portional to the maximum transport rate only if the same intermediate determined the rates of both processes, which is possible but not particularly likely in a reaction involving several intermediates. Furthermore transport is a cyclic process depending on movement across the membrane and back, in contrast to the inhibition reaction.

In view of the complex nature of the measured binding constants it is also not surprising that the half-saturation constant for glucose in promoting FDNB reaction is not identical with that found for transport inhibition. However the comparable magnitude of the constants supports the contention that both are related to binding at the carrier site.

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Inhibition of Sugar Transport in Erythrocytes by Fluorodinitrobenzene*

R. M. Krupka

ABSTRACT: Inactivation of sugar transport in erythrocytes by 1-fluoro-2,4-dinitrobenzene (FDNB), previously believed to be second order with respect to FDNB and carrier (fourth order overall), is now shown to be first order in both. Apparent second-order behavior for FDNB was due to a secondary, physical action of this compound, which accelerates the reaction with carrier. Apparent second-order behavior for carrier resulted from a gradual decline in FDNB concentration during treatment of the cells. The physical action of FDNB was shared by various agents, including urea, alcohols, and detergents. The evidence shows that urea, urethane, and alcohols add reversibly at two regions in the carrier—one within the sugar site, causing competitive transport inhibition, and another outside bringing about a conformational change similar to that induced by sugars. It is suggested that the second action involves exposure of a hydrophobic region of the carrier, allowing it to pass through a lipid barrier in the membrane.

Inactivation of sugar transport in erythrocytes by 1-fluoro-2,4-dinitrobenzene (FDNB)[1] was reported to be proportional to the square of the concentrations of both FDNB and functional carrier sites, being fourth order overall (Bowyer and Widdas, 1958; Stein, 1969). The relationship for FDNB was taken to mean that inhibition ensues only when pairs of chemical groups in the carriers have reacted with two FDNB molecules. The dependence on carrier concentration could not be explained simply by the interaction of pairs of carriers, however, and suggested that all the units in the system undergo cooperative changes in conformational state, as in the membrane model of Changea ux *et al.* (1967).

If these observations have been correctly interpreted they may provide a unique clue to the mechanism of biological transport. Their importance appears to justify a reexamination of the evidence, which has been undertaken here.

Methods

Determination of glucose exit rates from human red blood cells, as well as methods of determining rates of inactivation of transport by FDNB, were described previously (Krupka, 1971). The FDNB inactivation rate constant, k, was calculated from eq 1, where τ_0 and τ are sugar exit times for un-

$$2.3 \log (\tau/\tau_0) = kt \quad (1)$$

treated cells and cells suspended in 2.0 mM FDNB for t minutes, respectively. Exit times are expressed in units of minutes per millimole of sugar per liter of cell water (min mM^{-1}). The incubation medium consisted of 0.1 ml of red blood cells, 3.6 ml of glucose–salt–buffer solution (130 mM glucose in 0.9%

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[1] Abbreviation used is: FDNB, 1-fluoro-2,4-dinitrobenzene.

NaCl with 5 mM sodium phosphate buffer, pH 7.5), and 0.2 ml of a solution of FDNB in ethanol. The final pH was 7.0. All experiments were conducted at 25°.

Experimental Section

Relation of Inactivation Rates to FDNB Concentration. With cells incubated in 119 mM glucose and 5% ethanol the inactivation rate constants were 2.3×10^{-2}, 5.8×10^{-2}, and 21.4×10^{-2} min^{-1} with 1, 2, and 4 mM FDNB, respectively. From this it appears that the order may be fractional (between 1.5 and 2) rather than exactly 2. The present experiments were carried out in 5% ethanolic solutions rather than 1.9% as in the work of Bowyer and Widdas (1956), which could account for this difference in behavior.

Other observations suggested an alternative explanation for the near second-order behavior. Ethanol, included in the reaction mixture to increase the solubility of FDNB, accelerated inactivation. With 2 mM FDNB and 109 mM glucose the rate increased 53% when the ethanol concentration was raised from 2.5 to 7.5%, and another 100% from 7.5 to 12.5 ethanol. In similar experiments without glucose the corresponding increases were 64 and 90%. In both experiments the overall increase between 2.5 and 12.5% ethanol was 3.1-fold. Accelerations brought about by ethanol and glucose (the latter increases the rate 2.5-fold) are thus additive, as in the case of urethane and glucose (Krupka, 1971), and it follows that ethanol and urethane act by different mechanisms than transported sugars. A further point is that the dependence on urethane or ethanol concentration is higher than first order.

In view of this it seemed possible that only one FDNB molecule is required for inactivation of the carrier but that a second promotes reaction in the same way as do ethanol and urethane. To test this hypothesis several substances resembling FDNB, but lacking a reactive chemical function, were tested. Two of these (nitrobenzene and *m*-dinitrobenzene) did accelerate the reaction, even though they produced no irreversible inhibition themselves. When the total of FDNB plus *m*-dinitrobenzene concentrations was held constant and FDNB varied, all trace of second-order kinetics vanished (Figure 1). Instead of curving upward the plot of k against FDNB concentration was at first linear, while at high concentrations k approached a maximum and the system appeared to become saturated. FDNB may therefore be absorbed to the carrier before reacting. An alternative explanation, that the limits of FDNB solubility were exceeded at the highest concentrations, was eliminated; after equilibration at 25° with salt—buffer containing 5% ethanol (the medium used in inactivation experiments) the solution contained 12.6 mM FDNB, which is well above the 4 mM limit in the experiment.

Apparent saturation would also appear if *m*-dinitrobenzene is more efficient in promoting reaction of carrier than FDNB itself, though these compounds should probably have similar physical effects. The inactivation reaction may be written as eq 2, where C and C′ are active and inactive carrier. The apparent

$$C + FDNB \xrightarrow{k_i \cdot f(X)} C' \qquad (2)$$

rate constant is proportional to some function of the concentration of X, f(X), where X may be dinitrobenzene, FDNB, or a related compound. In this case the measured rate constant is equal to

$$k = k_i[C][FDNB] \cdot f(X) \qquad (3)$$

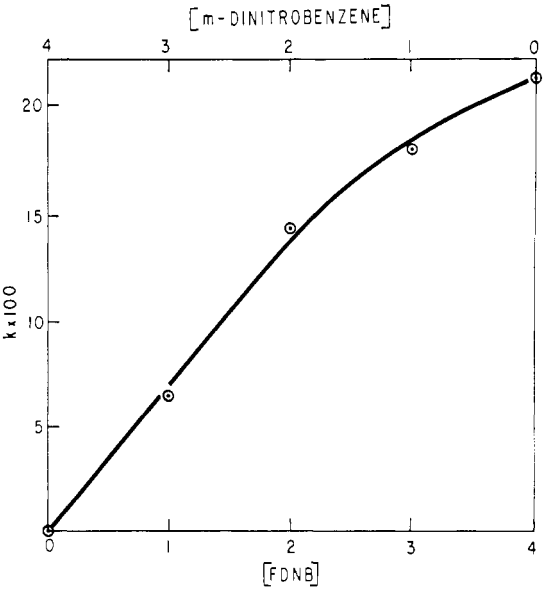

FIGURE 1: Inactivation constant, k, for varying FDNB concentrations in the presence of *m*-dinitrobenzene. The total of FDNB and *m*-dinitrobenzene concentrations is 4.0 mM. The incubation medium included 109 mM glucose and 5% ethanol. Units of k are min^{-1}, and of FDNB and *m*-dinitrobenzene are millimolar.

and the relative size of f(X) with a given concentration of X is proportional to k/[FDNB]. With 2 and 4 mM FDNB alone k was 5.8×10^{-2} and 21.6×10^{-2} min^{-1}, respectively. With 2 mM FDNB together with 2 mM *m*-dinitrobenzene $k = 13.6 \times 10^{-2}$ min^{-1}. The relative values of k/[FDNB] in these three cases are therefore 2.9, 5.4, and 6.8. Thus f(X) for 2 mM FDNB contributes a factor of 5.4/2.9 = 1.9, and 2 mM *m*-dinitrobenzene a factor of 6.8/2.9 = 2.3. The physical effect of the latter may therefore be larger than that of FDNB, though the difference is not great.

In conclusion, higher than first-order dependence on FDNB concentration is almost certainly the result of a first-order chemical reaction with a physical effect of FDNB superimposed. The apparent saturation with FDNB may not be real.

Relation to Carrier Concentration. Earlier work had also shown the rate of inactivation to be proportional to the square of remaining transport activity (Bowyer and Widdas, 1956; Stein, 1969). This conclusion was based on conformity with a second-order law, according to which the rate of carrier loss is

$$\frac{-d[C]}{dt} = k[C]^2 \qquad (4)$$

and from this

$$1/[C] = 1/[C_0] + kt \qquad (5)$$

where $[C_0]$ and [C] are the initial carrier concentration and that after t minutes of treatment with FDNB. As glucose exit times are inversely proportional to carrier concentration, τ should be proportional to the duration of treatment, t. Figure 2 shows appropriate plots for 2 mM FDNB and 119 mM glucose or 203 mM urethane with 119 mM glucose. The relationship is actually nonlinear. The apparent inactivation constant (the slope) increases with time, showing that the rate does not

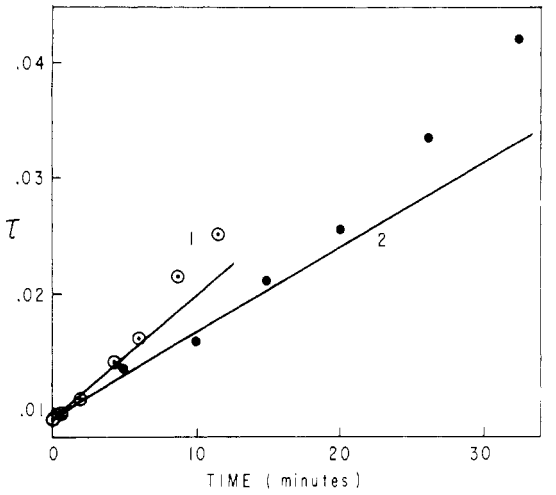

FIGURE 2: Glucose exit times, τ, for cells treated for various periods with 2 mM FDNB. Incubation medium contained 203 mM urethane and 119 mM glucose (curve 1), or 119 mM glucose (curve 2).

fall as quickly as expected. In other words the behavior is less than second order. When the data are replotted according to a first-order law (eq 1) a linear relationship is seen at first but later inactivation lags somewhat. The behavior would therefore appear to be intermediate between first and second order with respect to carrier concentration.

An alternative explanation for the behavior, not previously considered, is that FDNB declined in concentration, either through reaction with the large amount of protein in erythrocytes, or through hydrolysis catalyzed by carbonic anhydrase (Henkart *et al.*, 1968), which occurs in red blood cells.

This idea was tested by determining the inhibitory power of FDNB solutions after incubation with red blood cells. Suspensions were prepared in the usual way with 0.2 ml of cells in 3.8 ml of 119 mM glucose and 2 mM FDNB. After various periods of preincubation the cells were sedimented in a clinical centrifuge. The supernatant solution was mixed with 0.2 ml of cells equilibrated with glucose, and transport rates for the new cells were measured periodically. The inactivation constant, k, calculated by a first-order law (eq 1) progressively declined as preincubation times were extended, falling about 35% in 1 hr and 55% in 2 hr (Figure 3). This agrees with the inhibition observed in a single reaction solution, where the

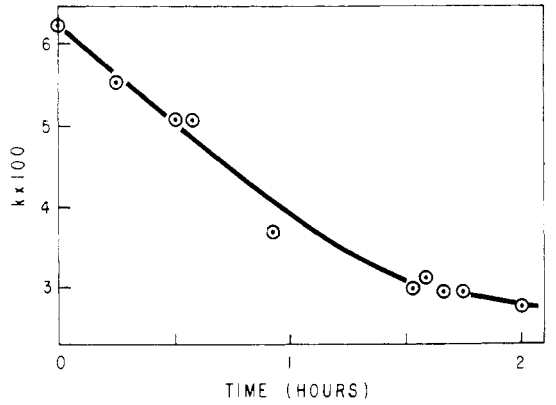

FIGURE 3: Inactivation constants, k, for solutions of 2 mM FDNB and 119 mM glucose preincubated for various periods of time with red blood cells (0.2 ml of cells + 3.8 ml of solution).

TABLE I: Effects of Various Agents on FDNB Inactivation and on Glucose Exit Times.[a]

	FDNB Inactivation		Transport Inhibition	
Compound	Concn (mM)	$k \times 10^2$ (min^{-1})	Concn (mM)	τ/τ_0
None		5.8		1.0
Nitrobenzene	2.4	9.7	3.5	2.1
	9.6	31		
m-Dinitrobenzene	2.0	14.5		
2,4-Dinitrophenol	6.8	8.1		
2,4-Dinitroaniline	Saturating	15		
Indole	4.3	19.8	2.1	2.7
Indole-3-acetic acid	28.8	12.0		
Methanol	620	7.4		
Ethanol	430	7.4	850	1.5
1-Butanol	270	55.0	100	2.1
Isobutyl alcohol	270	41.0		
Urethane	200	13.1	120	2.0
	1000	62		
Urea	1000	11.3	1000	2.2
Dimethylurea	120	6.5	350	2.2
Guanidine hydrochloride	100	6	32–320	1.0
Triton X-45	6.2×10^{-2}	9.4	3.7×10^{-2}	2.3
Triton X-100	2.2×10^{-2}	7.9	5.2×10^{-3}	1.8
Sodium lauryl sulfate	2.2×10^{-3}	9.3	2.5×10^{-3}	1.6
Cetyldimethylbenzylammonium chloride	7.7×10^{-3}	8.6	7.8×10^{-3}	1.8

[a] Left: inactivation rate constants, k, for 2 mM FDNB in the presence of 119 mM glucose, 5% ethanol, and various additives. Right: relative glucose exit times, τ/τ_0, into media containing these same compounds.

slope of a first-order plot fell off with time (Krupka, 1971, Figure 1), indicating the k dropped approximately 25% after 1 hr and 45% after 2 hr.

Summing up, inactivation has the appearance in our experiments of being higher than first order with respect to carrier concentrations, though lower than second. Nevertheless reaction is actually first order, with the complication that the FDNB concentration gradually falls, lowering the inactivation rate.

Effects of Urethane and Various Other Agents. A variety of physical agents was tested, first with respect to FDNB inactivation and then sugar transport.

Summaries of the findings are given in Table I. The capacity for accelerating FDNB inhibition was always accompanied by an inhibition of sugar transport. Upward curvature in plots of the inactivation constant, k, or glucose exit rates against the concentration of urethane in the medium showed that several urethane molecules are involved in these actions (Krupka, 1971). Similar inhibitions of transport were observed with urea, Triton X-100, and nitrobenzene (Figure 4).

Inhibitions by all these agents were instantly reversible. When 0.5-ml aliquots of cell suspension containing detergents or urea were injected into 65 ml of salt solution, the rates of

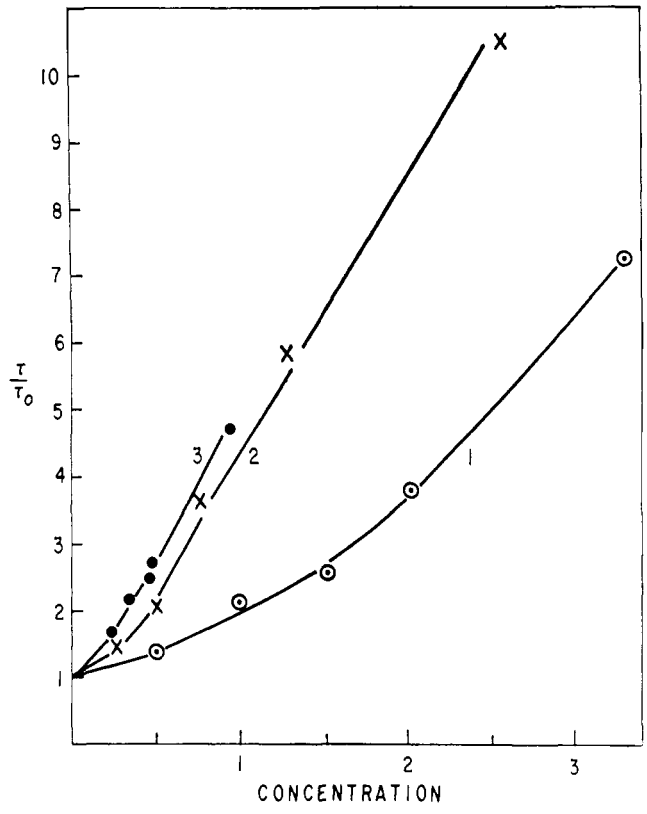

FIGURE 4: Relative glucose exit times, τ/τ_0, in the presence of reversible transport inhibitors. Curve 1: urea, molar concentration units; curve 2: Triton X-100, molar concentration $\times 10^{-5}$; curve 3: nitrobenzene, molar concentration $\times 10^{-3}$.

glucose exit were identical with those seen without inhibitor. As glucose moves out of the cells in roughly 1 min, reversal of inhibition must have been very rapid. Their enhancement of FDNB reactivity was also rapidly nullified by dilution. Cells were incubated with glucose and detergent or urea for about 5 min and then were sedimented. The supernatant was discarded, and the cells were resuspended in 3.8 ml of a glucose solution containing 2 mM FDNB. Glucose exit rates were measured after 1 min and periodically thereafter. While the original concentrations of detergent or urea would have at least doubled the inactivation rate, there was no increase after dilution in this way.

A possible explanation of apparent second-order behavior for FDNB inhibition and its stimulation by various agents is that only part of the added FDNB is available to react with carrier, the rest being sequestered in a hydrophobic phase of the membrane. Doubling the amount of FDNB could then more than double the available concentration, and addition of a compound able to replace FDNB in the hydrophobic phase, such as dinitrobenzene or urethane, would release FDNB and increase the reaction rate. For this mechanism to be correct (in fact it is not) a large proportion of added FDNB would have to be immobilized, considering that 1 M urethane increases the inactivation rate more than 10-fold, and 9.6 mM nitrobenzene 5-fold, and that these factors would become still larger at higher concentrations (Krupka, 1971; Figure 4). The figure for urethane implies that more than 90% is sequestered. Sedimented cells should then carry down most of the FDNB present, and the supernatant solution should have low inhibitory activity toward fresh cells. This prediction was not borne out. Experiments like those described above

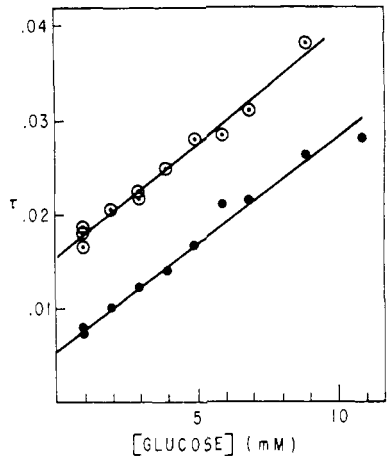

FIGURE 5: Inhibition of glucose exit by 124 mM urethane. Lower line, uninhibited exit times; upper line, with urethane. The calculated binding constant for urethane is 65 mM.

were performed, in which inactivation by supernatants was compared with the original inhibitory power. An immediate reduction of about 10% was observed in the inactivation rate constant. If less than 10% of the FDNB remained, as the hypothesis requires, there would have been virtually no inhibition at all, in view of the dependence on the square of FDNB concentration. Effects of these agents may therefore largely depend on interactions with the carrier itself.

Transport rates were also measured in the presence of fixed inhibitor and varying glucose concentrations in the external medium, to determine whether inhibition is competitive or noncompetitive (Sen and Widdas, 1962; Miller, 1969). Surprisingly urethane and urea acted as competitive, nontransported inhibitors, as shown by parallel lines in a plot of exit times vs glucose concentration (Figures 5 and 6). Inhibition by ethanol, butanol, and indole was also largely competitive, though not precisely so (Figure 7). Detergents (sodium lauryl sulfate, cetyldimethylbenzylammonium chloride, and Triton X-100) inhibited noncompetitively (Figure 8). It may be noted, with regard to the interpretation of such plots, that pure competitive inhibition is characterized by equal increases in the half-saturation constant for glucose, K, and the reciprocal of the maximum transport rate, V. In noncompetitive inhibition, V, falls but K is unaltered, while in intermediate cases both constants may change, but V more than K.

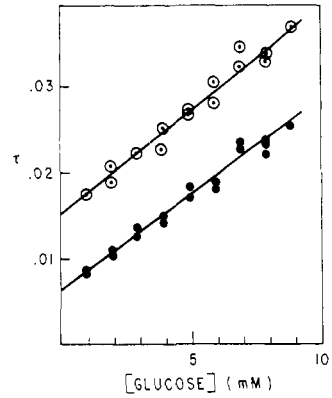

FIGURE 6: Inhibition of glucose exit by 1.22 M urea. Lower and upper lines are uninhibited and inhibited exit times, respectively. The calculated K_i for urea is 0.87 M.

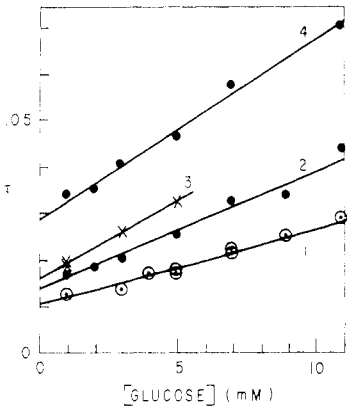

FIGURE 7: Inhibition of glucose exit by 5% ethanol (1), 0.9% 1-butanol (2), 2.1 mM indole with 1.2% ethanol (3), or 1.8% 1-butanol (4). The glucose binding constants (glucose concentrations where the exit times are doubled) are 6.2, 5.5, 4.7, and 7.5 mM, respectively, in these four cases. The constant in the absence of inhibitor is approximately 2.4 mM.

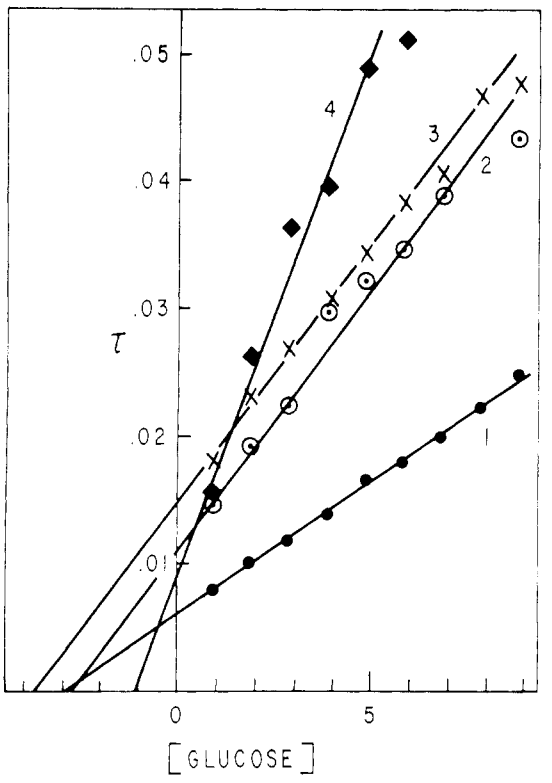

FIGURE 8: Inhibition of glucose exit by detergents. Exit times in the absence of inhibitor, curve 1; with 4.9×10^{-5} M Triton X-100, curve 2; with 4.2×10^{-6} M sodium lauryl sulfate, curve 3, and with 3.9×10^{-6} M cetyldimethylbenzylammonium chloride, curve 4. In pure noncompetitive inhibition the extrapolated lines for inhibited and uninhibited exit times should intersect on the horizontal axis to the left of the origin.

Considering that inhibition of transport was often competitive, it was surprising to learn that accelerations of FDNB reaction produced by glucose and these nonspecific agents additive. For example, the rate constant k in the presence of 1.0 M urea was 3.0×10^{-2} min^{-1} and in its absence 2.4×10^{-2} min^{-1}. With 119 mM glucose, k was 6.1×10^{-2} min^{-1}, and with 119 mM glucose plus 1.0 M urea it was 11.0×10^{-2} min^{-1}. That is, urea and glucose acted independently, indicating that both bind to carrier at once. Similar observations were made for ethanol, as described above, and for urethane (Krupka, 1971). This anomaly might be explained if inhibition of transport is a mixture of competitive and noncompetitive types rather than purely competitive, *i.e.*, if the ternary complex CSI is formed, though less readily than complexes with sugar or inhibitor alone (CS or CI). The inhibitor concentraion required in the FDNB experiment, where the glucose level is high, would then be substantially greater than in transport experiments, where it is low. In fact the concentrations were very similar. With identical salt and ethanol concentrations, 0.86 M urea reduced the transport rate by 50% and 1 M urea doubled the FDNB inactivtion rate (Table I). Urethane at 0.11 M halved the transport rate, and at 0.16 M doubled the inactivation rate. In order to give the nearly competitive kinetics seen in Figures 5 and 6, CSI must be at least five times more difficult to form than CI, since the intercepts in these plots are altered at least five times more by the inhibitor than are the slopes. In purely noncompetitive inhibition, where I adds to CS as easily as to C, slopes and intercept are increased to exactly the same extent (see Miller, 1969). There is therefore a real discrepancy in these experiments, which will be discussed later.

Discussion

Despite its seeming complexity the reaction of FDNB with carrier turns out to be simple: first rather than second order with respect to both FDNB and carrier sites. The support that experiments with FDNB formerly gave to theories involving interaction between pairs of carriers (Stein, 1969) must therefore be withdrawn.

The secondary, physical action of FDNB, which leads to increased reactivity of FDNB with carrier, and which also led to confusion about the order of reaction, is now seen to be shared by a wide variety of compounds. This behavior is of special interest because transported sugars have the same effect, though as a result of specific, 1:1 complex formation with carrier instead of nonspecific interactions of several molecules. Once bound, transported sugars induce a conformational change in the carrier, and the new form, which reacts rapidly with FDNB, is probably an intermediate in transport (Krupka, 1971). Insight into the nature of the specific conformational transition may perhaps be gained by considering the nonspecific action of substances as diverse as alcohols and detergents.

There are several reasons for thinking the conformations induced by the latter and by well-transported sugars are fundamentally alike. First, the new conformation reacts rapidly with FDNB, indicating that the site of this reaction becomes exposed. Second, the conformational change is completely and immediately reversible. The energy barrier between the two forms must therefore be low, suggesting that both are within the range of normal conformations. Still more pertinent is the observation that though glucose and urethane or urea can add to carrier at the same time (as shown by the additivity of their effects on FDNB reaction) their inhibition of transport is competitive. It necessarily follows that the complex with glucose and urethane or glucose and urea must undergo normal transport, which it could hardly do if the conformation stabilized by urethane or urea were much different from that induced by glucose. If the ternary complex was inactive, inhibition of transport would of course be noncompetitive, as it is with detergents. These agents must therefore disrupt

carrier structure more drastically, and though the change is reversible, the complex is not transported.

Agents such as urea, alcohols, and detergents have one obvious property in common—all increase the water solubility of nonpolar substances, such as fatty acid chains in lipids, and hydrophobic side chains in proteins, which are normally buried within the protein structure (Tanford, 1970). Indeed there is a correlation between lipophilicity and inhibition of transport or promotion of reaction with FDNB. For example, the partition coefficient of 1-butanol in an olive oil–water system is 6.5 times that of ethanol (Stein and Danielli, 1956) and in line with this the FDNB inactivation constant is 7.5 times larger with 2.5% butanol plus 2.5% ethanol than with 5% ethanol, and sugar transport rates are reduced somewhat more by 0.9% butanol in the external medium than 5% ethanol. Furthermore, detergents act at very low concentrations, without regard to charge type, and urea and urethane at much higher concentrations, as in protein denaturation. Effective urea concentrations are considerably lower than those that cause complete unfolding of protein (8 M), but like those altering enzyme activity as in the case of invertase (Chase and Krotkov, 1956).

An ionizable group reduces the potency of small molecules, *e.g.*, indole-3-acetic acid, dinitrophenol, and guanidine hydrochloride, compared with indole, dinitrobenzene, and urea (Table I), suggesting that penetration into a hydrophobic mileu may be required. Dinitrophenol and indoleacetic acid should, and do, have partial activity, since they exist partly in the un-ionized form, whereas guanidine is completely ionized and inactive. On the other hand, charged and uncharged detergents act similarly, presumably because the long hydrophobic chains can reach nonaqueous regions of the carrier while the attached ion remains outside, in the water phase.

These findings may suggest the broad outlines of a carrier mechanism. To recapitulate, the carrier undergoes a conformational change following glucose binding and the altered form is an intermediate in transport. A similar conformation is stabilized by various agents capable of increasing the aqueous solubility of nonpolar substances. The form induced by glucose may therefore differ from the initial form in having hydrophobic groups more exposed to a polar environment, and may be able to penetrate a lipid barrier in the membrane, with transfer of sugar. According to this mechanism, free carrier should cross the membrane less easily than the complex, in agreement with experiments on sugar exchange. Transport was found to accelerate when sugar is present on both sides of the membrane rather than on one side only, and hence the rate limitation under the latter condition may be movement of free carrier (Levine *et al.*, 1965; Mawe and Hempling, 1965).

With respect to FDNB inhibition it was seen that glucose and urea (or urethane) can add to carrier at the same time and hence must bind at separate sites. Contrariwise, competitive inhibition of transport shows that once urea or urethane become bound, glucose cannot. These observations are reconciled if urea binds at two places, at the substrate site to block transport, and elsewhere to accelerate FDNB reaction, but without effect on transport. An acceptable hypothesis should explain why concentrations producing the two effects are comparable, and why any of a wide range of agents should directly compete with sugars, which are decidely hydrophilic. It should also indicate why several molecules are involved in competitive inhibition, and why saturation of the site does not occur (Figure 4).

Inhibitors related to phloretin, it may be noted, are also hydrophobic and probably do bind at or near the sugar site. The latter may therefore be composed of polar groups that specifically attract hydroxyls in sugar, in conjunction with a nonpolar region. Hydrophobic attraction, which is relatively nonspecific and independent of the shape of interacting molecules, can probably account for all the observations. In any case, comparable effects were seen only at lower concentrations, those doubling exit times and inactivation rates (and therefore relevant in the earlier discussion). With higher levels of urethane there was a wide divergence, transport being much more sensitive than FDNB reaction (Krupka, 1971; Figure 4).

It follows that there may be two hydrophobic regions, one at the carrier site, which becomes unavailable when sugar is bound, and another outside this site, which is exposed in the process of transport.

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