radioactivity determined as described elsewhere [14].

Results and Discussion

It has been shown before that uptake of sugar analogues into *Chlorella vulgaris* cells proceeds by a strictly energy-coupled transport system [15, 16]. Thus, uptake is severely inhibited by uncoupling agents even below concentration equilibration of the sugar and no net efflux occurs, when loaded cells are poisoned. Similar observations have been made with other eucaryotic cells like yeast and *Neurospora* for the transport of ions [25], sugars [8], and amino acids [17, 18]. The effect has also been described for ion transport in *Chlorella* [1]. Therefore, it seems that these cells do not have a facilitated diffusion system in series to an active transport system as has been proposed for example for β-galactoside transport in *E. coli* [10, 29]. When nystatin is added to such cells [18, 19, 25] they rapidly lose part of their accumulated substances. This is also the case with 6-deoxyglucose accumulated by *Chlorella* when 6 μg nystatin/ml are added to the cells (Fig. 1). The addition of FCCP to cells considerably accelerates efflux. Under these conditions *Chlorella*, therefore, resembles qualitatively *E. coli*, which loses galactose [9] or TMG [11] for example, when poisoned with uncouplers or azide. In the absence of nystatin the addition of FCCP produced no effect on the level of accumulated sugar, which in this experiment amounts to 50-fold the outside concentration.

The result could be explained in the following way: net efflux occurs through holes produced by nystatin and the stimulation of efflux by FCCP in the presence of low amounts of nystatin could mean that pumps are still active to counteract part of the outflow. These remaining pumps are blocked by FCCP. The following two results, however, strongly indicate that net 6-deoxyglucose efflux does not proceed through holes produced by nystatin. Thus, the kinetics of efflux (Fig. 2) have, for at least 20 min, the characteristic of a zero-order rather than a first-order reaction. This is expected for a

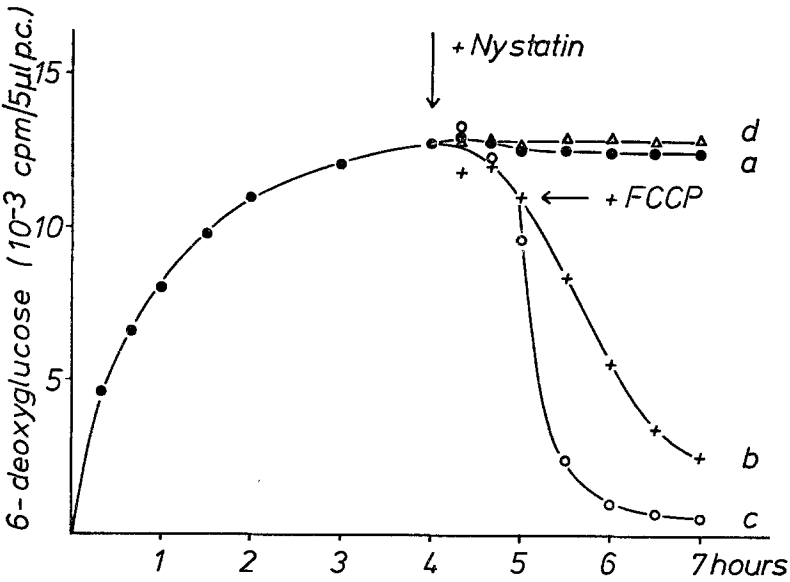

Fig. 1. Efflux of 6-deoxyglucose after addition of nystatin and FCCP. Cells (10 µliters of packed cells/ml) were preloaded with (^{3}H)-6-deoxyglucose (3×10^{-3} M; 55 µC/mmole). After 4 hr, nystatin (final concentration 6 µg/ml) was added to samples *b* and *c* and 5×10^{-5} M FCCP to sample *d*; sample *a* is the control. After another hour, FCCP at a final concentration of 5×10^{-5} M was added to *c*

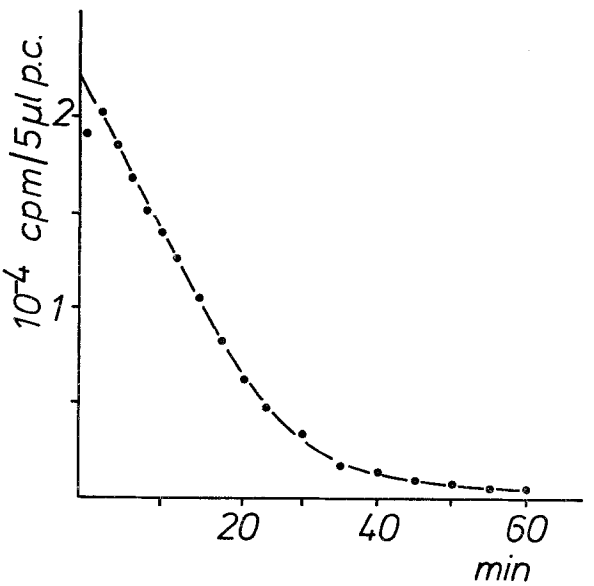

Fig. 2. Net efflux caused by nystatin; a zero-order reaction. Algae (10 µliters of packed cells/ml) were preloaded with (^{3}H)-6-deoxyglucose (10^{-2} M, 240 µC/mmole) for 3.5 hr. The cells were then harvested, washed once and resuspended in a large volume of incubation buffer with 100 µg nystatin/ml yielding a cell concentration of 0.2 µliter of packed cells/ml. 10,000 cpm/5 µliters of packed cells correspond to an intracellular concentration of 53 mM

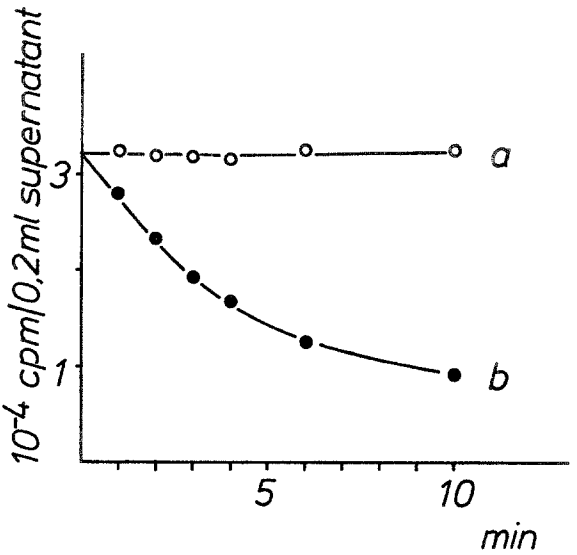

Fig. 3. Inhibition of 6-deoxyglucose-uptake by glucose in nystatin-treated cells. Thick suspensions of algae (0.63 ml packed cells/ml) were incubated with 100 µg nystatin/ml for 30 min. Then to sample *b* (^{3}H)-6-deoxyglucose was added (4×10^{-4} M; 250 µC/mmole); to sample *a* a mixture of (^{3}H)-6-deoxyglucose (4×10^{-4} M; 250 µC/mmole) and of glucose (1×10^{-2} M) was added. The decrease in radioactivity in the supernatant after centrifugation was determined

carrier-mediated process as long as the carrier is saturated or close to saturation. Furthermore, 6-deoxyglucose influx into cells treated with high nystatin concentrations can be completely inhibited by glucose (Fig. 3) even below concentration equilibration.

It then follows that the results shown in Fig. 1 need to be explained in a different way. Normally the carrier "moves" to the inside at a significant rate only in the presence of substrate and energy [13, 15, 16]. Since the addition of an uncoupler stops the carrier translocation and movement of carrier is required for both influx and efflux [14], net efflux does not take place under these conditions. In the presence of sufficient nystatin the carrier or the carrier membrane interaction is changed in such a way as to allow the empty carrier to move in and out and in addition does not require energy. This carrier now should allow facilitated diffusion. In the presence of low amounts of nystatin part of the carrier will still be strictly coupled and another part will be transformed. The addition of FCCP will block the former. Any pumping would stop and net efflux would increase.

If the above explanation were correct it should be possible to show the existence of facilitated diffusion in nystatin-treated cells. This has indeed been possible with an overshoot experiment (Fig. 4). The uptake of (^{3}H)-6-

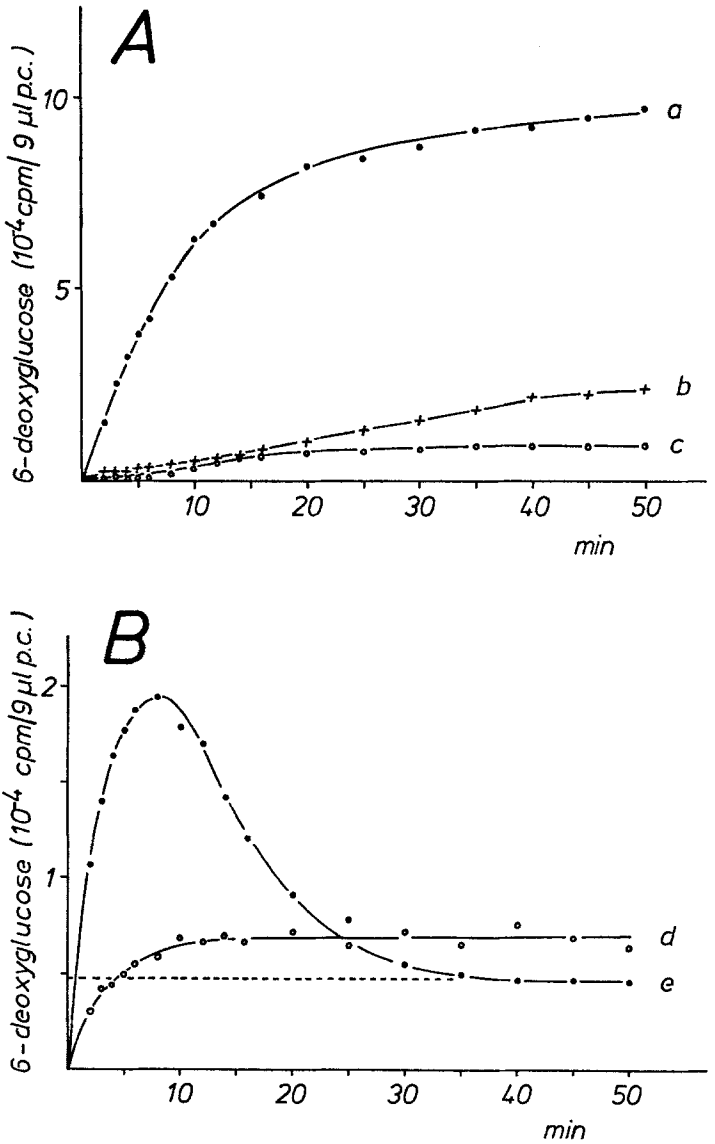

Fig. 4. Overshoot experiment. The algae (36 µliters of packed cells/ml) were preloaded in sodium phosphate buffer containing 10^{-1} M 6-deoxyglucose and 12 µg nystatin/ml for 2 to 3 hr. Then the cells were centrifuged and washed once with ice-cold buffer to remove external 6-deoxyglucose. During this washing procedure more than 75% of the internal 6-deoxyglucose remained in the cells. The cells were resuspended in buffer containing (^{3}H)-6-deoxyglucose (1×10^{-5} M; 160 mC/mmole) to give 4.5 µliters of packed cells/ml. Part *A* — cells not treated with nystatin: curve (*a*)-cells not preloaded (preloaded ones give similar-uptake kinetics; *see* Ref. [10]); curve (*b*)-with FCCP 5×10^{-5} M present, cells preloaded; curve (*c*)-with FCCP 5×10^{-5} M present, cells not preloaded. Part *B* — cells treated with nystatin (12 µg/ml) before and after dilution: curve (*d*)-with FCCP 5×10^{-5} M, cells not preloaded; curve (*e*)-with FCCP 5×10^{-5} M, cells preloaded. The broken line indicates concentration equilibrium

deoxyglucose was measured in the presence of nystatin and FCCP. The uptake reaches a plateau value slightly above the concentration equilibration. When the uptake of the sugar analogue was measured under the same condition, except that cells preloaded with nonradioactive 6-deoxyglucose were used, an overshoot of radioactivity was observed which reached about three times the equilibrium concentration (Fig. 4*B*). The fact that the radioactivity in the nonpreloaded sample is somewhat above concentration equilibration as compared to the amount of radioactivity in the preloaded sample past the overshoot transient, can be explained by assuming that a small amount of the radioactive sugar is not free but adsorbed on (and may be in) the cells. In the preloaded sample this amount will not show up. The control (Fig. 4*A*) without nystatin and FCCP accumulates sugar under these conditions 200-fold. 5×10^{-5} M FCCP inhibits the rate of influx by more than 90% and preloading does not lead to an overshoot when only FCCP is present (Fig. 4*A*). The overshoot experiment also excludes the existence of holes but in addition can be considered as evidence for the second explanation given above, since it is the most decisive experiment to demonstrate facilitated diffusion [24, 28].

The possibility that nystatin itself acts directly as a carrier has been excluded. Since the hexose uptake system of *Chlorella* is inducible [26] it was possible to compare the rate of uptake in the presence of nystatin of induced cells with that of noninduced cells. Clearly, the cells had to be induced to observe a significant rate of uptake.

Finally it has been observed that the K_m for 6-deoxyglucose influx increases drastically due to the presence of nystatin. Although it has not been possible for technical reasons to obtain an exact K_m value (it is difficult to measure influx rates below concentration equilibrium and in addition at high substrate concentrations osmotic effects were already observed), the K_m is certainly higher than 1×10^{-2} M. This value has to be compared with the K_m of 2×10^{-4} for untreated cells [14]. The K_m for 6-deoxyglucose efflux, however, does not change significantly in the presence of nystatin, as can be seen from Fig. 2 (a K_m value of 2×10^{-2} can be estimated from these data, which is close to the value obtained without nystatin; *see* Ref. [14]).

The experiments reported here have two consequences, which might be of general importance and not restricted to algae only. First, the assumption that polyene antibiotics produce holes in sterol containing biomembranes should be reconsidered in each individual case where outflow of substrate has been found. Possibly holes arising are smaller than 5 Å, as has been estimated from experiments with black films [6]. Secondly, the results with

Chlorella can be taken as an indication for a role of sterols in active transport processes, since it seems safe to assume that also in *Chlorella* nystatin acts due to its binding to sterols. *Chlorella vulgaris* contains ergostenol, chondrillastenol and chondrillasterol [23]. Sterols might be responsible, therefore, to prevent the movement of the carrier in the absence of substrate and energy. It has been demonstrated that artificial membranes become less fluid and less permeable above transition temperature for a number of substances in the presence of cholesterol [2, 3, 6]. Polyene antibiotics partly revert the effect of cholesterol [6, 22]. One can imagine, therefore, that also parts of the membrane itself, here the carrier molecules, become more mobile when sterols present in the membrane have reacted with nystatin. On the other hand, the results reported also allow the interpretation that sterols may play a more direct part in the functioning of eucaryotic active transport systems.

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