BBA 75712

ON THE MECHANISM OF MUTUAL INHIBITION AMONG SODIUM-DEPENDENT TRANSPORT SYSTEMS IN THE SMALL INTESTINE. A HYPOTHESIS

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(Received February 19th, 1971)
(ADDENDUM by P. HONEGGER AND G. SEMENZA received May 10th, 1971)

SUMMARY

1. Amino acids and sugars, although transported in the small intestine by different carriers, mutually inhibit each other's uptakes. Various mechanisms have been suggested for this interaction among heterologous substrates.

2. The present paper indicates that both the inhibition of substrate influx and the stimulation of substrate efflux can be due to a local decrease of Na^+ (and perhaps K^+) gradient(s) across the brush border membrane. The hypothesis suggested here accommodates satisfactorily all observed interactions taking place at the luminal pole of the cell.

3. The equations for Na^+-dependent unidirectional flux, and for the trans inhibition by Na^+ are reported for the general case.

Glucalogues (*i.e.* D-glucose and its analogues), monoamino-monocarboxyl acids, monoamino-dicarboxyl acids and diamino-monocarboxyl acids are considered to be transported through the brush border membrane of enterocytes by different systems. However, various laboratories have reported in recent years that the net uptake of some amino acids is reduced by the presence of some sugars in the medium, and *vice versa.*

The actual reports from the various laboratories are sometimes conflicting and the nature of the underlying mechanisms has been the subject of considerable debate. "At least four hypotheses have been put forward: (a) inhibition due to the formation of toxic metabolites[1,2], (b) competition for energy[3-7], (c) stimulation of the efflux of the substrate form the cell[8,9], and (d) an inhibition between the two groups of substrates at the level of the brush border membrane[10-12], which is probably an allosteric effect due to the proximity of related binding sites in a polyfunctional matrix[10,13,14]. The originators of these various theories have defended them with considerable vigour." (quoted from ref. 15).

Undoubtedly much of the controversy is due to differences in methodology, and/or differences in the species used: for example, mucosal–serosal transfer may measure, under some conditions, a different step from the uptake at the brush border pole; the intestine of some species (*e.g.* rat) may be less viable than others, or more sensitive to energy depletion or to toxic metabolites. BINGHAM *et al.*[4], for example, has shown that metabolisable sugars, even when supplied from the serosal side, are capable of increasing the mucosal–serosal transfer of both methionine and proline in rat small intestine during incubations lasting 30 min. An additional complication is shown by the observation[5] that much of the glucose stimulation of amino acid mucosal–serosal transfer is related to the increase in trans-intestinal water flow induced by this sugar. An inhibition occurring at the level of the brush border can, therefore, be masked by these effects, unless methods singling out unidirectional fluxes are used (see below).

It is not the purpose of the present paper to review the extensive literature in the field. This has been done by others recently[14–17] but rather to discuss those phenomena of partial mutual inhibition among transport systems, which, because of the experimental conditions used, may be presumed to take place in the brush border region. I shall try to discuss a hypothesis which seems to account for the phenomena recorded. The major facts on which it is based are:

(a) Although the intestinal uptake of some substrates (say, of the sugars of the glucose group) has always been considered as taking place through a transport system independent of the transport system of other substrates (say, of neutral amino acids), the first group of substrates do inhibit, albeit partially, the net uptake of the second group of substrates and *vice versa.*

In addition, substrates belonging to the one group elicit the net exit from preloaded tissue of substrates belonging to the second group, and *vice versa,* although less efficiently than the exit of substrates belonging to the same group[15,18].

(b) The substrates for which this kind of mutual interaction is known have Na^+ as a co-substrate for transport*. Thus, Na^+ dependence seems to be a necessary prerequisite. Other conditions must, however, be fulfilled, because monoamino-dicarboxyl acids and sugars do not interact similarly (in the hamster)[12], although they all have Na^+ as a co-substrate. (The Na^+ dependence of intestinal uptake of monoamino-dicarboxyl acids is a recent acquisition: see ref. 21, rabbit ileum.)

(c) Simple binding to the carrier is not enough, because competitive inhibitors of sugar transport, the K_i of which is also Na^+ dependent[22,23], do not inhibit amino acid uptake (phlorizin)[10], or inositol uptake (L-fucose)[24], respectively, although the substrates of the same Na^+-dependent sugar carrier (*e.g.* glucose, galactose) do so.

(d) Phlorizin, in addition to inhibiting intestinal uptake of glucalogues, both prevents their inhibition of amino acid uptake[10] and inhibits the glucalogue-associated Na^+ influx[25].

FEATURES OF THE PRESENT HYPOTHESIS

These phenomena of mutual interaction between different transport systems

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* D-Fructose (30 mM), as reported recently[15], provokes a small but significant inhibition of phenylalanine uptake (4.5 mM) in rat intestinal slices (incubation during 5 min), but not in intestinal slices from other species. It remains to be seen whether this phenomenon bears any relation with the Na^+ dependence of intestinal fructose uptake in rat small intestine, which GRACEY *et al.*[19] have reported a few months ago. One more observation which deserves to be further investigated is the (week) stimulation of phenylalanine exit by mannitol[15]. This substance, which had long been regarded as a non-absorbable marker for extracellular space, is now recognized to be absorbed, albeit to a limited extent[20]. It is not known yet whether Na^+ is a co-substrate for mannitol uptake.

can be explained easily if the following two conditions are both fulfilled:

(a) The substrates involved have Na^+ as a co-substrate for transport, and their effects on Na^+ influx are additive[26,27].

(b) The transport systems involved are located close to each other in the brush border region.

The Na^+ entering along with a substrate (say, an amino acid) creates a local increase in Na^+ concentration at the intracellular face of its carrier. This increase in Na^+ concentration extends by diffusion to reach the cellular face of another neighbouring carrier, for example of a Na^+-dependent carrier for sugars. (If the entry of Na^+ is paralleled by the exit of K^+, a local decrease of K^+ concentration will also occur in analogous fashion.)

The new Na^+ concentration reached is conceivably in dynamic equilibrium; except for a very short initial time, the Na^+ influx through the brush border membrane is equal to the sum of Na^+ efflux through the brush border membrane *plus* the diffusion of Na^+ into the rest of the cell. Due to the short distances (perhaps some hundred Å) and to the large area/volume ratio of the microvilli, the steady-state concentration of Na^+ responds very quickly to changes in Na^+ fluxes, particularly Na^+ influx (similar considerations can be made for the concentration of K^+, if Na^+ influx is accompanied by K^+ efflux).

The very architecture of the microvillus — very long and thin, and with a number of Na^+-dependent transport systems presumably packed in the membrane — makes likely that the Na^+ entered builds up a local hyperconcentration before diffusing through the length of the microvillus and the terminal webb.

In analogous fashion, opposite changes of Na^+ (and possibly K^+) concentrations presumably occur in the unstirred layer at the outer face of the brush border membrane. Although centred around what is presumed to happen at the internal face of the membrane, the following discussion can be extended also to accommodate the events at the outer face.

The changes in Na^+ (and/or K^+)concentrations can affect neighbouring Na^+-dependent carriers (that of glucalogues, in the example), in either one or both the following ways: (a) stimulate the Na^+-dependent substrate efflux (Na^+-dependent efflux has been demonstrated for both amino acids sand (sugars), and (b) inhibit the Na^+-dependent substrate influx.

Whether one or both phenomena actually take place in a given case depends on the kinetic parameters of the transport system involved (see next paragraph).

This hypothesis (which we will call "the trans-Na hypothesis" for short but which refers to both mechanisms) obviously has some points in common with other hypotheses previously put forward — notably with that of READ[9]. At the end of his paper he suggested that "the localized increase in sodium would enhance the probability for efflux of amino acid from the cell and lower the net transport". His idea does not seem to have been followed up or subjected to quantitative evaluation, although — as I hope to show in the following — it does account for a large number of data published both before and after READ's[9] paper.

BROWN AND PARSONS[28], as well as KOOPMAN AND SCHULTZ[29] and ARMSTRONG *et al.*[30] have demonstrated that in some cases changes in the total Na^+ and K^+ contents of intestinal tissue do occur during exposure to some of the solutes which are absorbed by Na^+-dependent systems, the changes being most notable in the case of galactose.

The changes in local cation concentrations which are regarded as relevant for the trans-Na hypothesis, however, are supposed to occur in a limited area of the brush border region and may well escape detection by the usual analytical procedures, in which the whole enterocyte, or even the whole small intestine, have to be analyzed. KOOPMAN AND SCHULTZ[29], in fact, failed to detect any change in the so-called "intracellular" Na^+ concentration following preincubation of small intestine in the presence of either lysine, glucose or 3-methylglucose.

The trans-Na hypothesis, it should be pointed out, is not to be regarded as an alternative to the polyfunctional-carrier hypothesis[10], and much less so to the proposed second (translocation) step in carrier-mediated transport[23]. Rather, it suggests a chemical link between the primary phenomenon and the final events propounded by the latter hypotheses.

Finally, the trans-Na hypothesis is related to the energy-limitation hypothesis of NEWEY AND SMYTH[3] the energy being, in our case, the difference in chemical potentials of Na^+ (and K^+) across the brush border membrane.

KINETICS

(*a*) *Na^+-dependent substrate efflux.* The kinetics of cis stimulation are well known and need not be discussed in detail. Eqn. A-1 (see APPENDIX) for the Na^+-dependent substrate influx (J_s^i) in one of the possible models (non-compulsory reaction sequence) can be easily re-written for the Na^+-dependent substrate efflux (J_s) for the same model, by exchanging the dashed symbols with non-dashed ones, and *vice versa*. The major condition to be fulfilled for a Na^+ stimulation of substrate efflux is that the permeability coefficient of the Na^+–substrate-loaded carrier (P_3) should not be negligibly small. The kind of kinetic stimulation by Na^+ (major effect on the apparent maximum velocity or on the apparent Michaelis constant) will depend in the intrinsic properties of the transport system involved (compulsory or non-compulsory reaction sequence, relative magnitude of the permeability coefficients, *etc.*) (Similar consideration can be made for the effect of decreased K^+ concentration.) Na^+-dependent substrate efflux from the brush border membrane was demonstrated conclusively by CURRAN *et al.*[36] last year.

(*b*) *Trans inhibition of substrate influx by Na^+.* This case will be considered in some detail, because some cases of mutual inhibition of uptake have been attributed, and probably rightly so, to inhibition of substrate influx. In subsequent paragraphs some examples of mutual inhibition among substrates will be discussed as if they were related to a trans inhibition of substrate influx, although the experimental evidence available at this moment may not exclude a stimulation of Na^+-dependent substrate efflux. The reader should not believe that I regard the Na^+-dependent substrate efflux as an unlikely mechanism (sugar-stimulated alanine efflux was actually demonstrated in at least one case[8]), the reason is simply that the detailed kinetics of trans inhibition of substrate influx are perhaps less obvious.

In the APPENDIX I report the rate equation for unidirectional substrate flux (J_s^i) in a Na^+-dependent system (A-1). For the sake of simplicity I have considered one model only, and the effect of Na^+ only. The effect of K^+ (which in addition to having other effects, presumably completes for the Na^+-binding site[16,31]) is easy to include, without changing the fundamental conclusions of the treatment. Finally, the trans effect of the substrate itself could also be considered, again without altering the fundamental conclusions.

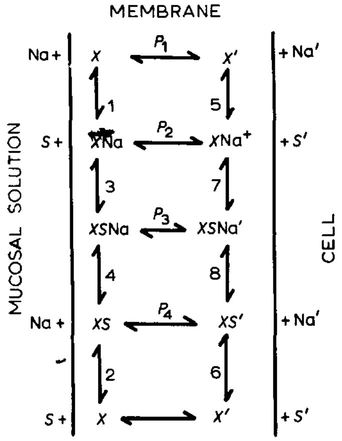

Fig. 1. A general model for the formation of a ternary complex comprising a membrane site (X), a solute (S) and sodium. The sequence of formation of the ternary complex is not specified and all forms of X are permitted to translocate across the membrane.

Simple inspection of Eqns. A-2 and A-3 immediately indicates that a negative trans effect of Na^+ can only be expected if $P_2 < P_1$ ($\varepsilon > 1$) that is, if the permeability coefficient to the Na^+-loaded carrier is smaller than the permeability coefficient of the free carrier. This is logical enough, since a small P_2 (the permeability coefficient of the Na^+-loaded carrier) accompanied by a large Na′ will make the carrier "stick" on the internal face of the membrane once it is in the form XNa′ (see Fig. 1). Although P_1 and P_2 have never been experimentally determined, different permeability coefficients for various other forms of intestinal carriers have already been reported, and they actually build a fundamental feature of the model of GOLDNER *et al.*[32] for sugar transport in rabbit ileum.

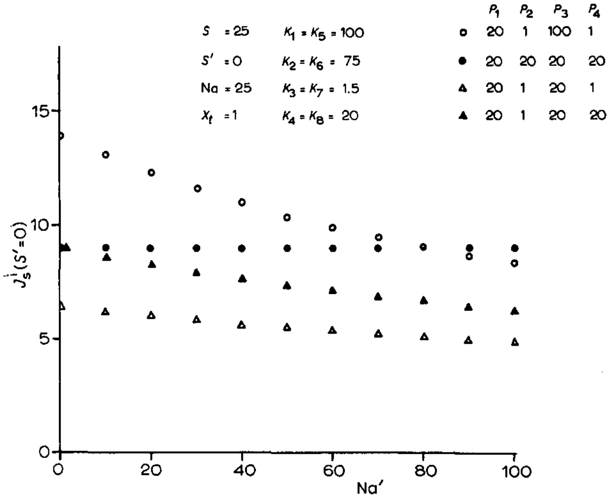

Fig. 2. Simulation of the trans inhibition of substrate influx at zero trans substrate concentration (J_s ($S' = 0$)) by increasing trans concentrations of Na^+ (Na′), from Eqn. A-2. One single set of dissociation constants and of *cis* concentrations of substrate and sodium, but four different sets of permeability constants were considered, as indicated in the inset. For explanation of symbols, see Fig. 1.

Fig. 2 shows how the unidirectional flux of the substrate is affected by increasing trans concentrations of Na^+. For purposes of comparisons, four sets of P values and one single set of dissociation constants were considered.

Comparison between Eqns. A-5 and A-7 indicates that the trans-concentration of Na^+ (Na′) can affect the apparent K_t values of the uni-directional fluxes. However, even under extreme conditions, *i.e.* at both $P_2 \ll P_1$ and at very high trans-concentrations of Na^+ the apparent K_t never becomes infinitely large.

Comparison of Eqns. A-6 and A-8 indicates the trans-concentration effect of Na^+ on the apparent maximum uni-directional fluxes (J_s^i). At very large Na′, if P_2 is very small in comparison with P_1, large Na′ values will strongly reduce the apparent maximum J_s^i. How much the apparent maximum J_s^i will decrease will also depend on the P_2/P_1 and P_4/P_1 ratio, *i.e.* on the intrinsic properties of the system involved.

From these simple kinetic considerations it is clear that the trans effect of Na^+ depends on a number of parameters. The inhibition belongs to the so-called "mixed type", with prevalence, however, of the decrease of maximum J_s^i rather than of the increase in apparent K_t. Both a "purely non-competitive" and a "partially competitive" inhibition can be expected, and both types have actually been reported. For example, the inhibition of inositol net uptake by glucalogues in hamster small intestine is purely non-competitive[24]. On the other hand, the mutual inhibition of amino-acids and glucalogues in the hamster[10], as well as that of valine or cycloleucine net uptake by galactose in the rat[7], or the dogfish[9], respectively, are partially competitive; *i.e.* the apparent K_t never becomes infinitely large, even at infinitely large concentrations of the inhibitor.

TIME INDEPENDENCE OF THE RATE OF SUBSTRATE UPTAKE AND OF THE EXTENT OF INHIBITION

Sugar net uptake into intestinal strips (from sugar alone in the medium) remains usually linear for some 10–20 min, whereas sugars inhibit amino acid uptake from the very beginning of the incubation[13]. One may wonder why sugars do not inhibit their own net uptake (*i.e.* why their uptake does not deviate from linearity) at an earlier time. Again, this phenomenon is satisfactorily explained by the trans-Na hypothesis, because the Na^+ fluxes of amino acids and sugars are additive[26,27], *i.e.* when sugars and amino acids are both present together in the incubation medium, more Na^+ enters the brush borders (and more K^+ gets out). The new steady-state Na^+ (and/or K^+) concentrations deviate more markedly from the original concentrations. It is reasonable to assume that in general the new steady-state concentrations of the cations are reached almost instantaneously and remain essentially constant thereafter (see above); this is probably why the mutual inhibition between amino acids and sugars may be present from the very beginning of the incubation[13] and remains essentially constant thereafter.

LACK OF MUTUAL INHIBITION IN PREINCUBATION EXPERIMENTS

Contrary to what happens when amino acids and sugars are both present during the incubation, preloading with amino acids usually does not cause inhibition of sugar uptake during the subsequent incubation period, nor does preloading with sugars inhibit subsequent amino acid uptake[8, 10]. This lack of inhibition by preloading is satisfactorily explained by the present hypothesis, because the local perturbation of Na^+ (K^+) concentration(s) is assumed to be unstable and needs to be sustained by an uninterrupted Na^+ influx (respectively K^+ efflux) (see above).

LACK OF SYMMETRY IN INHIBITION OF UPTAKE

Glucalogues are non-competitive inhibitors of inositol net uptake[24], but inositol does not inhibit the net uptake of glucalogues. This lack of symmetry can be explained most easily on the basis of the trans-Na hypothesis. In fact, although Na^+ dependent, the absorption of inositol does not bring about a detectable increase in the mucosal–serosal potential difference. Since the maximum velocity of uptake of inositol is very small (some 1 % of the maximum velocity of glucalogue uptake) the most plausible explanation is that Na^+ is a co-substrate of inositol uptake (as in the case of glucalogue uptake), but that "the number of carriers available for *myo*-inositol is not enough to translocate Na^+ at a rate required to give a significant change in transmural potential difference" (quoted from ref. 24). Within the framework of the present hypothesis, the inositol-associated Na^+ influx (and K^+ efflux) is thus too small to induce a sizable change in the steady-state concentration of Na^+ (and K^+) in the neighbourhood of the internal face of the glucalogue carrier, and to affect the second (translocation) step in carrier transport suggested by CASPARY *et al.*[23]. It remains to be seen whether the new cation concentration of Na^+ and K^+ induced by inositol entry may nevertheless be sufficient to make the K_i of glucalogues somewhat larger than their own transport K_m (ref. 24).

An alternative mechanism (which, however, probably need not apply in the inositol–glucalogue interaction) whereby Substrate A can inhibit the unidirectional flux of Substrate B, without itself being affected by Substrate B in a symmetrical fashion, is the following:

In the Na^+-dependent transport system for Substrate B, $P_2 < P_1$: the unidirectional flux of B is, therefore, sensitive to a trans effect from the Na^+ which entered *via* the Na^+-dependent transport system for Substrate A or *via* other systems. If, however, in the A system $P_2 = P_1$, the unidirectional flux of A will not be affected by the Na^+ which entered *via* the transport system for substrate B, or by way of other systems.

The trans-Na hypothesis, therefore, also accommodates in a most simple fashion the cases asymmetrical inhibition.

TIME DEPENDENCE OF THE INHIBITION

In some cases the inhibition of amino acid uptake by a sugar (*e.g.* cycloleucine uptake by galactose in the intestine of the dogfish[9]) is negligible at short incubation times (2 min), but detectable at somewhat longer times (10 min), and is evident after preincubation with galactose, even when the sugar is not present during the actual incubation with cycloleucine. These observations do not require any major change in the trans-Na hypothesis, except that — possibly for anatomical reasons — the building up and vanishing of the perturbed Na^+, K^+, concentrations in the brush border region of dogfish intestine are slower processes than in mammals. Alternatively, the amino acid transport system of the dogfish may be less senstitive to the trans effect of

Na^+ (*i.e.* it may have a large K_5), so that higher values of Na′ have to be reached. Finally, the experiments on the dogfish were carried out at 20°, whereas those on the hamster at 37°.

DIFFERENCES AMONG SPECIES AND ALONG THE INTESTINE

As ROBINSON AND ALVARADO[15] have shown recently the differences among species are more quantitative than qualitative in nature. In addition to differences in methodology — and thus differences in the rate-limiting steps possibly measured — as well as to differences in tissue viability or sensitivity towards toxic metabolites (factors likely to influence long-term experiments), species differences as well as differences in ileum *vs.* jejunum[7] may be due to (a) different spatial relationship among the Na^+-dependent transport systems within the brush border membrane — the perturbation of Na^+,K^+, concentrations may not extend over large distances; (b) different permeability coefficients and/or Na^+,K^+ dissociation constants in different species (a number of examples are known). As it was pointed out above, the trans effect of Na^+ on substrate influx requires that $P_2 < P_1$. The stimulation of substrate efflux by the Na^+ present at the intracellular face of the brush border membrane, on the other hand, is independent of this condition; it merely required that the permeability coefficient of the Na^+–substrate-loaded carrier (P_3) should be different from zero. It remains to be seen whether these considerations (or a different methodology[15]) explain the inhibition of amino acid net uptake by sugars (in hamster small intestine[13]) as opposed to the stimulation of amino acid efflux by sugars (in rabbit ileum[8]).

At any rate, one should not be surprised to find species differences or differences between ileum and jejunum in a phenomenon likely to be of rather limited physiological significance, such as this, and thus presumed to be subjected to limited selective pressure.

AN EXPERIMENTAL TEST OF THE TRANS-Na HYPOTHESIS

The value of a hypothesis is judged from its capacity to account satisfactorily for a large number of phenomena in a unitarian framework. The previous paragraphs have hopefully succeeded in showing that the trans-Na hypothesis does so; the unitarian framework being the coupling of solute transport with Na^+ transport[34] and almost universally accepted[16, 35].

The trans-Na hypothesis predicts that the phenomena of mutual inhibition discussed above should not take place in the absence of both Na^+ and K^+ at both sides of the membrane.

It is doubtful whether it is possible at all to obtain undamaged Na^+- and K^+-free small intestinal tissue with the procedures presently available. Table I (ADDENDUM) reports, nevertheless, an attempt in this direction. The small intestine was significantly depleted of Na^+ by appropriate preincubation, and the incubation itself was carried out in the absence of Na^+. The uptake of 6-deoxy-D-glucose during a rather short time was measured in the presence and in the absence of phenylalanine; everted small intestinal rings, which were mounted in frames so as to expose to the medium the mucosal side alone, were used. Therefore, any exchange between tissue and medium through ways other than the mucosal surface can be ruled out safely.

As it can be seen, decreasing the Na^+ content of both the medium and the tissue brings about a significant reduction — both in per cent and in absolute terms — of phenylalanine inhibition of 6-deoxy-D-glucose uptake. This observation, therefore, lends support to the trans-Na hypothesis.

APPENDIX

Assuming that (a) Na^+ and substrate are non-obligatory co-substrates, (b) the carrier binds one substrate and one Na^+, (c) the ternary complex substrate–Na^+–carrier can form either from $X\mathrm{Na} + S$ or from $XS + \mathrm{Na}^+$ (no compulsory reaction sequence), (d) the rate-limiting step is given by the translocation of the carrier through the membrane (or its physical equivalent), so that carrier, substrate and Na^+ can be regarded as being in equilibrium at each face of the membrane; (e) in the steady state there is no steady buildup of carrier anywhere in the membrane (*i.e.* that the total velocity of translocation of carrier molecules in its various forms is the same in both directions), the unidirectional flux of the substrate into the cell (J_s^i) is given by:

$$J_s^i = \frac{X_t(P_3 + P_4K_4/\mathrm{Na})}{1 + \frac{K_3}{S} + \frac{K_4}{\mathrm{Na}} + \frac{K_3K_1}{S\cdot\mathrm{Na}} + \left(1 + \frac{K_7}{S'} + \frac{K_8}{\mathrm{Na}'} + \frac{K_7K_5}{S'\cdot\mathrm{Na}'}\right) \dfrac{P_3 + P_2\frac{K_3}{S} + P_4\frac{K_4}{\mathrm{Na}} + P_1\frac{K_3K_1}{S\cdot\mathrm{Na}}}{P_3 + P_2\frac{K_7}{S'} + P_4\frac{K_8}{\mathrm{Na}'} + P_1\frac{K_7K_5}{S'\cdot\mathrm{Na}'}}} \qquad \text{(A-1)}$$

where X_t is the total carrier concentration, the P's are the permeability coefficients and the K's the equilibrium constants, as defined in Fig. 1 and in the following equations:

$$\frac{X\cdot\mathrm{Na}}{X\mathrm{Na}} = K_1 \qquad \frac{X\cdot S}{XS} = K_2 \qquad \frac{X\mathrm{Na}\cdot S}{X\mathrm{Na}S} = K_3 \qquad \frac{XS\cdot\mathrm{Na}}{X\mathrm{Na}S} = K_4$$

$$\frac{X'\cdot\mathrm{Na}'}{X\mathrm{Na}'} = K_5 \qquad \frac{X'\cdot S'}{XS'} = K_6 \qquad \frac{X\mathrm{Na}'\cdot S'}{X\mathrm{Na}S'} = K_7 \qquad \frac{XS'\cdot\mathrm{Na}'}{X\mathrm{Na}S'} = K_8$$

From Eqn. A-1 the unidirectional flux of the substrate into the cell at $S' = 0$ and $\mathrm{Na}' \neq 0$ ($J^i_{s(S'=0)}$) (*i.e.* at negligible trans-substrate concentration, and at non-negligible trans-Na^+ concentration) is given by:

$$J^i_{s(S'=0)} = \frac{X_t(P_3 + P_4K_4/\mathrm{Na})}{1 + \frac{K_3}{S} + \frac{K_4}{\mathrm{Na}} + \frac{K_3K_1}{S\cdot\mathrm{Na}} + \frac{\mathrm{Na}' + K_5}{P_2\mathrm{Na}' + P_1K_5}\left(P_3 + P_2\frac{K_3}{S} + P_4\frac{K_4}{\mathrm{Na}} + P_1\frac{K_3K_1}{S\cdot\mathrm{Na}}\right)} \qquad \text{(A-2)}$$

or, by putting $\varepsilon = (\mathrm{Na}' + K_5)/(P_2\,\mathrm{Na}'/P_1 + K_5)$,

$$J^{\mathrm{i}}_{\mathrm{s(S'=0)}} = \frac{X_{\mathrm{t}}(P_3 + P_4K_4/\mathrm{Na})}{1 + \varepsilon\dfrac{P_3}{P_1} + \dfrac{K_4}{\mathrm{Na}}\left(1 + \varepsilon\dfrac{P_4}{P_1}\right) + \dfrac{K_3}{S}\left[1 + \varepsilon\dfrac{P_2}{P_1} + \dfrac{K_1}{\mathrm{Na}}(1 + \varepsilon)\right]} \tag{A-2}$$

Clearly, a trans effect of Na^+ on the unidirectional flux of the substrate into the cell requires that $P_1 \neq P_2$. In fact, for $P_1 = P_2$ (and thus $\varepsilon = 1$), Eqn. A-2 becomes

$$J^{\mathrm{i}}_{\mathrm{s(S'=0)}} = \frac{X_{\mathrm{t}}(P_3 + P_4K_4/\mathrm{Na}}{1 + \dfrac{P_3}{P_1} + \dfrac{K_4}{\mathrm{Na}}\left(1 + \dfrac{P_4}{P_1}\right) + 2\dfrac{K_3}{S}\left(1 + \dfrac{K_1}{\mathrm{Na}}\right)} \tag{A-3}$$

where $J^{\mathrm{i}}_{\mathrm{s(S'=0)}}$ is independent of Na'. An identical equation can be obtained, for $P_1 = P_2$, from Eqn. A-4 for the unidirectional flux of the substrate into the cell at zero trans concentrations of both substrate and Na^+ ($J^{\mathrm{i}}_{\mathrm{s(S'Na=0)}}$) by putting $\mathrm{P}_1 = P_2$. Eqn. A-4 can be derived from Eqn. A-1:

$$J^{\mathrm{i}}_{\mathrm{s(S',Na'=0)}} = \frac{X_{\mathrm{t}}(P_3 + P_4K_4/\mathrm{Na})}{1 + \dfrac{P_3}{P_1} + \dfrac{K_4}{\mathrm{Na}}\left(1 + \dfrac{P_4}{P_1}\right) + \dfrac{K_3}{S}\left(1 + \dfrac{P_2}{P_1} + 2\dfrac{K_1}{\mathrm{Na}}\right)} \tag{A-4}$$

Eqns. A-1 to A-4 are all michaelian in S. The apparent K_{t} for substrate transport for Eqn. A-2 is equal to:

$$K_3\frac{P_1 + \varepsilon P_2 + (1 + \varepsilon)P_1K_1\mathrm{Na}}{P_1 + \varepsilon P_3 + (P_1 + \varepsilon P_4)K_4/\mathrm{Na}} \tag{A-5}$$

Its apparent maximum velocity ($J^{\mathrm{i}}_{\mathrm{s(S'=0,S=\infty)}}$) is given by:

$$J^{\mathrm{i}}_{\mathrm{s(S'=0,S=\infty)}} = \frac{X_{\mathrm{t}}(P_3 + P_4K_4/\mathrm{Na})}{1 + \varepsilon\dfrac{P_3}{P_1} + \dfrac{K_4}{\mathrm{Na}}\left(1 + \varepsilon\dfrac{P_4}{P_1}\right)} \tag{A-6}$$

The apparent K_{t} and the apparent maximum velocity for substrate transport at zero trans concentrations of both Na^+ and substrate can be calculated from Eqn. A-4 itself, or from Eqns. A-5 and A-6. In fact, at zero Na', $\varepsilon = 1$. The apparent K_{t} of Eqn. A-4 is given by:

$$K_3\frac{P_1 + P_2 + 2P_1K_1/\mathrm{Na}}{P_1 + P_3 + (P_1 + P_4)K_4/\mathrm{Na}} \tag{A-7}$$

The apparent maximum velocity of Eqn. A-4 ($J^{\mathrm{i}}_{\mathrm{s(S',Na'=0;S=\infty)}}$) is:

$$J^{\mathrm{i}}_{\mathrm{s(S',Na'=0;S=\infty)}} = \frac{X_{\mathrm{t}}(P_3 + P_4K_4/\mathrm{Na})}{1 + \dfrac{P_3}{P_1} + \dfrac{K_4}{\mathrm{Na}}\left(1 + \dfrac{P_4}{P_1}\right)} \tag{A-8}$$

Comparison of Eqns. A-5 and A-6, with Eqns. A-7 and A-8 respectively, indicates how the trans concentration of Na^+ (Na′) affects the apparent K_t and the apparent maximum velocity of the unidirectional substrate flux. For Na′ to affect them, ε must be $\neq 1$. It is equal to 1 under at least one of two conditions: $Na' = 0$, or $P_2 = P_1$. For Na′ to inhibit the unidirectional flux of the substrate, $\varepsilon > 1$. The extent of this inhibition depends, therefore, on the P_2/P_1 ratio, on the trans concentration of Na^+ relative to K_5, and also, as shown by Eqn. A-6 on the P_3/P_1 and P_4/P_1 ratios.

ADDENDUM

by P. HONEGGER AND G. SEMENZA

As indicated above, the trans-Na hypothesis predicts that no inhibition of sugar uptake by amino acids should be detectable in the absence of Na^+ and K^+ at both sides of the membrane. In the experiment reported in Table I the effect of the Na^+ concentration alone was tested: intracellular Na^+ was drastically reduced by preincubating everted small intestinal rings in the cold in a Krebs–Henseleit buffer where all the Na^+ had been substituted with choline (5.4 mM K^+ was still present), and containing ouabain (data not shown). This glycoside is known to be effective if provided from the serosal side[37].

For the incubation proper, the rings were mounted in frames[38], so that the mucosal side only was exposed to the medium containing the sugar (6-deoxy-D-glucose) ± phenylalanine. The concentration of the amino acid chosen (20 mM) is known to be effective in media of standard composition[15]. Experimental details are reported in the legend of Table I.

As it can be seen (Exp. 1), at low intracellular Na^+, at very low extracellular Na^+ (traces were still detected) and in the presence of ouabain: (i) 6-deoxyglucose uptake is very much reduced; (ii) a further inhibition by phenylalanine is almost undetectable. The decrease in sugar uptake brought about by preincubation in Na^+-free medium and ouabain is not irreversible (Expt. 2).

An experiment showing the lack of an effect can seldom be given one single explanation. Ours is no exception. The increase in K_m values of substrates in the absence of Na^+; a change in allosteric properties of a polyfunctional carrier in the absence of Na^+, and possibly other mechanisms as well, could also account for the results of Table I.

Even so, these observations find a straightforward explanation in the trans-Na hypothesis.

TABLE I

EFFECT OF L-PHENYLALANINE ON 6-DEOXY-D-GLUCOSE UPTAKE IN Na^+-DEPLETED HAMSTER SMALL INTESTINE

Hamster of either sex (weight, approx. 120 g) were killed by a blow in the neck; their small intestines were excised, rinsed with cold saline, everted and cut in pieces some 2 cm long. The pieces were randomised as recommended by CRANE AND MANDELSTAM[39]. *Preincubation* (30 min at approx. 0°, under continuous gassing with O_2–CO_2 (95:5, v/v): Either in normal Krebs–Henseleit buffer, or in a buffer where all Na^+ was substituted with choline. The concentrations of all other cations, including K^+ (5.4 mM), and of the anions were the same in both media. The Na^+-free, choline-substituted Krebs–Henseleit buffer contained, in addition, 0.1 mM ouabain (Fluka). *Incubation* (4 min at 37°): After the preincubation, the everted intestinal rings were mounted in appropriate frames[38], so as to expose to the medium the mucosal side only. The incubation media had the same composition as in the preincubation step; in addition, they contained 1 mM ^3H-labeled 6-deoxy-D-glucose (Koch and Light; ^3H labeling was carried out by Amersham, and the product was purified by paper chromatography); inulin (5 mg/ml, Merck); and, in some samples, 20 mM L-phenylalanine (Fluka). At the end of the incubation, the tissues were rinsed with cold buffer, punched[38], blotted, weighted, homogenized in 1 ml $ZnSO_4$. After addition of 1 ml of corresponding $Ba(OH)_2$ (ref. 40) and centrifugation, inulin[41], Na^+ and K^+ (by flame photometry), and ^3H-labeled 6-deoxyglucose (by scintillation counting) were determined. The tissue concentrations were referred to the tissue water, assumed to be 80% of the fresh weight[39]. The uptake values of 6-deoxyglucose were corrected for the inulin space (less than 5%).

	(*n*)	*6-Deoxyglucose uptake μmoles·4 min^{-1}·ml^{-1} tissue water ± S.E.*	% ± *S.E.*
Expt. 1			
(a) Preincubation and incubation in normal Krebs–Henseleit buffer:			
6-Deoxyglucose alone	(8)	0.919 ± 0.095	100
6-Deoxyglucose + phenylalanine	(8)	0.591	67.6 ± 5.1*
(b) Preincubation and incubation in Na^+-free buffer:			
6-Deoxyglucose alone	(8)	0.049 ± 0.006	100
6-Deoxyglucose + phenylalanine	(8)	0.048	92.8 ± 7.8*
Expt. 2			
(a) Preincubation and incubation in normal Krebs–Henseleit buffer:			
6-Deoxyglucose alone	(4)	0.656 ± 0.035	100
(b) Preincubation in Na^+-free buffer, incubation in normal buffer:			
6-Deoxyglucose alone	(4)	0.493 ± 0.037	75
(c) Preincubation in normal buffer, incubation in Na^+-free buffer:			
6-Deoxyglucose alone	(4)	0.217 ± 0.039	33
(d) Preincubation and incubation in Na^+-free buffer:			
6-Deoxyglucose alone	(4)	0.030 ± 0.010	4.6

* $0.01 < P < 0.005$.

ACKNOWLEDGEMENTS

The author is indebted to Drs. F. Alvarado and J. W. L. Robinson for communicating a number of unpublished observations, to Dr. H. C. Curtius for the determinations of Na^+ and K^+, and to Dr. E. Berger for the calculations of Eqn. A-2.

The financial assistance of the Schweiz. Nationalfonds zur Förderung der wissenschaftlichen Forschung is gratefully acknowledged.

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