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THE KINETICS OF THE ACTIVE AND DE-ENERGIZED TRANSPORT OF *O*-METHYL GLUCOSE IN *USTILAGO MAYDIS*

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Summary

The kinetics of the uptake and efflux of 3-*O*-methyl-glucose in sporidia of *Ustilago maydis* were measured, both in active cells and in cells whose metabolic activity had been inhibited by azide and iodoacetate.

The de-energized transport system proved to be carrier mediated with apparent affinity constants 13 ± 2 mM outside (K_0) and 18 ± 2 mM inside (K_1). The apparent maximum rate constants for the same system were 0.66 ± 0.05 mmol/l cell water per min for uptake (V_+) and 0.53 ± 0.04 mmol/l cell water per min for efflux (V_-). For the active system $K_0 = 0.08 \pm 0.01$, $K_1 > 40$, $V_+ = 9.7 \pm 0.5$ and $V_- = 1.1 \pm 0.9$ (in equivalent units). These results are discussed in the context of the carrier mechanism as proposed by Regen and Morgan (Regen, D.M. and Morgan, H.E. (1964) Biochim. Biophys. Acta 79, 151—166).

The antifungal compound carboxin had no effect on de-energized transport but was shown to decrease both K_0 and V_+ in the active system. Phloretin and phlorizin were also found to be without effect on de-energized cells but the former enhanced while the latter inhibited active uptake.

Introduction

Our knowledge of the kinetics of sugar transport systems in fungi appears to be limited to a few reports on yeast [1,2], *Neurospora crassa* [3], *Aspergillus nidulans* [4] and *Necosmospora vasinfecta* [5]. To increase the information available on these important organisms the following study was made on the basidiomycete, *Ustilago maydis*. The reasons for choosing this particular species

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include its economic importance as a pathogen on corn and its ease of growth on artificial media. Furthemore, in liquid culture it exists as single yeast-like cells or 'sporidia' which are easily pipetted, filtered and centrifuged.

In the present work, the kinetics of transport by the cells of the non-metabolizable glucose analogue, 3-*O*-methyl-glucose is examined both in normal cells, where transport is active and in de-energized cells, where facilitated transport alone occurs. In essence this work follows the plan utilized by Winkler and Wilson [6] in their study of galactoside transport in *Escherichia coli*.

Materials and Methods

The substrate, 3-*O*-methyl-α-D-glucopyranoside was obtained from the Sigma Chemical Co., St. Louis, Mo. and was mixed in appropriate proportions with its radioactive analogue obtained from the New England Nuclear Co.

U. maydis, ATCC 14826 was provided by Dr. G.A. White of this laboratory and was maintained on agar slants containing the culture medium of Coursen and Sisler [7]. The same medium in liquid form was used to grow sporidia for experimental purposes, harvesting them during log phase growth by centrifugation. They were then washed and resuspended to the required density in 30 mM phosphate buffer at pH 6.5 in preparation for the following experiments.

Determination of intercellular water volume

The total volume of a sample of cells was obtained by the exclusion of (1) inulin at room temperature and (2) *O*-methyl glucose at 0°C. The dry weight of the sample was also obtained, and on the assumption that this dry material would have a density of approximately 1 in its hydrated state, the volume of cell water was found by difference. This was then related to the original absorbance (A_{540nm}) in such a way that the volume of cell water per ml suspended cells could be derived from measurement of A.

Determination of the nature of the accumulated sugar

Sporidia were allowed to accumulate *O*-methyl glucose from a 1.0 mM solution. After 120 min the cells were centrifuged and washed with buffer at 0°C, and extracted with 200 μl ethanol. The extracts were chromatographed on Whatman No. 1 paper, developing with a mixture of *n*-butanol/ethanol/water (52 : 32 : 16; v/v).

Transport measurements

Active cells. *O*-Methyl glucose was added to a stirred suspension of cells of known absorbance (0.07—0.7) and a temperature of 30°C. 1 ml samples were removed at measured intervals, injected into 10 ml ice-cold buffer and filtered under suction through glass fiber filters (Reeve Angle 934AH). The filters were washed three times with cold buffer, air dried, placed in counting vials, 10 ml scintillation fluid added and the radioactivity counted. The amount of *O*-methyl glucose in the cells was obtained by a direct comparison of the sample count to that of a known volume of supernatant. Dividing this by the cell water volume gave the internal concentration as reported below.

To measure efflux, a sporidial suspension was first loaded with *O*-methyl

glucose by incubating the cells in a solution of known concentration. After 2 h, when the internal substrate concentration had reached a steady level (Fig. 1) samples were taken for assay and the remaining cells centrifuged (2000 $\times g$, 20 min). The cells were then rapidly resuspended in a large volume (in which the intercellular solution clinging to the cells was diluted an estimated 70 000 times) and samples taken at known times for assay.

De-energized cells. Cells are incubated for 30 min as above but at 20 times the absorbance and in the presence of 7.5 mM potassium azide and 2.5 mM iodoacetic acid. This reduces metabolism to the point where no active transport occurs (see below). Influx and efflux measurement procedures were otherwise identical to those for active cells.

Rate measurement

Since the exit and entrance curves of both active and de-energized cells proved to be approximately linear for at least the first 5 min, the initial rates of transport were taken as the change in internal concentration after 5 min divided by that time. These rates, therefore, have the units, mmol/l cell water per min.

Results

Cell water volume

Identical results were obtained in the determination of cell water volume with either inulin or *O*-methyl glucose as the excluded substance. The cell water averaged 71 ± 2% of the total cell volume.

Nature of the accumulated sugar

Two radioactive peaks appeared in the chromatograph of cell extracts. The larger of these had the same R_F value as pure *O*-methyl glucose while the smaller one was produced by material remaining at the origin. This latter peak contained only 3.4 ± 1% of the total radioactivity however, so it may be concluded that virtually all substrate present in the cells is unchanged *O*-methyl glucose.

Measurements of the active transport kinetics

Uptake. Fig. 1 presents a typical time course of uptake. Note that after an hour the internal concentration had reached an equilibrium value approximately 400 times the outside concentration, i.e. the accumulation coefficient, α = concentration inside/concentration outside = 400. This coefficient was determined as a function of the concentration outside and the results presented in Fig. 2. The fact that α can exceed 2000 and that *O*-methyl glucose is unchanged on uptake shows that we are dealing here with a true active transport system.

This being the case, we were able to proceed with the measurements of the various kinetic constants in accordance with the procedures outlined by Winkler and Wilson [6].

The apparent affinity constant (K_0) and maximum rate constant (V_+) for uptake were obtained from the initial flux velocities at varying external sub-

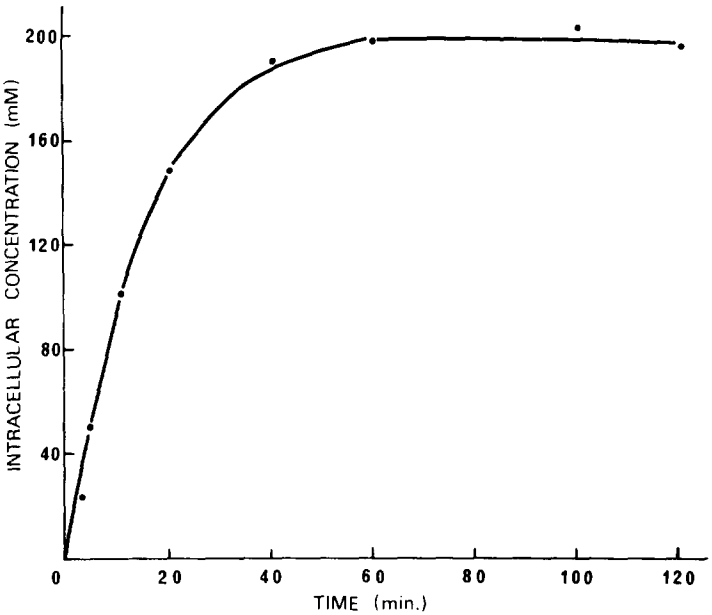

Fig. 1. Time course of uptake of 0.50 mM *O*-methyl glucose by normal sporidia.

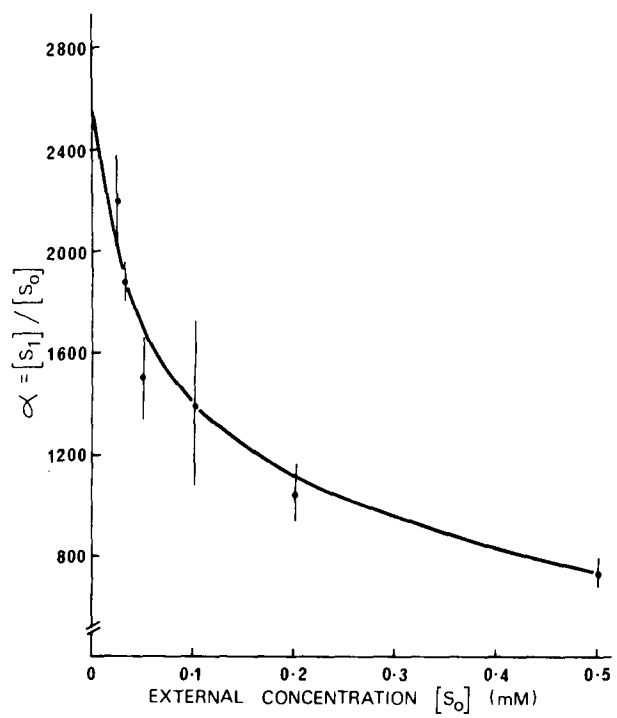

Fig. 2. Plot of the ratio α (inside concentration. $[S_1]$/outside concentration, $[S_0]$) versus the external concentration. Error bars are ±S.D.

strate concentrations using the method of Hofstee [8].

Efflux. The drop in internal concentration as a function of time was followed for cells which had been equilibrated with substrate for 2 h and resuspended in a sugar-free medium. The results of two such experiments are shown by the solid lines in Fig. 3A and B and demonstrate that approximately 70% of the substrate leaves the cells fairly readily but the remainder appears to be fixed in some way. Experiments in which the cells were resuspended in fresh sugar-free media after 4 h revealed no further loss in the retained substrate. On the other hand, this material was totally displaced by the addition of either the usual concentration of metabolic inhibitors (Fig. 3A) or the non-radioactive compound (Fig. 3B).

Initial efflux measurements were obtained at various internal concentrations of substrate in an attempt to obtain the kinetic constants for efflux of that portion of the substrate which does exit rapidly. This process was not readily saturated, even at high concentrations, indicating either that efflux was passive rather than carrier mediated, or if carrier mediated, the value of K_1 was very high (Table I); or put another way, that the carrier has a low affinity for substrate inside the cell.

Effect of inhibitors on active transport

Azide and iodoacetate. Sherald and Sisler [9] found that both azide and antimycin A caused a stimulation of oxygen uptake by *U. maydis*, in which respect these compounds appear to act as uncouplers. We were unable to confirm these results with azide, finding instead a steady decline in Q_{O_2} with increasing concentration. Thus with azide, 17% inhibition occurred at 0.5 mM and 90% throughout the range 2.5—15 mM. This appears to be the maximum inhibition attainable by azide, the remaining 10% oxygen consumption probably occurring via the alternate pathway whose presence has been demonstrated in this organism [9].

Since azide appear to totally inhibit electron transport, the further addition of iodoacetate to suppress glycolysis should eliminate all energy sources within the cell, thus preventing active transport of *O*-methyl glucose. This supposition was tested by incubating cells in the presence of 7.5 mM azide and 2.5 mM

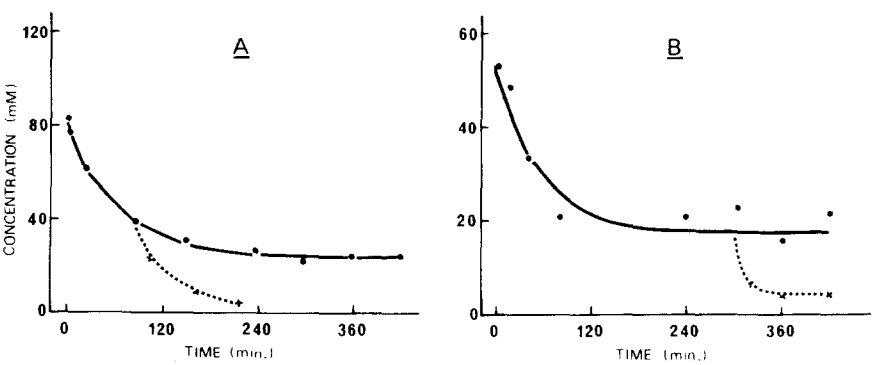

Fig. 3. Time course of efflux from active sporidia. Cells were loaded for 90 min, then resuspended in a large volume of sugar-free solution (zero time on graphs). Solid line, untreated efflux; broken lines, efflux following addition of (A) 7.5 mM azide + 2.5 mM iodoacetic acid and (B) non-radioactive sugar.

TABLE I

KINETIC CONSTANTS FOR THE 3-*O*-METHYLGLUCOSIDE TRANSPORT SYSTEM IN U. MAYDIS

Errors are standard deviations.

State of cells	Maximum velocity constants (mmol/l cell water per min)		Affinity constants (mmol/l)	
	Uptake (V_+)	Efflux (V_-)	Outside (K_0)	Inside (K_1)
Normal	9.7 ± 0.5	1.1 ± 0.9	0.08 ± 0.01	>40
De-energized	0.66 ± 0.05	0.53 ± 0.04	13 ± 2	18 ± 2

iodoacetate for 30 min followed by the addition of various amounts of *O*-methyl glucose and measurement of its appearance within the cells. Again the internal concentration increased with time reaching a final steady value after about 80 min. However, the final concentration reached in this case was much lower than that obtained in unpoisoned cells, and in fact did not rise above the outside concentration. This indicates that poisoned cells are still able to transport the substrate but are unable to do so against a concentration gradient, so that $\alpha = 1$ for all concentrations tested.

Antimycin A. This compound, like azide, is a potent respiratory inhibitor in a variety of organisms. Its effect on *O*-methyl glucose transport was tested by incubating sporidia with 5 μg/ml of the compound, then measuring the uptake of *O*-methyl glucose from a solution of 2.5 mM. In this case active transport was not completely suppressed since the value of α was 2.4.

Phloretin and phlorizin. Both these compounds have been shown to inhibit sugar transport in a number of organisms. In human erythrocytes, phloretin is the more powerful inhibitor of glucose transport, while in kidney tubules, the reverse is true. In the present system, cells incubated with 0.05 mM phloretin showed stimulation of uptake (Fig. 4) whereas those in the presence of 0.1 mM phlorizin showed a high degree of inhibition for about 40 min followed by a period of normal uptake. Since phloretin is the aglucone of phlorizin the explanation of this peculiar behavior, at least in part, is probably that while phlorizin is an effective inhibitor, its glucose moiety is slowly removed by enzymic hydrolysis reducing it to phloretin, a much less effective compound. Similar measurements on de-energized sporidia showed no effect by either compound. Phloretin was tested at a concentration of $5 \cdot 10^{-5}$ M and phlorizin at the concentrations 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M.

Carboxin. U. maydis is sensitive to the antifungal compound carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) which inhibits its growth to the extent of 50% at 0.5 μM and 80% at 2 μM. When tested against *O*-methyl glucose uptake in active sporidia, 2 μM carboxin caused the apparent affinity constant K_0 to decrease to 58% of its inhibited value, and the maximum rate constant V_+ to decrease to 50% of its uninhibited value. The same concentration of carboxin had no effect on de-energized transport, however.

Effect of exchanging Na^+ for K^+

To determine whether ions play a specific role in the active uptake of

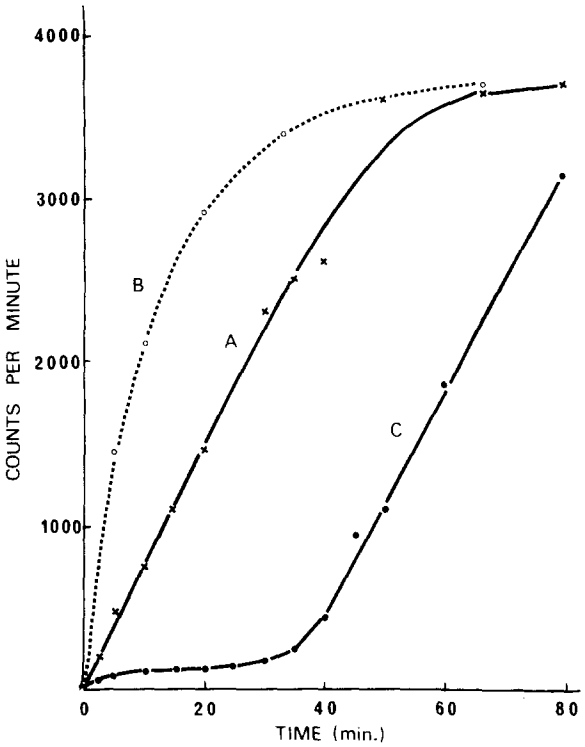

Fig. 4. Effect of phloretin and phlorizin on *O*-methyl glucose uptake by active sporidia. Normal sporidia were incubated with 0.1 mM *O*-methyl glucose, either alone (curve A) or in the presence of 0.05 mM phloretin (curve B) or 0.1 mM phlorizin (curve C).

O-methyl glucose, the initial rate of transport was measured, first in potassium phosphate buffer and then in sodium phosphate buffer of equivalent concentration and pH. The rates for these two tests were found to be identical, within experimental errors.

Proton movements during substrate uptake

West and Mitchell [10] were able to demonstrate a stoichiometric uptake of protons (or efflux of OH^-) in association with the uptake of β-galactosides by *Escherichia coli*. Their procedure consisted of measuring changes in the pH of an unbuffered solution during movement of substrate from it into de-energized cells. These experiments were repeated here using 30 mM KCl, as the suspending medium, contained in a stoppered thermostated vessel into which pH electrodes and a bubbler were inserted. Nitrogen gas was passed through the solution to free it of air and both uninhibited and de-energized cells were used. Recordings of the pH as a function of time were made and when this appeared to have reached a steady rate of change substrate was injected into the suspension. The system was calibrated by adding to the suspension an amount of hydrochloric acid equivalent in molar content to the *O*-methyl glucose taken up by the cells. A number of such experiments was performed but in no case did the pH change resulting from injection of the substrate exceed 10% of the change caused by addition of the acid.

Measurements of the kinetic constants of de-energized cells

The initial rates of uptake and efflux were measured as a function of substrate concentration as before, but this time using cells which had first been de-energized by a 30 min incubation with 7.5 mM azide and 2.5 mM iodoacetate. The kinetic constants derived from these data are listed in Table I.

Measurement of the kinetic constants for exchange in de-energized cells

The rate of efflux of radioactive *O*-methyl glucose from preloaded de-energized cells into buffer containing non-radioactive substrate at the same concentration as that in which the cells were loaded was measured at a number of loading concentrations. This data led to an estimate of 2.2 ± 0.3 mmol · l^{-1} · min^{-1} for the apparent maximum rate constant for exchange (V_e) and 70 ± 20 mM for the apparent affinity constant (K_e).

Discussion

The mechanism of the transport system

Following the rationale of Winkler and Wilson [6], we would consider the transport of *O*-methyl glucose in *U. maydis* to be basically a facilitated mechanism which is acted upon by metabolism in such a way as to render it active. If this is so, then, by studying the kinetics of the system in both its active and passive states one possibly could deduce at what point energy interacts with the carrier system. To see how this might be done let us examine the generalized carrier mechanism [11] as depicted schematically in Fig. 5, along with the transport rate equation derived from it. Here we see that eight separate steps are involved in transport, each with its own rate constant.

From the rate equation it is obvious that during uptake when the internal concentration reaches its steady value (as in Fig. 1) $d[S_1]/dt = 0$, and $A[S_0]$

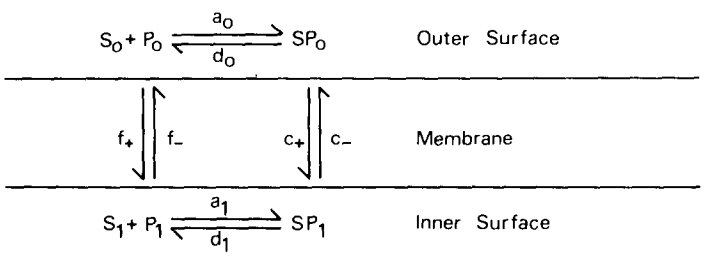

Fig. 5. Generalized carrier mechanism [11]. Substrate S combines with carrier P to form complex SP at both the outer surface (subscript '0') and inner surface (subscript '1') of the membrane. The rate constants are: a_0 and a_1 for complex association, d_0 and d_1 complex dissociation, f_+ and f_- for free carrier transfer and c_+ and c_- for compared carrier transfer. This mechanism leads to the transport rate equation.

$$\frac{d[S_1]}{dt} = \frac{A[S_0] - B[S_1]}{D + E[S_0] + \{F + G[S_0]\}[S_1]}$$

where $[S_0]$ is the outside substrate concentration, $[S_1]$ inside substrate concentration, T total mol carrier per 1 cell water, A is $a_0c_+d_1f_-T$, B is $a_1c_-d_0f_+T$, D is $(c_-d_0 + c_+d_1 + d_0d_1)(f_- + f_+)$, E is $a_0[d_1(c_+ + f_-) + f_-(c_- + c_+)]$, F is $a_1[d_0(c_- + f_+) + f_+(c_- + c_+)]$ and G is $a_0a_1(c_- + c_+)$.

must equal $B[S_1]$. This leads to an expression for the accumulation coefficient,

$$\alpha = \frac{[S_1]}{[S_0]} = \frac{A}{B} = \frac{a_0 c_+ d_1 f_-}{a_1 c_- d_0 f_+}$$

Thus, the degree to which a system is active, as expressed by the magnitude of α, is determined by the ratio of the product of the rate constants of all those processes acting in a clockwise direction in Fig. 5, to that of the reverse processes. From this it follows that the effect of metabolism on a system which is facilitated when de-energized, could be either to increase the rate constants of one or more of the clockwise steps and/or decrease one or more of the constants of the counter-clockwise processes.

Winkler and Wilson measured the initial rates of uptake and loss of galactosides by *E. coli* as a function of the external and internal substrate concentration, respectively, and plotted the results in accordance with Michaelis-Menten kinetics to derive the apparent affinity and maximum rate constants for the two processes. This can be shown to be valid in terms of the generalized mechanism as follows.

Uptake experiments. Since the measurements of $[S_1]$ are made soon after the addition of substrate to the outside solution, the internal concentration can be considered to be negligible, reducing the rate equation to

$$\frac{d[S_1]}{dt} = \frac{(A/E) \cdot [S_0]}{(D/E) + [S_0]}.$$

By analogy with the Michaelis-Menten equation the apparent maximum rate and affinity constants for uptake will be

$$V_+ = \frac{A}{E} = \frac{c_+ d_1 f_- T}{d_1(c_+ + f_-) + f_-(c_- + c_+)}$$

and

$$K_0 = \frac{D}{E} = \frac{(c_- d_0 + c_+ d_1 + d_0 d_1)(f_- + f_+)}{a_0[d_1(c_+ + f_-) + f_-(c_- + c_+)]}$$

Efflux experiments. Although there are a number of possible explanations for biphasic efflux of the type shown in Fig. 3, perhaps the most likely is that of Spoerl [12], who provided good evidence that a similar phenomenon occurring in yeast resulted from vacuolar sequestering of the substrate. Accepting this explanation in the present study allows us to ignore the slower phase, which represents substrate retained by the vacuoles, while deriving our parameters from the faster components arising from transport of substrate out of the cell.

During efflux, the external concentration $[S_0] = 0$ and since the rate measurements are made over a relatively short period, $[S_1]$ is approximately constant. Under these conditions the rate of efflux is

$$\frac{-d[S_1]}{dt} = \frac{(B/F) \cdot [S_1]}{(D/F) + [S_1]}$$

from which, as before, the apparent rate constants are seen to be

$$V_- = \frac{B}{F} = \frac{c_- d_0 f_+ T}{d_0(c_- + f_+) + f_+(c_- + c_+)}$$

and

$$K_1 = \frac{D}{F} = \frac{(c_- d_0 + c_+ d_1 + d_0 d_1)(f_- + f_+)}{a_1[d_0(c_- + f_+) + f_+(c_- + c_+)]}$$

According to Winkler and Wilson [6] the only effect of metabolism in their system was to increase K_1 or, in other words, to decrease the affinity of the carrier for the substrate at the inner membrane surface. An examination of the above expressions for the apparent constants reveals that the simplest method of accomplishing this is to reduce the rate of formation of the complex at the inner surface by diminishing a_1 which, since it does not appear in any other K or V expression, will affect K_1 only. At the same time the value of α, which must be one for a de-energized system, will also be increased, as required for a change to active transport.

With *U. maydis*, *O*-methyl glucose is taken up by uninhibited sporidia to produce internal concentrations which may exceed 2000 times that outside and since the accumulated compound is essentially unchanged chemically, there seems little doubt that the mechanism causing this uptake is a true active transport system. Furthermore, de-energized transport must be facilitated (or carrier mediated) since it obeys Michaelis-Menten kinetics and is apparently symmetrical, since as the data in Table I indicates, $V_+ = V_-$ and $K_0 = K_1$.

The effect of metabolism on this system is much more complex than on the *E. coli* system, as we can see from Table I, where all apparent constants are affected. The effect on the efflux rate V_- is relatively slight, however and this provides an important clue to the mechanism. This fact allows us to make the simplifying assumption that if V_- is unchanged then none of the processes contributing to V_- will be changed. (We cannot totally ignore the possibility that a number of changes may occur in such fashion as to compensate each other, but this explanation seems much less likely). This allows us to state that only those processes whose constants do not appear in the full expression of V_- qualify as sites of energy coupling, which narrows the possibilities to those steps whose constants are a_0, a_1, d_1, and f_-. Looking next at the outside affinity constant (K_0) which is reduced by a factor of 160 (which is much greater than for either of the other constants) we note that the easiest method of accomplishing this is to increase a_0 by this same factor. This will have no effect on the other apparent constants but will increase α by two orders of magnitude and since its final value must exceed 1000, one of the remaining constants (a_1, d_1 or f_-) must also be increased 10-fold. At the same time we must look for an increase in both V_+ and K_1, and if we wish to increase these constants together with α through an increase in only one rate constant, the association constant a_1 is ruled out since it does not appear in the expression for V_+. We are left to decide then whether the remaining effect is confined to f_- alone, to d_1 alone, or divided between the two.

In discussing the generalized carrier mechanism, Hoare [13] has made the important observation that if the apparent rate constant for exchange transport

(V_e) exceeds that for net transport, carrier reorientation must be the slow step. In terms of the mechanism in Fig. 5 this means that if V_e/V_- (or V_e/V_+) > 1 then a_0, a_1, d_0 and d_1 must exceed in magnitude the constants f_+, f_-, c_+ and c_-. That this condition applies in the present case is indicated by the fact that in de-energized cells, V_e/V_- is approximately 4, and we may therefore simplify the expressions for V_+ and K_1, of de-energizied cells only, to the forms.

$$V_+ = \frac{c_+ f_- T}{(c_+ + f_-)} \quad \text{and} \quad K_1 = \frac{d_1(f_- + f_+)}{a_1(c_- + f_+)}.$$

Since d_1 does not now appear in the expression for V_+, our conditions are not satisfied by increasing it alone. They are satisfied however by an increase in f_- providing c_+ initially exceeds f_- (which is to say that in the de-energized state the complexed carrier is transferred more rapidly than the free carrier) and f_+ does not greatly exceed f_-.

To summarize our conclusions then, we can say that the mechanism in Fig. 5 conforms adequately with the data in Table I upon introduction of the following assumptions: 1, In the de-energized state the rate of transfer of the free carrier between the two surfaces is low relative to that for the complex; 2, metabolism increases the rate of association of the substrate with the carrier at the outer membrane surface by a factor in excess of 100; 3, the rate of transfer of the free carrier from the inner to the outer surface is increased 10-fold by metabolism.

These changes would produce a 1000-fold increase in α, a 100-fold increase in K_0, a 10-fold increase in V_+ and K_1 and no significant change in V_-, in harmony with the data in Table I.

Next let us consider the possible means by which metabolic energy is linked to the transport system rendering it active. Since the accumulated substrate is unaltered chemically this cannot be by group translocation. Nor can it result from sodium or potassium cotransport since the flux is indifferent to these two ions, and finally, since substrate movement did not give rise to pH changes, the driving force cannot be proton motive, and must, for the present, remain unspecified.

The effect of inhibitors

Carboxin. This compound has been shown to inhibit the metabolic activity of the cell by interfering with the succinic dehydrogenase system [14,15]. If this were its only effect on *U. maydis* however, one would predict that it would result in the same changes as those produced by the metabolic inhibitors used to de-energize the sporidia, that is, to increase K_0 and decrease V_+. In fact, although the value of V_+ moves in the expected direction, that for K_0 is opposite. The effect of this compound therefore cannot be simply to reduce the availability of metabolic energy alone, nor can it be the result of the a direct attack on the carrier in one of its passive forms since the de-energized cells exhibit no inhibition. We are thus left with the conclusion that it interacts with the carrier in its active form only, in such a way as to increase the apparent affinity for the substrate at the outer surface. It is perhaps of interest to note, however, that since the two systems attacked by carboxin, namely succinic dehydrogenase and the sugar carrier, are membrane bound, one is

tempted to conclude that this compound produces a general effect on membranes resulting in distortion of the membrane structure and of the more sensitive membrane-bound systems.

Phloretin and phlorizin. The fact that neither of these compounds shows any effect on the transport in de-energized cells indicates that neither directly attacks the carrier, at least in its passive forms. In the case of phlorizin then, we are left with two other possibilities, either it attacks the activated form of the carrier rendering it incapable of further conformational changes, or it inhibits energy coupling. In either case the inhibition is obviously reversible since enzymatic degradation of phlorizin appears to restore active transport fully.

It is interesting to note from Fig. 4 that phloretin enhances the rate of active uptake of *O*-methyl glucose. An explanation of this intriguing phenomenon is certainly not obvious and is likely to demand much further work before it is found.

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