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Role of Substrate Binding Forces in Exchange-Only Transport Systems: II. Implications for the Mechanism of the Anion Exchanger of Red Cells

R.M. Krupka
Agriculture Canada, Research Centre, London, Ontario, Canada N6G 2V4

Summary. The transition-state theory of exchange-only membrane transport is applied to experimental results in the literature on the anion exchanger of red cells. Two central features of the system are in accord with the theory: (*i*) forming the transition state in translocation involves a carrier conformational change; (*ii*) substrate specificity is expressed in transport rates rather than affinities. The expression of specificity is consistent with other evidence for a conformational intermediate (not the transition state) formed in the translocation of all substrates. The theory, in conjunction with concepts derived from the chemistry of macrocyclic ion inclusion complexes, prescribes certain essential properties in the transport site. Separate subsites are required for the preferred substrates, Cl^- and HCO_3^-, to account for tight binding in the transition state ($K_{diss} \simeq 1$ μM). Further, the following mechanism is suggested. A substrate anion initially forms a loose surface complex at one subsite, but in the transition state the subsites converge to form an inclusion complex in which the binding forces are greatly increased through a chelation effect. The conformational change at the substrate site, which is driven by the mounting forces of binding, sets in train a wider conformational change that converts the carrier from an immobile to a mobile form. Though simple, this composite-site mechanism explains many unusual features of the system. It accounts for substrate inhibition, partially noncompetitive inhibition of one substrate by another, and "tunneling," which is net transport under conditions where exchange should prevail, according to other models. All three types of behavior result from the formation of a ternary complex in which substrate anions are bound at both subsites. The mechanism also accounts for the enormous range of substrate structures accepted by the system, for the complex inhibition by the organic sulfate NAP-taurine, and for the involvement of several cationic side chains and two different protein domains in the transport site.

Key Words anion exchange · transport · carriers · binding forces · band 3 protein · coupled transport

Introduction

Exchange-only carrier systems exhibit coupled transport in its most elementary form. As coupling mechanisms underlie all active transport systems, in which the flow of one substrate is driven by the chemical potential of another, the study of exchange transport may offer the simplest route to an understanding of the principles involved in active transport, at the molecular level.

The fundamental insight given by the transition-state theory of exchange-only transport (Krupka, 1989) is that coupling depends on the utilization of substrate binding forces to convert the carrier from an immobile to a mobile state, and that in order to harness these forces the transport site forms an initial complex with the substrate, which is loose, and a transition-state complex, which is tight. A coupling mechanism dependent on charge neutralization by a bound substrate ion could be ruled out. An alternative mechanism, which can account for coupling with both charged and neutral substrates, depends on a conformational change rather than charge neutralization in the transport protein. In this case, the transition state in carrier reorientation has an altered conformation stabilized by substrate binding forces.

In the following sections, the behavior of the anion exchanger of red cells is first shown to be consistent with some of the implications of the theory. A mechanism is then suggested for transport, based on the transition-state theory together with findings from the field of host-guest chemistry on the strength and specificity of macrocyclic ion inclusion complexes. The red cell system, having been intensively studied in many laboratories, provides a ready test of the new hypothesis. The mechanism is found to provide a simple explanation for various seemingly unrelated properties of the anion exchanger.

The Expression of Substrate Specificity

The kinetic theory of exchange-only transport outlined in the preceding paper predicts that substrate

specificity could be expressed in several ways. In the most likely case, it is seen in the maximum transport rate, not affinity. In a special case, where the rate for different substrates depends on how well they induce the formation of a transport intermediate and where a good substrate converts the carrier largely to this form, specificity would be seen in affinity alone, or both rate and affinity. The experimental observations accord with the first case (*see* Knauf, 1979). The physiological substrates Cl^- and HCO_3^- are transported rapidly, and at comparable rates (Lowe & Lambert, 1983). Other halide ions are more slowly transported, fluoride and bromide at about 1/10 the Cl^- rate, and iodide at about 1/250. Sulfate and phosphate are very slowly transported, about 1/10,000 as fast (at the pH maximum for sulfate, 6.4, the fraction is roughly 1/4600 (Brahm, 1977; Kaufmann, Eberl & Schnell, 1986). In contrast, the half-saturation constants for all the ions are similar, between 10 and 90 mM. The values for Cl^- and HCO_3^-, 65 and 43 mM, respectively, fall in the middle of this range. Again, for a group of phosphate analogs the maximum flux varies by a factor of 1300 but the half-saturation constant by a factor of only 5 (from 24 to 124 mM) (Lobatka & Omachi, 1987).

The basis of selectivity is not size alone, for bulkier ions are transported, though slowly; examples are tetrathionate (Deuticke et al., 1978), benzenesulfonate, phthalate, *p*-aminobenzenesulfonate (Aubert & Motais, 1975), pyridoxal phosphate (Nanri, Hamasaki & Minakami, 1983), and even NAP-taurine (Cabantchik et al., 1976; Rothstein, Knauf & Cabantchik, 1977; Knauf et al., 1978*a*). Hence the breadth of the channel through which the substrate is conducted would not seem to determine the specificity for ions no larger than sulfate or phosphate. Something else is probably responsible, which from the theoretical analysis would be the ability of the substrate to stabilize the transition-state in carrier reorientation (Krupka, 1989).

Conformational Changes Related to Carrier Mobility

Canfield and Macey (1984) have produced evidence for an extensive conformational change in the transport protein as it passes through the transition state in carrier reorientation, and Ginsburg, O'Connor and Grisham (1981) have detected an intermediate in carrier reorientation having an altered conformation. First the evidence on the transition state: the activation volume for sulfate exchange is large and positive, too large to be explained either by the formation of an ion complex, by subunit interactions in the Band 3 protein, or by reversible denaturation. The conclusion, therefore, was that a conformational change affecting a large part of the carrier protein occurs in the transition state (Canfield & Macey, 1984).

A stable intermediate in transport having an altered conformation was detected by EPR spectroscopy, with a spin-labeled derivative of the anion-carrier protein (Ginsburg et al., 1981). The extent of the conformational change was directly related to the transport activity of the system at varying pH. Also, the altered conformation was induced by Cl^- binding to the carrier, with a half-saturation constant similar to that for transport. Both observations argue that the altered carrier form is an intermediate in transport. At saturating Cl^- concentrations, 1/4 of the carrier was converted to this form, but with different substrates the fraction in the altered form did not determine the maximum transport rate. With sulfate, for example, the conformational change was 40% as large as with Cl^-, though the maximum flux is about 5000 times lower. These findings—only partial conversion of the carrier to the intermediate by a good substrate, and comparable conversions by good and poor substrates—agree with the finding that substrate specificity is expressed in the maximum rate of transport and not affinity (*see* the previous section and Krupka, 1989).

Substrate Binding Forces in the Transition State

The ratio of exchange transport to slippage, $\bar{\bar{V}}_S/\bar{V}_{Si}$, which is required to calculate K_{ts}, the virtual dissociation constant in the transition state (Krupka, 1989), is estimated to be 4×10^4 for Cl^- at 25°C (Fröhlich, 1984; Fröhlich & King, 1987). The half-saturation constants for Cl^- and HCO_3^- are roughly 50 and 30 mM, respectively at 25°C (Lowe & Lambert, 1983). K_{ts}, equal to $\bar{K}^S_{So}\,\bar{V}_{Si}/\bar{\bar{V}}_S$ (where $\bar{K}^S_{So}$ is taken to be the experimentally measured K_m value), is therefore about 1.3 μM for Cl^- and 0.8 μM for HCO_3^-.

Whereas a broad range of structures is bound with similar affinity in the initial complex, the structural requirements for interaction in the transition state are severe. A comparison of HCO_3^-, $HPO_3^=$ (phosphite), $H_2PO_2^-$ (hypophosphite), and $H_2PO_4^-$ (phosphate) is instructive. The first three anions are of similar size, and contain oxygen, which is presumably involved in the binding interactions, and yet the rates of transport (at the optimum pH and 37°C) fall in the order 1 : 1/300 : 1/500 (Lobatka & Omachi, 1987, 1988). The figure for phosphate is 1/

12,000 (Brahm, 1977; Kaufmann et al., 1986). The half-saturation constants for the ions are 43, 59, 99 and 52 mM, respectively. Thus phosphite and hypophosphite are bound about as well as HCO_3^- in the initial complex but several hundred times more weakly in the transition state (*see* Table 1). Phosphate, slightly larger, has the same affinity in the initial complex but 12,000 times lower affinity in the transition state.

A comparison of ortho, meta and para substituted benzene sulfonates (Aubert & Motais, 1975) is also informative. Here the affinity and transport rate are inversely related, suggesting that if the intrinsic binding forces are realized in the initial complex they are unavailable to stabilize the transition state in carrier reorientation. The high affinity and negligible transport rate of certain large organic anions may be explained in the same way, as in the case of stilbene disulfonates such as H_2DIDS, which has a half-saturation constant of 0.1 μM (Shami, Rothstein & Knauf, 1978) but is not transported.

Table 1. Calculated dissociation constants for various substrates in the transition state,[a] based on experimental half-saturation constants (K_m) and maximum exchange rates (V)[b]

Substrate	K_m(mM)	V (relative to Cl^-)	Calculated K_{ts} (mM)
Cl^-	65	1.0	0.0016
F^-	88	0.097	0.023
Br^-	32	0.12	0.0067
I^-	10	0.004	0.063
HCO_3^-	43	1.1	0.0010
$HPO_3^=$	59	0.0037	0.40
$H_2PO_2^-$	99	0.0022	1.13
$SO_4^=$	40	0.00022	4.5
$HPO_4^=$	52	0.000083	16

[a] The dissociation constant in the transition state is calculated as $K_{ts} = K_m \overline{V}_{Si}/\overline{\overline{V}}_S$, where $\overline{V}_{Si}$ and $\overline{\overline{V}}_S$ are the maximum rates of net exit and exchange, respectively (Krupka, 1989). Their ratio, $\overline{\overline{V}}_S/\overline{V}_{Si}$, is reported to be 4×10^4 for Cl^- (Fröhlich & King, 1987), and the ratio for the other substrates is found from the relative rates of exchange. The rate of slippage, $\overline{V}_{Si}$, a measure of the rate of movement of the free carrier, should have the same value for all the substrates. Net exit due to "tunneling" (Fröhlich, 1988) involves a different process and does not enter into these calculations.

[b] Estimates of K_m and of the maximum transport rate V for most of the substrates are taken from reviews by Knauf (1979) and Lowe & Lambert (1983). $SO_4^=$ and, $HPO_4^=$: Brahm (1977); Kaufmann et al. (1986); $HPO_3^=$ and $H_2PO_2^-$: Lobatka & Omachi (1987, 1988).

The Physiological Substrates, Chloride and Bicarbonate Ions

Considering how specific the anion exchanger is (Table 1), it is striking that the two preferred substrates, Cl^- and HCO_3^-, are so dissimilar in structure and physical properties. Cl^- and HCO_3^- appear to differ more from one another than either does from some of the poorer substrates. For example, HCl, HBr and H_2SO_4 are strong acids, whereas H_2CO_3 and H_3PO_4 are weak acids. The single negative charge is spherically distributed in Cl^- but in HCO_3^- is shared by two oxygen atoms with a fixed geometry: in this respect Cl^- resembles Br^- and F^-, while HCO_3^- resembles nitrate, sulfate and phosphate. Cl^- and HCO_3^- also differ greatly in size. The van der Waals radii are as follows: F^-, 1.35 Å; Cl^-, 1.80 Å; Br^-, 1.95 Å; I^-, 2.15 Å; HCO_3^-, 2.83 Å (the last figure is the C–O bond length plus the van der Waals radius of O). Other characteristics listed in Table 2, including the molar volume and properties related to ionic hydration, show the same trends.

Lessons From Host-Guest Chemistry

The physical principles determining the strength and specificity of ion complexes have been elucidated through studies of macropolycyclic molecules, in the field aptly named host-guest chemistry (Cram, 1988; Lehn, 1988). Such principles serve as a guide in relating the reaction sequence involved in carrier reorientation, as deduced from the transition-state theory of exchange transport, to molecular interactions that develop between the substrate and the carrier site. A macropolycyclic molecule, referred to as the "host" or "molecular receptor," can recognize and bond a smaller molecule or ion, called the "guest" or "substrate." The receptor takes the form, in the complex, of a hollow sphere or hemisphere whose surface is defined by chains of atoms, mainly carbon, together with oxygen, nitrogen or both.

The requirements for strong binding in the complex include multiple sites in the receptor that cooperatively interact with the substrate, and contact between the receptor and the substrate over a large area, best achieved when the receptor encloses the substrate completely, establishing the maximum number of noncovalent interactions. Of equal importance is preorganization of the receptor: "the more highly hosts and guests are organized for binding and low solvation prior to their complexation, the more stable will be their complexes" (Cram, 1988).

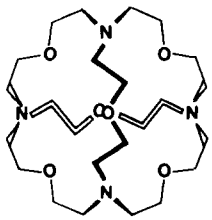
In its uncomplexed form, the flexible macrotricyclic cryptand I contains neither cavities nor binding sites. Binding is a complicated process involving

Table 2. Comparisons of some physical properties of bicarbonate and halide ions in aqueous solution

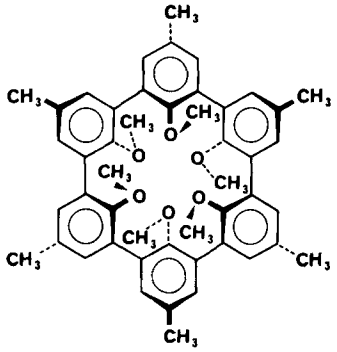
	HCO_3^-	F^-	Cl^-	Br^-	I^-
Partial molar volume ($cm^3\ mol^{-1}$)[a]	28.5	3.3	23.7	30.3	41.4
Partial molar entropy ($cal\ K^{-1}mol^{-1}$)[a]	28.2	0.5	19.0	24.9	31.2
Ionic mobility ($cm^2\ sec^{-1}V^{-1} \times 10^4$)[b]	4.61	5.74	7.91	8.10	7.97
pK*a* of conjugate acid	6.37	3.45	(−4.7)	(−7.7)	(−10.7)

[a] Conway, 1981.
[b] Chang, 1981.

desolvation, complementary steric and coulombic interaction, and conformational changes in the receptor (Lybrand, McCammon & Wipff, 1986). In the complex with K^+, I rearranges into a sphere enclosing the ion, which simultaneously interacts with six O atoms in the rings. The required ordering and desolvation of the receptor molecule detracts from the potential affinity for the substrate, and as a result completely preorganized receptors, such as the rigid spherand II, bind substrates still more tightly. Thus the affinity of II for Li^+ is 4000 times greater than the affinity of I for K^+. A still more dramatic effect of preorganization is seen in the comparison of II with its open-chain (i.e., acyclic) analog, where affinity declines by a factor of 10^{12} (Cram, 1988). The unfavorable entropy change involved in ordering the receptor molecule may account for factors of this magnitude, just as in intramolecular catalysis where free rotation around each bond between reacting groups could plausibly contribute an unfavorable factor of about 10 to the formation of the activated complex (Page & Jencks, 1971).



(I)



(II)

While preorganization contributes to binding power, complementarity between the receptor and the substrate determines specificity. For example, K^+, unlike Na^+, makes contact with all the heteroatoms (O and N) in a cryptospherand receptor and as a result is bound 10^4 times more strongly than Na^+ (Cram, 1988).

The dissociation rates of macrocyclic complexes are extremely low. The constants for Na^+ and K^+ with a macrobicyclic receptor were 30 and 40 sec^{-1}, respectively at 25°C (Lehn, 1973), and the rates for macrotricyclics, which bind the ions more tightly, should be lower still. The turnover number of the anion exchanger is much higher, about 10^4 sec^{-1} (Brahm, 1977).

Anions, of more direct concern here than cations, are also bound by macrocyclic host molecules such as I (Lehn, 1978). They add to the protonated form of the receptor, which is positively charged, whereas cations add to the unprotonated form. The tightness and specificity of the complex is determined by factors such as those outlined above. The number of rings and the size of the rings is important, so that the complex of Cl^- with the macrotricyclic I is far more stable than that with a macrobicyclic. In I, the ion fits into a closed, spherical, rigid cavity and bonds tetrahedrally with four positively charged ammonium groups, while in the bicyclic it lies half-exposed in a trough or pit. I forms the tightest complex with Cl^- found so far, with a dissociation constant of 30 μM; it binds bromide ion 1000 times more weakly and fails to bind iodide, nitrate, trifluoroacetate or sulfate (Graf & Lehn, 1976). A macrobicyclic analog of I binds Cl^- and Br^- equally, but with a dissociation constant of 20 mM. Clearly, affinity and specificity are interdependent: where the affinity is high the specificity is high, and where the affinity is low the specificity is low.

Structural Requirements in the Transport Site

The physical principles derived from host-guest chemistry have obvious implications for the design

of the substrate site in a coupled transport system. Significantly, transition-state theory and host-guest chemistry suggest a similar conclusion: the carrier first forms a loose complex with the substrate and then a tight complex, and the conversion of one to the other provides the driving force in the formation of a mobile carrier from the initially immobile state.

1. Tight binding of Cl^- and HCO_3^-, which have virtual dissociation constants in the transition state of about 1 μM, would only be achieved in some type of inclusion complex. A precedent for the idea of an enclosed state is found in other transport systems. In the choline system of red cells, half-sites matching opposite surfaces of the choline molecule appear to converge upon the substrate during transport (Devés & Krupka, 1979), and in muscle, Ba^{2+} can be trapped inside the closed K^+ channel, suggesting that the occluded form is an intermediate in passage across the membrane (Miller, 1987).

2. As a rule, affinity increases with the number of binding interactions. For example, a macrocyclic receptor with six oxygens in the rings was found to have 10^4–10^5 times greater affinity for Na^+ or K^+ than a receptor with four oxygens, as if each site increased the affinity by a factor of about 100 (Lehn, 1973). To form a very tight complex, the transport site should contain several subsites interacting with the substrate anion.

3. The rate of dissociation of an inclusion complex is far lower than the turnover number for the anion exchanger. It follows that the initial complex between the carrier and the substrate could not be an inclusion complex, which would limit the rate of dissociation of the substrate following the translocation step. Hence, the substrate site should be capable of initially forming a loose surface complex; the required inclusion complex could be formed subsequently.

4. Whereas a rigid receptor such as II preserves its structure in forming the inclusion complex, a flexible receptor such as I undergoes extensive reorganization, transforming itself into a hollow sphere in which binding sites converge upon an interior space. The behavior of the flexible receptor suggests how binding forces could induce a protein conformational change: by analogy, the substrate site in the carrier could be incompletely preorganized in the initial state, but with the substrate bound could rearrange to form an inclusion complex, accompanied by shifts in other regions of the transport protein.

5. Tight binding in the transition state (the calculated dissociation constant being about 1 μM) depends on close complementarity between receptor and substrate and, therefore, involves a high degree of specificity. The substrates, Cl^- and HCO_3^-, were seen to be unlike in many respects—in the distribution and density of charge, affinity for a proton, hydration, shape and size. Consequently, no single constellation of binding sites can be expected to form a tight complex with both. The macrotricyclic I illustrates the point, for it strongly binds Cl^- (K_{diss} = 30 μM) but fails to bind nitrate, which is isosteric with HCO_3^- (Glasstone, 1940) and is transported nearly as well (Gunn, Wieth & Tosteson, 1975). It follows that distinct binding regions in the transport site would almost certainly be required for Cl^- and HCO_3^-.

Milanick and Gunn (1984) have drawn attention to a common dimension in Cl^- and HCO_3^- that might account for their selection by the transport site: the diameter of Cl^- and the "thickness" of HCO_3^- (which is flat, with a trigonal planar structure). However, the sharp discrimination between Cl^- and nitrate by the host compound I demonstrates that high affinity depends on overall structure, not a single dimension. That the transport site is likely to contain a region specific for the planar HCO_3^- configuration is consistent with a point made by Milanick (*personal communication*) regarding oxygen-containing substrates. HCO_3^-, nitrate, phosphite and hypophosphite, with a planar configuration, it was noted, are more rapidly transported than sulfate or phosphate, with tetrahedrally disposed O atoms (*see* Table 1). But the transport site is highly sensitive to the size of the ions as well, reflecting the similar dimensions of C and N (diameter 3.36 and 3.10 Å; covalent bond length to O, 1.23 and 1.21 Å, respectively) and the somewhat larger size of S and P (diameter 3.60 and 3.70 Å; covalent bond length to O, 1.43 and 1.65 Å, respectively). Thus HCO_3^- and nitrate are transported 300–500 times faster than the planar phosphorus acids, but the latter only 10–40 times faster than the tetrahedral sulfate and phosphate (*see* Table 1). The site is without doubt sensitive to both shape and size. The more remarkable, then, that in spite of their differences Cl^- and HCO_3^- are equally received by the carrier.

A Mechanism for the Anion-Exchange Carrier

There is evidence that the anion transport mechanism corresponds to the carrier model (Cabantchik, Knauf & Rothstein, 1978; Gunn & Fröhlich, 1979, 1982; Fröhlich, 1982; Jennings, 1982*c*; Eidelman & Cabantchik, 1983; Macara & Cantley, 1983). In this mechanism, a substrate site is alternately exposed on opposite sides of the membrane; a bilateral model, with sites simultaneously exposed on both

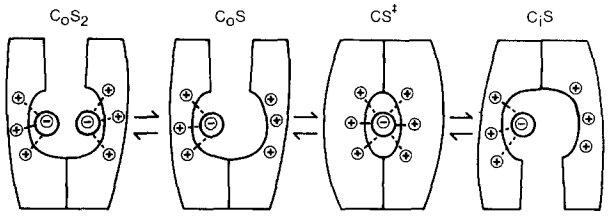


Fig. 1. A diagrammatic representation of the composite-site transport model. The carrier-substrate complex exists in outward-facing and inward-facing conformations, C_oS and C_iS respectively, as in the ordinary carrier model. The transport site contains two clusters of cationic side chains, each capable of forming an initial surface complex with a substrate anion. The two halves of the site, in which these subsites are located, may be composed of separate domains in the transport protein. In the course of carrier reorientation from one side of the membrane to the other, a transition state $CS^{\ddagger}$ is formed, in which the two halves of the site contract, allowing the subsites to interact simultaneously in an inclusion complex. The binding forces are now augmented through a chelate effect, which stabilizes the transition state, thereby increasing the mobility of the substrate complex relative to the free carrier. Consequently, the tightness of coupling of opposing substrate flows is directly related to the magnitude of the binding forces that develop in the transition state. At high concentrations, substrate anions can add to both subsites, forming a ternary complex C_oS_2 or C_iS_2 (*see* Fig. 2). Electrostatic repulsion between the ions has two effects: the second ion is more weakly bound than the first, and the transition state is formed with difficulty, making transport of the ternary complex very slow. Formation of the ternary complex accounts for substrate inhibition, partially noncompetitive inhibition between substrates, and the phenomenon called tunneling. The diagram should not be taken to imply that the transport protein is actually symmetrical, or that carrier reorientation necessarily involves a single step with no intermediates

sides, could be ruled out. The rate-limiting step in transport was shown to be carrier reorientation rather than substrate dissociation (Falke, Kanes & Chan, 1985). The new model proposed here simply builds on the carrier model by incorporating a composite transport site with individual subsites for Cl^- and HCO_3^-. The mechanism is shown diagrammatically in Fig. 1. For the sake of simplicity, the transition state, in which the composite site encloses the substrate, is represented as having a structure intermediate between the inward-facing and outward-facing carrier conformations. In forming the initial loose complex, different substrates may add indiscriminately in these binding regions, or alternatively they may add specifically at the matching subsite, halides at a Cl^- subsite and oxygen-containing anions at a HCO_3^- subsite. The available evidence, considered below in connection with NAP-taurine inhibition, favors the second possibility. In the transition state, in any case, the substrate would develop its strongest interactions at the matching subsite, though in the proposed inclusion complex the other subsite contributes to binding as it closes over the substrate. By hypothesis, Cl^- and HCO_3^- are held in the same crypt; whether the shape of the crypt is the same with either substrate need not be specified. Cl^- could be bound as a hydrate occupying about the same volume as HCO_3^-, with the O of H_2O interacting at the HCO_3^- subsite in the transition state.

Amino acid side chains with specificity for individual anions seem likely to be present at the Cl^- and HCO_3^- subsites. For example, the guanidine group of arginine can form two hydrogen bonds with a carboxylate ion (Lehn, 1988), and the positive charge in the imidazole ring of histidine, which is shared by two ring N atoms, might interact with the negative charge shared by two O atoms in HCO_3^-. A cluster of lysine ammonium groups could specifically bind Cl^-, by analogy with the tight macrocyclic complex of I in which Cl^- is bonded to four tetrahedrally placed ammoniums. Hydrogen bonds with neutral groups such as –NHCO– and –COOH could also participate.

With respect to the tertiary structure of the protein, separate subdomains in the folded polypeptide chain are likely to form the two halves of the proposed composite transport site. The region between domains is a dynamic location in proteins, probably because of the flexibility of the structure at this point (Creighton, 1984). Hexokinase, in which the substrate is bound between adjacent domains, may be a helpful model, for following binding the enzyme undergoes a conformational change in which the two domains swing together to enclose the substrate (Bennett & Steitz, 1980).

Though the proposed transport mechanism is built on the carrier model, it is the functional implications of the composite site, not the carrier model, that are to be tested here. A site with the same properties, if incorporated into another transport model, should have similar effects.

The Structure of the Transport Site

Evidence for two separate cationic subsites within the anion transport site has been summarized by Macara and Cantley (1983). Both arginine (Wieth, Bjerrum & Borders, 1982; Julien & Zaki, 1987) and lysine (Jennings, 1982*b*) have been shown to be present. Moreover, two lysine residues associated with the transport site have been reported to be part of a cluster of at least five basic amino acid residues; the positive charge in the vicinity of the lysines reduces their pK_a values to 8.5 and 8.8 from a normal value of about 10.5, showing that the groups are in close proximity (Passow et al., 1979). The electrostatic repulsion between the ammonium ions

could be relieved either by loss of a hydrogen ion, accounting for the fall in pKa, or by gain of an anion from solution, for which the affinity will be unusually high. Other workers (Brock, Tanner & Kempf, 1983) demonstrated a group of six cationic amino acids in a section of the protein involved in transport. These residues were divided into two clusters with sequences Arg-Lys-Phe-Lys and Lys-Leu-Arg-Arg. The clusters were separated by seven residues including serine, asparagine and tyrosine, all of which could conceivably form hydrogen bonds with a substrate anion. Such findings support the suggestion, based on the structures of macrocyclic ion-inclusion complexes, that four to six cationic groups should simultaneously interact with the substrate in the transition state. The involvement of several lysine residues at the transport site could explain how modification of one or two of these groups, by reagents that leave the positive charge intact, only partially blocks transport (Jennings 1982*a*, 1982*b*; Jennings et al., 1985).

A dominant theme emerging in structural studies of the anion exchanger is the complexity of the binding sites. For example, inhibitors fall into three classes, those interacting with the transport site, with the channel leading to this site, or with an altered carrier form believed to be an intermediate in translocation (Falke & Chan, 1986). Arginine-specific reagents and stilbene disulfonates react at sites within the transport site, which are distinct but which interact with one another (Julien & Żaki, 1988). Of special interest is the finding that certain inhibitory sites encompass two separate subdomains in the transport protein (Salhany, Rauenbuehler & Sloan, 1987). All twelve membrane-spanning helices in the polypeptide chain are contained in these subdomains, five in one and seven in the other (Kopito & Lodish, 1985). Such observations—on distinct sites involved in transport, on interactions between the sites, and on interacting subdomains in the transport protein—are clearly in accord with the composite-site transition-state model.

The Diversity of Substrates

As Stein (1986) has remarked, the anion exchanger handles an enormous range of substrate structures, from the tiny fluoride ion to much larger molecules containing two separate negative charges, such as benzylmalonate and tetrathionate. The composite-site model offers a simple explanation. In the open conformation, with the subsites separated, the transport site should accommodate anions far larger than the natural substrates. To be translocated, however, the ions would have to be enclosed within converging segments of the site. If the substrate's size or charge interferes with this contraction, the carrier might attain a partially mobile conformation. Indeed, the larger anions are only slowly transported. Phosphate, for example, moves about 10,000 times more slowly than Cl^-, little better than slippage.

Even large molecules containing two sulfate groups, such as the stilbene disulfonates, would not be debarred from entering the transport site in its open conformation. Assuming the two anionic substituents interact simultaneously with cationic groups in the transport site (Cabantchik, 1983) while the rest of the molecule enters into favorable nonpolar interactions, the high affinity and specificity of these compounds as inhibitors (Stein, 1986) would be understandable. Their size would prevent the site from closing to form the mobile transition state. Clearly, the lack of selectivity for substrates is only apparent, being a property of the transport site in its open conformation, when the carrier is immobile, and not of the site in the crucial transition state in carrier reorientation.

Substrate Inhibition

A transport site made up of two cationic subsites should be able to bind two substrate anions at the same time, and if the ternary carrier-substrate complex is only slowly transported, substrate inhibition would be predicted. Indeed substrate inhibition is characteristic of the anion exchanger and is seen with all substrates, good and poor (Knauf, 1979).

There is little doubt that a ternary complex of two substrate anions should be relatively immobile, since doubly charged anions like sulfate and phosphate are transported at a very low rate. In the proposed mechanism (Fig. 1), a single substrate anion is sandwiched between two sections of the transport site in the mobile binary complex; in a ternary complex, electrostatic repulsion between the substrate ions should hinder the approach of the half-sites. Electrostatic repulsion should also reduce the affinity for the second anion, and therefore, rates should only decline at relatively high substrate concentrations, as is the case.

The observed mixed competitive and noncompetitive inhibition of one substrate by another (Dalmark, 1976) is also predicted by this mechanism. Mixed inhibition results if addition of the substrate anion to the transport site reduces but does not abolish affinity for a second anion.

Substrate inhibition has usually been explained by a separate modifier site, outside the transport

site (Dalmark, 1976; Knauf et al., 1978*a*,*b*). The normal function of this site would be problematical, since substrate inhibition is seen only at nonphysiological concentrations. Macara and Cantley (1981) have already proposed an alternative. Their mechanism for substrate inhibition was similar to that suggested here, though the transport mechanism was different, particularly in the role assigned to substrate binding forces in carrier activation.[1]

Leaks: Slippage and Tunneling

With a substrate present on only one side of the membrane, any real carrier system must allow net transport to occur, for net transport, which in exchange systems is called slippage, depends only on movement of the free carrier. If the free carrier were completely immobile (and the rate of slippage zero), an infinitely strong binding force with the substrate would be required to convert the carrier to the mobile form (Krupka, 1989). Consequently even exchange would be impossible. Thus if exchange is possible, net transport is possible too.

According to the ordinary carrier model, the rate of slippage, because it depends on the return of the free carrier, should decline when substrate is added on the opposite side of the membrane. Here the carrier returns in the form of the substrate complex rather than free. At a sufficiently high substrate concentration, exchange should predominate and net transport should be eliminated. Instead, net transport is observed to persist. This unexpected flow of substrate is inhibited in a specific manner by stilbene disulfonates, showing that the anion exchanger is responsible (Kaplan, Pring & Passow, 1983; Knauf, Law & Marchant, 1983; Fröhlich, 1984).

To explain the behavior, Knauf and Fröhlich and their co-workers proposed that the substrate can move through the closed carrier channel, somehow circumventing the normal barrier, and without any carrier conformational change occurring. The phenomenon has been called "tunneling" (Fröhlich, 1988). To explain all the observations, it was also necessary to postulate that the substrate enters the channel at both the open end, where the substrate site is exposed, and at the closed end, where it is not; paradoxically, substrate exit seemed more rapid through the outward-facing than the inward-facing carrier (Fröhlich, 1988). In this mechanism, substrate on either side of the membrane binds to both the inward-facing and outward-facing carrier forms, but here the interpretation conflicts with evidence for the carrier model in which a substrate site is exposed on only one side at a time. A bilateral model, with sites exposed on both sides at once, had already been ruled out (*see above*).

A far simpler solution to the problem of "tunneling" is offered by the composite-site model, which actually predicts the observed behavior. "Tunneling" simply depends on a low rate of translocation of the ternary complex containing two substrate anions. Slow transport of this complex is expected, considering that doubly charged anions such as phosphate are slowly transported (Schnell & van der Mosel, 1979), as well as the still larger tetrathionate (Deuticke et al., 1978), with its two negative charges separated by 6–7 Å. "Tunneling" and substrate inhibition are, in this view, expressions of the same property of the transport site, the ability to bind two substrate anions at once.

The mechanism would be as follows. In net exit experiments, the internal substrate concentration is necessarily higher than that outside, and the ternary complex is, therefore, more abundant inside than outside. Translocation of the internal ternary complex brings about the exit of two substrate ions. The carrier tends to return as the binary complex, bearing a single substrate ion, with a net exit of one anion per cycle.[2]

The hypothesis can be tested by examining the predictions of a rate equation based on the composite site model (Fig. 2):

$$\frac{v}{C_t} = \frac{\left(f_2 + 2f_3 \frac{[S_o]}{K_o}\right)\frac{[S_o]}{K_{So}} M - \left(f_{-2} + 2f_{-3}\frac{[S_i]}{K_i}\right)\frac{[S_i]}{K_{Si}} N}{\left\{1 + \frac{[S_o]}{K_{So}}\left(1 + \frac{[S_o]}{K_o}\right)\right\} M + \left\{1 + \frac{[S_i]}{K_{Si}}\left(1 + \frac{[S_i]}{K_i}\right)\right\} N} \quad (1)$$

[1] In the mechanism of Macara and Cantley (1981), the substrate's path inward does not reverse its path outward, one cationic site being involved in entry and the other in exit. In this respect, the model is certainly incorrect, for it violates the principle of microscopic reversibility (*see* Krupka et al., 1966). Both sites or neither would have to be involved in both directions, a requirement not easily reconciled with the proposed gating mechanism. Aside from this, the mechanism appears to make contradictory demands on the effects of the postulated ionic forces. The gate, containing an anionic side chain, is held firmly shut by electrostatic bonding to one of two cationic sites, and is allowed to open only when a substrate anion binds at the same site, displacing the gate. To do so, the substrate should interact with the cationic site about as strongly as the gate does. Paradoxically, the substrate, once having displaced the gate, readily dissociates, as is necessary for transport, whereas, to account for tight coupling, the gate anion must be assumed not to dissociate.

[2] Gutknecht and Walter (1982) have pointed out that "tunneling" could result from the exchange of ternary and binary substrate complexes, as in the present model, but the carrier mechanism giving rise to this behavior was not considered.

Here, v is the transport rate, and C_t the total carrier concentration; also,

$$M = f_{-1} + ([S_i]/K_{Si})\,(f_{-2} + f_{-3}[S_i]/K_i)$$
$$N = f_1 + ([S_o]/K_{So})\,(f_2 + f_3\,[S_o]/K_o).$$

To decide whether Eq. (1) agrees with the observed behavior it will be helpful to reduce it to a simpler form applying to particular conditions. In the case of exit from cells containing a high substrate concentration into a suspending medium containing a much lower substrate concentration, we can set $[S_i]/K_{Si} \gg 1$ and $[S_i] \gg [S_o] \ll K_o$. Also, the binary substrate complex is far more mobile than the free carrier in an exchange-only system, and as the ternary complex too is relatively immobile, f_2, $f_{-2} \gg f_1, f_{-1}, f_3, f_{-3}$ (*see* Fig. 2). The equation then becomes:

$$\frac{v}{C_t} \simeq \frac{f_1 f_{-2} + f_{-3}\dfrac{[S_i]}{K_{Si}}\left(2f_1 + f_2\dfrac{K_{Si}[S_o]}{K_i K_{So}}\right)}{f_{-2} + f_2\dfrac{[S_o]}{K_{So}} + \dfrac{[S_i]}{K_i}\left(f_1 + f_{-3} + f_2\dfrac{[S_o]}{K_{So}}\right)}. \qquad (2)$$

Equation (2) shows that even if the external substrate is present at a low concentration ($[S_o] \ll K_{So}$) it can affect the net exit rate, for with $f_2 \gg f_1$ or f_{-3} the terms in $[S_o]$ in the expressions $(2f_1 + f_2 K_{Si}[S_o]/K_{So}K_i)$ and $(f_1 + f_{-3} + f_2[S_o]/K_{So})$ can still be important.[3] Equation (2) also shows that if the ternary complex is immobile ($f_{-3} = 0$), the net rate falls to zero as $[S_o]$ increases; i.e., there is no tunneling. With $f_{-3} > 0$, the rate begins to level off at concentrations of S_o high enough to saturate the carrier and form the binary complex C_oS, but not high enough to form the ternary complex C_oS_2 ($K_o \gg [S_o] > K_{So}$). This is the behavior observed. From Equation (2) the rate should level off when

$$\frac{v}{C_t} \simeq \frac{f_{-3}}{1 + K_i/[S_i]}. \qquad (3)$$

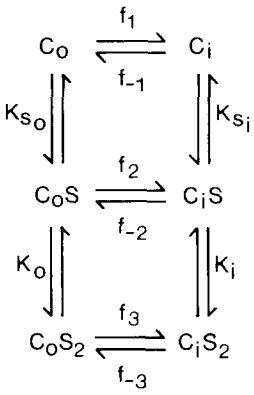


Fig. 2. Kinetic scheme for the composite-site carrier model in which a binary carrier-substrate complex, C_oS or C_iS, is formed at ordinary substrate concentrations, and a ternary complex, C_oS_2 or C_iS_2, at high concentrations. This scheme accounts for many of the experimental observations, provided that the binary complex is by far the most mobile species and the ternary complex is more mobile than the free carrier ($f_2, f_{-2} \gg f_3, f_{-3} > f_1, f_{-1}$)

At still higher concentrations of the external substrate, where the ternary complex C_oS_2 becomes important, the rate of net transport should of course decline; for when $[S_o]$ becomes sufficiently large the numerator of Eq. (1), and therefore the rate, is reduced to zero.

For the sake of clarity, the implications of the model should be pursued a little further. The equations predict that intermediate concentrations of the external substrate ($K_o \gg [S_o] > K_{So}$) could either depress the net exit rate (as observed), or elevate it, depending on the level of $[S_i]$ and on the relative sizes of f_1 and f_{-3}. To see why, we note that in the absence of external substrate the exit rate, from Eq. (2), would be

$$v/C_t \approx f_1. \qquad (4)$$

Then, as substrate is added outside the rate is altered, leveling off at the value given in Eq. (3). If $[S_i]$ is too low to convert more than a small fraction of the carrier to the ternary complex C_iS_2, the rate (given by Eq. (3)) should fall as external substrate is added: if $[S_i] \ll K_i$ the effect is to depress the rate to nearly zero. But if $[S_i]$ is high enough to convert all the inward-facing carrier to the ternary complex ($[S_i] \gg K_i$), adding external substrate brings the rate to $v/C_t \approx f_{-3}$ (Eq. (3)); under these conditions the rate rises if f_{-3} if larger than f_1, and falls if it is smaller.

In other experiments, in which the internal substrate concentration was varied over a wide range, the net exit rate increased linearly (Knauf et al., 1983), possibly up to a maximum (Fröhlich, 1984).

[3] For technical reasons, it is almost impossible to reduce the external substrate concentration to zero. In the experiments of Fröhlich (1984) for example, the concentration was lowered by suspending the packed cells in either 50 or 100 volumes of saline solution. If the cells were loaded in 150 mM Cl^- and suspended in 100 volumes of pure saline solution, and if the extracellular volume in packed cells is 4% of the total, the final extracellular concentration would be 0.06 mM. With a K_m for Cl^- of about 40 mM at 25°C, the rate of transport inward would be roughly $v = V/(1 + K_m/[S]) = V/670$. Given that the exchange rate (V) is 4×10^4 times larger than the rate of slippage, the substrate complex would then move inward $4 \times 10^4/670 = 60$ times faster than the free carrier. In the experiments of Knauf et al. (1983), the dilution was 10-fold greater, and the factor would be 6 instead of 60. In experiments in which the internal concentration is raised, these factors would be proportionately larger.

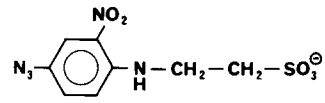
The intercept on the v axis was low, implying, as Fröhlich pointed out, that the rate of slippage, the intercept in this plot, is much lower than the rate of tunneling. Setting $f_{-3} \gg f_1$ (where f_1 governs the rate of slippage and f_{-3} the translocation of the ternary complex), and given that the term $f_2 K_{Si}[S_o]/(K_i K_{So})$ is large[3] relative to $2f_1$, Eq. (2) reduces to a form in agreement with the observed behavior:

$$\frac{v}{C_t} \simeq \frac{f_2 f_{-3}\,[S_i][S_o]/(K_i K_{So})}{f_{-2} + [S_i]\,(f_{-3} + f_2\,S_o/K_{So})/K_i}. \qquad (5)$$

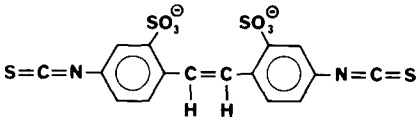
A finding predicted by the ternary complex model, but not by the earlier "tunneling" mechanism, involves a comparison of the rates of net exit of several substrates. At a fixed concentration, the rate was correlated with the substrate inhibition constant rather than the K_m value in exchange transport, which is understandable if substrate inhibition and net exit depend on the same ternary complex. The results were as follows. The permeabilities for HCO_3^-, Cl^-, Br^-, and I^- were in the ratio 1 : 1.3 : 1.9 : 6.5 (Hunter, 1977) and the corresponding substrate inhibition constants (i.e., the constants for noncompetitive inhibition) were 585, 335, 160 and 60 mM (Dalmark, 1976). For comparison, the half-saturation constants in exchange are 16, 65, 32 and 10 mM, respectively (Knauf, 1979). Net exit is seen to be related to the formation of the ternary, not the binary, complex.[4]

Inhibition by NAP-Taurine and Stilbene Disulfonates

NAP-taurine (III) and stilbenedisulfonates (IV) are powerful inhibitors of the anion exchanger, whose inhibition is initially reversible; in time isocyano-



(III)



(IV)

stilbene disulfonates react covalently with the carrier, and NAP-taurine does so following photoactivation of the arylazide group. The ordinary carrier model fails to explain the complex behavior of NAP-taurine, which may be summarized as follows. Inside the cell NAP-taurine inhibits Cl^- exchange competitively (Knauf et al., 1978*a*). Outside, it inhibits Cl^- exchange noncompetitively (Knauf et al., 1978*a*,*b*) but sulfate exchange competitively (Fröhlich & Gunn, 1982). Further, it competes with Cl^- for the substrate inhibition site (Dalmark, 1976; Knauf et al., 1978*a*). NAP-taurine is a slowly transported substrate (Cabantchick et al., 1976; Rothstein et al., 1977; Knauf et al., 1978*a*) and, therefore, despite its noncompetitive inhibition of Cl^- transport, must be capable of adding to the transport site on both sides of the membrane. Unlike NAP-taurine, stilbene disulfonates inhibit Cl^- transport competitively, and from this and other evidence appear to be bound at the substrate site

[4] The observation that inhibitors can have different effects on exchange and net transport has been cited as evidence that tunneling does not involve a carrier conformational change (Fröhlich, 1988). Actually, such behavior can be explained by the carrier model. A reversible inhibitor like phloretin, which exists partly as an anion at neutral pH (pKa = 7.3; Jennings & Solomon, 1976), should inhibit exchange more than net exit, as observed, if it is slowly transported. Its transport is to be expected, in view of the wide range of organic anions accepted by the system. After rapidly equilibrating across the cell membrane by simple diffusion (Jennings & Solomon, 1976; Fannin et al., 1981), phloretin could hypothetically add to both the inner and outer carrier sites. By adding to the inner, it would reduce the rate of outward Cl^- translocation in both exchange and net exit experiments. Binding to the outer site would have different effects in the two experiments, however. In exchange, the effect is the same as on the inside, reducing the translocation rate. In net exit, where external Cl^- is absent, phloretin would add to the outer carrier form and be translocated inward. Now net exit is rate limited by return of the carrier, outside to inside, which would be faster if the carrier is in the form of a substrate complex with phloretin than if free, and as a result the inhibition of net exit by internal phloretin would be diminished. In fact, if the carrier-phloretin complex were translocated rapidly enough, phloretin could accelerate net exit, even though it inhibited exchange. Such effects of membrane-permeant substrate analogs have been explored by Devés and Krupka (1987). An interpretation of this kind is not affected by the observation that the kinetics of phloretin inhibition are mixed competitive and noncompetitive with respect to external Cl^- (Fröhlich & Gunn, 1982, 1987), for here external substrate cannot compete with internal phloretin, and a degree of noncompetitiveness is expected (*see* Krupka & Devés, 1983). Besides, the present model suggests that two transported substrates can add to the carrier site at the same time, making the inhibition of one by the other noncompetitive. Even an irreversible inhibitor, such as NAP-taurine, could inhibit exchange more than net transport, if it allows the carrier to undergo reorientation slowly. Here we would have to assume that one of the steps in carrier reorientation is hindered in the derivative with the inhibitor, and that this hindered rate is faster than the rate-limiting step in translocation of the free carrier, but slower than the rate-limiting step in translocation of the Cl^- complex. Exchange would then be slowed, with little effect on net exit. The SH reagent tetrathionate may be an example of such an inhibitor, since it noncompetitively inhibits glucose transport in red cells, but reduces the rate by only 25% (Krupka, 1985).

(Knauf, 1979). The two inhibitors compete for the carrier (Macara & Cantley, 1981; Fröhlich & Gunn, 1982) and covalently label the same 17K peptide in Band 3, suggesting that they are bound close together (Knauf et al., 1978*b*).

In the past, the inhibition by NAP-taurine has usually been explained in terms of a modifier site, separate from the transport site, on the external surface of the membrane. The binding of NAP-taurine at this site was taken to explain its noncompetitive inhibition of Cl^- transport, while substrate binding at the same regulatory site explained substrate inhibition. The hypothesis is awkward for several reasons. It fails to explain why inhibition, though noncompetitive with one substrate, is competitive with another. It fails to explain why NAP-taurine competes with stilbene disulfonates, which are believed to bind at the substrate site, and why both inhibitors are covalently bound to the same region of the carrier. It fails to explain how NAP-taurine can be a transported substrate. Finally, the physiological role of the regulatory site is perplexing because, as noted above, Cl^- and HCO_3^- only inhibit at nonphysiological concentrations.

The composite-site carrier model could offer an explanation for the behavior, provided that, in the initial complex, Cl^- adds preferentially at one subsite and oxygen-containing anions at the other. That is, Cl^- and HCO_3^- are assumed to add directly to the region of the transport site with which they interact most strongly in the transition state. The single negatively charged sulfate substituent in NAP-taurine should then interact at the HCO_3^- subsite, which has a preference for oxygen-containing anions. As NAP-taurine is bound 1000 times more strongly than sulfate, it must also attach outside this site, probably in an adjacent nonpolar region. The sulfate ion should also be bound at the HCO_3^- subsite and would compete with NAP-taurine. Cl^-, if preferentially bound at the other site—the Cl^- subsite—could fail to compete with NAP-taurine. On this hypothesis, NAP-taurine adds at the transport site rather than elsewhere and can, therefore, be transported, though very slowly. Stilbene disulfonates, which contain two negative charges separated by about 10 Å, may interact at both subsites (Cabantichik, 1983), and as a result would be competitive with all substrates, as well as with NAP-taurine.

Broadly speaking, therefore, the composite-site mechanism provides a basis for explaining the results. Other puzzling aspects of the behavior could also be explained, for example the observation that while the interaction between Cl^- and NAP-taurine is strictly noncompetitive (Knauf et al., 1978*a*), that between Cl^- and HCO_3^- is mixed competitive and noncompetitive (Dalmark, 1976). If the conformational change induced by substrates (Ginsburg et al., 1981) involves some initial drawing together of the subsites, resulting in increased electrostatic replusion, this would hinder addition of the second substrate anion after addition of the first. Mixed competitive and noncompetitive inhibition would result. If NAP-taurine induces some different conformational change, addition of Cl^- at the other site could be unhindered, making the inhibition purely noncompetitive. It would be unprofitable to speculate further, but it may be noted that such hypotheses give rise to testable predictions.

NAP-taurine must interact differently at the inner and outer surfaces of the membrane, since it is competitive inside and noncompetitive outside, with respect to the Cl^- flux. At the inner site, but not at the outer, NAP-taurine presumably extends toward the Cl^- subsite from its anchoring point in the HCO_3^- subsite, causing the inhibition to be competitive. There is no doubt that the inner and outer sites do differ substantially. Certain stilbene disulfonates are bound inside but not outside (e.g., 4,4′-diacetamido-stilbene-2,2′disulfonic acid), while many others are bound on both sides (e.g., 2-(4′-aminophenyl)6-methyl-benzene thiazole-3′,7-disulfonic acid) (Zaki et al., 1975). Besides, the system has a 15-fold asymmetry in Cl^- transport (Gunn & Fröhlich, 1982). The observations argue for some rearrangement of the substrate binding region during carrier reorientation. Dissimilar specificity at inner and outer transport sites is seen in other systems, for example the glucose and choline carriers of red cells, where even close analogs of the substrate may be bound exclusively on one side of the membrane (Barnett, Holman & Munday, 1973; Devés & Krupka, 1984).

Concluding Remarks

In working towards a molecular mechanism for the anion exchanger we are guided by transition-state theory and host-guest chemistry, as discussed above, and also by the principle of microscopic reversibility, which tells us that under the conditions of exchange transport the inward pathway traversed by the substrate and the outward pathway must be identical (*see* Krupka, Kaplan & Laidler, 1966). These principles have combined to suggest the broad outlines of a mechanism for the anion exchanger. The mechanism either predicts, or gives a simple explanation for, many features of the system. It explains the way in which substrate specificity is expressed and the wide diversity of substrates. It predicts the occurrence of substrate inhibition and noncompetitive inhibition, and the

phenomenon called tunneling. It calls for a carrier conformational change in the transition state. It requires a complex structure in the transport site, involving multiple subsites. In addition, it shows how the interaction of the substrate at these sites can induce the conformational change involved in transport.

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