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# COMBINED EFFECTS OF MALTOSE AND DEOXYGLUCOSE ON FLUORODINITROBENZENE INACTIVATION OF SUGAR TRANSPORT IN ERYTHROCYTES*

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## SUMMARY

Deoxyglucose greatly accelerates the rate of reaction between fluorodinitrobenzene and the sugar transfer system of human erythrocytes, whereas maltose reduces the rate. Both sugars act by forming a 1:1 complex with carrier. It is shown that maltose, which does not enter the cells, competes with deoxyglucose both outside the cells and inside. The fluorodinitrobenzene concentration within the cells is identical to that in the suspending medium. These observations are inconsistent with a transport model involving a pair of carrier units in a single pore, each restricted to one surface of the membrane, where each unit undergoes conformational changes independently of the other. They are consistent with three models: (i) a single carrier alternately exposed at either membrane surface, though other evidence militates against this; (ii) a symmetrical pair of carrier units undergoing linked conformational changes following sugar binding; (iii) a highly unsymmetrical mechanism in which only the external member of a carrier pair is inactivated by fluorodinitrobenzene or activated towards fluorodinitrobenzene by bound sugar.

## INTRODUCTION

Sugar transfer through erythrocyte cell membranes appears to be especially suitable for study leading to a basic understanding of biological transport mechanisms, for reasons of simplicity: the transported substrates are non-ionic, eliminating need for counter-ion movement or ion exchange, and the process is independent of energy metabolism and should therefore entail minimal transport machinery. The kinetics of this system have been investigated in great detail, but surprisingly, considerable doubt has been cast on all transport models so far tested[1-5]. The actual mechanism may be rather complex, and this hampers selection of a particular model; but any hypothesis quantitatively explaining the many observations on hand will have a better chance than otherwise of being correct in essentials. In this light experiments eliminating particular classes of mechanism can contribute to an eventual solution

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Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.
[*] Contribution No. 457.

of the problem. The study presented here eliminates one such class and suggests directions that might be profitably explored.

1-Fluoro-2,4-dinitrobenzene (FDNB) reacts with the sugar transfer system in erythrocyte membranes, inactivating it irreversibly[6,7]. Inhibition develops much more rapidly when well transported sugars such as 2-deoxyglucose and glucose are bound to the carrier, but lags in the presence of certain reversible inhibitors of transport, notably maltose, which does not penetrate the cell[8]. If, as postulated earlier, both effects depend on sugar binding at the transport site, an opportunity is presented to detect possible trans-membrane effects of bound maltose and transported sugars—in particular to see if maltose, restricted to the medium outside the cells, competes for carrier with sugar inside, with respect to FDNB inhibition. In transport studies maltose does compete with sugars in the external solution[8]. Such a study seemed called for because apparent sugar binding constants are substantially altered, depending on the sugar concentration at the opposite membrane surface, but the mechanism by which this occurs is not known[2]'

To assist interpretation of experiments, transport mechanisms may be divided into two types: substrate at one membrane face may effectively interact either with all carrier in the membrane, (Mechanism I) or only with carrier permanently exposed at the same surface (Mechanism II). The first mechanism includes the simplest of transport models, in which each carrier element is alternately exposed at either side of the membrane (Mechanism Ia). It also includes models in which the two members of a pair of conformationally-linked carrier elements reside in opposing surfaces of the membrane (Mechanism Ib, see Fig. 1). By this it is meant that complex formation between substrate and one member of a carrier pair induces a conformational change in both members. Evidence that conformational changes are induced by substrate binding was presented earlier[8,9]. The second type of mechanism (Mechanism II) includes those with pairs of non-interacting carrier elements, with the two members on opposite sides of the membrane (Fig. 1). Here the conformation assumed by one member of the pair is not influenced by the conformation of the other. Maltose should protect all the carrier from FDNB in the first case, but only half of it, that in the external membrane surface, in the second.

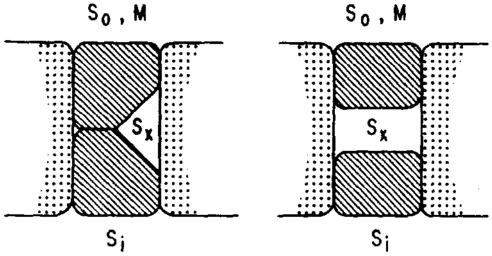

Fig. 1. Transport mechanisms involving a pair of carriers combined in a single pore, each member confined to one surface of the membrane. For transport both members of the pair must function. In Mechanism Ib (left) the members are structurally linked: substrate binding to either induces a conformational change in both. In Mechanism II (right) the members are independent, *i.e.* conformational changes are restricted to the unit forming a complex. In both cases a substrate pool may exist within the membrane ($S_x$). Substrate binding sites, which could be located on the side of the carrier unit adjoining this internal pool as well as on the membrane surfaces, are not shown. Both maltose and transported substrate are present in the external solution (M and $S_0$, respectively) but only substrate penetrates to the intra-membrane pool ($S_x$) and the cell interior ($S_i$)

In the first type of mechanism the total rate of inactivation, $-\mathrm{d}[\mathrm{C_t}]/\mathrm{d}t$, may be written in terms of three carrier species with their corresponding rate constants; free carrier, complex with maltose, and complex with transported sugar:

$$-\frac{\mathrm{d}[\mathrm{C_t}]}{\mathrm{d}t} = k_1[\mathrm{C}] + k_2[\mathrm{CM}] + k_3[\mathrm{CS}] \tag{1}$$

The reaction constants $k_1$, $k_2$ and $k_3$ are first order and include terms for the concentration of FDNB, which is held constant. The total concentration of carrier, $\mathrm{C_t}$, is given by the sum of these species:

$$[\mathrm{C_t}] = [\mathrm{C}] + [\mathrm{CM}] + [\mathrm{CS}] = [\mathrm{C}](1 + [\mathrm{S}]/K + [\mathrm{M}]/K_\mathrm{M}) \tag{2}$$

where $K$ and $K_\mathrm{M}$ are dissociation constants for transported sugar, S, and maltose, M. Following a previous derivation[8] for the measured inactivation rate constant $k$ (Eqn 7 below) it can be shown that

$$k = \frac{k_1 + k_2[\mathrm{M}]/K_\mathrm{M} + k_3[\mathrm{S}]/K}{1 + [\mathrm{M}]/K_\mathrm{M} + [\mathrm{S}]/K} \tag{3}$$

Hence in either the presence or absence of S, saturating M reduces the inactivation rate constant to $k_2$.

In this derivation it has been assumed that carrier molecules at the two membrane surfaces are identical with respect to FDNB inhibition and substrate binding, but a more general treatment (Appendix) shows this does not alter the form of the equations. It was also assumed that the external and internal concentrations of transported sugar are equal, which is fully justified because cells were equilibrated with sugar prior to treatment with FDNB*.

In the second mechanism, involving non-interacting carrier pairs, the carrier concentrations in the two membrane faces are equal:

$$[\mathrm{C_t}] = [\mathrm{C_i}] + [\mathrm{C_iS}] = [\mathrm{C_o}] + [\mathrm{C_oM}] + [\mathrm{C_oS}] \tag{4}$$

(S is available on both sides of the membrane but maltose, M, only outside). As before, the total inactivation rate is the sum of rates for all species:

$$-\frac{\mathrm{d}[\mathrm{C_t}]}{\mathrm{d}t} = k_\mathrm{i}[\mathrm{C_o}] + k_2[\mathrm{C_oM}] + k_3[\mathrm{C_oS}] + k'_1[\mathrm{C_i}] + k'_3[\mathrm{C_iS}] \tag{5}$$

and

$$k = \frac{k_1 + k_2[\mathrm{M}]/K_\mathrm{M} + k_3[\mathrm{S}]/K_\mathrm{o}}{1 + [\mathrm{M}]/K_\mathrm{M} + [\mathrm{S}]/K_\mathrm{o}} + \frac{k'_1 + k'_3[\mathrm{S}]/K_\mathrm{i}}{1 + [\mathrm{S}]/K_\mathrm{i}} \tag{6}$$

where primed $k$ values are for inactivation rates on the inner membrane surface, and unprimed on the outer; $K_\mathrm{i}$ and $K_0$ are substrate dissociation constants for the inner and outer sides of the membrane.

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* More complete derivations for Mechanism I, allowing for different carrier properties at the two membrane faces and for the possible existence of an intra-membrane sugar pool in Mechanism Ib, is given in the Appendix. It is shown that the form of the resulting equations is identical to Eqn 3, except in one special case: when two sugar molecules add to the carrier pair simultaneously, second order behavior results.

The two models are readily distinguished except in two special cases, when Eqn 6 reduces to the form of Eqn 3: (i) FDNB inactivates only carrier in the outer membrane surface ($k'_1 \simeq k'_3 \simeq 0$); (ii) sugar accelerates inactivation of carrier in the outer surface only ($k'_3 = k'_1$).

## EXPERIMENTAL METHODS

Measurement of sugar exit rates from human red blood cells using a light-scattering apparatus was described before[8]. For treatment with FDNB 0.2 ml cells were suspended in 3.6 ml of a solution containing sugar in 0.9 % NaCl and 5 mM sodium phosphate buffer, pH 7.5, together with 0.2 ml of ethanolic FDNB solution, giving a final concentration of 2 mM FDNB. The final pH was 7.0 and the temperature was maintained at 25 °C. Reaction was stopped by cooling and dilution, and the sedimented cells were equilibrated with a solution containing 125 mM glucose. Rate assays, done in duplicate or triplicate, agreed to within 5 %. The inactivation rate constant was calculated from a first order relationship:

$$2.3 \log (\tau/\tau_0) = kt \tag{7}$$

where $\tau_0$ and $\tau$ are sugar exit times for untreated cells and those suspended in 2.0 mM FDNB for $t$ min, respectively.

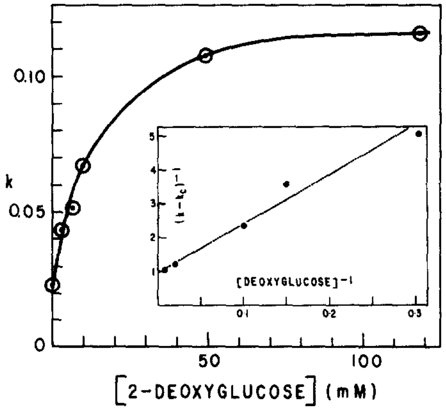
## EXPERIMENTAL RESULTS

The two mechanisms described above may be distinguished if inactivation rate constants are measured at increasing maltose concentrations, in the presence of a transported sugar (S) at its half-saturating concentration. In the first mechanism, but not the second, maltose should reduce the inactivation rate below one half, since it competes with both internal and external sugar. A half saturating concentration is chosen because here the sugar can substantially increase $k$ but is easily displaced by maltose at concentrations above its half-saturating value. For differences to be large the preferred sugar is 2-deoxy-D-glucose, which accelerates FDNB reaction more than any other sugar tested[8].

The dependence of $k$ on deoxyglucose concentration is shown in Fig. 2, from which a half saturation constant of roughly 10 mM is found. In these experiments 0.2 ml of packed red blood cells were suspended in a total volume of 4 ml, as described under Methods. Incubation with 50 mM sugar was for 15 min, and with lower concentrations, 30 min. At the highest concentration, 118 mM, 0.5-ml samples of the suspension were withdrawn at short intervals and deoxyglucose exit rates measured directly[8]. The inactivation rate constant at this highest concentration was $11.7 \cdot 10^{-2}$ min$^{-1}$, based on 8 measurements in a 10-min period, with 3 different incubation mixtures.

The inset in Fig. 2 shows a reciprocal plot of the same data, $(k-k_c)^{-1}$ *vs* [deoxyglucose]$^{-1}$, where $k$ and $k_c$ are FDNB inactivation rate constants in the presence and absence of sugar. The approximate linearity indicates 1:1 complex formation between carrier and deoxyglucose[8].

Experiments on maltose were carried out in a similar manner, except that the incubation period was always 30 min. The calculated inactivation constant $k$ fell continuously as the maltose concentration rose (Fig. 3, lower curve) and appeared to approach a minimum value about 56 % of that in the absence of maltose. The latter is $2.3 \cdot 10^{-2}$ min$^{-1}$. The half saturating maltose concentration, taken as that giving half-maximal protection (*i.e.* 22 %), is then about 75 mM.



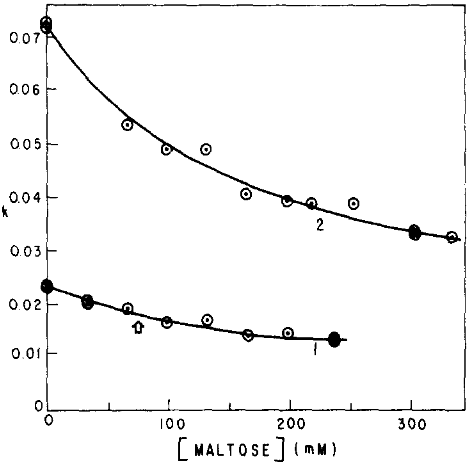


Fig. 2. Inactivation rate constant ($k$) as a function of the deoxyglucose concentration. The half saturation concentration is approximately 10 mM. Inset shows a reciprocal plot of the same data, with $k$ and $k_c$ defined as constants in the presence and absence of sugar, respectively.

Fig. 3. Inactivation rate constant ($k$) at increasing maltose concentrations, either in the absence of any other sugar (Curve 1) or together with 9.9 mM deoxyglucose pre-equilibrated with the cells (Curve 2). The arrow on Curve 1 points to the apparent half saturation constant for maltose (75 mM).

Another series of runs was carried out under conditions identical to those described above, except for the inclusion of 9.9 mM deoxyglucose equilibrated with the cells. Treatment with FDNB was in this case for 15 min, a shorter period being necessary because inactivation is rapid in the presence of deoxyglucose. Duplicate reaction mixtures were set up for zero maltose, and the calculated values of $k$ were $7.15 \cdot 10^{-2}$ and $7.26 \cdot 10^{-2}$ min$^{-1}$. Their average can be used, together with the maximum value of $k$ with deoxyglucose, $11.7 \cdot 10^{-2}$ min$^{-1}$, to improve the estimate of the half saturation constant. The fraction of carrier in the form of complex with deoxyglucose is given by $[S]/K(1+[S]/K)$, which equals the increase in $k$ with 9.9 mM sugar divided by that with a saturating concentration: $(7.2-2.3)/(11.7-2.3) = 0.521$. Hence $[S]/K = 1.1$, rather than exactly 1.

The observed inactivation rates may be compared with the predictions of the two mechanisms (Eqns 3 and 6). Eqn 3 may be rearranged to give the following relationship:

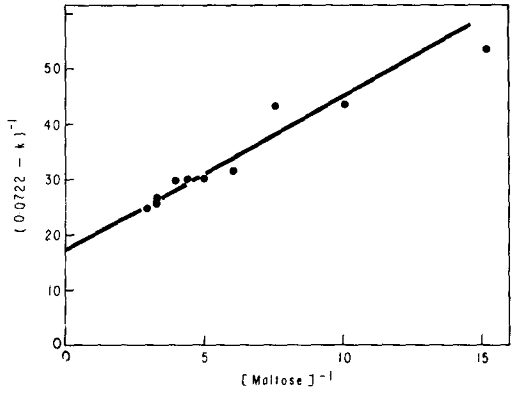
$$\left\{\frac{k_3 + k_1K/[S]}{1 + K/[S]} - k\right\}^{-1} = \left\{\frac{k_3 + k_1K/[S]}{1 + K/[S]} - k_2\right\}^{-1} \left\{1 + \frac{K_M(1 + [S]/K)}{[M]}\right\} \tag{8}$$

Since $(k_3 + k_1K/[S])/(1 + K/[S])$ can be calculated from known constants, the term on the left may be plotted against $1/[M]$. From the straight line drawn through the points (Fig. 4) $k_2$ and $K_M$ were found to be $1.1 \cdot 10^{-2}$ min$^{-1}$ and 80 mM respectively, in excellent agreement with the limiting value of $k$ at high maltose concentrations in the absence of deoxyglucose $(1.3 \cdot 10^{-2})$ and with the maltose concentration giving an approximately half maximal effect, 75 mM (Fig. 3). In a symmetrical type II mechanism (Eqn 6), $k_{1\,\mathrm{app}} = 2k_1$ and $k_{3\,\mathrm{app}} = 2k_3$, where $k_{1\,\mathrm{app}}$ and $k_{3\,\mathrm{app}}$ are experimental values. An equation similar to Eqn 8 may be derived:

$$\left\{\frac{k_{3\mathrm{app}} + k_{1\mathrm{app}}K/[\mathrm{S}]}{1 + K/[\mathrm{S}]} - k\right\}^{-1} = \left\{\frac{k_3 + k_1K/[\mathrm{S}]}{1 + K/[\mathrm{S}]} - k_2\right\}^{-1} \left\{1 + \frac{K_\mathrm{M}(1 + [\mathrm{S}]/K)}{[\mathrm{M}]}\right\} \qquad (9)$$

The minimum value of the intercept (taking $k_2$ as zero) is now calculated to be 28. This is obviously higher than any possible intercept based on the experimental points in Fig. 4, which rules out the mechanism.

A possible explanation of these findings is that FDNB fails to inhibit internally because its concentration within the cells remains low. Either it may penetrate slowly or be rapidly lost inside the cells as a result of chemical reaction or hydrolysis catalyzed by carbonic anhydrase[10]. This possibility was tested by observing the rates of FDNB reaction with the cell constituents, with either intact or lysed cells. Reaction with proteins *etc.* may be followed from the spectral absorption of the product. Cells (0.2 ml) were suspended as usual in a total volume of 4 ml with salt-buffer solution containing



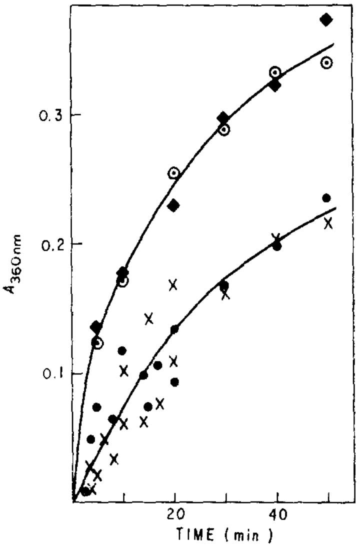


Fig. 4. Plot according to Eqn 8 for inactivation by FDNB in the presence of deoxyglucose with varying maltose concentrations, in molar units (data cf Curve 2, Fig. 3). The expression in the ordinate is

$$\frac{k_3 + k_1K/[\mathrm{S}]}{1 + K/[\mathrm{S}]} - k$$

where $k_1 = 2.30 \cdot 10^{-2}$ min$^{-1}$, $k_3 = 11.7 \cdot 10^{-2}$ min$^{-1}$, and $[\mathrm{S}]/K = 1.10$. The first term, which is equal to the inactivation rate at this deoxyglucose concentration in the absence of maltose, is calculated to be 0.0722 min$^{-1}$, in agreement with the measured value shown in Fig. 3.

Fig. 5. Course of reaction of FDNB with intact or lysed erythrocytes, as measured by optical density at 360 nm (see text). Curve 1, 2 mM FDNB: ×, intact cells; ●, lysed cells. Curve 2, 4 mM FDNB: ⊙, intact cells; ◆, lysed cells.

2 or 4 mM FDNB and 5 % ethanol. Aliquots (0.1 ml) were withdrawn at intervals, added to 5 ml water, and read at 360 nm against a control taken immediately after addition of FDNB. The absorbance gradually rose, reflecting the spectral shift produced by reaction with amino groups (Fig. 5). In a companion experiment lysed cells were treated in exactly the same manner. The same volume of cells (0.2 ml) was suspended first in 1.8 ml distilled water, and several minutes later, when lysis was virtually complete, 1.8 ml of a salt–buffer solution was added to give the same final concentrations of all constituents as before. The difference was that the cell contents were now dispersed throughout the solution, and no longer separated from it by the cell membrane. The rate of FDNB reaction was essentially the same in the two experiments (Fig. 5). The effective internal concentration of FDNB must therefore have been identical to that in the external solution.

## DISCUSSION

The increased rate of inactivation in the presence of glucose was shown to be dependent upon 1:1 complex formation with carrier[8] and the deoxyglucose effect has the same characteristics (see inset, Fig. 2). The large reduction in rates of FDNB reaction in the presence of deoxyglucose and increasing maltose concentration, and the close conformity with a model involving competition between these sugars for a single site of attachment (Eqns 3 and 8), shows that protection by maltose against FDNB also depends on 1:1 complex formation. This strengthens an earlier conclusion that transported sugars and certain non-transported inhibitors such as maltose stabilize different carrier conformations though all become bound at the same site[8]. The comparative smallness of the effect of maltose in the absence of deoxyglucose (Fig. 3, lower curve) makes it difficult to test the hypothesis with these data alone*.

The symmetrical form of Mechanism II (an unlinked carrier pair as in Fig. 1) is inconsistent with the observations, but Mechanism I fits the data very well. The latter includes the classical model involving a carrier that shuttles back and forth through the membrane. However kinetic experiments, in which measured sugar binding constants were shown to differ widely depending on sugar concentrations at the trans membrane surface, have demonstrated the inadequacy of this mechanism in its simplest form[1,3] as well as in a form involving different conformational states at either membrane surface[2]. It may be noted that these same experiments do not necessarily rule out a model as broadly defined as Mechanism II, because sugar in the pool within the membrane ($S_x$ in Fig. 1) can influence either member, and the size of the pool is directly related to sugar concentrations at both membrane surfaces. By contrast maltose should not enter the internal pool, because it is known not to undergo transport[11]. Transmission of a maltose effect through the membrane would therefore depend on a structural linkage between carrier elements. The present observations together with the kinetic experiments noted above suggest then, that if a symmetrical mechanism operates it may involve pairs of linked carriers confined to opposing membrane faces (Mechanism Ib, Fig. 1). The model proposed by Lieb and Stein[4] fits into this category.

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* It may be noted that protection by maltose cannot be explained by osmotic effects of a non-penetrating sugar, since other non-penetrating sugars such as sucrose gave no protection[8]. This is because sucrose is not bound by the carrier, as shown by its failure to inhibit transport.

An alternative explanation entails extreme asymmetry in the carrier, where only the external member of a pair is inactivated by FDNB ($k'_1 = k'_3 \simeq 0$) or only the external member is activated by substrate towards FDNB ($k'_1 = k'_3$). As seen above the FDNB concentrations inside the cells and outside are equal, so that a FDNB gradient could not account for such asymmetry. Certain other forms of non-symmetry are unlikely: if only the internal and external sugar binding constants, $K_i$ and $K_0$, differed significantly, the saturation curve for sugar as reflected in rates of FDNB inhibition would deviate from the simple form shown with deoxyglucose (Fig. 2) and glucose[8], as it would if sugar protected internal carrier from FDNB ($k'_3 < k'_1$). On the other hand if only internal carrier reacted with FDNB or was activated by bound substrate, protection by maltose and competition between it and deoxyglucose would require that the effect of maltose binding be transmitted through the membrane. An explanation involving non-symmetry and excluding Mechanism I appears then to be somewhat improbable because of its special requirements, though it cannot be ruled out.

## APPENDIX

Eqns 1–3 represent a greatly simplified version of Mechanism I. They are nevertheless correct in general form, which justifies their use in the analysis. General treatments of three forms of Mechanism I are now given.

### (i) *The simple carrier, or shuttle model*

$$\begin{tikzcd}
 & C_i \arrow[r, shift left, "K_i"] \arrow[d, shift left, "k_c"] & CS_i \arrow[l, shift left] \arrow[d, shift left, "k_s"] \\
CM_o \arrow[r, shift left] & C_o \arrow[l, shift left, "K_M"] \arrow[u, shift left, "k_{-c}"] \arrow[r, shift left] & CS_o \arrow[l, shift left, "K_o"] \arrow[u, shift left, "k_{-s}"]
\end{tikzcd} \qquad (10)$$

In this transport scheme the subscripts i and o designate the interior and exterior membrane surface respectively; $K_i$ is the dissociation constant for carrier and sugar at the inner face and $K_0$ at the outer, and $K_M$ that for maltose at the outer surface. The rate constant for carrier movement from the inner to the outer surface is $k_c$, and for the reverse direction is $k_{-c}$; similarly for movement of the sugar complex outward and inward the constants are $k_s$ and $k_{-s}$.

The net rate of sugar transport across the cell membrane is given by

$$\frac{-d[S_i]}{dt} = k_s[CS_i] - k_{-s}[CS_o] = 0 \qquad (11)$$

In these experiments cells were always pre-equilibrated with the transported sugar, which is to say that the net transport rate was zero, and also that the internal and external sugar concentrations were equal. A second limitation on this system is that total transport of carrier across the membrane in one direction must equal that in the reverse:

$$k_c[C_i] + k_s[CS_i] = k_{-c}[C_o] + k_{-s}[CS_o] \qquad (12)$$

Combining Eqns 10 and 11 gives

$$k_c[C_i] = k_{-c}[C_o],$$

and

$$[C_i]/[C_o] = \frac{k_{-c}}{k_c} = K_c \qquad (13)$$

Assuming equilibrium between the sugars and carrier an expression may now be written for the total carrier concentration:

$$\begin{aligned}[C_t] &= [C_i] + [CS_i] + [C_o] + [CS_o] + [CM_o] \\ &= [C_o]\left\{1 + K_c + \frac{[M]}{K_M} + \left(\frac{K_c}{K_i} + \frac{1}{K_o}\right)[S]\right\} \qquad (14)\end{aligned}$$

The inactivation rate in the presence of FDNB is as follows, by analogy with Eqns 1 and 5:

$$-\frac{d[C_t]}{dt} = k_1[C_o] + k'_1[C_i] + k_2[CM] + k_3[CS_o] + k'_3[CS_i] \qquad (15)$$

The experimental inactivation rate constant is therefore

$$k = \frac{k_1 + K_c k'_1 + k_2[M]/K_M + [S]\left(\dfrac{k_3}{K_o} + \dfrac{k'_3 K_c}{K_i}\right)}{1 + K_c + [M]/K_M + [S]\left(\dfrac{1}{K_o} + \dfrac{K_c}{K_i}\right)} \qquad (16)$$

Eqns 16 and 3 are of identical form, so that analysis based on Eqn 3 will also apply to Eqn 16. Only the physical significance of the constants is altered. It will be noted then, that agreement of experiments with Eqn 3 is an agreement with all forms of the shuttle mechanism, whether symmetrical or not and whether FDNB reacts equally well with carrier at the external and internal faces of the membrane, or unequally.

(*ii*) *The conformationally-linked dimer model* (*Fig.* 1)

$$\begin{tikzcd}
 & CM_o \arrow[d, shift left, "K_M"] & \\
SC_i \arrow[r, shift left, "K_i"] \arrow[rr, bend right=40, "k_s"'] & C \arrow[l, shift left] \arrow[u, shift left] \arrow[r, shift left, "K_o"] & CS_o \arrow[l, shift left] \arrow[ll, bend right=60, "k_{-s}"']
\end{tikzcd} \qquad (17)$$

This transport scheme allows for binding of a single sugar molecule to the carrier pair, but not more than one. That is, binding at one surface of the membrane is assumed to induce a conformational change that prevents binding at the site on the opposite surface. As before net flux of sugar is zero:

$$\frac{-\mathrm{d}[\mathrm{S_i}]}{\mathrm{d}t} = k_\mathrm{s}\,[\mathrm{SC_i}] - k_{-\mathrm{s}}[\mathrm{CS_o}] = 0 \tag{18}$$

It is not specified whether an intermediate pool exists within the membrane, but if it does the substrate concentration there, or more properly activity, must equal that outside, under equilibrium conditions. If the binding constants for sugar within this pool are $K'_\mathrm{i}$ and $K'_0$, then

$$[\mathrm{SC_i}] = [\mathrm{C}]\,[\mathrm{S}]/K_\mathrm{i} + [\mathrm{C}]\,[\mathrm{S}]/K'_\mathrm{i} = [\mathrm{C}]\,[\mathrm{S}]/K''_\mathrm{i} \tag{19}$$

where $1/K_\mathrm{i} + 1/K'_\mathrm{i} = 1/K''_1$. Similarly $[\mathrm{CS_0}] = [\mathrm{C}][\mathrm{S}]/K''_0$. It is clear that the existence of such a pool does not alter the generality of the above transport scheme. The equation for the inactivation rate constant is now found to be

$$k = \frac{k_1 + \dfrac{k_2[\mathrm{M}]}{K_\mathrm{M}} + [\mathrm{S}]\left(\dfrac{k_3}{K''_\mathrm{o}} + \dfrac{k'_3}{K''_\mathrm{i}}\right)}{1 + \dfrac{\mathrm{M}}{K_\mathrm{M}} + [\mathrm{S}]\left(\dfrac{1}{K''_\mathrm{o}} + \dfrac{1}{K''_\mathrm{i}}\right)} \tag{20}$$

Again the form of this equation is identical to that of Eqn 3.

*(iii) A conformationally-linked dimer model allowing two sugar molecules to be bound simultaneously*

$$\begin{tikzcd}
\mathrm{CM_o} \arrow[r, shift left, "K_\mathrm{M}"] \arrow[d, shift left] & \mathrm{C} \arrow[l, shift left] \arrow[r, shift left, "K^\mathrm{o}"] \arrow[d, shift left] & \mathrm{CS_o} \arrow[l, shift left] \arrow[d, shift left] \arrow[dl, shift left, "k_{-\mathrm{s}}"] \\
\mathrm{S_iCM_o} \arrow[u, shift left, "K''_\mathrm{i}"] \arrow[r, shift left] & \mathrm{S_iC} \arrow[l, shift left] \arrow[u, shift left, "K_\mathrm{i}"] \arrow[r, shift left] \arrow[ur, shift left, "k_\mathrm{s}"] & \mathrm{S_iCS_o} \arrow[l, shift left] \arrow[u, shift left, "K'_\mathrm{i}"']
\end{tikzcd} \tag{21}$$

Net flux is given by $k_\mathrm{s}[\mathrm{S_iC}] - k_\mathrm{s}^{-}[\mathrm{CS_0}] = 0$. The species with sugar bound at both-surfaces, $\mathrm{S_iCS_0}$, cannot produce any net transport, and that containing both transported sugar and the inhibitor maltose, $\mathrm{S_iCM_0}$, is inactive. The expression for $k$ now contains second order terms in sugar:

$$k = \frac{k_1 + \dfrac{[\mathrm{M}]}{K_\mathrm{M}}\left(k_2 + k'_2\dfrac{[\mathrm{S}]}{K''_\mathrm{i}}\right) + [\mathrm{S}]\left(\dfrac{k_3}{K_\mathrm{o}} + \dfrac{k'_3}{K_\mathrm{i}}\right) + \dfrac{k_4[\mathrm{S}]^2}{K_\mathrm{o}K'_\mathrm{i}}}{1 + \dfrac{[\mathrm{M}]}{K_\mathrm{M}}\left(1 + \dfrac{[\mathrm{S}]}{K''_\mathrm{i}}\right) + [\mathrm{S}]\left(\dfrac{1}{K_\mathrm{o}} + \dfrac{1}{K_\mathrm{i}}\right) + [\mathrm{S}]^2/K_\mathrm{o}K'_\mathrm{i}} \tag{22}$$

where $k_1$, $k'_1$, $k_2$, $k'_2$, $k_3$, $k'_3$, and $k_4$ are rate constants for FDNB reaction with individual carrier species, as before. This equation predicts a second order relation between the inactivation rate and the concentration of transported sugar, and also a change in the apparent half saturation constant for maltose in the presence of transported sugar. As neither effect is observed it is unlikely that this mechanism is correct.

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