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The Kinetic Approach In Studies Of Mediated Membrane Transport

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THE KINETIC APPROACH IN STUDIES OF MEDIATED MEMBRANE TRANSPORT

By

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To my grandfather for his

example of total dedication to science

ABSTRACT

The objective of this work is to formulate kinetic tests capable of establishing the elementary features of transport mechanisms. A theoretical analysis and its experimental application to the choline and glucose transport systems of human erythrocytes is presented.

1. From a new kinetic analysis of the reversible inhibition of transport, both competitive and non-competitive kinetics are shown to be a normal consequence of a competitive inhibition mechanism.

2. Once these special features of transport kinetics are recognized, reversible inhibition studies can give new kinds of information about transport systems, regarding relative inhibitor affinity on the two membrane surfaces, inhibitor penetration into the cell, and rate-limiting steps in transport.

3. Rejection criteria are formulated for each of five distinguishable transport models, only one of which is of the classical carrier type.

4. A simple test is presented to distinguish two fundamentally different kinds of transport mechanism: (a) those with substrate binding sites exposed simultaneously at both membrane surfaces, and (b) those with a substrate site that is exposed alternately at the two surfaces. The test is applied to the glucose and choline systems, and in both cases the first mechanism is unambiguously rejected.

5. A simple method is described for determining affinities

and maximum transport rates for unlabeled substrate analogs. The method is based on the trans effect of the analog on the movement of a single labeled substrate, and is shown by kinetic analysis to be applicable to any transport system.

6. Affinities and maximum transport rates are determined for a series of 17 choline analogs. The structural requirements of the two main steps in transport - binding at the carrier site and translocation through the membrane - are strikingly different. Whereas affinity is very high for some analogs with long alkyl substituents on the nitrogen atom, the translocation step is seriously retarded by even the slightest enlargement of the substrate, relative to choline. Reduction in substrate size, however, has little or no effect on the rate. In binding, the substrate is only partly enclosed, whereas during translocation it is completely enclosed by complementary surfaces in the carrier.

7. Irreversible inhibitors are shown in theory to be potentially important experimental tools, and are applied in studies of the choline transport system. Analysis of the inactivation of transport by the inhibitor N-ethyl maleimide in the presence of a variety of substrate analogs leads to the following conclusions:

(a) Separate inward- and outward-facing carrier forms, having different conformations, are detected.

(b) When undisturbed, the carrier is usually about equally distributed between these two forms, but the distribution varies in different cell samples.

(c) The conformation of the carrier changes when the substrate is bound.

(d) The substrate specificities of the inward- and outward-facing carrier forms are different, and the difference depends on parts of the site that interact directly with choline, the normal substrate. Hence two physically distinct sites may be suggested.

(e) Dissociation of substrate is a rapid, and not rate-limiting, step in transport.

8. All the experimental evidence combines to support a model having the behavioral features of the classical carrier, and to reject a number of more recent, and seemingly more sophisticated, mechanisms.

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CHAPTER 1

INTRODUCTION

KINETIC STUDIES OF MEMBRANE TRANSPORT

The importance of the kinetic approach in investigations of the transport of small molecules across the cell membranes has been lucidly expressed by Christensen (1) in his book "Biological Transport". He writes:

> "Kinetics heretofore has been one of the most important sources of information about biological transport. Much of our present understanding has come from comparing the rates of migration of substances, and from observing the effect of their structures and concentration on that rate. Investigators have tended to suppose from time to time that they were about to exhaust the further information that could be gained by kinetics, and that more direct methods of observing the stages of the transport event would become necessary. Because the more direct methods have generally been rather unsuccessful, we have had the opportunity to learn at each stage that the kinetic approach has had still more to offer. We may note, however, that studies of the effect of substrate structure on reaction course and rate continue for enzymes that have been available in crystalline form for decades; consider, for example, the flow of important new information coming steadily from experimental studies on the reaction of chymotrypsin with substrate analogs, despite the availability of this enzyme in crystalline form for many years. Undoubtedly, we must steadily apply kinetics more thoughtfully and to distinct steps as they become distinguishable; but given those conditions I do not believe that we are about to exhaust the information kinetics can give us about transport".

THE CLASSICAL CARRIER MODEL

I. The Original Proposal

The kinetic treatment of mediated transport, that is, transport which depends on a membrane component present in limiting amounts, has most often been based on a model originally suggested by Widdas (2) in 1952, which today is known as the "classical carrier model". The existence of carriers was suggested in order to explain observations which were not predicted by the theory governing simple diffusion. These observations included saturability, differences in the permeabilities of substrate analogs that were unrelated to lipid solubility, competition between structurally related substances, and irreversible inhibition by protein reagents.

The most important postulate of the classical model is the oscillation of the carrier molecule between the two membrane surfaces so that it can accept a substrate molecule at one face and later release it at the other. Thus, two different carrier forms, an inward-facing and an outward-facing form, can be distinguished.

II. The Generalized Model

When originally proposed by Widdas, the model was assumed to be completely symmetrical, in the sense that the rates of movement of the free carrier and the carrier substrate-complex were supposed to be the same, and also in

the sense that affinities for the substrate on the two membrane faces were identical. Later the concept was extended to include the more general case in which there are no such restrictions (3). Moreover, experimental evidence for asymmetry in both rates and affinities has been found in various systems (4-6). Differences in affinity on the two membrane faces suggest the existence of a carrier with two different binding sites, one to accept substrate from the interior and one to accept substrate from the exterior. Nevertheless, if the classical model in its generalized form is valid, then these two sites must never be exposed simultaneously, making it necessary to distinguish two different free carrier forms.

The capacity of the free carrier to reorientate between these two positions is a strict requirement for net transport. Obligatory exchange rather than net transport results when the free carrier cannot undergo reorientation (7). In either system, the mechanism may be fundamentally the same.

The carrier concept has also been broadened in terms of the physical mechanism that is believed to be responsible for transport. While Widdas originally described the carrier as a ferryboat or shuttle, today it is understood that any physical mechanism, whose behaviour accords with the characteristic kinetic scheme, can be considered a classical carrier, regardless of the physical mechanism. It is this more general formulation that we shall refer to as the classical

carrier model.

III. Alternative Models

No other model has been subjected to so many rigorous tests as the classical carrier. Most other models have been formulated either to provide alternative explanations for the observations upon which the classical carrier was based, or to account for certain possible inconsistencies between data in the literature and the predicted behaviour. Rarely, however, have the new mechanisms been subjected to further tests and they have remained in the literature as possible alternatives to the classical model, generally with little justification. The greatest power of the kinetic method lies in its ability to reject hypotheses, rather than to provide final proof of their correctness, and the failure to determine rejection criteria for these models contributes to their weakness.

Another consequence of the new models' having arisen to explain apparent discrepancies in some particular item of the behaviour is that they themselves necessarily become complex and therefore have a tendency to be improbable (8,9). In some cases it seems possible that the inconsistencies may result from error in the experimental design or may be artifacts, owing for example to a reversible inhibitor trapped inside the cell. Indeed, it has been shown that such

an inhibitor would explain the observed asymmetry in the glucose carrier in red blood cells (10).

In order that tests be available for alternative mechanisms a number of different transport models are described and analyzed below (Chapter 4). These models all account for the saturation and specificity phenomena observed in biological transport. They are defined according to kinetic characteristics rather than their physical mechanism, since different mechanisms may predict identical behaviour in transport, and in consequence would not be distinguished by such tests.

In the formulation of new tests and models special attention is given to the following deficiencies that appear to be common in the kinetic treatment of transport.

(1) There is lack of agreement about the symbols representing the microscopic constants in transport schemes, and this presents a needless obstacle to understanding the kinetic treatment given by different authors.

(2) The meaning of the symbols is sometimes not at all obvious when kinetic equations are examined. For example, the same symbols may stand for substrate association and dissociation constants and also for constants defining carrier reorientation rates, making it difficult to read the equations without constant reference to the transport scheme.

(3) A special difficulty in naming the experimental parameters of transport is that there are many possible experimental arrangements by which rates may be measured (e.g. zero trans exit, infinite trans exit, equilibrium exchange, etc.).When the experimental parameters are named, there is usually no indication of the experiment to which they refer, so that their significance and their inter-relationships are not easily seen.

(4) Finally, there is often a failure to summarize the kinetic characteristics of models and to enunciate specific tests in a readily accessible way, which ideally would bypass the mathematical derivation.

In this study, a new notation is suggested which, it is hoped, may facilitate the kinetic description of models and the design of experiments. The conclusions are presented in tables that should be easily understood and that may be used to plan and interpret experiments without reference to the mathematical analysis.

TESTING CARRIER MODELS

In the course of the present study, a number of tests for carrier models are formulated. These involve three different approaches which depend upon either a) substrates.b) non-transported reversible inhibitors or c) irreversible inhibitors. It is shown how each kind of study can give evidence about different aspects of the mechanism. Moreover conclusions may be greatly strengthened

when supporting evidence can be obtained in several independent tests.

I. The Use of Substrates

Kinetic studies involving substrates have in the past been the main source of information about carrier mechanisms. In fact, the observation in non-active systems of trans acceleration (11,12) and of counter transport (13,14) was the strongest evidence in favour of the classical model. Studies of substrate specificity, however, have not been given the attention they deserve. Even though the relationship between structure and affinity has been looked at in many systems (1), the effect of alterations in structure on the translocation process itself has been little studied. Again Christensen (1) has elegantly expressed this need:

> "The effects of solute structure on transport deserve much wider exploitation than they sometimes receive. The interest in this subject is often too purely a biological one: How widely does the system serve? How many of the ordinary amino acids, how many of the principal sugars, purines, nucleotides, or dicarboxylic acids (etc.) are transported by a given system? How are those omitted taken care of? The biochemist brings to transport study the responsibility for thinking chemically about what the differences in substrate structure mean, with reference not only to the structure of the receptor site, but also to the discrimination and description of steps subsequent to the binding step. The potentialities of this approach, represented for example by the continuing studies of the operation of chymotrypsin and other enzymes that have been available for years in pure state, should convince the student that this is not simply an indirect "black box" approach to be pursued only until

> we have the skill and courage to isolate the macromolecular components of the transport systems. Instead the approach must continue at rising levels of sophistication as the components of systems are recognized".

We have now examined the effects of modifications of substrate structure on both affinity and the translocation process. For this purpose, the choline transport system of red blood cells has been chosen (Chapter 7). This system is especially suitable, since a great variety of choline analogs can be obtained by substituting alkyl chains of various lengths in place of the methyl groups attached to the nitrogen atom, producing a series of substrate analogs of gradually increasing size.

In the past, the measurement of transport rates has almost always depended on the availability of labeled substrates. A new method is described in Chapter 6 which obviates this requirement, and which allows the determination of affinities and transport rates for unlabeled analogs. This method is of general application and should therefore broaden the scope of transport studies.

II. The Use of Reversible Inhibitors

Reversible inhibitors can be a powerful tool in elucidating transport mechanisms, but this potential does not seem to have been recognized. Thus, no adequate treatment of the kinetics of reversible inhibition appears to be available. The reason may be that the kinetic theory for

reversible inhibition of transport is often adopted with little modification from enzyme kinetics in its simplest form; and whereas in an enzyme a competitive inhibitor and the substrate have the same accessibility to the active site, in a carrier it is common for inhibitor and substrates to be present in different compartments (inside or outside the cell). For example when substrate efflux is measured with a non-transported inhibitor in the external medium, which binds at the substrate site, the kinetic pattern is expected to be non-competitive, even though the mechanism is competitive. This is because a substrate and inhibitor in different compartments are prevented from competing with one another. Though this fact may seem obvious to everyone, and though an adequate kinetic treatment of carrier systems is easily worked out by conventional methods, the full consequences have been overlooked in many experimental studies and even in the treatment of transport kinetics given in recent textbooks. For example, in "Biological Transport" by H.N. Christensen, (1) the treatment of the kinetics of inhibition is taken directly, and explicitly, from enzyme kinetics, the tacit assumption being that as in simple enzyme models the transport "reaction" involves no isomerization step in the carrier, i.e., the carrier has only one form, with which substrates and inhibitors combine whichever compartment they occupy. Such a mechanism fails in many respects to describe the process of transport

and leads directly to the conclusion that a competitive inhibitor will under all circumstances produce competitive kinetics. This conclusion, as we have seen, is a serious mistake.

In "Cell Membrane Transport" by A. Kotyk and K. Janacek (15) the possibility that substrate and inhibitor may occupy different compartments is overlooked. As in the previous example, it follows, competitive inhibitors must produce competitive kinetics. In fact with both compartments taken into account we find that non-competitive, as well as mixed competitive and non-competitive kinetics commonly occur. In "Elementary Kinetics of Membrane Carrier Transport" by K.D. Neame and T.G. Richards (16) the kinetic equation given for transport rates with a reversible competitive inhibitor present in the solution outside the cell predicts that though the inhibitor interferes with the entry of the substrate into the cell it has no effect whatever upon substrate efflux, a conclusion which is made explicit in the text. This conclusion is of course in error as both analysis and experiment show (17). In fact the inhibition of efflux will be even stronger than the inhibition of influx.

In view of these difficulties, an elementary kinetic treatment of the subject is called for and is given in Chapter 4.

III. The Use of Irreversible Inhibitors

The usefulness of irreversible inhibitors in the study of transport mechanisms has been seriously questioned in a recent paper by Lieb and Stein (18). Even though such experiments are seen to be capable of providing rejection criteria for the classical carrier model, these authors conclude that we should not be tempted to ask questions about the details of the carrier mechanism, such as the following:

"i). Does the loaded carrier move at a different rate than the unloaded carrier? ii) Do the inward - and outward-facing forms of the carrier react with irreversible inhibitors at different rates? iii) Are intermediate forms inactivated at rates different from other forms?"

It is shown below that irreversible inhibitors, in combination with other tests, can be a powerful tool capable of answering the above questions. In the case of the choline transport system of red cells, the irreversible inhibitor N-ethyl maleimide can be used as a probe of carrier distribution in the membrane (as an inward-facing or outward-facing form). Such knowledge may be essential in answering the questions cited above.

OBJECTIVES OF THIS STUDY

The general aim of this entire work is to design tests that can distinguish among different carrier mechanisms and to apply them primarily to the choline transport system

in red blood cells.

The main questions posed are as follows:

1). Do the basic postulates of the classical carrier mechanism hold?

a) Can two different forms of free carrier be distinguished, as the carrier model demands?

b) If the existence of two different binding sites is suspected, are they alternately exposed as in the classical mechanism, or are they, on the contrary, simultaneously exposed at both membrane faces?

2). Which alternative transport models can be rejected?

3). What is the rate-limiting step in the transport process: translocation of the substrate across the membrane, or dissociation of substrate from the carrier?

4). What is required in the substrate structure for

a) binding

and

b) translocation through the membrane?

5). If two different free carrier forms can be distinguished, what is their normal partition between the inner and outer surfaces of the membrane?

6). Does the loaded carrier move at a different rate than the free carrier?

The answers to these questions have emerged from a combination of evidence obtained with substrates,

reversible inhibitors, and irreversible inhibitors. In doing this work we have intended not only to demonstrate how the choline transport system operates, but to suggest ways in which the kinetic approach may be used most effectively to reveal the underlying properties of a transport system. Even when the arduous task of isolating the components is complete, the kinetic approach will still be essential if the operation of the system,and not only the nature of its isolated components, is to be understood.

CHAPTER 2

THE POTENTIAL OF REVERSIBLE INHIBITION STUDIES

INTRODUCTION

The kinetic treatment of reversible inhibition of biological transport systems has been adopted with little modification from the field of enzyme kinetics. The result has not been altogether successful. According to the familiar rules of enzyme kinetics, when a substrate and inhibitor compete for the active site, inhibition is overcome by high substrate concentrations and is called competitive; when they are bound at different sites, inhibition persists at all substrate concentrations and is called non-competitive. This is not necessarily true of transport systems, where inhibition often persists despite addition of substrate and inhibitor to the same site. The reason is that the carrier operates between two aqueous compartments, outside and inside the cell. For experimental purposes, the substrate is present in only one compartment initially and if the inhibitor is present in the other, the substrate may be unable to displace it from the carrier, in which case the behaviour will be non-competitive.

These observations seem self-evident, but they have not always been appreciated. Our attention was forcibly drawn to the subject in the course of experiments with cytochalasin B. This substance reversibly inhibits glucose transport in erythrocytes in a manner which according to one

study is purely competitive (19)and according to others purely non-competitive(20,21). The discordant findings were based on different kinds of experiments, in which the procedures appeared to be thorough and the results convincing. There was no disagreement about specificity, in that other transport systems were unaffected, or about high affinity for the glucose carrier. Hence a single physical mechanism could well underlie the behaviour and the error be one of interpretation. Re-examination of the kinetics of transport inhibition showed that this possibility was indeed feasible. Our subsequent studies of cytochalasin B (17), demonstrate that it adds exclusively to the form of the glucose carrier which is present on the inner surface of the erythrocyte membrane. This circumstance, together with the ability of cytochalasin B to penetrate into the cell, independent of the carrier, accounts for the anomalies.

The general kinetic theory for reversible inhibition of transport is presented here in order to provide simple tests for competitive and non-competitive inhibition and for asymmetric binding of inhibitors. The latter phenomenon is important for our understanding of biological transport and there is growing evidence that it may not be unusual. For example a number of alkyl and phenyl derivatives of glucose and galactose, which are non-transported inhibitors of the hexose system, have vastly higher affinity for carrier on one side of the membrane than the other (4,5) and in addition, they readily penetrate the cell (22,23).

At the outset it is necessary to make a sharp distinction between a competitive or non-competitive mechanism, on the one hand, and competitive or non-competitive kinetics on the other. The former refers to the molecular mechanism involved - whether addition of substrate to the carrier excludes the binding of inhibitor (usually ascribed to involvement of the same binding site for both) or whether substrate and inhibitor may add simultaneously to the carrier (which must involve separate binding sites). The latter, the kinetic behaviour, refers to experimental observations on rates- whether addition of sufficient quantities of substrate overcomes the effect of an inhibitor. Mechanism and kinetics do not necessarily agree, as indicated above, but it will be seen that appropriate kinetic experiments can establish the actual mechanism.

In the first section below kinetic equations applying to a number of common transport experiments are derived. Later sections deal with the general conclusions which emerge and their application in transport studies. Finally, tables displaying the kinetic behaviour in different experiments are provided. These may be used directly in planning and interpreting experiments without recourse to the mathematical derivation.

Kinetic Theory

In order to analyze the kinetics of inhibition in various kinds of experiment, as well as the consequences

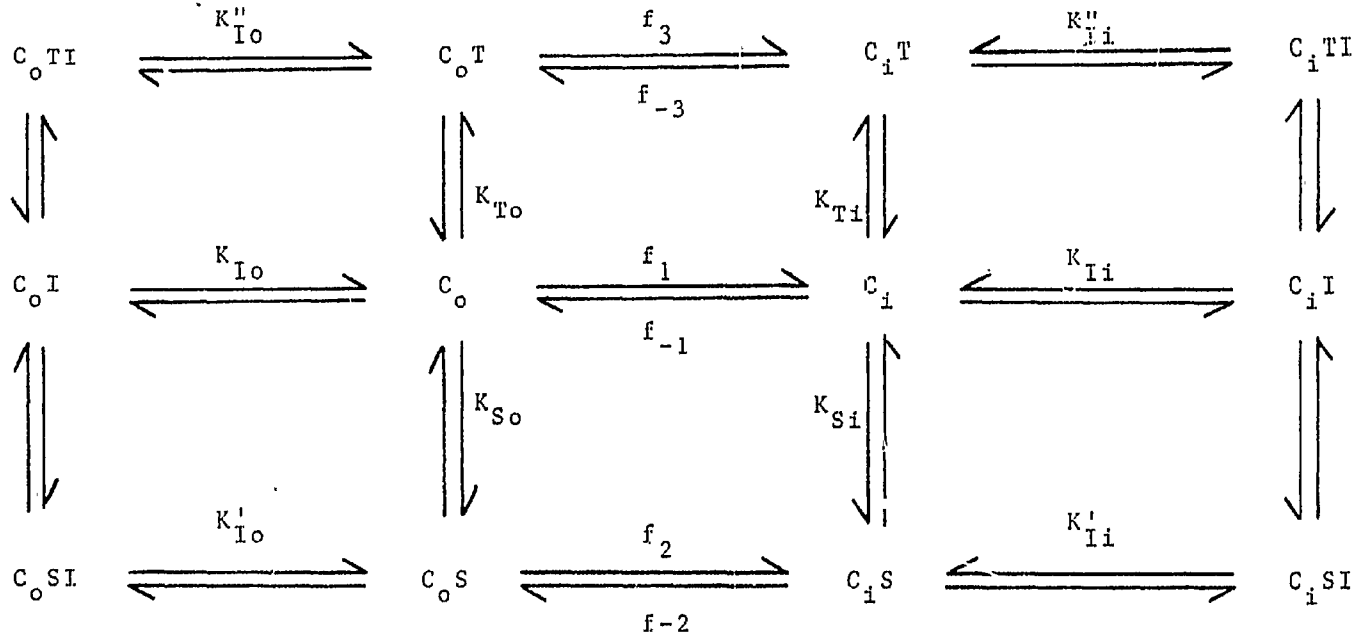
of asymmetric inhibitor binding, rate equations are derived for the general transport scheme shown in Fig. 2.1*. In this scheme S and T represent two different substrates; their inclusion makes it easy to derive rate expressions for equilibrium exchange (where S and T stand for radioactively labeled and unlabeled pools of the same substrate) as well as expressions for inhibition by competing substrates. The subscripts o and i designate the external and internal surfaces of the cell membrane: thus C_o and C_i are forms of the carrier in which the substrate binding site is exposed to the external or internal aqueous pool, and S_o and S_i represent substrate in these pools. Substrate or inhibitor may add to the free carrier, forming the complex C_oS, C_oI, etc., and the possibility of non-competitive inhibition is allowed for by inclusion of a ternary complex C_oSI, C_oTI etc. Rate constants for movement of bound substrate through

*Footnote:

Neither this formal scheme for transport, nor the use of the word "carrier" denotes anything about the details of the physical mechanism, other than the appearance of a substrate binding site alternately at the inner and outer membrane face, i.e., a given site is not simultaneously accessible to substrate in the internal and external pools. In this sense a "mobile carrier" is represented, and any physical model having this property is described kinetically by Fig. 2.1. Other transport schemes are possible, for example (i) a single form of free carrier and two forms of carrier-substrate complex(24); (ii) two forms of free enzyme and one of the complex(24); and (iii) the occurrence of a ternary complex, in which two molecules of substrate are bound simultaneously, one on either side of the membrane. The present model is the most common and has the required properties to explain the observations on cytochalasin. We shall therefore treat it in detail here and in a following chapter, investigate ways of distinguishing one model from another.

FIGURE 2.1

Transport scheme for two substrates S and T in the presence of an inhibitor I. Subscripts o and i refer to carrier forms on the outer and inner surfaces of the membrane respectively, and f $\pm$i are rate constants for reorientation of carrier in the membrane. In purely competitive inhibition the ternary complexes C_oSI, C_iSI, etc. do not form. ($K_{Io}' = K_{Ii}' = K_{Io}'' = K_{Ii}'' = \infty$). K_{So}, K_{To}, K_{Io}, etc. are equilibrium constants.


C_oTI
K''_{Io}
C_oT
f_3
f_{-3}
C_iT
K''_{Ii}
C_iTI
K_{To}
K_{Ti}
C_oI
K_{Io}
C_o
f_1
f_{-1}
C_i
K_{Ii}
C_iI
K_{So}
K_{Si}
K'_{Io}
f_2
f_{-2}
K'_{Ii}
C_oSI
C_oS
C_iS
C_iSI

the membrane are designated $f_{\pm i}$ + standing for inward diffusion, - for outward. The binding constants for addition of substrate and inhibitors to carrier on either the outer or inner membrane face, K_{So}, K_{Si}, K_{Io}, K_{Ii} etc. are defined as dissociation constants, having units of concentration.

In order that the analysis be as useful as possible, the rate equations should apply to both active and non-active systems. In the latter the substrate moves down its concentration gradient to achieve equilibrium, and in accordance with this, the principle of detailed equilibrium dictates the following relationship among the constants in Fig. 2.1:

$$\frac{K_{So}}{K_{Si}} = \frac{f_{-1}f_2}{f_1 f_{-2}} \qquad (2.1)$$

$$\frac{K_{To}}{K_{Ti}} = \frac{f_{-1}f_3}{f_1 f_{-3}} \qquad (2.2)$$

In an active system cellular energy is made to impinge upon one or more of the steps designated by the constants, causing the relationship to deviate from equality and the ultimate substrate concentrations attained on either side of the membrane to become unequal. If constants α and β are defined as

$$\alpha = \frac{f_{-1}\, f_2\, K_{Si}}{f_1\, f_{-2}\, K_{So}} \qquad (2.3)$$

$$\beta = \frac{f_{-1} f_3\, K_{Ti}}{f_1\, f_{-3}\, K_{To}} \qquad (2.4)$$

and

they are found to equal the ratio of final steady-state concentrations of substrate inside and outside the cell: $([S_i]/[S_o])_{(final)}=\alpha$ and $([T_i]/[T_o])_{(final)}=\beta$. In an equilibrating system α and β equal unity.

In deriving a rate equation we shall make one important simplifying assumption, that the slow step in the transport process is passage through the membrane itself rather than diffusion up to the carrier site or formation and dissociation of the carrier-substrate complex. This assumption is reasonable in view of the great speed of complex formation and dissociation of enzymes and their substrates (25). It is also supported by the observation of accelerated exchange in the leucine (26), choline (27) and glucose (28) transport systems of erythrocytes. *See footnote next page.

The general rate law for diffusion of substrate across the membrane is derived as follows. An equation is first written expressing the steady-state condition that the sum of movements of all forms of carrier in the inward direction must equal that in the outward:

$$f_1[C_o] + f_2[C_oS] + f_3[C_oT] = f_{-1}[C_i] + f_{-2}[C_iS] + f_{-3}[C_iT] \qquad (2.5)$$

Next, the total concentration of carrier in all forms is a constant, C_t:

$$C_t = [C_o] + [C_oS] + [C_oT] + [C_oI] + [C_oSI] + [C_oTI] + [C_i] + [C_iS] + [C_iT] + [C_iI] + [C_iSI] + [C_iTI] \tag{2.6}$$

These equations, together with the dissociation constants for complex formation defined in the following manner, $[C_o][S_o]/[C_oS] = K_{So}$ etc., enable us to write down an expression for the rate of transport of substrate S:

$$\frac{d[S_i]}{dt} = f_2[C_oS] - f_{-2}[C_iS]$$

$$= \frac{\frac{f_1 f_{-2} C_t}{K_{Si}}\left(\alpha[S_o] - [S_i]\right) + \frac{f_{-2} f_3 C_t}{K_{Si} K_{To}}\left(\frac{\alpha}{\beta}[S_o][T_i] - [S_i][T_o]\right)}{\left(1 + \frac{[S_o]}{K_{So}} + \frac{[T_o]}{K_{To}} + \frac{[I_o]}{K_{Io}} + \frac{[S_o][I_o]}{K_{So}K'_{Io}} + \frac{[T_o][I_o]}{K_{To}K''_{Io}}\right) M + \left(1 + \frac{[S_i]}{K_{Si}} + \frac{[T_i]}{K_{Ti}} + \frac{[I_i]}{K_{Ii}} + \frac{[S_i][I_i]}{K_{Si}K'_{Ii}} + \frac{[T_i][I_i]}{K_{Ti}K''_{Ii}}\right) N} \tag{2.7}$$

where $$M = \left(f_{-1} + \frac{f_{-2}[S_i]}{K_{Si}} + \frac{f_{-3}[T_i]}{K_{Ti}}\right)$$

$$= \frac{f_{-1}}{f_1}\left(f_1 + \frac{f_2[S_i]}{\alpha K_{So}} + \frac{f_3[T_i]}{\beta K_{To}}\right)$$

and $$N = \left(f_1 + \frac{f_2[S_o]}{K_{So}} + \frac{f_3[T_o]}{K_{To}}\right)$$

$$= \frac{f_1}{f_{-1}}\left(f_{-1} + \frac{\alpha f_{-2}[S_o]}{K_{Si}} + \frac{\beta f_{-3}[T_o]}{K_{Ti}}\right)$$

Footnote:

Completely general rate equations which are not dependent on this assumption will be presented in Chapter 3 . It will then be shown that the observation of non-competitive kinetics, under conditions specified below, constitutes a simple test for equilibrium complex formation.

APPLICATION TO EXPERIMENT

The kinetics of transport are most often and most conveniently studied by measuring rates in the early stages of the reaction - initial rates under conditions specified at the very beginning of the process. We shall deal with five different kinds of experiments, all depending on initial rate measurements. Different experiments will be seen to supply different kinds of information, and their combination to allow a determination of the molecular nature of the inhibition, whether competitive or non-competitive, and of the symmetry or asymmetry of inhibitor binding.

Initial rates of transport are most commonly studied under one of five different conditions of substrate distribution as follows: (a) "Zero trans efflux", in which substrate is present inside the cell only and its initial rate of appearance outside is determined; (b) "Zero trans influx", the reverse of this, in which substrate is initially present only on the outside and its rate of penetration is measured; (c) A "Sen-Widdas experiment", with substrate at a saturating concentration inside and at a lower, variable concentration outside. The net rate of exit is measured. (d) "Equilibrium exchange", where the substrate is present inside and outside the cell, one pool being labeled with radioactive substrate. The diffusion of the latter across the membrane is followed. (e) "Infinite trans influx". Cells

loaded with a saturating concentration of unlabeled substrate are placed in a medium containing a lower concentration of labeled substrate. The initial rate at which label appears inside the cell is measured.

I. Competitive Mechanisms

A. Non-transported Competitive Inhibitors

We shall consider each of these experiments in turn, confining our attention to a competitive mechanism, i.e. one in which the ternary complexes in Fig. 2.1are not formed: $K'_{Io} = K''_{Io} = K'_{Ii} = K''_{Ii} = \infty$

a). Zero trans efflux.

We set $[T_o] = [T_i] = [S_o] = 0$:

$$\frac{1}{v} = \frac{1}{f_{-2}Ct}\left\{1 + \frac{f_{-2}}{f_1} + \frac{f_{-2}}{f_1}\frac{[I_o]}{K_{Io}} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{f_{-1}}{f_1} + \frac{[I_i]}{K_{Ii}} + \frac{f_{-1}}{f_1}\frac{[I_o]}{K_{Io}}\right)\right\} \quad (2.8)$$

where v is the velocity at which the substrate S diffuses across the membrane. Rates of transport at varying substrate concentrations $[S_i]$ and at more than one inhibitor concentration may be analyzed by means of a reciprocal plot, 1/v against 1/[S] , i.e.,a Lineweaver-Burk plot, as well as by any other linear transformation. The intercept in the reciprocal plot is the reciprocal of the maxium rate, and the ratio of slope to intercept is the half-saturation constant for substrate, $[S_i]_{1/2}$. Let us consider three different conditions of inhibitor distribution or affinity.

(i) Inhibitor is present inside the cell and outside, at equilibrium, and has affinity for carrier on both membrane surfaces: The substrate independent and dependent terms in eqn(2.8) are increased in the presence of inhibitor, i.e.,both the intercept and slope. This is characteristic of non-competitive inhibition, since no matter how high the substrate concentration, the maximum velocity is reduced by the inhibitor.*

(ii) Inhibitor is restricted to the external pool because of inability to pass through the cell membrane; or equivalently, the inhibitor though present on both sides, becomes bound only to those substrate sites exposed to the external solution, owing to asymmetry in carrier structure. Again both intercept and slope increase, as in non-competitive inhibition.

(iii) Inhibition is restricted to the internal membrane surface because of asymmetry in either the distribution or affinity of the inhibitor. The intercept in the reciprocal plot is now constant though the slope changes. In other words, V_{max} is constant while K_m increases, as in pure

Footnote:

This definition of non-competitive inhibition is adopted here for sake of simplicity, but would include both pure non-competitive inhibition and mixed competitive and non-competitive inhibition.

competitive inhibition.

(2.9).

(b)Zero trans influx. $[T_o]=[T_i] = [S_i] = 0$:

$$\frac{1}{v} = \frac{1}{f_2 C_t}\left\{1 + \frac{f_2}{f_{-1}} + \frac{f_2[I_i]}{f_{-1}K_{Ii}} + \frac{K_{So}}{[S_o]}\left(1 + \frac{f_1}{f_{-1}} + \frac{[I_o]}{K_{Io}} + \frac{f_1[I_i]}{f_{-1}K_{Ii}}\right)\right\}$$

Under the above three conditions of inhibition on both sides of the membrane, outside only, or inside only, the kinetic form of the inhibition will be non-competitive, competitive or non-competitive, respectively, as inspection of eqn. 2.9 shows.

(c) Sen-Widdas exit experiment (Infinite cis). $[T_o] = [T_i] = 0$; $[S_i]/K_{Si} >> 1$; $[S_i] >> [S_o]$:

(2.10).

$$\frac{1}{v} = \frac{1}{f_1 C_t}\left\{1 + \frac{f_1}{f_{-2}} + \frac{[I_o]}{K_{Io}} + \frac{f_1 K_{Si}\ [I_i]}{f_{-2}[S_i]\ K_{Ii}} + \frac{[S_o]}{K_{So}}\left(1 + \frac{f_2}{f_{-2}} + \frac{f_2 K_{Si}\ [I_i]}{f_{-2}\ [S_i]\ K_{Ii}}\right)\right\}$$

(i) Inhibitor binds on both sides of the membrane. Compared to the term in $[I_o]$, those in $[I_i]$ should be negligible because of being multiplied by $K_{Si}/[S_i]$ which is very much smaller than unity. Hence in a plot of $1/v$ against $[S_o]$ the intercept increases but the slope is constant. The ratio of intercept to slope in such a plot is equal to the half-saturation constant for external substrate, and the intercept to the reciprocal of the maximum exit rate. The inhibitor reduces V_m and, by exactly the same factor, increases K_m. The inhibition has in this sense the features of both competitive and non-competitive inhibition,

owing to the peculiar distribution of substrate across the membrane. Since it does not correspond to any case of enzyme inhibition, including "mixed competitive and non-competitive" in which V_m and K_m may change by different factors, we may for present purposes describe it as "competitive - non-competitive", meaning that it is competitive with respect to external substrate, non-competitive with respect to internal. (ii) Inhibition outside: The kinetic behaviour is identical to that above, where the inhibitor is capable of binding on either side. (iii) Inhibition inside: Both the slope and intercept are increased, and by a similar factor. Hence V_{max} declines while K_m is nearly constant; inhibition is non-competitive.

(d) Equilibrium exchange (or exchange in the final steady state).

We let S_i be labeled substrate, T_o the same substrate unlabeled; $[S_o] = [T_i] = 0$; $[S_i] = \alpha[T_o]$:

$$\frac{1}{v} = \frac{1}{f_{-2}Ct}\left\{1 + \frac{f_{-2}}{f_2} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{f_{-1}}{f_1} + \frac{[I_i]}{K_{Ii}} + \frac{f_{-1}}{f_1}\frac{[I_o]}{K_{Io}}\right)\right\} \qquad (2.12).$$

In either case, active or non-active, inhibition is overcome at high substrate concentration and is therefore of the pure competitive type.

(e) Infinite trans influx (non-equilibrium exchange). Measurement of initial rates of zero trans entry may be difficult because the internal substrate concentration rapidly attains

a level where efflux becomes important, diminishing the net rate of entry. However, if cells loaded with a high substrate concentration are placed in a medium containing labeled substrate, the label is diluted inside the cell and return flux is negligible. The rate equation for this case is found by setting $[T_i]$ as saturating, $[S_o]$ lower and variable, and $[T_o] = [S_i] = 0$:

$$\frac{1}{v} = \frac{1}{f_2 Ct}\left\{1 + \frac{f_2}{f_{-3}} + \frac{f_2}{f_{-3}}\frac{K_{Ti}}{[T_i]}\frac{[I_i]}{K_{Ii}} + \frac{K_{So}}{[S_o]}\left(1 + \frac{f_1}{f_{-3}} + \frac{[I_o]}{K_{Io}} + \frac{f_1 K_{Ti}}{f_{-3}[T_i]}\frac{[I_i]}{K_{Ii}}\right)\right\} \quad (2.13).$$

If S and T represent molecules of the same substrate this may be re-written as:

$$\frac{1}{v} = \frac{1}{f_2 Ct}\left\{1 + \frac{f_2}{f_{-2}} + \frac{f_2 K_{Si}}{f_{-2}[S_i]}\frac{[I_i]}{K_{Ii}} + \frac{K_{So}}{[S_o]}\left(1 + \frac{f_1}{f_{-2}} + \frac{[I_o]}{K_{Io}} + \frac{f_1 K_{Si}}{f_{-2}[S_i]}\frac{[I_i]}{K_{Ii}}\right)\right\} \quad (2.14).$$

The system behaves in the same way as in the Sen-Widdas experiment: inhibition is competitive if the inhibitor adds to carrier on both sides or on the outside, non-competitive if only on the inside.

B. Competing Substrates

Of the above experiments, only zero trans efflux and zero trans influx are of special interest here, for they enable us to distinguish substrate analogs which undergo transport from those that do not, even where both rapidly diffuse through the membrane without intervention of the carrier.

(a) Zero trans efflux. We determine the rate of exit of S, with $[S_o] = 0$, in the presence of a second substrate T:

$$\frac{1}{v} = \frac{1}{f_{-2}Ct}\left\{1 + f_{-2}\left(\frac{1 + \frac{[T_o]}{K_{To}}}{f_1 + f_3\frac{[T_o]}{K_{To}}}\right) + \frac{K_{Si}}{[S_i]}\left[1 + \frac{[T_i]}{K_{Ti}} \div \left(f_{-1} + f_{-3}\frac{[T_i]}{K_{Ti}}\right)\left(\frac{1+\frac{[T_o]}{K_{To}}}{f_1+f_3\frac{[T_o]}{K_{To}}}\right)\right]\right\} \quad (2.15).$$

(i) Analog on both sides: If the values of f_1 and f_3 are not too different, the slope but not the intercept increases, and inhibition is competitive. If the analog is not transported, and f_3 and f_{-3} are small relative to f_1 and f_{-1}, then as seen before slope and intercept increase and inhibition is non-competitive.

(ii) Analog outside: There is now little or no inhibition, or the rate of exit of substrate S may actually increases.

(iii) Analog inside: The slope but not the intercept increases, giving pure competitive inhibition.

(b) Zero trans influx. $[S_i]=0$

$$\frac{1}{v} = \frac{1}{f_2Ct}\left\{1 + f_2\left(\frac{1 + \frac{[T_i]}{K_{Ti}}}{f_{-1} + f_{-3}\frac{[Ti]}{K_{Ti}}}\right) + \frac{K_{So}}{[S_o]}\left[1 + \frac{[T_o]}{K_{To}} + \left(f_1 + f_3\frac{[T_o]}{K_{To}}\right)\left(\frac{1 + \frac{[T_i]}{K_{Ti}}}{f_{-1} + f_{-3}\frac{[T_i]}{K_{Ti}}}\right)\right]\right\} \quad (2.16)$$

With analog on both sides or only on the outside, inhibition is competitive; if only on the inside there is little or no inhibition, or rate enhancement.

II. Non-Competitive Mechanisms

When the mechanism is non-competitive there is no ambiguity; for in all experiments, as may be demonstrated by means of eqn. 2.7, the kinetic behaviour is also non-competitive. As a result, such inhibitors are likely to provide little information about transport mechanisms. Depending on the symmetry of the system in binding inhibitor or substrate, inhibition may not be of the pure non-competitive type, where slope and intercept in a reciprocal plot are raised to exactly the same degree.

III. Studies of Active or Passive Transport

The forms of the rate expressions are identical whether $\alpha = 1$ (in non-active transport) or $\alpha >> 1$ (in active transport).

IV. Rules for Interpreting Inhibition Kinetics

The detailed conclusions of the preceding sections are summarized in Table 2.1. Several more general rules follow:
(i) A competitive mechanism can give rise to non-competitive kinetics, but not vice-versa. Hence if a substance inhibits non-competitively in some experiments but competitively in

TABLE 2.1.

Inhibition behaviour in various transport experiments as dependent on the symmetry of binding. The inhibitor or substrate analog can compete with the substrate for the carrier site, and may become bound to the carrier forms (a) on both the external and internal membrane surfaces, (b) only on the external surface, or (c) only on the internal surface (columns 3-5). The competitive analog may or may not undergo transport on the carrier (column 2). C-N (competitive - non-competitive) is a designation peculiar to the Sen-Widdas experiment, since the behaviour indicates competition with substrate in the external medium, non-competition with that inside the cells. C = competitive kinetics; N = non-competitive or mixed competitive and non-competitive kinetics.

TABLE 2.1.

Experiment	Competitive Analog	Site of Inhibition		
		Both Membrane Surfaces	Outer Membrane Surface	Inner Membrane Surface
Zero Trans Efflux	Non-transported	N	N	C
	Transported	C	No Inhibition	C
Zero Trans Influx	Non-transported	N	C	N
	Transported	C	C	No Inhibition
Sen-Widdas Exit	Non-trasnported	C-N	C-N	N
Equilibrium exchange or Steady state exchange	Non-transported	C	C	C
Infinite Trans Influx	Non-transported	C	C	N

another the mechanism must be competitive.

(ii) The only unambiguous test of a competitive as against a non-competitive mechanism is provided by equilibrium exchange or exchange in the final steady state. If conclusions are to be drawn from a single type of experiment this one should be chosen. Otherwise it is the best foundation for interpreting other experiments.

(iii) If the mechanism is competitive, the inhibition pattern in zero trans exit and zero trans entry experiments establishes the sidedness of inhibition (whether binding occurs on both sides of the membrane, exclusively outside, or exclusively inside); whether the inhibitor undergoes transport on the carrier (i.e., is a competing substrate); and with one exception where doubt remains, whether it penetrates into the cell either with or without assistance from the carrier. The expected patterns of inhibition in these experiments and in a third, infinite trans entry, are summarized in Table 2.2. The latter experiment serves to add confirmation to the conclusions reached from the first two, and could as well be replaced by a Sen-Widdas exit experiment, which has essentially the same properties.

(iv) A competing substrate which is transported at a rate comparable to that of the substrate under investigation

TABLE 2.2.

Inhibition kinetics observed in three different transport experiments as related to the properties of the inhibitor: whether it undergoes transport on the carrier, whether it adds to carrier forms on both membrane surfaces or only on the inner or outer face, and whether it enters the cell (either by passive diffusion or via the carrier). Inhibitor and substrate add to the same carrier site except in the last case (bottom) where different sites are involved and the kinetics are non-competitive in all experiments.
C = competitive; N = non-competitive; 0 = no inhibition.

Experiment			Characteristics of Inhibitor		
Zero Trans Exit	Zero Trans Entry	Infinite Trans Entry	Carrier Transport	Sidedness of Inhibition	Penetration into Cell
N	N	C	No	Both	Yes
N	C	C	No	Outer	?
C	N	N	No	Inner	Yes
C	C	C	Yes	Both	Yes
0	C	C	Yes	Outer	Yes
C	0	0	Yes	Inner	Yes
N	N	N	Non-competitive mechanism		

never inhibits non-competitively. However, a very poorly transported substrate would resemble a non-transported analog in giving non-competitive, or partially non-competitive, inhibition.

V. Asymmetry in Carrier Systems

Strong asymmetry even in simple equilibrating transport systems may not be unusual, judging by observations on cytochalasin B and other inhibitors of glucose transport in erythrocytes. The observations on cytochalasin B are as follows: (i) competitive binding of D-glucose and cytochalasin B to membrane preparations was demonstrated (19). (ii) Non-competitive inhibition of transport was found in Sen-Widdas experiments (20,21). (iii) Non-competitive inhibition of glucose entry was shown (20). Rules summarized above indicate that (a) where both competitive and non-competitive behaviour is seen, the mechanism cannot be non-competitive; (b) where a competitive mechanism produces non-competitive kinetics in entry and Sen-Widdas experiments, the inhibitor must enter the cell and become bound exclusively to sites on the inner membrane surface. The theory predicts pure competitive inhibition in zero

trans exit experiments, and this has now been found (17)

As noted earlier, various alkyl derivatives of hexoses have been shown to be far more strongly bound to carrier on one side of the membrane than the other (4,5). These compounds readily enter the cells, though not by means of the carrier(22,23).Another interesting example, this time of structural asymmetry in the carrier, rather than its binding site, is that of the choline transport mechanism in erythrocytes, which undergoes reaction with N-ethyl maleimide (NEM) (and inactivation) only when located on the inner surface of the membrane (Chapter 8).

Asymmetry is requisite in active transport systems, where substrate disequilibrium is imposed through the agency of the carrier and at the expense of metabolic energy. An element of asymmetry impressed in the carrier may appear as a shift in any of the constants in the equation for α(eqn.2.3) relative to the non-energized state of the system. Non-transported substrate analogs able to pass through the cell membrane could be useful in studying such problems, since such shifts should be manifest in altered inhibition behaviour.

So far we have spoken only of extreme asymmetry, where the inhibitor is bound far more strongly on one side of the membrane than the other. With moderate asymmetry different affinity constants are found on the two membrane

surfaces. Table 2.3 summarizes the experimental parameters which are determined in initial rate experiments. One parameter is obtained in purely competitive inhibition (or in the competitive - non-competitive inhibition of a Sen-Widdas experiment) and two parameters in non-competitive inhibition, one obtained from ratios of slopes in plots of 1/v against 1/[S] and one from ratios of intercepts. In non-competitive inhibition which is formally pure, slope and intercept are raised by the inhibitor to exactly the same degree, and the two parameters are equal. In mixed competitive and non-competitive inhibition the parameters are unequal. It is seen that in nearly all cases the apparent inhibition constants are combinations of binding constants and ratios of flux constants, as are substrate binding constants.

Where an inhibitor binds preferentially but not exclusively on one side of the membrane we expect mixed competitive and non-competitive inhibition, to a degree that varies with the experiment. Such behaviour can be explained by postulating two different kinds of binding site, one competitive with substrate and the other non-competitive, though asymmetric addition is the simpler hypothesis and is easily tested. This hypothesis provides a simple explanation for observations on series of structurally related inhibitors with similar physical properties in which some members inhibit in a competitive, others in a mixed and still others in a non-competitive fashion, as

TABLE 2.3.

Half-saturation constants in various experiments for inhibitors having different characteristics and for substrate (see text for equations). In competitive inhibition only one constant is measured. In non-competitive inhibition there are two, one constant obtained from the change in slope in a reciprocal plot ($1/v$ x $1/[S]$)-the so-called competitive component - and another from the change in intercept - the so-called non-competitive component. When these are equal inhibition is described as purely non-competitive, but as "mixed competitive and non-competitive" when the non-competitive component is considerably smaller than the competitive. The influence of the relative values of reorientation constants (f_1, f_{-1}, f_2 and f_{-2}) upon the ratio of the inhibition parameters will not be dealt with here, but must be taken into consideration in testing for predominant but not exclusive inhibitor addition on one side of the membrane.

TABLE 2.3

Experiment	Substrate Constant	Measured Inhibition Parameter	Site of Inhibition: Both Sides, $K_{Io} = K_{Ii}$	Site of Inhibition: Both Sides, $K_{Io} \neq K_{Ii}$	Site of Inhibition: Outside, $[I_i]=0$ or $K_{Ii} = \infty$	Site of Inhibition: Inside, $[I_o]=0$ or $K_{Io} = \infty$
Zero Trans Efflux	$\frac{K_{Si}(1+f_{-1}/f_1)}{1+f_{-2}/f_1}$	Competitive	K_{Io}	$\frac{1+f_{-1}/f_1}{1/K_{Ii}+f_{-1}/f_1K_{Io}}$	$K_{Io}(1+f_1/f_{-1})$	$K_{Ii}(1+f_{-1}/f_1)$
		Non-competitive	$K_{Io}(1+f_1/f_{-2})$	$K_{Io}(1+f_1/f_{-2})$	$K_{Io}(1+f_1/f_{-2})$	-
Zero Trans Influx	$\frac{K_{So}(1+f_1/f_{-1})}{(1+f_2/f_{-1})}$	Competitive	K_{Io}	$\frac{1+f_1/f_{-1}}{1/K_{Io}+f_1/f_{-1}K_{Ii}}$	$K_{Io}(1+f_1/f_{-1})$	$K_{Ii}(1+f_{-1}/f_1)$
		Non-competitive	$K_{Io}(1+f_{-1}/f_2)$	$K_{Ii}(1+f_{-1}/f_2)$	-	$K_{Ii}(1+f_{-1}/f_2)$
Sen-Widdas Exit	$\frac{K_{So}(1+f_1/f_{-2})}{(1+f_2/f_{-2})}$	Competitive	$K_{Io}(1+f_1/f_{-2})$	$K_{Io}(1+f_1/f_{-2})$	$K_{Io}(1+f_1/f_{-2})$	$\frac{K_{Ii}[Si](1+f_{-2}/f_1)}{K_{Si}}$
		Non-competitive	-	-	-	$\frac{K_{Ii}[Si](1+f_{-2}/f_2)}{K_{Si}}$
Equilibrium Exchange	$\frac{K_{Si}(1+f_{-1}/f_1)}{(1+f_{-2}/f_2)}$	Competitive	K_{Io}	$\frac{(1+f_{-1}/f_1)}{1/K_{Ii}+f_{-1}/f_1K_{Io}}$	$K_{Io}(1+f_1/f_{-1})$	$K_{Ii}(1+f_{-1}/f_1)$
		Non-competitive	-	-	-	-
Infinite Trans Entry	$\frac{K_{So}(1+f_1/f_{-2})}{(1+f_2/f_{-2})}$	Competitive	$K_{Io}(1+f_1/f_{-2})$	$K_{Io}(1+f_1/f_{-2})$	$K_{Io}(1+f_1/f_{-2})$	$\frac{K_{Ii}[S_i](1+f_{-2}/f_1)}{K_{Si}}$
		Non-competitive	-	-	-	$\frac{K_{Ii}[S_i](1+f_{-2}/f_2)}{K_{Si}}$

was the case for inhibition of glucose transport in erythrocytes by steroid (29) and pyridine (30) derivatives. The relative affinities for the glucose carrier on the two membrane surfaces has in fact been shown to be very sensitive to small changes in substrate structure (4,5).

VI. Reversible Inhibitors as tools for Discriminating among Transport Mechanisms.

The occurrence of non-competitive kinetics with an inhibitor whose mechanism is competitive (as established by competitive binding studies or inhibition of exchange flux) can be expected of certain transport models, the present case being one, but not of others. For example if there is only one form of free carrier and that form has sites on both sides of the membrane, then substrate on one side may actually compete with inhibitor on the other side. Further, with certain models only one inhibitor molecule may add to the carrier at a time, while with others the availability of sites on both sides of the membrane could enable two to bind. Such behaviour is readily demonstrated in kinetic studies, and hence reversible competitive inhibitors capable of penetrating the cell should be of great value in confirming or excluding particular mechanisms. This aspect of the problem will be treated in Chapter 4.

Summary

Kinetic equations are derived for reversible inhibition of both active and facilitated transport systems for five common experimental arrangements. It is shown that the unique features of transport kinetics may be exploited to give new kinds of information. It is also shown that the familiar rules of enzyme kinetics, though often applied to transport, can be seriously misleading. The analysis leads to the following general conclusions:

(1) A competitive mechanism frequently gives rise to non-competitive kinetics, depending on the experimental design, but a non-competitive mechanism never produces competitive kinetics. (2) Inhibition studies on exchange diffusion at equilibrium in non-active systems or in the final steady state in active systems are the only unambiguous kinetic tests to distinguish competitive from non-competitive mechanisms. (3) Substrate analogs that are bound to the carrier and transported are readily distinguished by inhibition kinetics from those not transported, even though both may rapidly enter the cell by another route. (4) Even in non-active systems competitive inhibitors commonly have far different affinities for the substrate sites on the two membrane faces: where sufficient non-polarity allows their penetration into the cell, inhibition kinetics readily establish such sidedness in their action. (5) Inhibition

kinetics of the mixed competitive and non-competitive type result from moderately asymmetrical binding of inhibitor at the substrate site. (6) Asymmetry is a necessary feature of active transport; hence studies of inhibition kinetics should provide important insights into its mechanism.

CHAPTER 3

A NEW TEST FOR THE RATE-LIMITING STEPS IN TRANSPORT

INTRODUCTION

Experimental studies of the reversible inhibition of transport have revealed that the kinetics of inhibition may be either competitive or non-competitive, depending on whether the substrate and the inhibitor are on the same side of the membrane or on opposite sides, respectively. (Ref. 17 and Chapter 2). This is true even if the inhibitor is a substrate analog bound at the substrate site. The crucial assumption made in deriving the kinetic equations which predict this behaviour was that dissociation of the substrate from the carrier occurs at a far more rapid rate than carrier reorientation in the membrane. We now show that the observation of non-competitive kinetics is in fact inconsistent with rate-limiting dissociation of the carrier-substrate complex.

In Chapter 4 other transport models are suggested that do not lead to the prediction of non-competitive inhibition, even when dissociation is a rapid step in transport. Accordingly the observation of non-competitive inhibition represents an additional piece of evidence for a model with the kinetic properties of the classical carrier.

THE KINETIC APPROACH IN STUDIES OF MEDIATED MEMBRANE TRANSPORT

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RATE EQUATIONS

The appropriate equations may be written down directly from the more general equation for transport of a substrate (S) in the presence of an inhibitor (I) which was derived for the scheme in Fig. 3.1 and is shown in Appendix 1 (eqn. A.6).

For the sake of simplicity, only the case of zero trans efflux of substrate from pre-loaded cells into a solution containing a non-transported substrate analog will be considered. In the proposed experiment the internal concentration of substrate S is varied, the external concentration of the inhibitor T is fixed, and initial rates are measured under conditions where the external substrate concentration is negligible. With these assumptions, the general rate equation reduces to:

$$-\frac{d[Si]}{dt} = \frac{\bar{V}_{Si}[Si]/\bar{K}_{Si}}{1 + \frac{[Io]}{\bar{K}_{Io}} + \frac{[Si]}{\bar{K}_{Si}}\left(1 + \frac{[Io]}{\tilde{K}_{Io}^{S}}\right)} \qquad (3.1)$$

where

$$\bar{V}_{Si} = \frac{k_{-1}\ f_1\ f_{-2}\ Ct}{k_{-1}\ \{(f_1 + f_{-2}) + f_1\ (f_2 + f_{-2})\}}$$

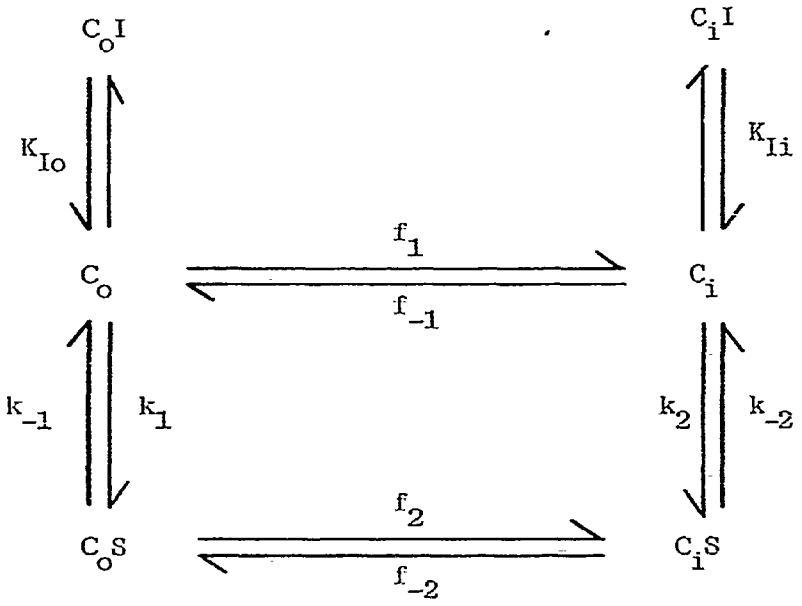
$$\bar{K}_{Si} = \frac{(f_1 + f_{-1})\ \{k_{-1}\ f_{-2} + k_{-1}\ k_{-2} + k_{-2}\ f_2\}}{k_2\ \{k_{-1}\ (f_1 + f_{-2}) + f_1(f_2 + f_{-2})\}}$$

$$\bar{K}_{Io} = K_{Io}\ (1 + f_1/f_{-1})$$

$$\tilde{K}_{Io}^{S} = K_{Io}\left\{1 + \frac{f_1}{f_{-2}} + \frac{f_1}{k_{-1}}\left(1 + \frac{f_2}{f_{-2}}\right)\right\}$$

FIGURE 3.1

Scheme for the transport of substrate S in the presence of an inhibitor I. Subscripts o and i refer to carrier forms on the outer and inner surfaces of the membrane respectively; $f \pm_i$ are rate constants for reorientation of carrier in the membrane, and $k +_i$ and k_{-i} are association and dissociation constants, respectively. K_{Io} and K_{Ii} are the equilibrium constants for carrier-inhibitor complex formation.


C_oI
C_iI
K_{Io}
K_{Ii}
f_1
C_o
C_i
f_{-1}
k_{-1}
k_1
k_2
k_{-2}
f_2
C_oS
C_iS
f_{-2}

and where $K_{Io} = k_{-3}/k_3$, Ct is the total carrier concentration. The conventions used in naming the constants are fully explained in Appendix 1. In brief, the conventions are as follows: carrier reorientation constants are designated by the letter f, and constants for carrier-substrate complex formation by k. A bar above a constant indicates a zero trans experiment, and the superscript $^{\infty S}$ indicates an infinite trans experiment, the trans substrate being S. The location of this substrate is indicated by the attached subscript, i or o, meaning internal and external, respectively. As examples, the constants $\overline{V}_{Si}$ and $\overline{K}_{Si}$ are the maximum rate and the affinity constant for S in a zero trans exit experiment.

INHIBITION PATTERNS

The results of an exit experiment are plotted according to some linear rearrangement of e.g. (3.1) for example the familiar reciprocal plot of 1/v against 1/[S]. From the ratio of slopes in this plot the competitive constant $\overline{K}_{Io}$ is calculated, and from the ratio of intercepts, the non-competitive constant, $K_{Io}^{\infty S}$. When the inhibitor increases the slope and intercept by an identical factor these two constants are equal ($\overline{K}_{Io} = K_{Io}^{\infty S}$), and the inhibition appears to be of the pure non-competitive type. If the intercept is unchanged in the presence of the inhibitor, while the slope increases, $K_{To}^{\infty S} \to \infty$ and the

inhibition is purely competitive. If the intercept is raised more than the slope, the non-competitive component of the inhibition is stronger than the competitive component ($\tilde{K}^{S}_{Io} < \bar{K}_{Io}$), and the inhibition approaches the "uncompetitive" type.

RATE-LIMITING STEPS IN RELATION TO THE TYPE OF INHIBITION

I. Zero Trans Exit

Let us now consider the inhibition patterns which are predicted by eqn (3.1) with different assumptions about the relative magnitudes of the rate constants. This is most easily accomplished by inspection of an equation which gives the ratio of the non-competitive and the competitive inhibition constants

$$\frac{\tilde{K}^{S}_{Io}}{\bar{K}_{Io}} = \frac{1 + \frac{f_1}{f_{-2}} + \frac{f_1}{k_{-1}}\left(1 + \frac{f_2}{f_{-2}}\right)}{1 + \frac{f_1}{f_{-1}}} \qquad (3.2)$$

It is to be noted that the nature of the inhibition depends entirely on the kinetic constants for the carrier system and is completely independant of constants for inhibition binding. This is understandable because the inhibitor's actual mechanism (as opposed to the observed kinetic pattern) is purely competitive and involves no non-competitive addition to the carrier whatever.

a) Let substrate dissociation be far more rapid than carrier recrientation ($k_{-1} >> f_1$):

$$\frac{\tilde{K}_{Io}^{S}}{\overline{K}_{Io}} = \frac{1 + \frac{f_1}{f_{-2}}}{1 + \frac{f_1}{f_{-1}}} \qquad (3.3)$$

If $f_{-2} \simeq f_{-1}$, this ratio is approximately equal to unity and the inhibition is purely non-competitive; if $f_{-2} > f_{-1}$, the inhibition tends to be uncompetitive. With a very poor substrate ($f_{-1} > f_{-2}$) mixed competitive and non-competitive inhibition would result.

b) Let substrate dissociation be the rate-limiting step ($f_1 >> k_{-1}$):

$$\frac{\tilde{K}_{Io}^{S}}{\overline{K}_{Io}} = \frac{f_1 \left(1 + \frac{f_2}{f_{-2}}\right)}{k_{-1}\left(1 + \frac{f_1}{f_{-1}}\right)} >> 1 \qquad (3.4)$$

the inhibition is necessarily competitive.

c) Let the dissociation and reorientation steps occur at comparable rates ($f_1 \overset{\sim}{-} k_{-1}$):

$$\frac{\tilde{K}_{Io}^{S}}{\overline{K}_{Io}} = \frac{2 + \frac{1}{f_{-2}}\left(f_1 + f_2\right)}{1 + f_1/f_{-1}} \qquad (3.5)$$

The ratio is likely to be larger than unity, making the non-competitive component of the inhibition weaker than the competitive component, and the inhibition "mixed

competitive and non-competitive".

II. Zero Trans Entry

We now summarize the expected behaviour with the locations of the substrate and inhibitor reversed. Equations for the ratios of inhibition constants are conveniently written down from the equations given above, by reversing the sense in which each constant applies (see Figure 3.1)

$$\frac{{}^{\sim S}K_{Ii}}{\bar{K}_{Ii}} = \frac{1 + \frac{f_{-1}}{f_2} + \frac{f_{-1}}{k_{-2}}\left(1 + \frac{f_{-2}}{f_2}\right)}{1 + \frac{f_{-1}}{f_1}} \qquad (3.6)$$

(a) $k_{-2} >> f_{-1}$

$$\frac{{}^{\sim S}K_{Ii}}{\bar{K}_{Ii}} = \frac{1 + \frac{f_{-1}}{f_2}}{1 + \frac{f_{-1}}{f_1}} \qquad (3.7)$$

(b) $f_{-1} >> k_{-2}$

$$\frac{{}^{\sim S}K_{Ii}}{\bar{K}_{Ii}} = \frac{f_{-1}}{k_{-2}} \frac{\left(1 + \frac{f_2}{f_{-2}}\right)}{\left(1 + \frac{f_1}{f_{-1}}\right)} >> 1 \qquad (3.8)$$

(c) $f_{-1} \gtrsim k_{-2}$

$$\frac{\tilde{K}_{Ii}^{S}}{K_{Ii}} = \frac{2 + \frac{1}{f_2}\left(f_{-1} + f_{-2}\right)}{1 + \frac{f_{-1}}{f_1}} \qquad (3.9)$$

THE INTERPRETATION OF EXPERIMENTAL OBSERVATIONS

We shall now consider whether any unambiguous conclusions may be drawn about the underlying mechanism from inhibition patterns. We assume that the inhibitor has already been shown to act in competition with the substrate, by means of equilibrium exchange experiments, for example, and that its action is confined to one side of the membrane. This requirement is easily achieved in the case of a non-transported and non-penetrating substrate analog, which must remain outside the cell membrane, and less easily achieved for an inhibitor acting on the inner surface of the membrane. Methods for establishing whether a penetrating inhibitor binds on only one side of the membrane or on both sides were discussed in Chapter 2. Fortunately, penetrating inhibitors bound to the carrier site on only the inner membrane surface are available for both the glucose (17) and choline (see Chapter 9) transport systems of erythrocytes.

The observation of purely competitive inhibition by a trans inhibitor can be interpreted without difficulty as meaning that either (1) dissociation of the carrier

substrate complex is rate-limiting in transport, or (2) the transport mechanism is fundamentally different, in its kinetic character, from the classical carrier. We note that many proposed mechanisms, despite their being physically unlike the original "ferryboat" concept, behave in exactly the same way; e.g. a gated channel. Where competitive kinetics actually result from the operation of a mechanism of a different kind, this may be demonstrated by other experimental tests (Chapter 4). If the system passes all other tests for the classical model, then slow dissociation of the complex would be a necessary conclusion.

On an intuitive level, the competitive inhibition seen under conditions of slow substrate dissociation is easily understood as a consequence of the substrate's ability to occupy both the cis and trans substrate binding site when it is present at a saturating concentration in only the cis compartment. If dissociation is rapid, the concentration of the substrate-complex on the trans side is vanishingly small, and the carrier on the trans side exists either in the free form or in the form of a complex with internal inhibitor. If dissociation is slow, on the other hand, then the trans carrier accumulates as the substrate complex and is therefore inaccessible to the inhibitor.

Let us now turn to the observation of non-competitive kinetics. This observation rules out dissociation as the rate-limiting step in transport. It also makes it unlikely that substrate dissociation and carrier reorientation occur at similar rates, but of this conclusion we cannot be certain without additional knowledge about the relative magnitudes of the rate constants in the transport scheme. However, dissociation may be shown without ambiguity to be a very rapid and non-rate-limiting step, if non-competitive inhibition is demonstrated in both directions - in substrate exit with an inhibitor outside, and in substrate entry with an inhibitor inside. On the assumption that dissociation and reorientation occur at equal rates, the observation of non-competitive inhibition in both directions entails that the expressions in both equations (3.5) and (3.9) must equal unity.

$$\frac{2 + (f_1 + f_2)\ /f_{-2}}{(1 + f_1/f_{-1})} = 1 \qquad (3.10)$$

and

$$\frac{2 + (f_{-1} + f_{-2})\ /f_2}{(1 + f_{-1}/f_1)} = 1 \qquad (3.11)$$

From (3.10), $f_1/f_{-1} = 1 + (f_1 + f_2)/f_{-2}$; and from (3.11), $f_{-1}/f_1 = 1 + (f_{-1} + f_{-2})/f_2$. For the first of these two equalities to hold, $f_{-2} >> f_2$, but in this

case the second must be very large, i.e., $1 + (f_{-1} + f_{-2})/f_2 \gg 1$. Non-competitive inhibition in both entry and exit experiments is therefore impossible unless dissociation is a rapid step. Here the following relationship holds (eqn 3.3 and 3.7).

$$\frac{1 + f_1/f_{-2}}{1 + f_1/f_{-1}} = 1 = \frac{1 + f_{-1}/f_2}{1 + f_{-1}/f_1} \qquad (3.12)$$

which is clearly possible.

In summary, competitive inhibition by a trans inhibitor shows that substrate dissociation is rate-limiting if the mechanism is of the classical carrier type. Non-competitive inhibition by a trans inhibitor in both exit and entry experiments demonstrates that carrier-reorientation steps alone must be rate limiting. Mixed competitive and non-competitive inhibition in these experiments shows that carrier reorientation must limit the transport rate but does not exclude the possibility that dissociation of the substrate must also play a part in limiting the rate.

SUMMARY

A simple test to determine the rate-limiting steps in transport is described, which depends on the inhibition pattern produced by a competitive inhibitor on the trans side of the membrane with respect to the substrate. When the mechanism has been shown in other tests to correspond to that of a classical carrier, the observation of competitive inhibition under these conditions provides unequivocal evidence that dissociation of the substrate from the carrier complex determines the rate. If non-competitive inhibition of both influx and efflux is found, carrier reorientation alone must be rate-limiting. If mixed competitive and non-competitive inhibition is observed. carrier reorientation must limit the rate, but substrate dissociation may also participate in the rate-determining processes.

CHAPTER 4

TESTING TRANSPORT MODELS WITH SUBSTRATES AND REVERSIBLE INHIBITORS

INTRODUCTION

Studies of membrane transport have relied, for their interpretation, upon an almost unquestioned acceptance of some version of the classical carrier model, the essence of which is that a substrate binding site in an entity called the carrier is alternately, and not simultaneously, exposed at either surface of the cell membrane. Where other models are alluded to, they have usually been either variations on this theme, in which different physical devices produce the same effect, or simple pores, which probably lack the complexity required to explain many transport phenomena. It is possible to account for transport, however, by models distinctly different from these; and yet, perhaps surprisingly, no systematic attempt appears to have been made to define the alternatives and to design tests by which they may be recognized or eliminated. This is undertaken here. We begin by considering hypothetical mechanisms and grouping them according to expected uniformities in their kinetic behaviour. Then, through analysis of the classes arrived at in this way, we seek criteria by which each may be ruled out. Previously such criteria, when applied to the simple carrier or the pore, have depended solely upon the behaviour of substrates (31). We now investigate the behaviour of reversible inhibitors,

and find them to be as incisive a diagnostic tool as substrates, capable of providing information that substrates cannot give. Together, inhibitors and substrates serve to discriminate among a variety of possible mechanisms.

The potential usefulness of reversible inhibitors in such studies was suggested by our analysis of competitive and non-competitive inhibition of transport, based on a general form of the classical carrier model. Earlier, Lieb and Stein(31,32)had presented a treatment which aimed at establishing rejection criteria for the simple pore and the simple carrier models, involving only the behaviour of substrates.

In the following analysis, simple kinetic tests are invoked, which taken together can distinguish among a number of transport mechanisms. These are (1) the inhibition pattern in a zero trans exit experiment when an inhibitor is present outside the cell; (2) the dependence of the transport rate upon the concentration of an inhibitor present both inside and outside; and (3) the effect of substrate on the trans side of the membrane upon unidirectional flux. Five different transport models are considered and appropriate rate equations are derived, from which it is shown how each model presents different kinetic behaviour. The results are summarized in tables that should be useful in planning and interpreting experiments.

THE REPRESENTATION OF TRANSPORT MECHANISMS

In discussing physical models for transport it will be convenient to use the following descriptive terms; carrier, pore, gated channel, shuttle and mobile carrier:

(i) Carrier. In view of its long usage in a general sense, a carrier will be taken to encompass all mechanisms in which there is specific, saturable transport of substrate.

(ii) Pore. A continuous, open passage extends through the membrane and is accessible to substrate from both sides. Transport involves no moving element other than the substrate itself, and does not necessarily depend on the presence of substrate binding sites.

(iii) Gated Channel. A moveable barrier in a channel shifts back and forth past a fixed substrate binding site. Hence only bound substrate molecules can reach the far side of the barrier and in this way undergo transport.

(iv) Shuttle. The substrate binding site moves past a fixed barrier in the membrane. In consequence, binding to this site is required for passage.

(v) Mobile Carrier. Any specific saturable transport system dependant on the operation of a mobile element. This definition includes both the gate and the shuttle as defined above.

A reading of the literature on transport reveals an unfortunate lack of agreement as to the meaning of these terms. Part of the difficulty stems from a seemingly clear

mechanistic implication of the word "carrier", which has detracted from its acceptance as a general term. In addition the actual mechanism, when fully understood, may not correspond to a single categgry. A mechanism involving elements of both a gate and a shuttle, for example, is easily envisaged; one such is shown in Model 3 below. There is a tendency also to designate a gated channel as a pore, even though its properties would be distinct from those of an open passage through the membrane.

Transport mechanisms may be represented in two different ways, which should be distinguished:

(a) Physical models. The transport process is represented diagrammatically, for example as a shuttle or a gated channel.

(b) Kinetic models. The key intermediates in transport and the rates of their interconversion are represented mathematically. Distinguishable physical models may reduce to the same kinetic description, as is the case for certain shuttles and gated channels.

THE SELECTION OF KINETICALLY DISTINGUISHABLE MECHANISMS

In addition to the classical carrier model, alternative mechanisms involving either a simple pore or a mobile carrier having two substrate binding sites simultaneously accessible to substrate appear to call for close examination, and it would be helpful to have simple kinetic tests available by which they could be confirmed or excluded. Various such mechanisms have been invoked in

experimental studies. For example pore models have often been favoured in explaining fluxes of inorganic ions (33) as well as of larger molecules. Systems which depend on more than one substrate binding site have also been suggested and in the case of the dicarboxylate transporter of E. coli there is clear evidence, both genetic and structural, for two separable membrane binding proteins involved in transport (34). Striking differences in the affinities of substrate analogs on the internal and external membrane surfaces have led to the suggestion that different inner and outer components may exist in the glucose carrier of erythrocytes (5).

The treatment will be limited to models with no more than one binding site exposed to substrate either inside or outside the cell, that is, no more than two when both membrane faces are counted. Such systems give rise to simple hyperbolic substrate saturation curves, in agreement with observations on many facilitated transport systems, e.g. the much studied glucose carrier of erythrocytes (28). Models involving two or more sites accessible to substrate in one pool would exhibit more complex kinetics.

Transport systems may be static as in the case of a pore, or may involve a mobile element, as in a mobile carrier. Among those depending on a mobile element we shall deal with (a) mechanisms with two forms of free carrier

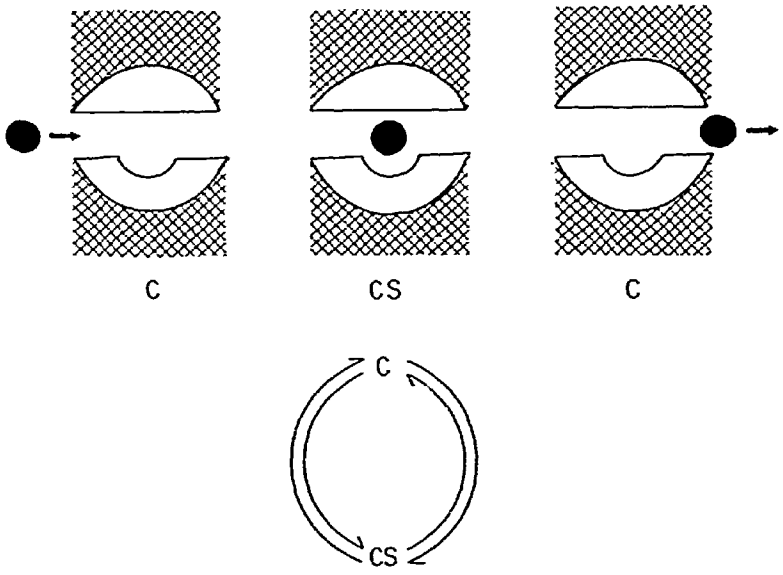
and a substrate site alternately exposed to the two substrate pools; and (b) mechanisms with one form of free carrier, having substrate sites simultaneously accessible to both compartments. Among models based on the pore, in which the free carrier has only one form, those accommodating either one or two substrate molecules are considered.

TRANSPORT MODELS

The models to be treated are described below. In each case the physical process is represented diagrammatically. The corresponding kinetic scheme is given in a simplified form, in which only those intermediates assumed to exist in significant amounts are shown. In general the physical representation chosen is not a unique determinent of the kinetic scheme.

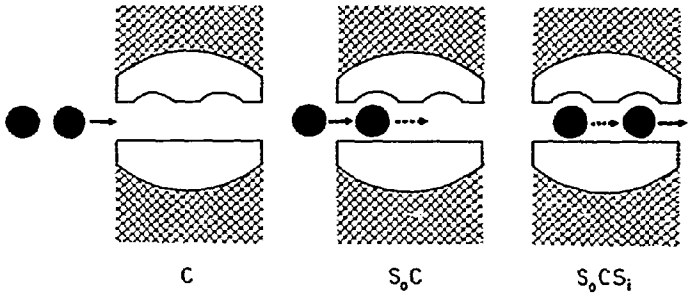
Model 1. A simple pore is represented which can be occupied by one substrate molecule at a time. The carrier exists in only one form and is accessible to substrate on both sides of the membrane.

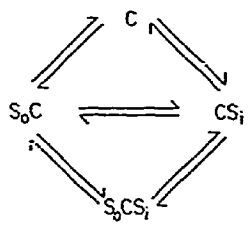
Occupancy applies only to that region of an opening through the membrane which limits the rate of substrate diffusion. This region could be very short even though a pore of uniform diameter extending across the whole membrane would accommodate many substrate molecules. To account for substrate specificity, there must be severe restrictions upon the molecular structures permitted to enter this passage, which for this reason would be the point of slowest



movement, i.e. the rate-limiting step in diffusion. In addition, it would be impossible for two substrate molecules to pass one another at this point, with the result that flux in one direction would oppose flux in the other. Competitive inhibition is not easily explained, but would occur if an inhibitory substrate analog is bound outside the restrictive channel or if it partly enters this region but owing to steric restraints does not proceed further.

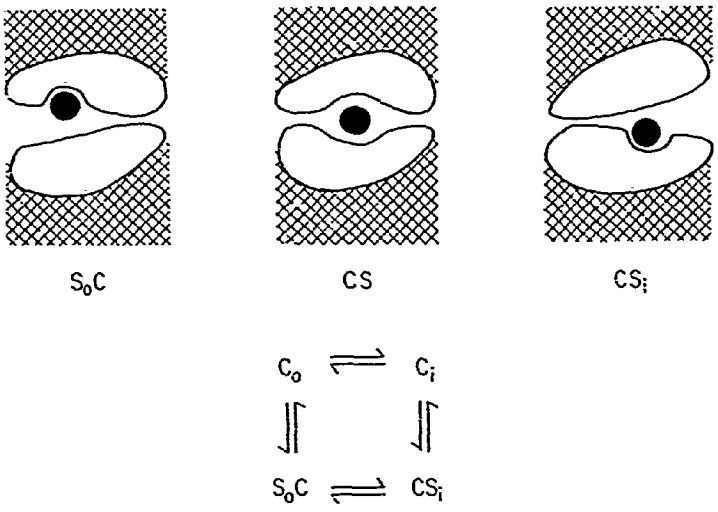
Model 2. This is a simple pore with room for two substrate molecules, which in other respects is like the pore in Model 1. Because the substrate must fit the pore closely





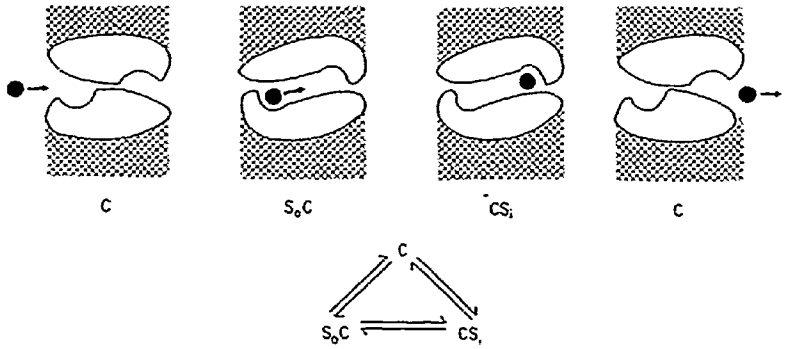
there is interference between molecules moving in opposite directions. The kinetic treatment of this model should apply as well to longer pores which accommodate more than two substrate molecules, for in any case the predicted behaviour depends on the ability of molecules to enter the pore simultaneously from opposite compartments.

Model 3. The carrier exists in two forms, one in equilibrium with substrate in the external pool, the other with substrate in the internal pool, and their interconversion is the essential step in translocation of substrate through the membrane.



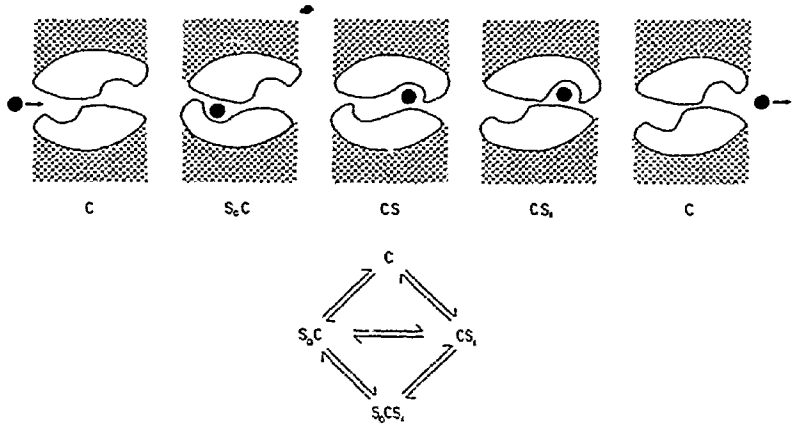
The nature of this reorientation step is necessarily left undefined. It may involve operation of a gate or a shuttle or a combination of the two, as in the diagram. The intermediate form of the carrier (CS) is assumed to be kinetically unimportant.

Model 4. A gated channel mechanism is represented having only one form of free carrier, with binding sites simultaneously available to substrate in both internal and external pools. Addition of a substrate molecule on either side induces a conformational change associated with the transport process which occludes the other site (S_oC and CS_i);



in consequence a ternary complex of carrier and two substrate molecules is not formed. This conformational change is the essential step in transport and therefore should not occur with a competitive inhibitor. For this reason addition of an inhibitor at one site does not occlude the other; hence two inhibitor molecules may be bound simultaneously. The simplified kinetic scheme omits the initial complex since this is assumed to form transiently.

Model 5. This is a gated channel mechanism similar to the previous one, except that two substrate molecules may add simultaneously to sites on opposite sides of the membrane. The kinetic scheme appears to be the same as that for the second pore model (Model 2), but on account of the difference in mechanism, substrate molecules moving in opposite directions may now assist one another rather than interfere.



The dominant properties of the five models are summarized in Table 4.1

TESTS OF THE MODELS

I. Selection of Inhibitors

A word should be said about the properties of the inhibitors to be used in these experiments. First, inhibitor and substrate, when present in the same compartment, must compete for binding to the carrier. Second, some experiments depend on an inhibitor present in the external medium only, while others involve an inhibitor inside the cell as well. The way in which these requirements may be fulfilled is now considered.

TABLE 4.1. .

CHARACTERISTICS OF TRANSPORT MODELS

Model	Number of Sites per Free Carrier Form	Number of Free Carrier Forms	Involvement of Carrier Rearrangement Step	Number of Substrate Molecules Bound
1	1	1	-	1
2	2	1	-	2
3	1	2	+	1
4	2	1	+	1
5	2	1	+	2

The analysis of the kinetics of reversible inhibition for the classical carrier model, in Chapter 2, showed that the only unambiguous test of whether substrate and inhibitor can add to the same binding site is inhibition of equilibrium exchange (or exchange in the final steady state in active systems). The reason is obvious, since in this case substrate is always in a position to compete with the inhibitor. In experiments in which substrate is initially present on only one side of the membrane it may be unable to reach sites accessible to an inhibitor on the other side. Whatever the mechanism the kinetics of inhibition must be competitive in an equilibrium exchange experiment if the inhibitor binds at the substrate site.

An inhibitor restricted to the external solution is easily found. Inhibitors able to penetrate the cell are probably less common, but are known, as in the example of a number of glucose derivatives (22,23). It is sometimes found that such inhibitors, though having access to both sides of the membrane, bind preferentially to carrier on one side (5,17). A combination of two inhibitors may then be employed in the proposed experiments, one adding to external, the other to internal sites. Where inhibitors with the required properties are unavailable it may be possible to seal a non-penetrating inhibitor inside cell ghosts or membrane vesicles.

II. Kinetic Analysis

Kinetic equations are now derived for the above models. The kinetic schemes are expanded to include two substrates, S (radioactive) and T (unlabeled), and a reversible competitive inhibitor I. The rate-limiting step is assumed to be movement of substrate through the membrane rather than dissociation from the binding site in all schemes except the first, where this would not be possible. Equations are given for initial rates (v) of unidirectional exit of substrate S under the conditions of inhibitor and substrate distribution required in the proposed experimental tests:

(a) Inhibitor is present outside (trans). $[I_i] = 0$; $[S_o] = [T_o] = [T_i] = 0$

(b) Inhibitor is present on both sides. $[I_i] = [I_o] = [I]$; $[S_o] = [T_o] = [T_i] = 0$

(c) Unlabeled substrate is present outside (trans). Inhibitor is absent. $[I_o] = [I_i] = 0$; $[S_o] = [T_i] = 0$

The equation derived for this third condition is applied to three different experimental situations: (i) the internal substrate concentration is held constant and the external concentration varied; (ii) the external concentration is constant and the internal varied; and (iii) the external and internal concentrations which are equal, are varied.

Scheme 1. (one-site, pore; Figure 4.1)

Condition (a): Inhibitor outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{\bar{K}_{Si}}{[S_i]}\left(1 + \frac{[I_o]}{K_{Io}}\right)\right\} \qquad (4.1).$$

where $\bar{K}_{Si} = (f_{-1} + f_{-2})/f_2$. Inhibition is competitive, since only the slope of a reciprocal plot (1/v against $1/[S_i]$) is increased by the inhibitor.

Condition (b): Inhibitor both sides

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{\bar{K}_{Si}}{[S_i]}\left[1 + [I]\left(\frac{1}{K_{Ii}} + \frac{1}{K_{Io}}\right)\right]\right\} \qquad (4.2).$$

Inhibition is again competitive.

Condition (c): Unlabeled substrate outside (trans).

$$\frac{1}{v} = \frac{}{f_{-1}C_t}\left\{1 + \frac{\bar{K}_{Si}}{[S_i]}\left(1 + \frac{[T_o]}{\bar{K}_{To}}\right)\right\} \qquad (4.3).$$

where $\bar{K}_{To} = (f_{-4} + f_{-3})/f_3$

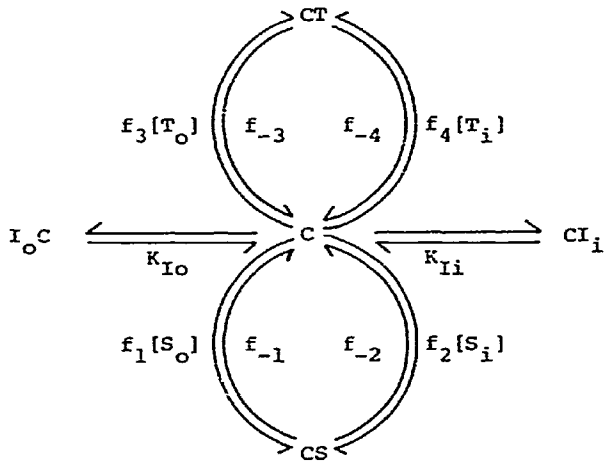
(i) $[S_i]$ constant, $[T_o]$ varied: When $[T_o] >> [S_i]$, $v \to 0$.

(ii) $[T_o]$ constant, $[S_i]$ varied: Unidirectional efflux of substrate S is inhibited by external substrate, T_o, in a purely competitive manner.

(iii) Equilibrium exchange: $[S_i] = [T_o] = [S]$. As S and T represent the same substrate, $\bar{K}_{To} = \bar{K}_{So}$; and equation (4.3)

FIGURE 4.1

Scheme 1. General transport scheme for the one-site simple pore model (1), showing two substrates, S and T. The same substrate complex, CS, is formed whether substrate enters the pore from the external or internal solutions (S_o or S_i respectively). This is also true for substrate T. An inhibitor, being unable to pass through the pore, forms a complex on one side of the membrane distinguishable from that on the other (I_oC and CI_i).


CT
$f_3[T_o]$
f_{-3}
f_{-4}
$f_4[T_i]$
I_oC
C
CI_i
K_{Io}
K_{Ii}
$f_1[S_o]$
f_{-1}
f_{-2}
$f_2[S_i]$
CS

becomes

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left(1 + \frac{f_1}{f_2} + \frac{\bar{K}_{Si}}{[S]}\right) \qquad (4.4).$$

The relationship is linear. The maximum rate of equilibrium exchange is less than the maximum rate of zero trans exit by the factor $(1 + f_1/f_2) = (1 + \bar{K}_{Si}/\bar{K}_{So})$, as comparison of eqns(4.4) and (4.1) shows.

Scheme 2. (Two-site, pore; Figure 4.2).

Condition (a): Inhibitor outside.

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{[I_o]}{K_{Io}} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{[I_o]}{K_{Io}}\right)\right\} \qquad (4.5).$$

Inhibition is purely non-competitive since slope and intercept are raised equally by the inhibitor.

Condition (b): Inhibitor both sides

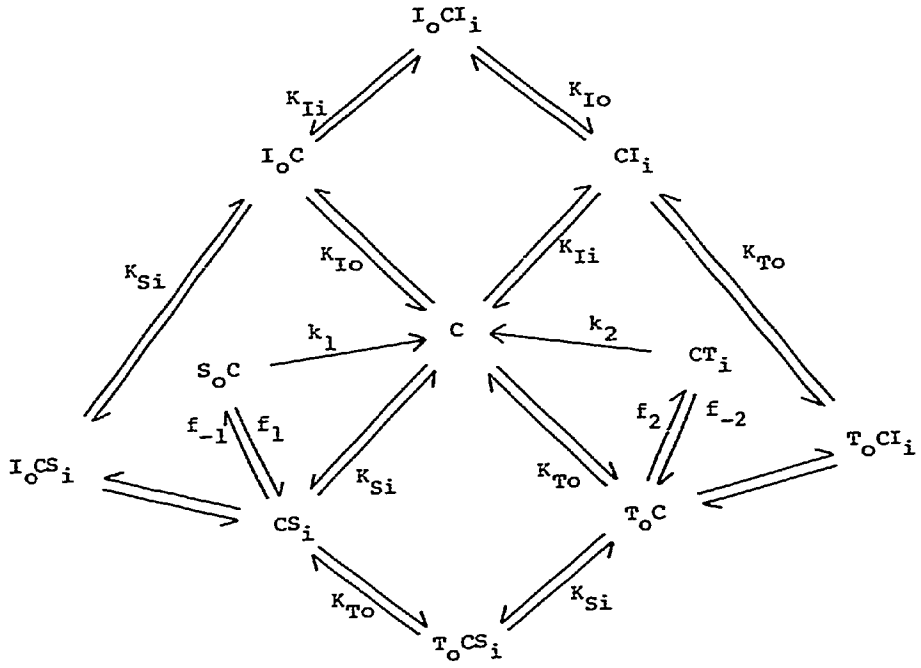
$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{[I]}{K_{Io}} + \frac{K_{Si}}{[S_i]}\left[1 + [I]\left(\frac{1}{K_{Io}} + \frac{1}{K_{Ii}}\right) + \frac{[I]^2}{K_{Ii}K_{Io}}\right]\right\} \qquad (4.6).$$

Inhibition is mixed competitive and non competitive, in that slope and intercept are both increased, but the slope more than the intercept. Dependence on the inhibitor concentration involves a square term, which is apparent in plots of 1/v against [I].

Condition (c): Unlabeled substrate outside (trans).

FIGURE 4.2

Scheme 2. Transport scheme for the two-site, simple pore model (2), showing two substrates S and T and an inhibitor I. A ternary complex of carrier with two substrate molecules or one substrate and one inhibitor may be formed. The subscript i denotes a complex formed from substrate or inhibitor inside the cell, and o from outside. To simplify the full kinetic scheme as far as possible for our purpose, it is assumed that substrate T is present only externally (T_o) and substrate S only internally (S_i). The inhibitor is present in both compartments (I_o and I_i).


I_oCI_i
K_{Ii}
K_{Io}
I_oC
CI_i
K_{Si}
K_{Io}
K_{Ii}
K_{To}
k_1
C
k_2
S_oC
CT_i
f_{-1}
f_1
f_2
f_{-2}
I_oCS_i
K_{Si}
K_{To}
T_oCI_i
CS_i
T_oC
K_{To}
K_{Si}
T_oCS_i

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left(1 + \frac{K_{Si}}{[S_i]}\right)\left(1 + \frac{[T_o]}{K_{To}}\right) \qquad (4.7).$$

(i) $[S_i]$ constant, $[T_o]$ varied: As $[T_o] \to \infty$, $v \to 0$

(ii) $[T_o]$ constant, $[S_i]$ varied: Efflux of substrate S is non competitively inhibited by T_o.

(iii) Equilibrium exchange: $[S_i] = [T_o] = [S]$; $K_{To} = K_{So}$:

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left(1 + \frac{K_{Si}}{K_{So}} + \frac{K_{Si}}{[S]} + \frac{[S]}{K_{So}}\right) \qquad (4.8).$$

Equilibrium exchange is subject to substrate inhibition, and as $S \to \infty$, $v \to 0$.

Scheme 3. (One-site, mobile; Figure 4.3).

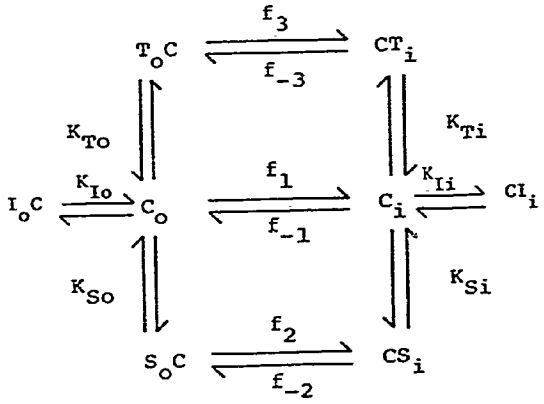
Condition (a): Inhibitor outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-2}C_t}\left\{1 + \frac{f_{-2}}{f_1}\left(1 + \frac{[I_o]}{K_{Io}}\right) + \frac{K_{Si}}{[S_i]}\left[1 + \frac{f_{-1}}{f_1}\left(1 + \frac{[I_o]}{K_{Io}}\right)\right]\right\} \qquad (4.9).$$

This model can account for a variety of inhibition patterns. Pure non-competitive behaviour results when $f_{-2} = f_{-1}$. If $f_{-2} > f_{-1}$, as in the case of accelerated exchange, inhibition tends towards the uncompetitive type. If $f_{-1} > f_{-2}$, where exchange is slower than zero trans flux, inhibition becomes partly competitive. A further complication is that if the rate-limiting step in transport is dissociation of the carrier-substrate complex, inhibition will always be competitive

FIGURE 4.3.

Scheme 3. The classical mobile carrier model (3), involving one substrate binding site and two forms of free carrier, one in which the site is exposed to the external, the other to the internal, solution. Transport depends on a carrier reorientation step in which there is interconversion of the two carrier forms. The significance of subscripts i and o is as in the previous schemes.


f_3
T_oC
CT_i
f_{-3}
K_{To}
K_{Ti}
K_{Io}
f_1
K_{Ii}
I_oC
C_o
C_i
CI_i
f_{-1}
K_{So}
K_{Si}
f_2
S_oC
CS_i
f_{-2}

as shown in the previous chapter. However slow dissociation is unlikely, as shown elsewhere in this thesis.

Condition (b): Inhibitor both sides.

$$\frac{1}{v} = \frac{1}{f_{-2}C_t}\left\{1 + \frac{f_{-2}}{f_1}\left(1 + \frac{[I]}{K_{Io}}\right) + \frac{K_{Si}}{[S_i]}\left[1 + \frac{f_{-1}}{f_1} + [I]\left(\frac{1}{K_{Ii}} + \frac{f_{-1}}{f_1 K_{Io}}\right)\right]\right\} \quad (4.10).$$

Depending on the values of the kinetic constants, the inhibitor may show various degrees of non-competitiveness.

Condition (c): Unlabeled substrate outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-2}C_t}\left\{1 + f_{-2}\left(\frac{1 + [T_o]/K_{To}}{f_1 + f_3[T_o]/K_{To}}\right) + \frac{K_{Si}}{[S_i]}\left[1 + f_{-1}\left(\frac{1 + [T_o]/K_{To}}{f_1 + f_3[T_o]/K_{To}}\right)\right]\right\} \quad (4.11).$$

(i) $[S_i]$ constant, $[T_o]$ varied: At low values of $[S_i]$, where $K_{Si}/[S_i] \gg 1$, relative rates in the presence of saturating levels of T_o and in the absence of T_o are given by:

$$\frac{v_{[To]\to\infty}}{v_{[T_o]=0}} = \frac{(1 + f_{-1}/f_1)}{(1 + f_{-1}/f_3)} \quad (4.12).$$

This ratio may be greater or less than unity, depending on the relative sizes of f_3 and f_1: when f_3 is the larger, exchange is more rapid than zero trans flux, and when smaller, less rapid.

(ii) $[T_o]$ constant, $[S_i]$ varied. Let T and S represent the same substrate, so that $f_3 = f_2$. The characteristic pattern of acceleration or inhibition by the unlabeled

trans substrate, as exhibited in reciprocal plots, depends, on the ratio of f_{-1} and f_{-2} (whereas the occurrence of acceleration as against inhibition is determined by f_1/f_2). When $f_{-2}>f_{-1}$, the most probable circumstances in the case of acceleration ($f_2>f_1$), the pattern tends towards the uncompetitive, the intercept falling more than the slope (in the case of inhibition, rising more). When $f_{-1}>f_{-2}$ which is probable in inhibition, the pattern is competitive, the slope being altered more than the intercept. If $f_{-1} = f_{-2}$ the effect is of the non-competitive type, with slope and intercept identically altered.

(iii) Equilibrium exchange: $[S_i] = [T_o] = [S]$; $K_{To} = K_{So}$, $f_3=f_2$, $f_{-3}=f_{-2}$.

$$\frac{1}{v} = \frac{1}{f_{-2}C_t}\left\{1 + \frac{f_{-2}}{f_2} + \frac{K_{Si}}{[S]}\left(1 + \frac{f_{-1}}{f_1}\right)\right\} \qquad (4.13).$$

The relationship between 1/v and 1/[S] is now linear. The ratio of V_{max} for exchange to that for zero trans efflux (from eqns.4.9 and4.13)is equal to $(1 + f_{-2}/f_1)/(1 + f_{-2}/f_2)$, a quantity which we shall find useful in discriminating among the models.

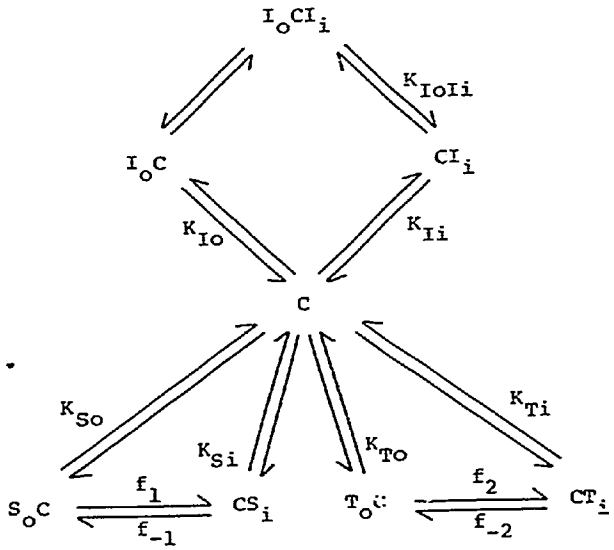
Scheme 4. (Two site, mobile, with site occlusion; Fig. 4.4)

Condition (a): inhibitor outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{K_{Si}}{[S_i]}\left(1 + \frac{[I]_o}{K_{Io}}\right)\right\}$$

FIGURE 4.4.

Scheme 4. Mechanism involving a mobile element and one form of free carrier, as in model 4, with substrate sites exposed to both pools, external and internal (subscripts o and i respectively). Substrate may add to the external or internal site but not to both at the same time, since a conformational change related to transport occludes the second site following binding of substrate. Inhibitors, not undergoing transport, do not induce this conformational change. Hence a ternary complex may form, a carrier with two inhibitor molecules.


I_oCI_i
K_{IoIi}
I_oC
CI_i
K_{Io}
K_{Ii}
C
K_{So}
K_{Si}
K_{To}
K_{Ti}
S_oC
f_1
f_{-1}
CS_i
T_oC
f_2
f_{-2}
CT_i

Competitive inhibition.

Condition (b): Inhibitor both sides.

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{K_{Si}}{[Si]}\left[1 + [I]\left(\frac{1}{K_{Ii}} + \frac{1}{K_{Io}}\right) + \frac{[I]^2}{K_{Ii}\,K_{IoIi}}\right]\right\} \quad (4.15).$$

Competitive inhibition, non-linear in [I].

Condition (c): Unlabeled substrate outside (trans).

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{K_{Si}}{[S_i]}\left(1 + \frac{[T_o]}{K_{To}}\right)\right\} \quad (4.16).$$

(i) $[S_i]$constant, $[T_o]$ varied: When $[T_o] >> [S_i]$, $v \to 0$.

(ii) $[T_o]$constant, $[S_i]$ varied: Competitive inhibition.

(iii) Equilibrium exchange: $[S_i] = [T_o] = [S]$: $K_{To} = K_{So}$:

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left(1 + \frac{f_{-1}}{f_1} + \frac{K_{Si}}{[S]}\right) \quad (4.17).$$

The ratio of the maximum rates of exchange and zero trans exit (from eqn.4.13)) is now $1/(1 + f_{-1}/f_1)$.

Scheme 5. (Two-site, mobile, without site occlusion; Fig.4.5).

Condition (a): Inhibitor outside (trans)

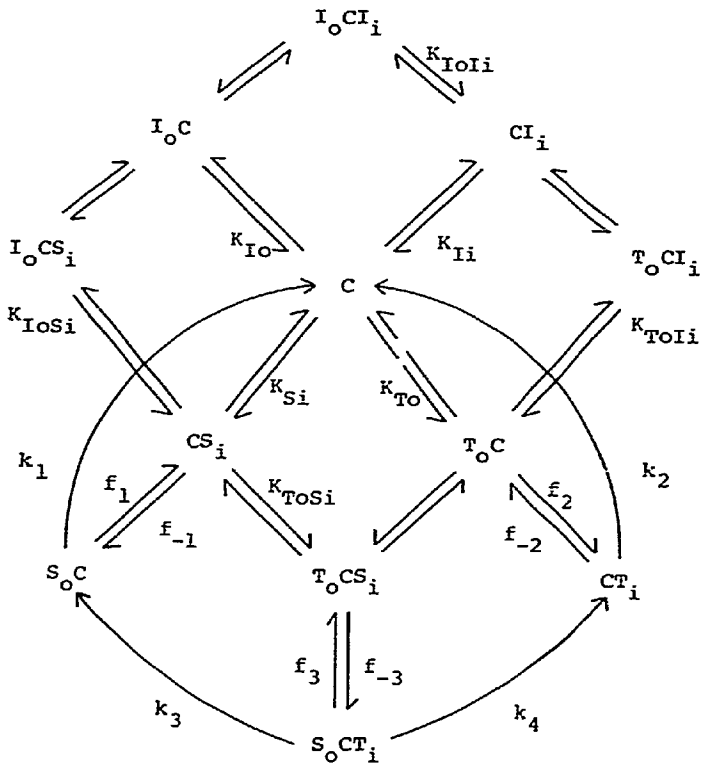
$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{\left(1 + \frac{[I_o]}{K_{IoSi}}\right) + \frac{K_{Si}}{[S_i]}\left(1 + \frac{[I_o]}{K_{Io}}\right)\right\} \quad (4.18).$$

Non-competitive inhibition

Condition (b): Inhibitor both sides.

FIGURE 4.5.

Scheme 5. Mechanism involving a mobile element and one form of free carrier with a site exposed in both pools, as in model 5, where a ternary complex may form with two substrate molecules, two inhibitor molecules, or one substrate and one inhibitor. Movement of substrate may occur in the complex of two substrate molecules (f_3 and f_{-3}). For the sake of simplicity it is assumed that substrate T is present only externally (T_o) and substrate S only internally (S_i), as in the treatment of scheme 2.


I_oCI_i
K_{IoIi}
I_oC
CI_i
I_oCS_i
K_{Io}
K_{Ii}
T_oCI_i
C
K_{IoSi}
K_{ToIi}
K_{Si}
K_{To}
CS_i
T_oC
k_1
f_1
K_{ToSi}
f_2
k_2
f_{-1}
f_{-2}
S_oC
T_oCS_i
CT_i
f_3
f_{-3}
k_3
k_4
S_oCT_i

$$\frac{1}{v} = \frac{1}{f_{-1}C_t}\left\{1 + \frac{[I]}{K_{IoSi}} + \frac{K_{Si}}{[S_i]}\left[1 + [I]\left(\frac{1}{K_{Io}} + \frac{1}{K_{Ii}}\right) + \frac{[I]^2}{K_{Ii}\ K_{IoIi}}\right]\right\} \quad (4.19).$$

Non-competitive inhibition, non linear in [I].

Condition (c): Unlabeled substrate outside (trans).

$$\frac{1}{v} = \frac{1 + \frac{[T_o]}{K_{ToSi}} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{[T_o]}{K_{To}}\right)}{C_t\left(f_{-1} + f_{-3}[T_o]/K_{ToSi}\right)} \quad (4.20).$$

(i) $[S_i]$ constant, $[T_o]$varied. When $[S_i]/K_{Si} << 1$ the ratio of rates with saturating trans substrate ($[T_o] \to \infty$ and in the absence of T_o is equal to f_{-3}/f_{-1}, which could vary from zero to greater than one.

(ii) $[T_o]$ constant, $[S_i]$ varied. Inhibition of acceleration by trans substrate is non-competitive, provided that K_{To} and K_{SiTo} are of similar size.

(iii) Equilibrium exchange. $[S_i]=[T_o]=[S]$; $K_{To}=K_{So}$; $K_{ToSi}=K_{SoSi}$:

$$\frac{1}{v} = \frac{1 + \frac{[S]}{K_{SoSi}} + \frac{K_{Si}}{[S]}\left(1 + \frac{[S]}{K_{So}}\right)}{C_t\left(f_{-1} + f_{-3}\,[S]/K_{SoSi}\right)} \quad (4.21).$$

This relationship is obviously non-linear in [S] (unless $f_{-3}=f_{-1}$ and $K_{SoSi} = K_{So}$, when the presence of trans substrate has no effect on the rate).

DISCUSSION

I. Inhibitors

The usefulness of competitive inhibitors in testing transport models is apparent from Table 4.2, which summarizes the behaviour of the five systems. It is seen that a trans inhibitor (i.e. inhibitor and substrate occupying opposite sides of the membrane) produces non-competitive inhibition in some systems, and competitive inhibition in others. In all cases the dependence of the rate on the inhibitor concentration is linear. With inhibitor present on both sides, inhibition may be competitive, non-competitive, or a combination of the two (mixed). The dependence on the inhibitor concentration now reveals the number of substrate binding sites available at a single moment on either membrane surface, being linear for some models and related to the square of the concentration in others. These tests distinguish all but two of the models, namely 2 and 5. It is to be noted that the simultaneous existence of internal and external binding sites should become obvious in inhibitor studies, but may be masked in experiments with substrates. The latter, however, reveal properties of the system not disclosed by inhibitors.

II. Unlabeled Trans Substrates

Tests with a trans substrate are summarized in Table 4.3. In three of the models (1, 2, and 4) inhibition

TABLE 4.2.

INHIBITION PATTERNS IN SUBSTRATE EXIT EXPERIMENTS

	Inhibitor Outside		Inhibitor Both Sides	
Model	Type	Concentration dependence	Type	Concentration dependence
1	competitive	[I]	competitive	[I]
2	non-competitive	[I]	mixed	$[I]^2$
3	non-competitive*	[I]	non-competitive**	[I]
4	competitive	[I]	competitive	$[I]^2$
5	non-competitive	[I]	mixed	$[I]^2$

*See discussion under eqn. (4.9)

**See discussion under eqn. (4.10)

TABLE 4.3.

EFFECTS OF TRANS SUBSTRATE (UNLABELED T_o) ON SUBSTRATE EFFLUX (LABELED S_1)

Model	Type of Effect	Characteristic Pattern*		$\frac{\bar{\bar{V}}}{\bar{V}_{So}} + \frac{\bar{\bar{V}}}{\bar{V}_{Si}}$ **	Dependence of Equilibrium Exchange Rates on [S] ***
		Inhibition	Acceleration		
1	Inhibition	competitive	-	1	hyperbolic
2	Inhibition	non-competitive	-	0	substrate inhibition
3	Can accelerate	competitive	uncompetitive	$1+\left\{\frac{1/f_1 + 1/f_{-1}}{1/f_2 + 1/f_{-2}}\right\}$	hyperbolic
4	Inhibition	competitive	-	1	hyperbolic
5	Can accelerate	non-competitive	non-competitive	$f_{-3}\left\{\frac{1}{f_1} + \frac{1}{f_{-1}}\right\}$	Sigmoid, hyperbolic, or substrate inhibition****

* The patterns seen with a trans substrate and a trans inhibitor (Table 4.2)are necessarily related since both depend on f_{-1}/f_{-2}.

** $\bar{\bar{V}}$, $\bar{V}_{So}$ and $\bar{V}_{Si}$ are maximum rates of exchange, influx and efflux, respectively. Equations for zero trans influx were omitted in the text, for the sake of brevity. They are easily found by inspection of equations for zero trans efflux and the corresponding transport schemes, since influx and efflux expressions mirror one another, with interchange of entry and exit rate constants and concentrations.

*** $[S] = [S_1] = [T_o]$

**** Hyperbolic if $f_{-1} = f_{-3}$, in which case a trans substrate has no effect on the rate (second column). Sigmoid if $f_{-3} > f_{-1}$, when a trans substrate accelerates efflux. Substrate inhibition if $f_{-1} > f_{-3}$.

is inevitable. In 5 inhibition is possible, but depending on rate constants acceleration may occur. In 3 either acceleration or the absence of any effect is expected of a good substrate, though inhibition is seen if loaded carrier reorientates more slowly than free carrier ($f_1 > f_2$).

In many respects the behaviour of 2 and 5 is similar, but the occurrence of acceleration in 5 but not 2 would distinguish between them. Further, a trans substrate in model 2 would reduce the rate to zero. In model 5 on the other hand retardation may be only partial.

The inhibition produced by trans substrate may be competitive or non-competitive in different models and this too serves to distinguish among them (second column). The characteristics of acceleration in models 3 and 5 may also differ (third column). In model 5 acceleration is purely "non-competitive", in the sense that at varying concentrations of the labeled substrate whose movement is followed the fractional increase in rate due to trans substrate is a constant. In model 3 the behaviour is governed by equation (4.11). Where T and S are identical substrates, $f_3=f_2$ and $K_{To}=K_{So}$. Clearly, acceleration occurs when $f_2>f_1$ (.i.e. loaded carrier reorientates faster than free carrier). The form that acceleration takes depends on the relative values of f_{-1} and f_{-2}, as inspection of the rate equation shows.

The expression $(\bar{\bar{V}} / \bar{V}_{So}) + (\bar{\bar{V}} / \bar{V}_{Si})$, i.e. the sum of the ratio of exchange and influx rates plus the ratio of exchange and efflux rates (fourth column), is also informative. In model 1 and 4 the value is unity, in 2 it is zero, in 3 it is greater than unity, and in 5 it may have any value depending on the relative rate constants in the scheme. Only model 5 can give a value between zero and one.

As shown in the last colum of Table 4.3, reciprocal plots for equilibrium exchange are non-linear in model 2 and may be non-linear in model 5.

III. Obligatory Exchange

Many of the transport systems of the mitochondrial inner membrane, as well as the anion transport system in red blood cells, function exclusively under conditions of exchange. Only models 3 and 5 can account for this behaviour. In model 3 obligatory exchange results if free carrier cannot undergo reorientation ($f_1=f_{-1}=0$), and in model 5 if substrate movement occurs only in the ternary complex of carrier with two substrate molecules ($f_1=f_{-1}=f_{-2}=0$).

IV. Limitations of Pore Models

After all relevant tests have been applied, ambiguity should remain in only one special case: schemes 2 and 5 are

indistinguishable if exchange in the latter system does not occur at saturating substrate concentrations, owing to a failure of substrate interchange in the CS_2 complex ($f_{-3}=0$). However any pore mechanism such as Scheme 2 suffers from a weakness which no semi-permeable membrane could afford except possibly in the case of small ions such as Na^+ and K^+. The difficulty is that all molecules smaller than the substrate should be able to navigate the channel (baring electrostatic repulsion). Since transport systems exist for compounds as large as disaccharides, nucleosides, uridine diphosphate glucose, arginine and phenylalanine, and ATP and ADP, high selectivity for smaller molecules would be difficult to account for (15). In any case specificity is not easily explained, since binding sites in a pore would retard molecules of high affinity rather than facilitate their movement. Binding sites would accord better with the specificity of inhibition by non-transported substrate analogs. Substrate specificity is more likely to be explained by a sieving action, as suggested by Keynes et al.(33), resulting from exclusion on the basis of either size or electrostatic charge.

In our analysis we have postulated that a simple pore could not exhibit accelerated exchange (Table 4.2), but it is alleged that unstirred layers of sufficient depth at the surface of the membrane could produce this behaviour

(35). A labeled substrate, once having traversed the pore, would remain for a time in the unstirred layer and could therefore return; an unlabeled trans substrate would compete in this back reaction and increase the net flux of label. However a layer of the required dimensions is found to be a practical impossibility (36-38). Even without this objection the hypothesis is questionable, for it depends on substrate molecules passing simultaneously in opposite directions within the rate-limiting segment of the pore. If this is not possible a trans substrate at high concentration eliminates flux altogether (eqn4.3 and4.7).

V. Active Transport

In the analysis of reversible inhibition for a mechanism of the classical carrier type 3 in Chapter 2, the characteristic kinetic patterns produced by an inhibitor were shown to be identical whether transport was facilitated (non-active) or concentrative (active). Similarly the patterns found here for other models should apply to active as well as facilitated transport systems.

SUMMARY

The kinetic behaviour of five models for biological transport, only one of which is based on the classical carrier mechanism, is investigated. All give hyperbolic substrate saturation curves in accord with experimental observations on many systems. Several simple kinetic tests with substrates and competitive inhibitors serve to exclude or confirm proposed models. The tests involve measuring rates of efflux of radioactive substrate in the presence of (i) a competitive inhibitor outside the cell; (ii) inhibitor inside and outside; and (iii) unlabeled substrate outside. Rules for testing hypothetical mechanisms are presented in tables which may be consulted directly, disregarding the mathematical derivation.

CHAPTER 5

TESTING TRANSPORT MECHANISMS FOR SIMULTANEOUS EXPOSURE OF SUBSTRATE BINDING SITES USING REVERSIBLE COMPETITIVE INHIBITORS

INTRODUCTION

Over the years a great many proposals have been made to explain membrane transport, and various physical devices have been suggested which could conceivably bring about a specific translocation of substrates across the membrane. Many of these models can account for certain essential features of mediated transport systems; for example they exhibit simple hyperbolic substrate saturation curves, and they can give rise to the phenomena of counter-flow (13,14) and trans-acceleration (11,12). Because of this, insufficient attention has been given to the problem of subjecting individual models to further tests that might differentiate among them. In the course of a theoretical study of competitive inhibition in relation to transport mechanisms, it became apparent that a very simple and fundamental new test may be applied to these models. This test reveals an essential property of any transport system, which is whether substrate sites are simultaneously or alternately exposed on the two membrane surfaces.

In dealing with this problem, we shall first consider transport mechanisms which have been proposed in the past,

and group them under two headings, according to whether the mechanism involves the exposure of either one or two substrate sites to the aqueous medium surrounding the membrane, at any given instant. A kinetic treatment will then be presented which allows us to distinguish between the members of the two groups.

TRANSPORT MECHANISMS

I. First Group: One Site Exposure

The earliest proposal of a carrier which could explain many aspects of the behaviour of mediated transport systems involved a moveable entity within the membrane that forms a complex with the substrate at one surface, and after migrating through the membrane, releases the substrate at the opposite side (see LeFevre, (39)). Since there is only one substrate site in this model, it was necessary that it be alternatively exposed on either side of the membrane. The carrier was regarded as moving transversely across the membrane.

A related mechanism involving a rotational motion of the carrier was suggested by LeFevre (40). Here the membrane component bearing the substrate binding site turns through 180^{o}, so that the site which is first exposed at one surface becomes exposed at the other.

A distinctly different version involves not a moveable binding site but a fixed site and a moveable gate

in a pore. Various representations of such a mechanism have been suggested, including those of Patlak (41,42) and Vidaver (43). Other models may combine movements of both the binding site and a restraining barrier located in a channel, as in the proposals of Klingenberg (44) and Singer (45), and in a model which we have suggested in the previous chapter (Model 3).

In the latter the possibility of two physically distinct sites located in different membrane proteins is allowed for. As a result of a conformational linkage between these two proteins only one site is exposed to the aqueous medium at any instant.

II. Second Group: Two Site Exposure

In this second group models have been gathered in which sites are simultaneously exposed on both sides of the membrane. These proposals are listed below. Since the details of the mechanisms are unnecessary for present purposes, they have been omitted.

We shall begin by considering the so called "hemiport" of Stein (46). This model postulates the existence of components which are restricted to either the inner or outer surfaces of the membrane. The inner and outer components are postulated to move independently in the plane of the membrane and occasionally to interact through it, forming a complex which simultaneously presents

substrate sites to the intracellular and extracellular solutions. As a result of the interaction a bound substrate molecule is transferred from one component to the other and thus undergoes transport.

In an elaboration of this concept, Lieb and Stein (8) proposed an "internal transfer oligomeric" model which pictures a tetramer of interacting subunits spanning the membrane. In two of these subunits the substrate sites are exposed to the medium, while in the other pair the sites face the core of the membrane. Transport depends on a linked conformational change, in which the orientation of the two pairs of subunits is interchanged. Again therefore substrate sites are postulated to be simultaneously accessible on both surfaces. LeFevre suggested an "introverting hemiport" model (9) which resembles the "internal transfer" model of Lieb and Stein in some of its properties but assumes that the inward-outward conformational transition in each monomer is completely independent of transitions in the opposing monomer in the other membrane face. The details of the mechanism may be omitted here, since its essential property for our purposes is the possession of simultaneously accessible substrate sites by each of the membrane-spanning dimers.

The "lattice-membrane" model of Naftalin (47) is related to the "bucket-brigade" of Danielli (48) and the the single file diffusion discussed by Heckmann (49)

but postulates a double file within a single pore. Although these three mechanisms may differ in detail they agree in one absolutely essential feature, the exposure of substrate sites at both membrane surfaces. An alternative model which was introduced and discussed in Chapter 4 also involves the simultaneous exposure of two binding sites (Model 5). In this case substrate translocation depends upon mutual displacements of a binding site and of a moveable barrier in a channel.

In treating these two groups of transport models, rate equations will be derived for a transport scheme of the classical carrier type, representing the first group, and for the last mentioned scheme above, representing the second group (Model 5, Chapter 4). Though the rather diverse models in the second group should have distinctive properties in substrate transport their response to inhibitors on the two sides of the membrane should be of the same form; and this allows us to distinguish them from the members of the first group.

KINETIC THEORY

General equations were derived earlier for enzyme rates in the presence of two reversible inhibitors (50). A simple relationship was demonstrated between the reaction rate measured with both inhibitors present, and the rates determined in the presence of each inhibitor alone or in

the absence of inhibitor. The relationships differed depending on whether the enzyme was able to bind only one of the inhibitor molecules at a time, or both. In the first case the relationship was linear in inhibitor concentration, and in the second, squared concentration terms entered in the equation. This made it easy to decide experimentally whether the enzyme contained one or two inhibitor binding sites. A similar test is applicable in carrier systems, and we shall specifically address the question of whether inhibitors in the external and internal solutions can simultaneously add to the carrier. With such experiments in mind kinetic equations will be derived for the transport mechanisms depicted in models 3 and 5. In order to simplify the treatment, only rate equations for efflux of substrate from pre-loaded cells (zero trans exit) will be presented. Equations for uptake (zero trans entry) are omitted, but owing to the symmetrical nature of the transport schemes, the exit and entry equations are of exactly the same form, and the general conclusions the same in either case.

I. First Group: The Classical Model (Model 3, Figure 4.3)

The rate equation for zero trans exit was shown earlier (Chapters 2 and 4) to be:

$$\frac{1}{v_{IoIi}} = \frac{1}{f_{-2}Ct}\left\{1 + \frac{f_{-2}}{f_1} + \frac{f_{-2}}{f_1}\frac{[I_o]}{K_{Io}} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{f_{-1}}{f_1} + \frac{[I_i]}{K_{Ii}} + \frac{f_{-1}}{f_1}\frac{[I_o]}{K_{Io}}\right)\right\} \qquad (5.1)$$

where the constants correspond to those in Figure 4·3 and where C_t is the total carrier concentration. $[I_i]$ $[I_o]$ are the concentrations of inhibitor inside the cell and outside, respectively.

Depending on the inhibitor concentrations, eqn.(5.1) reduces to the following forms:

a) $[I_i] = [I_o] = 0$

$$\frac{1}{v_o} = \frac{1}{f_{-2}Ct}\left\{1 + \frac{f_{-2}}{f_1} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{f_{-1}}{f_1}\right)\right\} \qquad (5.2).$$

b). $[I_i] = 0$; $[I_o] \neq 0$

$$\frac{1}{v_{Io}} = \frac{1}{f_{-2}Ct}\left\{1 + \frac{f_{-2}}{f_1} + \frac{f_{-2}}{f_1}\frac{[I_o]}{K_{Io}} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{f_{-1}}{f_1} + \frac{f_{-1}}{f_1}\frac{[I_o]}{K_{Io}}\right)\right\} \qquad (5.3)$$

c). $[I_o] = 0$; $[I_i] \neq 0$

$$\frac{1}{v_{Ii}} = \frac{1}{f_{-2}Ct}\left\{1 + \frac{f_{-2}}{f_1} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{f_{-1}}{f_1} + \frac{[I_i]}{K_{Ii}}\right)\right\} \qquad (5.4).$$

It is easily verified from eqns. (5.1 - 5.4) that the relationship among the rates measured under differert conditions can be expressed in the following way:

$$\left(\frac{v_o}{v_{IoIi}} - 1\right) = \left(\frac{v_o}{v_{Io}} - 1\right) + \left(\frac{v_o}{v_{Ii}} - 1\right) \qquad (5.5)$$

This equation may be rewritten in terms of apparent inhibitor constants for the external and internal inhibitors

$$\frac{v_o}{v_{IiIo}} = 1 + \frac{[I_o]}{(K_{Io})_{app}} + \frac{[I_i]}{(K_{Ii})_{app}} \qquad (5.6)$$

$(K)_{app}$ is here defined as the concentration of inhibitor required to reduce the rate of exit by half, at a given internal concentration of substrate.

II. Second Group: The Two-Site Carrier (Model 5, Figure 4.5)

In this model, sites for the substrate or inhibitor exist simultaneously on both sides of the membrane, making it possible for two molecules of the inhibitor to become bound, one at the external surface of the membrane, the other at the internal surface. A rate equation of the following form was previously derived for zero trans exit (Chapter 4): (5.7)

$$\frac{1}{v_{IiIo}} = \frac{1}{f_{-1}Ct}\left\{1 + \frac{[I_o]}{K_{IoSi}} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{[I_o]}{K_{Io}} + \frac{[I_i]}{K_{Ii}} + \frac{[I_o][I_i]}{K_{Ii}K_{IoIi}}\right)\right\}$$

again individual rate expressions may be written according to the distribution of the inhibitors

a). $[I_o] = [I_i] = 0$

$$\frac{1}{v_o} = \frac{1}{f_{-1}Ct}\left\{1 + \frac{K_{Si}}{[S_i]}\right\} \qquad (5.8).$$

b). $[I_i] = 0; \quad [I_o] \neq 0$

$$\frac{1}{v_{Io}} = \frac{1}{f_{-1}Ct}\left\{1 + \frac{[I_o]}{K_{IoSi}} + \frac{K_{Si}}{[S_i]}\left(1 + \frac{[I_o]}{K_{Io}}\right)\right\} \qquad (5.9).$$

c). $[I_o] = 0; \quad [I_i] \neq 0$

$$\frac{1}{v_{Ii}} = \frac{1}{f_{-1}Ct}\left\{1 + \frac{K_{Si}}{[S_i]}\left(1 + \frac{[I_i]}{K_{Ii}}\right)\right\} \qquad (5.10).$$

In this case rates under various conditions are found to be related by the following expression:

$$\left(\frac{v_o}{v_{IoIi}} - 1\right) = \left(\frac{v_o}{v_{Io}} - 1\right) + \left(\frac{v_o}{v_{Ii}} - 1\right) + \left(\frac{v_o}{v_{Io}} - 1\right)\left(\frac{v_o}{v_{Ii}} - 1\right) \qquad (5.11).$$

In terms of apparent half saturation constants for inhibition, this equation may be rewritten as:

$$\frac{v_o}{v_{IoIi}} = 1 + \frac{[I_o]}{(K_{Io})_{app}} + \frac{[I_i]}{(K_{Ii})_{app}} + \frac{[I_o][I_i]}{(K_{Io})_{app}(K_{Ii})_{app}} \qquad (5.12)$$

Equations (5.11) and (5.12) involve the assumptions that $K_{IoIi} = K_{I_o}$ (Figure 4); and what this means is that addition of inhibitor molecules on opposite sides of the membrane occurs without interference.

METHODS

I. Materials

Choline chloride (methyl-^{14}C, 30 m Ci/mmol) was obtained from New England Nuclear. The isotope was stored at - 10°C and the purity examined by paper chromatography (But: HCl:H_2O, (4:1:1)), with choline and trimethylammonium as standards. Other chemicals were of commercial reagent grade.

II. Choline Transport

A) Preparation of Cells:

Human blood obtained from an outdated blood bank supply was spun for 7 min at top speed in a clinical centrifuge and the plasma and buffy coat removed by aspiration. The cells were thoroughly washed with sterile salt solution (154 mM NaCl containing 5 mM sodium phosphate

buffer pH 6,8) and incubated (5% hematocrite) for 6 hours at 37° in a shaking bath to allow for exit of endogenous choline (13 µM). Cells were then packed by spinning for 15 to 20 min. in a clinical centrifuge. The same salt buffer solution was used at all stages of the experiment except during this long incubation period, when 0.1% glucose and 0.02% chloramphenicol (51) were added to the buffer.

B) Uptake Assay:

Cells (hematocrit 10%) were incubated at 37°C in a solution of 154 mM NaCl and 5 mM Na phosphate buffer, pH 6.8, and ^{14}C-choline(0.65µM).Samples (2 ml) were taken at intervals and added to tubes containing 5 ml of dibutyl phthalate and 10 ml of ice cold phosphate buffer. The cells were immediately spun in a clinical centrifuge for 10 min, and were found to sediment below the organic layer, which separated them from the aqueous radioactive solution above (52). The latter was removed by aspiration and the walls of the tube thoroughly washed with isotonic NaCl to eliminate contaminating radioactivity. Then, after removal of the dibutyl phthalate, the cells were precipitated (51) by adding 0.8 ml of buffer and 2.0 ml of 5% trichloracetic acid containing 1 mM choline. After centrifugation, 2 ml of the supernatant were removed and counted in 8 ml Aquasol.

The linearity of ^{14}-C choline (0.65 μM, 53 m Ci/mmol) uptake with time is illustrated by the results in Figure 5.1. The almost complete inhibition by the choline analog, dimethylamino ethanol (100 μM), whose half-saturation constant is of the order of 10 μM, demonstrates that influx is entirely due to the operation of the carrier, rather than to passive diffusion.

C) Analysis:

Uptake rates were calculated from plots of cpm against time by a least squares analysis carried out by a digital computer.

III. Glucose Transport

A) Preparation of Cells:

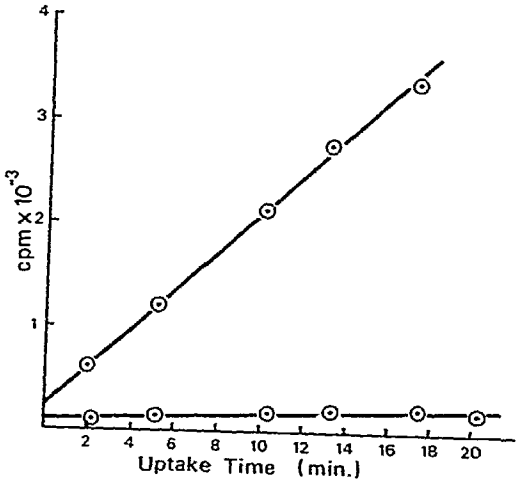
Cells were sedimented in a clinical centrifuge and washed twice in isotonic salt solution containing 17 mM sodium phosphate buffer pH 7.5. Cells (20% hematocrite) were incubated in 146 mM glucose solution at 37°C for at least one hour, after which they were transferred to a bath at 25°C.

B) Exit Assay:

An aliquote (50 μl) was injected into 25 ml of saline solutionat 25°C, and exit rates were determined by the light scattering method (53). Sugar exit times were computed according to the method of Sen and Widdas (53,54),

FIGURE 5.1

The uptake of ^{14}C-choline (0.65 μM, 53 mCi/mmol) either in the presence or absence (lower and upper lines respectively) of dimethylaminoethanol (100 μM), as a function of time. In this experiment the cells were washed, but not incubated for an extended period of time so that endogenous choline would therefore have been present internally.


4
3
2
1
cpm x 10^-3
2
4
6
8
10
12
14
16
18
20
Uptake Time (min.)

and were divided by the amount of sugar that escapes; the reciprocal of this figure, v, is the mmoles of sugar/l cell water transferred per minute at the initial rate.

RESULTS AND DISCUSSION

I. Glucose Transport

The main problem in this or any system is to find inhibitors that bind on the inner and outer surfaces of the cell membrane. Here the simplest solution to the problem is to use two different inhibitors. Phloretin, a non-penetrating inhibitor (55) binds on the outer surface in competition with glucose. Cytochalasin B, on the other hand, was shown to penetrate rapidly through the cell membrane and though present in both the internal and external solutions, to become bound to the carrier in competition with glucose on the inner surface only (17). This pair of inhibitors therefore provides us with the tool we need to distinguish between the two classes of carrier model. The effects of increasing concentration of phloretin and cytochalasin B on rates of glucose exit are shown in Figure 5.2 and 5.3, respectively.

The combined effects of the two inhibitors, at a constant concentration ratio which is approximately equal to the ratio of their apparent half saturation concentrations under these conditions, is shown in Figure 5.4. The line through the points is that predicted from eqns.

FIGURE 5.2.

Inhibition of glucose transport by phloretin alone. Rates in the presence and absence of the inhibitor are designated as v and v_o respectively. The line drawn through the points was calculated by a least squares analysis of the data. The calculated value of the inhibition constant $(K_{Io})_{app} = 0.62 \pm 0.017$.

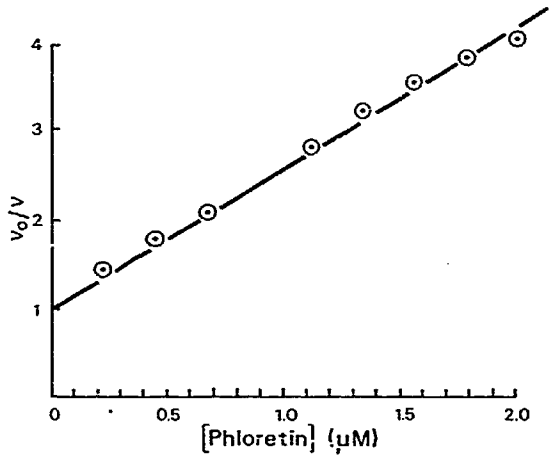

4
3
2
1
V_o/V
0
0.5
1.0
1.5
2.0
[Phloretin] (μM)

FIGURE 5.3

Inhibition of glucose transport by cytochalasin B alone. As in Figure 5.2, v_o/v is the ratio of rates in the absence or presence of the inhibitor. $(K_{Ii})_{app}=0.65\pm 0.018$.

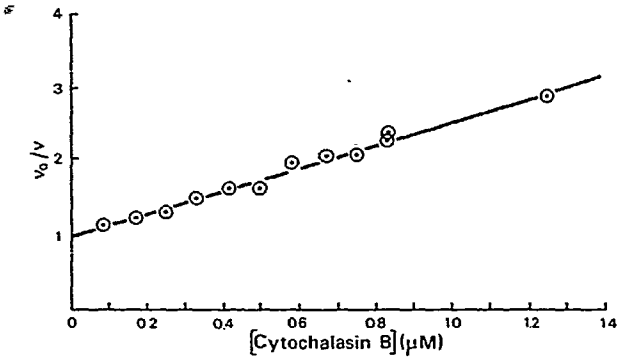
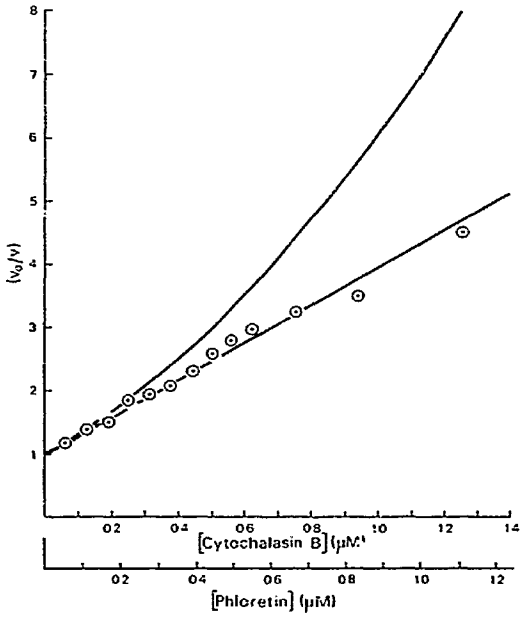

4
3
2
1
v_o/v
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
[Cytochalasin B](μM)

FIGURE 5.4

Inhibition of glucose transport by phloretin and cytochalasin B acting together. The ratio of phloretin and cytochalasin B is constant. The straight line drawn through the experimental points is calculated from the apparent inhibition constant derived from the data in Fig.5.2 and 5.3on the basis of equation 5.5 and 5.6 which were derived for the first group of models, where only one binding site is exposed at a time. Calculated slope of this line = 2970 ± 57.6. Slope calculated from the experimental points = 2900 ± 97.7. The curved line above is calculated on the basis of equations (5.11) and (5.12), corresponding to the second group of models, in which two sites are exposed simultaneously.


8
7
6
5
4
3
2
1
(V_o/v)
02
04
06
08
10
12
14
[Cytochalasin B] (μM)
02
04
06
08
10
12
[Phloretin] (μM)

(5.5) and (5.6), derived for the classical carrier scheme (Model 3, Figure 4.3). The behaviour predicted by the two-site model (Model 5, Figure 4.5) was calculated from eqns. (5.11) and (5.12) and is indicated by the curved line in the figure. This line is seen to curve upwards as the inhibitor concentrations rise, which is a consequence of the terms in eqns. (5.7) and (5.12) in which the two inhibitor concentrations are multiplied together. The classical scheme predicts a purely linear relationship. Thus linearity in the plot for two inhibitors is consistent with the one-site model and at odds with the two-site model. In addition, the close agreement between the predicted and observed rates demonstrates the quantitative adequacy of the classical model; while the disagreement in the case of the two-site scheme is sufficient grounds for rejecting all such mechanisms as a basis for glucose transport in erythrocytes.

II. Choline Transport

The problem of finding inhibitors which bind on both the inner and outer membrane surfaces is solved in much the same way as for the glucose system. Certain competitive inhibitors are not transported on the carrier and do not penetrate through the membrane, and their action must be restricted to the external surface. A convenient example of such an inhibitor is dimethyl-n-pentyl (2-

hydroxyethyl) ammonium ion (Chapter 7). Other inhibitors, which are tertiary amines, have been shown to enter the cells rapidly, though not via the choline carrier, and to become bound almost exclusively on the inner surface, just as in the case of cytochalasin B. Di-n-butylamino ethanol is an example of such an inhibitor (Chapter 9). Experiments with these two compounds are summarized in Table 5.1. It is seen that the rates in the presence of both inhibitors are in close agreement with the predictions of the one-site model, and without any doubt in disagreement with the predictions of the two-site model. We conclude therefore that neither the choline nor the glucose transport systems involve a mechanism in which substrate sites are simultaneously exposed to the solution on both sides of the membrane. These findings therefore unambiguously rule out a number of the prevailing models for transport in particular the "introverting hemiport" of LeFevre (9), the "internal transfer oligomeric model" of Lieb and Stein (8), and "lattice-membrane model" of Naftalin (35). All three of these mechanisms, it must be noted, were introduced to explain observations on the glucose transport system in erythrocytes, and experiments on this very system have provided the criteria by which they must be rejected. Whatever the details of the actual mechanism in these systems, one of its most fundamental properties is the disappearance of the site on one side

TABLE 5.1.

Inhibition of choline transport by competitive inhibitors acting on either the external or internal surface of the cell membrane: dimethyl-n-pentyl (2-hydroxyethyl) ammonium ion, I_o, and di-n-butyl (2-hydroxyethyl) ammonium ion, I_i, respectively. The concentration of the former, I_o, is 9.4 μM and the concentration of the latter 1.4 mM. The predicted values of the rate in the presence of both inhibitors at once, v_{IoIi}, are calculated from the rates in the presence of each inhibitor alone, v_{Io} and v_{Ii}, on the basis of equationsfor the one-site (eqns. (5.5) and (5.6))or the two-side models (eqns. (5.11) and (5.12)). The rate in the absence of inhibitors is designated as v_o; in the presence of dimethyl-n-pentyl (2-hydroxyethyl) ammonium ion, v_{Io}; in the presence of di-n-butyl (2-hydroxyethyl) ammonium ion, v_{Ii}; and with both inhibitors acting together at the same concentrations as when used alone, v_{IoIi}. Probable errors calculated by least squares analysis of plots of uptake vs. time.

TABLE 5.1.

EXPERIMENT	Rates of choline entry (μmoles min^{-1} /l (packed cells) $\times 10^4$					
	Experimental Values				Predicted values of v_{IoIi}	
	v_o	v_{Io}	v_{Ii}	v_{IoIi}	One-site Exposure	Two-site Exposure
1	71.73 ± 2.49	24.42 ± 2.01	26.66 ± 1.57	16.05 ± 1.35	15.5 ± 0.97	9.08 ± 0.69
2	70.74 ± 3.64	26.84 ± 2.28	28.90 ± 2.12	17.29 ± 0.98	17.3 ± 1.23	10.96 ± 0.69

of the membrane before the site on the other side is allowed to appear.

SUMMARY

A number of the mechanisms proposed to explain either facilitated or active transport have as a common feature the simultaneous exposure of substrate binding sites on both the inner and outer surfaces of the cell membrane. In other proposed mechanisms the sites are alternately exposed and therefore only one substrate site is available at a time. Both classes of model may correctly predict the form of substrate saturation curves, and both may account for accelerated exchange and counter-transport. A simple kinetic test is now proposed which is applicable in both active and facilitated transport systems and which clearly distinguishes between these two classes. The test depends on measurements of transport rates in the presence of reversible competitive inhibitors inside and outside the cell. Experiments are described on the glucose system of erythrocytes involving the inhibitors phloretin and cytochalasin B, and on the choline transport system of the same cells, with the inhibitors dimethyl-n-decyl(2-hydroxyethyl) ammonium and di-n-butyl(2-hydroxyethyl) ammonium ions. The results are in quantitative agreement with the one site models, which includes the classical carrier, and in disagreement with the two site models. All the mechanisms in the latter group must therefore be rejected as explanations for glucose or choline transport.

CHAPTER 6

A SIMPLE EXPERIMENTAL APPROACH TO THE DETERMINATION OF CARRIER TRANSPORT PARAMETERS FOR UNLABELED SUBSTRATE ANALOGS

INTRODUCTION

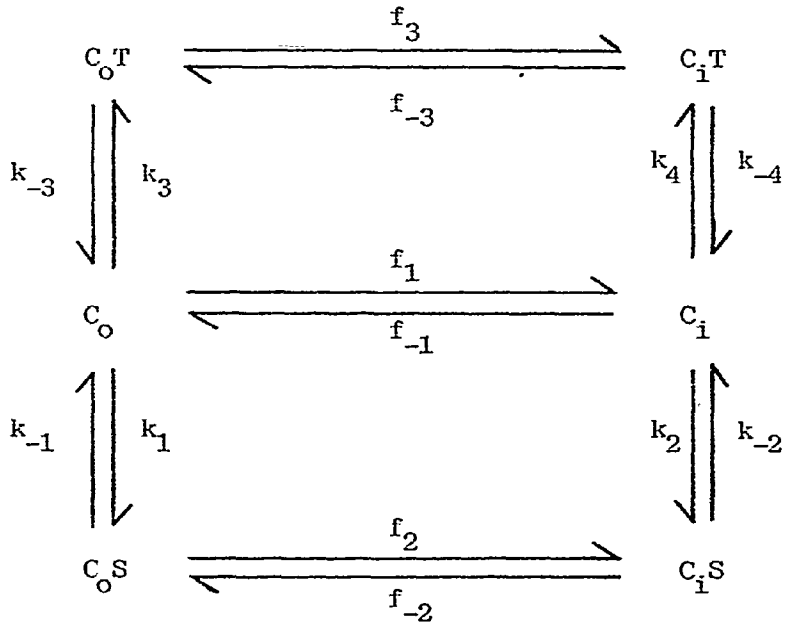
Transport studies often depend on the availability of labeled substrates, and this tends to limit their scope to substrates that are conveniently obtained in an appropriate form. Here a method is described in which the transport of an unlabeled analog is monitored by its effect upon the movement of a labeled substrate present on the trans side of the membrane. By this means both the affinities and transport rates of an unlimited number of analogs may be determined through the use of a single radioactive substrate. The method is of general application, whether the system is active or non-active and whether the rate-limiting step in substrate movement is passage across the membrane or dissociation from the carrier. We shall present the kinetic theory upon which this method is based and show how it can be applied in the case of the choline transport system in erythrocytes.

EXPERIMENTAL APPROACH

A rate equation based on the scheme in Figure 6.1 and applying to the proposed experiment, is given, in which the efflux of labeled substrate S from pre-equilibrated cells is determined in the presence of varying concen-

FIGURE 6.1

Transport scheme for two substrates, S and T. Subscripts o and i refer to carrier forms on the outer and inner surfaces of the membrane respectively; $f_{\pm i}$ are rate constants for reorientation of carrier in the membrane; and k_{+i} and k_{-i} are association and dissociation constants, respectively. $K_{To} = k_{-3}/k_3$; $K_{Ti} = k_{-4}/k_4$; $K_{So} = k_{-1}/k_1$; $K_{Si} = k_{-2}/k_2$.


C_oT
C_iT
f_3
f_{-3}
k_{-3}
k_3
k_4
k_{-4}
f_1
C_o
C_i
f_{-1}
k_{-1}
k_1
k_2
k_{-2}
f_2
C_oS
C_iS
f_{-2}

trations of unlabeled analog T in the external solution. The internal concentration of S is set at a very low value ($[S_i]/\bar{K}_{Si} \ll 1$), for under this condition we can show that the apparent affinity and maximum rate constants for T are identical to those obtained when its influx is observed directly.

$$v = \frac{-d[S_i]}{dt} = \frac{\dfrac{\bar{V}_{Si}}{\bar{K}_{Si}}[S_i] + \dfrac{\tilde{V}^{T}_{Si}}{\tilde{K}^{S}_{To}\,\bar{K}_{Si}}[S_i][T_o]}{1 + \dfrac{[T_o]}{\bar{K}_{To}}} \qquad (6.1)$$

Eqn. (6.1) may be written directly by simplification of eqn. (A.1) in the Appendix, by setting $[S_o] = [T_i] = 0$, and $S_i/\bar{K}_{Si} \ll 1$. The equation may be rearranged into a linear form suitable for analysis of the experiments, as shown below:

$$\frac{v}{v_o} = \left(\frac{v_\infty}{v_o}\right)_{T_o} + \bar{K}_{To}\left(\frac{1 - v/v_o}{[T_o]}\right) \qquad (6.2)$$

v_o is defined as the rate of exit of labeled substrate S into pure buffer solution, and v_∞ its exit rate when an analog T has been added to the external medium. From a plot of the data according to equation (6.2), the two experimental parameters $(v_\infty/v_o)_{To}$ and $\bar{K}_{To}$ are calculated from the intercept and slope respectively. For a substrate

$\overline{K}_{To}$ is the affinity constant in a zero trans entry experiment. In the case of an inhibitor, where f_3 and v_∞ equal zero $\overline{K}_{To}$ is equal to the half-saturation constant for inhibition of substrate entry (See Appendix 1)

The intercept, $(v_\infty / v_o)_{To}$, is directly proportional to the maximum transport rate of the analog under investigation; experimentally v_∞ is the exit rate for substrate S in the presence of a saturating concentration of substrate T outside the cell.

$$\left(\frac{v_\infty}{v_o}\right)_{To} = \overline{V}_{To} \; (1/f_1 + 1/f_{-1})/C_t \qquad (6.3)$$

where Ct is the total carrier concentration; and f_1 and f_{-1} are the rates of reorientation of the free carrier inward and outward, respectively (see Figure 6.1). The ratio of the parameters $(v_\infty / v_o)_{To}$ for two different substrates is equal to the ratio of their maximum zero trans entry rates,

$$\frac{[(v_\infty / v_o)_{To}]_1}{[(v_\infty / v_o)_{To}]_2} = \frac{(\overline{V}_{To})_1}{(\overline{V}_{To})_2} \qquad (6.4)$$

where the subscripts 1 and 2 refer to the two substrates.

APPLICATION TO THE CHOLINE TRANSPORT SYSTEM

I. Methods

A. Chemicals. The synthesis and characterization of choline analogs are described in the next Chapter.

B. Loading of cells with radioactive choline. Cells washed according to the procedure given in Chapter 5 were incubated (40% hematocrit) for 6 hours at 37°C in buffered saline solution containing ^{14}C-choline Cl (5-6 μM 30 or 53 mCi/mmol), 0.1% glucose and 0.02% chloramphenicol. The cells were centrifuged down and the external radioactivity removed by washing four times in ice cold buffer. The resulting concentration of choline was 3-4 μmoles per litre of packed cells. This is approximately 1/8 of the affinity constant on the inner side of the cell membrane (Chapter 9).

C. Assay

a) Exit Rate Measurements

Ice cold packed cells (1 ml) containing radioactive choline were added to 9 ml of buffer at 41°C with or without a choline analog; the resulting suspension, whose temperature is found to be at 37°C, was immediately transferred to a shaking bath at 37°C. Samples (3 ml) were removed at intervals and immediately centrifuged in the cold for 5 min. The supernatant (2 ml) was counted for radioactivity in 8 ml Aquasol. A correction for hemolysis was routinely applied, though it was almost always extremely small (<0.5%). The correction factor was obtained indirectly from the counting efficiency which was shown to be proportional to the O.D. at 540 nm. Corrections for counting efficiency were also applied.

b) Total Radioactivity in efflux suspension: $[S_i]_o$

Two ml of 5% trichloracetic acid was added to 1 ml of the cell suspension (52). After centrifugation the radioactivity in 2 ml of supernatant was counted in 8 ml of Aquasol.

II. Treatment of Experimental Data

A. Calculation of Rates:

Rate constants for transport are obtained from the slope of a plot of ln ($[S_i]_t/[S_i]_o$) against time calculated by means of a least squares fit to the data (Fig. 6.2).* $[S_i]_o$ is the initial substrate concentration and is calculated from the total radioactivity in the efflux suspension. $[S_i]_t$ is the concentration at the time of sampling and is found from the difference between $[S_i]_o$ and the concentration in the supernatant.

B. Determination of Affinities and Transport Rates:

The ratio of choline efflux rates in the presence or absence of an external substrate at a given concentration, (v/v_o), is plotted against a function of the absolute value of the reciprocal of the analog concentration, $(1-v/v_o)/[T_o]$ according to eqn. (6.2). Plots of this kind are shown in

*Footnote:

Exit rates are expected to be logarithmic when the internal substrate concentration is well below the half-saturation constant.

FIGURE 6.2

^{14}C-Choline Cl^- exit into solutions containing various unlabeled choline analogs. (1) Diethyl-n-decyl (2-hydroxyethyl) ammonium Br^-, 0.018 mM. (2) Dimethyl isopropyl (2-hydroxyethyl) ammonium Br^-, 2.3 mM. (3) Control: no analog present. (4) Diethyl methyl (2-hydroxyethyl) ammonium I^-, 0.38 mM. (5) Choline Cl^-, 0.052 mM. Cells were pre-loaded with 4 µmoles C^{14}-choline per 1. of cell water, 53 mCi/mmol.

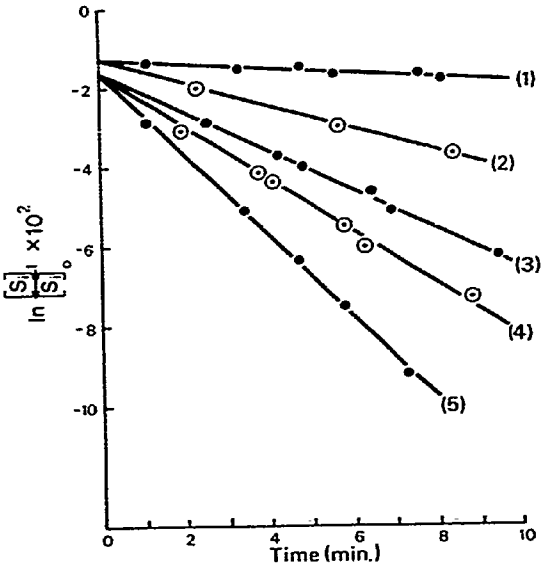

0
-2
-4
-6
-8
-10
$\ln \frac{[S]}{[S]_o} \times 10^2$
0
2
4
6
8
10
Time (min.)
(1)
(2)
(3)
(4)
(5)

Figure 6.3. All analogs give the predicted straight line, the slope being negative with analogs that increase the choline transport rate and positive with those that inhibit. Apparent affinities ($\overline{K}_{To}$) are calculated from the slope, and relative transport rates ($v_\infty / v_o)_{To}$, from the intercept in the ordinate.

All calculations were carried out automatically with the aid of a computer program written for the purpose (see Appendix 3). By means of this program the data were first corrected for counting efficiency and hemolysis, and initial rates determined from the experimental points by the method of least squares; then the experimental affinity and velocity constants, together with their standard deviations, were determined by a least squares fit to eqn. (6.2). Estimates of the calculated constants with probable errors are summarized in Tables (6.1) and (6.2).

DISCUSSION

Since the experimental approach is based on completely general rate equations the analysis must be valid whatever the rate-limiting steps in transport, whether diffusion through the membrane or dissociation of the carrier-substrate complex; and in both active and facilitated transport. It does not depend on the system's exhibiting accelerated exchange, which is seen in the object of our present investigation, choline transport. It should

FIGURE 6.3

Determination of affinities and transport rates for choline analogs (see eqn. 6.2). The factor A which multiplies the units in the abscissa was introduced so that different concentration ranges for various analogs could be plotted on the same scale.

(1) Choline Cl^-; A = 20. (2) Dimethylethyl (2-hydroxyethyl) ammonium I^-; A = 40. (3) Diethylmethyl (2-hydroxyethyl) ammonium I^-; A = 400. (4) Dimethyl isopropyl (2-hydroxyethyl) ammonium Br^-; A = 2000 (5). Dimethyl-n-decyl(2-hydroxyethyl) ammonium Br^-; A = 1. In each case the arrow marks the half-saturation concentration for the analog.

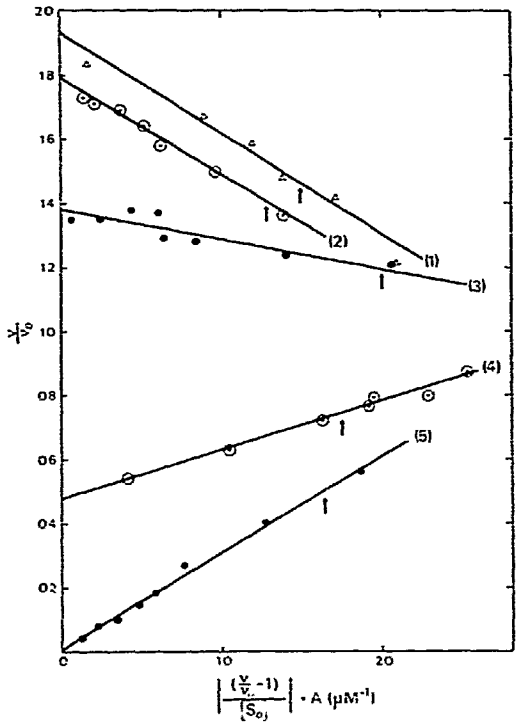

20
18
16
14
12
10
08
06
04
02
0
10
20
(1)
(2)
(3)
(4)
(5)
$\frac{1}{v_0}$
$\left|\frac{(\frac{V}{V_0}-1)}{[S_0]}\right|$ · A (μM⁻¹)

TABLE 6.1.

Rate constants with standard deviations for choline-^{14}C exit into solutions of unlabeled choline analogs present at saturating concentrations, calculated from the data in Fig. 6.2.

Analog	Rate constant (min^{-1})	Standard Deviation of Measurement
Choline Cl^-	1.01×10^{-2}	1.86×10^{-4}
Diethyl methyl (2-hydroxyethyl) ammonium I	6.30×10^{-3}	2.34×10^{-4}
Dimethyl isopropyl (2-hydroxyethyl) ammonium Br	2.81×10^{-3}	1.00×10^{-4}
Diethyl n-decyl (2-hydroxyethyl ammonium Br	6.03×10^{-4}	1.36×10^{-4}
---	4.90×10^{-3}	1.36×10^{-4}

TABLE 6.2

Transport parameters for choline analogs derived from the data in Fig. 6.3.

Analog	$\overline{K}_{To}$ (μM)	$(v_{oo}/v_o)_{To}$
Choline	6.33 ± 0.62	1.93 ± 0.043
Dimethyl ethyl (2-hydroxyethyl) ammonium I	12.4 ± 0.61	1.79 ± 0.012
Diethyl methyl (2-hydroxyethyl) ammonium I	35.2 ± 7.7	1.38 ± 0.019
Dimethyl isopropyl (2-hydroxyethyl) ammonium Br	303 ± 19	0.48 ± 0.017
Dimethyl-n-decyl (2-hydroxyethyl) ammonium Br	0.031 ± 0.009	0.012 ± 0.008

therefore broaden the scope of transport studies in all systems to include a great variety of substrate analogs.

I. Rationale of the Method

When expressions for choline exit rates in the presence or absence of an external substrate are examined, it becomes evident that the analog exerts its effect by altering the distribution of the free carrier on either side of the membrane, and either increasing or diminishing the supply of the internal carrier form, which combines with internal substrate and moves it out of the cell. This is most simply demonstrated by recourse to a form of equation (6.1) which contains individual rate constants rather than experimental parameters, and where dissociation of the carrier-substrate complex is assumed to be rapid compared to carrier reorientation steps (see Appendix 1):

$$v = \frac{-d[S_i]}{dt} = \frac{f_{-2}C_t(f_1 + f_3[T_o]/K_{To})\ [S_i]/K_{Si}}{f_1 + f_{-1} + (f_{-1} + f_3)\ [T_o]/K_{To}} \qquad (6.5)$$

where K_{Si} and K_{To} are dissociation constants for internal S and external T, respectively. Rates in the presence of a saturating concentration of T or in its absence are given by:

$$v_\infty = f_{-2}[CS_i] = f_{-2}\frac{[S_i][C_i]}{K_{Si}} = f_{-2}\frac{[S_i]}{K_{Si}}\left(\frac{f_3 C_t}{f_3+f_{-1}}\right) \qquad (6.6)$$

$$v_o = f_{-2}[CS_i] = f_{-2}\frac{[S_i]}{K_{Si}}[C_i] = f_{-2}\frac{[S_i]}{K_{Si}}\left(\frac{f_1 C_t}{f_1+f_{-1}}\right) \qquad (6.7)$$

$[C_i]$ is seen to be equal to a fraction of the total amount of carrier, C_t; in the first case this fraction is $f_3/(f_3+f_{-1})$, and in the second $f_1/(f_1+f_{-1})$. The value of $(v_\infty/v_o)_{To}$ therefore depends on the ratio of f_3 to f_1:

$$(v_\infty/v_o)_{To} = (1 + f_{-1}/f_1)/(1 + f_{-1}/f_3) \qquad (6.8)$$

When this ratio is greater than unity, the analog will accelerate the exit of internal substrate. The maximum possible acceleration, however, depends upon the distribution of the free carrier in the membrane in the absence of substrates or inhibitors, which is governed by the ratio of f_1/f_{-1}. For example if $f_1 = f_{-1}$ the maximum rate increase due to an analog is two-fold, whereas if $f_1 \ll f_{-1}$, so that the undisturbed carrier is mainly in the form of C_o, the rate increase may be very much larger than this. If the analog does not undergo transport ($f_3=0$), $(v_\infty/v_o)_{To}$ is zero in all cases.

Because of the upper limit on the value of $(v_\infty/v_o)_{To}$ this parameter is insensitive to differences in the value of f_3 (and hence to the rate of reorientation of carrier-substrate complex) when $f_3 \gg f_1$. Exactly the same limitation inheres in zero trans entry experiments with the same substrates, however, as is seen from the value of

$\overline{V}_{To}$ in Table A1, Appendix 1 . The only experiment capable of revealing the rate of movement of the carrier-substrate complex is equilibrium exchange, where reorientation of free carrier cannot limit the rate.

The experimental constant $(v_\infty / v_o)_{To}$ may also be intuitively understood as the ratio of maximum zero trans influx rates for the substrate T and for a hypothetical substrate whose complex with the carrier moves inwardly at exactly the same rate as free carrier, again assuming rapid dissociation of the carrier substrate complex. For this substrate, $f_3 = f_1$ and $\overline{V}_c = C_t / (1/f_1 + 1/f_{-1})$, while for substrate T, $\overline{V}_{To} = C_t(1/f_3 + 1/f_{-1})$ as may be verified from the rate expressions in Table A1, Appendix 1 . The ratio of $\overline{V}_{To}$ and $\overline{V}_c$ is exactly equal to $(v_\infty / v_o)_{To}$ given in eqn (6.8).

II. Rate Determinations with the Distribution of Substrates Reversed.

If with certain substrates the efficiency of the transport system is very great, it could be inconvenient to follow the strategy described here, which involves measuring exit in the presence of high external concentrations of an unlabeled substrate, for the latter would rapidly enter the cells and interfere with the efflux of the labeled substrate inside. In this circumstance it might be better to reverse the location of the substrates; first to incubate the cells with unlabeled analog and then

to measure rates of influx of labeled substrate present externally at a very low concentration. This procedure has another advantage in extremely efficient systems where sufficiently rapid sampling and quenching is difficult to achieve, for there is a transient build-up in the internal concentration of the labeled substrate, described as "counterflow", and this prolongs the period during which the entry of label is linear with time. The principles involved in analysis with the substrates reversed are the same, and the appropriate equation now has the form:

$$\frac{v}{\bar{v}_o} = \left(\frac{v_\infty}{v_o}\right)_{Ti} + \bar{K}_{Ti}\left(\frac{1 - v/v_o}{[T_i]}\right) \qquad (6.9)$$

where $\bar{K}_{Ti}$ is defined in Table A1, Appendix 1 and where

$$\left(\frac{v_\infty}{v_o}\right)_{Ti} = \bar{V}_{Ti}(1/f_1 + 1/f_{-1})/Ct \qquad (6.10)$$

The maximum velocities of exit for two substrates may be shown to be related to $(v_\infty / v_o)_{Ti}$ by an equation of exactly the same form as (6.8).

Important information on the symmetry of the system may be obtained if both $(v_\infty / v_o)_{Ti}$ and $(v_\infty / v_o)_{To}$ are determined for the same substrate. These parameters have the following relationship:

$$\frac{(v_\infty / v_o)_{To}}{(v_\infty / v_o)_{Ti}} = \frac{\bar{K}_{To}}{\bar{K}_{Ti}} \quad \beta = \frac{\bar{V}_{To}}{\bar{V}_{Ti}} \qquad (6.11)$$

In an equilibrating (non-active) system, $\beta = 1$, and the expression is equal to both the ratio of the apparent affinity constants, and the related ratio of maximum velocities of entry and exit (see Appendix 1).
In active transport, $\beta >> 1$; if $\overline{K}_{To}$ and $\overline{K}_{Ti}$ are determined independently, by the methods described here, then β may calculated from eqn. (6.11)

III. Experimental Tests of the Method

The validity of the method is demonstrated by several different observations, the first of which is that the relatively complex relationship between independent and dependent variables predicted by eqn. (6.2) is found to hold experimentally, as shown by linear plots for all analogs, ranging from good substrates to inhibitors (Fig. 6.2). Next, the affinity constants obtained here may be compared with those measured directly. The half-saturation constant, $\overline{K}_{To}$, determined from the rates of uptake of ^{14}C-choline into choline-free cells (zero trans entry) was found to be 6.54 $\pm$ 0.15 μM (Chapter 7). The result obtained by the present method is practically identical to this, 6.33 $\pm$ 0.62 μM. The affinity constant for an inhibitor was also determined directly: dimethyl-n-pentyl (2-hydroxyethyl) ammonium iodide placed in the external medium competitively inhibited the uptake of C^{14}-choline into choline-free cells, as expected, and

the inhibition constant was found to be 8.2 ± 1.6 μM (Chapter 7). The value of $\bar{K}_{To}$ obtained by the present method (Chapter 7) agrees well with this: 7.3 ± 1.2 μM. In quantitative terms, therefore, the method produces results which are true measures of the constants desired. Rates of transport have been checked only for the case of inhibitors. The present findings indicated that neither the dimethyl-n-pentyl nor diethyl-n-decyl analog undergoes transport. Even after an incubation with cells for 12 hrs. at 37°, at a concentration which was roughly 5 or 9 times their $\bar{K}_{To}$ values, respectively, these compounds failed to penetrate the cell membrane (Chapter 7), in complete agreement with the present results.

SUMMARY

A method is described that permits the determination of affinities and transport rates for unlabeled substrate analogs. It is based on the effect of the analog upon the rate of transport of a labeled substrate present at a low concentration on the trans side of the membrane. The method is shown to be widely applicable since it does not depend on assumptions about rate-limiting steps and holds for both active and non-active systems. Here it is applied in an experimental study of the facilitated diffusion system for choline in erythrocytes.

CHAPTER 7

THE BINDING AND TRANSLOCATION STEPS IN TRANSPORT AS RELATED TO SUBSTRATE STRUCTURE

INTRODUCTION

Structural analogs of substrates have made valuable contributions to the understanding of enzyme catalysis, but their potential in elucidating transport mechanisms has been little exploited. In the field of transport, studies of analogs have most often been concerned with the physiological implications of having shared or multiple transport systems for groups of substrates, or with the discovery of powerful inhibitors that could be used as drugs or biochemical tools. In some cases, analogs have been employed as an aid in exploring the shape of the binding site, but seldom has their use been directed towards the understanding of the translocation process itself, the step that follows binding. Potentially, a three-fold relationship may be established for a series of substrates: that between structure and affinity, between affinity and maximum transport rate, and between structure and rate. The first of these informs us about the shape and size of the binding site and the nature of its surroundings; the second about the dependance of the translocation process upon substrate binding forces within the site, and in adjacent regions of the carrier; and the third about the effects of steric restraints upon

penetration of the substrate into the channel through the membrane, which by some mechanism the carrier must provide.

Such an investigation will now be described. A facilitated (rather than an active) system has been chosen, because it involves the most fundamental form of a membrane carrier, uncomplicated by the superstructure of energy-channeling devices, but nevertheless possessing the essential features of great specificity, saturability, and the requirement for carrier reorientation steps in substrate translocation. It is to be noted that in the absence of an energy supply, active systems such as the lactose transporter of E. Coli (56) revert to this fundamental form, which has all the characteristics of the system when driven by energy except the capability of producing a concentration gradient.

Among facilitated systems, that for choline in erythrocytes is especially suitable, first because a great variety of structural analogs of the substrate may be prepared, and secondly because a potential means of probing carrier conformation, involving the irreversible inhibitor N-ethyl maleimide, has been described for this system (27).

A series of analogs was examined in which the fundamental ethanolamine structure is preserved, and which the three methyl groups on the quaternary nitrogen atom may be replaced by alkyl chains of various lengths. It is therefore expected that enlargement should produce crowding in the presumed cation binding locus, but not in the region of the carrier site that interacts with the hydroxyethyl chain.

METHODS

I. Preparation of Choline Analogs

Trialkyl(2-hydroxyethyl)ammonium halides (Table 7.1) were prepared by quaternarization of tertiary aminoalchols with alkyl iodides or bromides. Equimolar quantities of reactants were refluxed in dry methyl ethyl ketone at 80° C for approximately 8 hours (57). The products were purified by recrystallization in various solvents, followed by washing in ether and drying under vacuum, and were then characterized and shown to be homogeneous by means of melting points, paper chromatgraphy (Table 7.1) and NMR spectroscopy (Table 7.2).

One-dimensional ascending paper chromatography was carried out in butanol:acetic acid: water, (4:1:5) (58),

TABLE 7.1.

Synthesis of choline analogs: solvents used in recrystallization, melting points, and R_f values in ascending paper chromatography (butanol - acetic acid - water 4:1:5).

Series		Anion	Melting Point Determined	Melting Point Reported[57]	R_f	Recrystallization Solvent
$R-N^+(CH_3)_2-CH_2-CH_2-OH$	Ethyl	I^-	272	259	0.48	ethanol
	n-Propyl	Cl^-	124.5		0.54	acetone
	iso-Propyl	Br^-	278 (dec.)		0.50	ethanol
	n-Butyl	I^-	117	117	0.69	ethanol
	n-Pentyl	I^-	136		0.82	methyl-ethyl ketone
	n-Decyl	Br^-	139		0.93	acetone
	n-Dodecyl	Br^-	166		0.91	acetone
$R-N(C_2H_5)-CH_2-CH_2-OH + C_2H_5$	Methyl	I^-	278	279	0.54	ethanol
	Ethyl	I^-	283	283	0.57	ethanol
	n-Propyl	I^-	178.5	178	0.68	ethanol
	n-Butyl	I^-	116	112	0.75	methyl ethyl ketone
	n-Pentyl	I^-	75-79		0.83	methyl ethyl ketone
	n-Hexyl	Br^-	61.5		0.93	methyl ethyl ketone plus ether
	n-Decyl	Br^-	83.5		0.93	acetone
$R-N(C_3H_7)-CH_2-CH_2-OH + C_3H_7$	n-Propyl	I^-			0.83	methyl ethyl ketone

TABLE 7.2

Characterization of choline analogs by NMR spectroscopy. The relative positions of proton adsorption signals (δ) are assigned for individual groups in the molecule, as shown in the upper row in the table. The observed chemical shifts (ppm) and the number of protons calculated on the basis of the integrated spectrum are listed; in every case, the numbers of protons calculated for individual chemical groups agrees with the expected number for a given structure. External standard, tetramethylsilane. Spectrometers: Varian A60A for all spectra except those for the dimethylpropyl, dimethylbutyl and dimethyldodecyl analogs, where a Varian XL100 spectrometer was used.

		CH_3-C		CH_3-CH_2-C		C-CH_2-C		CH_3-N		C-CH_2-N		CH-N		C-CH_2-O	
Series	R	δ	H	δ	H	δ	H	δ	H	δ	H	δ	H	δ	H
CH_3 | R-N-CH_2-OH | + CH_3	Ethyl	1.85	3.00					3.65	6.20	3.95	3.70			4.52	2.10
	n-Propyl	1.54	2.83			2.35	1.93	3.65	6.15	3.94	3.98			4.53	2.17
	iso-Propyl	1.90	5.97					3.56	5.97	3.95	2.10	4.15	0.82	4.52	2.10
	n-Butyl	1.46	2.95			1.87	2.00	3.65	5.90	3.97	4.07			4.56	2.00
						2.25	2.00								
	n-Pentyl	1.40	3.20			1.92	6.06	3.65	5.98	3.95	4.06			4.54	1.72
	n-Decyl	1.40	2.90			1.80	15.80	3.65	6.00	3.95	4.00			4.52	2.20
	n-Dodecyl	1.36	2.74			1.79	17.70	3.65	6.10	4.01	4.00			4.52	2.32
						2.29	2.10								
C_2H_5 | R-N-CH_2-CH_2-OH | + C_2H_5	Methyl	1.84	5.89					3.58	3.04	3.95	5.98			4.52	2.14
	Ethyl	1.80	8.90							3.86	8.14			4.48	1.97
	n-Propyl			1.80	11.10					3.90	7.80			4.50	2.16
	n-Butyl			1.85	13.10					3.90	7.90			4.52	1.90
	n-Pentyl	1.36	2.80	1.80	12.30					3.85	7.96			4.49	1.88
	n-Hexyl	1.35	3.30	1.80	14.00					3.85	7.60			4.48	1.96
	n-Decyl	1.38	3.20	1.82	22.00					3.90	7.30			4.48	2.20
C_3H_7 | R-N-CH_2-CH_2-OH | + C_3H_7	n-Propyl	1.45	8.80			2.20	6.20			.83	8.03			4.50	1.94

and chromatograms were sprayed with an iodoplatinate reagent (59). In every case a single spot was observed having the R_f value given in Table 7.1.

NMR spectra (Table 7.2) were obtained in D_2O with tetramethylsilane as external standard. Chemical shifts (δ) are given in ppm. In the case of multiplets, the chemical shifts given correspond to the centre of the signal. The number of protons was calculated by multipying the relative area of the signal by the assumed number of protons in the molecule.

The chloride of dimethyl-n-propyl (2-hydroxyethyl) ammonium was prepared from the iodide, which did not crystallize. Equimolar amounts of analog and AgCl were shaken in ethanol for 2 days. The mixture was then filtered, the solvent evaporated and the product recrystallized from acetone.

II. Transport Studies

A. Determination of affinity and transport rate

The method used in determining the transport parameters for the analogs, as well as the theoretical basis

of the analysis, are described in detail in the previous Chapter. In brief, rates of efflux of ^{14}C-choline from pre-loaded cells were measured in the presence of various concentrations of an unlabeled choline analog in the external solution, and the results plotted in accordance with the following equation:

$$\frac{v}{v_o} = \left(\frac{v_\infty}{v_o}\right)_{T_o} + \bar{K}_{To}\left(\frac{1 - v/v_o}{[T_o]}\right) \qquad (7.1).$$

where v is the experimental rate of choline exit in the presence of a given concentration of analog, $[T_o]$, in the external solution, and v_o the measured exit rate in the absence of the analog. From the intercept of a plot of v/v_o against $(1 - v/v_o)/[T_o]$ we find $(v_\infty/v_o)T_o$, which was previously shown to be directly proportional to the maximum transport rate in zero trans entry. From the slope, we obtain $\bar{K}_{To}$, which is identical to the apparent affinity constant when entry of the analog is studied directly. In terms of the present experiment, v_∞ is the choline exit rate when the analog concentration is saturating.

B. Distribution of choline analogs during the efflux experiment

In the present assay the exit rates are measured under conditions where the analog is assumed to be re - strictedto the external solution. If analogs could enter the

cell with sufficient rapidity, either via the carrier in the case of substrates or by passive diffusion through the membrane in the case of inhibitors, they would compete with ^{14}C-choline inside the cell and give a misleading result.

Fortunately, this does not happen, as the following facts show. First, the plots of exit rate are linear with time in the case of both substrates and inhibitors (Chapter 6). Second, the inhibition of choline entry by dimethylpentyl(2-hydroxyethyl)ammonium bromide is purely competitive (see below). Competitive kinetics are expected only if the inhibitor is restricted to the external solution (Chapter 2). Were the inhibitor present inside the cell as well, inhibition would necessarily be non-competitive. Third, and most conclusively, neither the pentyl nor the decyl analog penetrated the cells even after a 12 hour incubation period (see below).

C. Uptake Assay

The experimental method was described in detail in Chapter 5.

RESULTS

I. Affinities and Transport Rates

The experimental affinity constants, $\overline{K}_{To}$, and the relative transport rates, $(v_\infty / v_o)_{To}$, for choline and

its analogs are shown in Table 7.3 (see Methods). The ratio f_3/f_{-1}, the relative rate of translocation of the carrier-substrate complex and the free carrier, as represented in the transport scheme in Fig. 6.1, was estimated from the $(v_\infty/v_o)_{To}$ values, on the basis of an equation derived previously (Chapter 6):

$$\left(\frac{v_\infty}{v_o}\right)_{T_o} = \frac{(1 + f_{-1}/f_1)}{(1 + f_{-1}/f_3)} \qquad (7.2).$$

For this purpose, the assumption was made that in the absence of substrate the free carrier is equally distributed in the inward and outward facing forms, so that $f_1 = f_{-1}$. Probable limits for f_3/f_{-1} were calculated from $(v_\infty/v_o)_{To}$ plus or minus one standard deviation. Using this value of f_3/f_{-1}, the true substrate dissociation constant, K_{To}, was estimated from the expression for $\bar{K}_{To}$ (Chapter 6):

$$\bar{K}_{To} = K_{To} \frac{(1 + f_1/f_{-1})}{(1 + f_3/f_{-1})} \qquad (7.3).$$

Again, f_1 and f_{-1} were assumed to be equal. Probable upper and lower limits for K_{To} were calculated from the upper and lower limits of f_3/f_{-1}*(footnote - see next page).

Trends in affinity and transport rates with increasing chain length for the dimethyl and diethyl series of choline

TABLE 7.3

Transport parameters for choline analogs. $\bar{K}_{To}$ is the observed half-saturation constant, and $(v_\infty/v_o)T_o$ is proportional to the maximum rate of transport (in zero trans entry): $(v_\infty/v_o)_{To} = \bar{V}_{To}(1/f_1 + 1/f_{-1})$. The ratio f_3/f_{-1} is the calculated rate of reorientation of the carrier-substrate complex relative to the free carrier, and K_{To} the calculated dissociation constant for the carrier-substrate complex (see text).

Structure	R	$\bar{K}_{To}$ (μM)	$(v_\infty/v_o)_{To}$	f_3/f_{-1}	K_{To} (μM)
$R\text{-}\overset{+}{N}(CH_3)_2\text{-}CH_2CH_2OH$	Methyl	6.33 ± 0.62	1.93 ± 0.043	17 - 73	57.0 - 234
	Ethyl	12.4 ± 0.61	1.79 ± 0.011	8 - 9	55.8 - 62.0
	n-Propyl	33.2 ± 6.4	1.32 ± 0.026	1.8 - 2.1	46.5 - 51.5
	iso-Propyl	303 ± 19	0.48 ± 0.017	0.30 - 0.[illegible]	197 - 202
	n-Butyl	30.1 ± 1.8	0.12 ± 0.022	0.05 - 0.08	15.8 - 16.25
	n-Pentyl	7.3 ± 1.2	0.04 ± 0.08	0.00 - 0.06	3.65 - 3.87
	n-Decyl	0.30 ± 0.009	0.012 ± 0.008	0.00 - 0.01	0.150 - 0.152
	n-Dodecyl	0.095 ± 0.010	0.019 ±0.019	0.00 - 0.02	0.0475 - 0.0485
$R\text{-}\overset{+}{N}(C_2H_5)_2\text{-}CH_2CH_2OH$	Methyl	35.2 ± 7.7	1.38 ± 0.019	2.1 - 2.3	54.6 - 58.1
	Ethyl	693 ± 65	0.12 ± 0.028	0.05 - 0.08	364 - 374
	n-Propyl	590 ± 44	0.004 ± 0.03	0.00 - 0.02	295 - 301
	n-Butyl	203 ± 32	0.009 ± 0.08	0.00 - 0.05	102 - 107
	n-Pentyl	51.8 ± 3.2	-0.07 ± 0.04	0.00	25.9
	n-Hexyl	17.1 ± 0.14	0.07 ± 0.003	0.035 - 0.038	8.85 - 8.88
	n-Decyl	1.95 ± 0.05	0.02 ± 0.006	0.007 - 0.013	0.98 - 0.99
$R\text{-}\overset{+}{N}(C_3H_7)_2\text{-}CH_2CH_2OH$	n-Propyl	1230 ± 170	0.049 ± 0.093	0.00 - 0.08	615 - 664
$CH_3\text{-}\overset{+}{N}(CH_3)_2\text{-}CH_3$	-	206 ± 25*	1.74 ± 0.13*		

*Footnote

The apparent affinity constant for tetramethyl-ammonium was determined from its effect at varying concentrations outside the cell on the rate of ^{14}C-choline entry. $(v_\infty/v_o)_{To}$ was obtained by measuring choline efflux in the presence of a single saturating concentration (1 mM) of analog on the outside. In the same experiment $(v_\infty/v_o)_{To}$ for choline was 1.88 ± 0.17

analogs are shown in Figs. (7.1) and (7.2) respectively. The difference in affinity between corresponding members of the dimethyl and diethyl series, based on calculated K_{To} values, is shown in Fig. (7.3) as a function of the number of carbon atoms in the alkyl chain. The difference is seen to be constant throughout the series.

II. Competitive Inhibition by a Non-Transported Choline Analog

Choline entry was found to be competitively inhibited by the dimethyl-n-pentyl analog (Fig.7.4). The $\bar{K}_{To}$ values for both choline (6.54 $\pm$ 0.15 μM) and the inhibitor (8.2 $\pm$ 1.6 μM) agree with those determined indirectly.

III. Comparison of the Sensitivity of Glucose and Choline Transport to Detergents and Choline Analogs

The effects of detergents and choline analogs on the transport of both choline and glucose are recorded in Table

*Footnote

The interpretation of $\bar{K}_{To}$ and $(v_{oo}/v_o)_{To}$ is independant of assumptions about rate-limiting steps in transport. Equations (7.2) and (7.3) and the calculated values of f_3/f_{-1} and K_{To}, however, depend on the condition that dissociation of the carrier-substrate complex is far more rapid than carrier reorientation steps. This assumption is supported by accelerated exchange for choline, and by observations to be described later on non-competitive inhibition by a trans inhibitor, and on inactivation rates for N-ethylmaleimide in the presence of choline analogs.

FIGURE 7.1

Observed half-saturation constants and maximum transport rates (zero trans influx, relative to choline) for choline analogs in the series dimethyl-n-alkyl (2-hydroxyethyl) ammonium halide . $\overline{K}_{T_o}$ is expressed in μM units. Transport parameters are plotted as a function of the number of carbon atoms in the chain. Dotted line, affinities; solid line, maximum transport rates relative to choline.

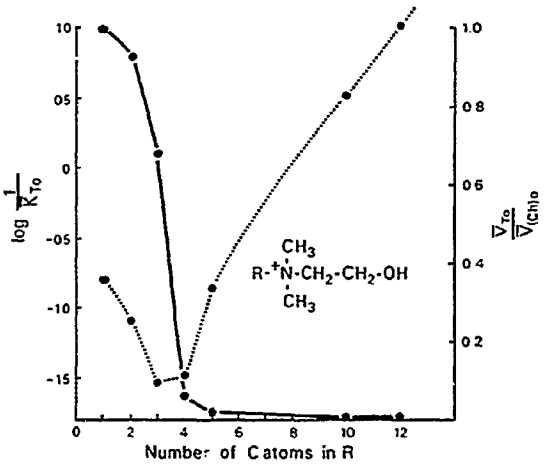

10
05
0
-05
-10
-15
log 1/K'To
1.0
0.8
0.6
0.4
0.2
V'To/V(Ch)o
CH3
R-+N-CH2-CH2-OH
CH3
0
2
4
6
8
10
12
Number of C atoms in R

FIGURE 7.2

Observed half-saturation constants and maximum transport rates (zero trans influx, relative to choline) for choline analogs in the series diethyl-n-alkyl (2-hydroxyethyl) ammonium halide . $\overline{K}_{T_o}$ is expressed in μM units. Transport parameters are plotted as a function of carbon atoms in the chain. Dotted line, affinities; solid line, maximum transport rates relative to choline.

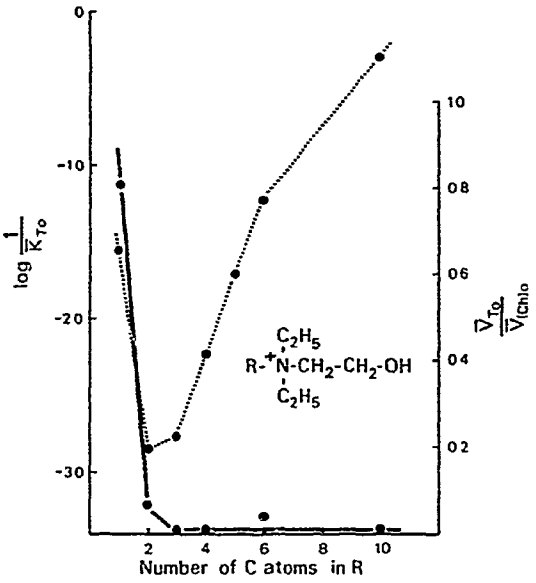
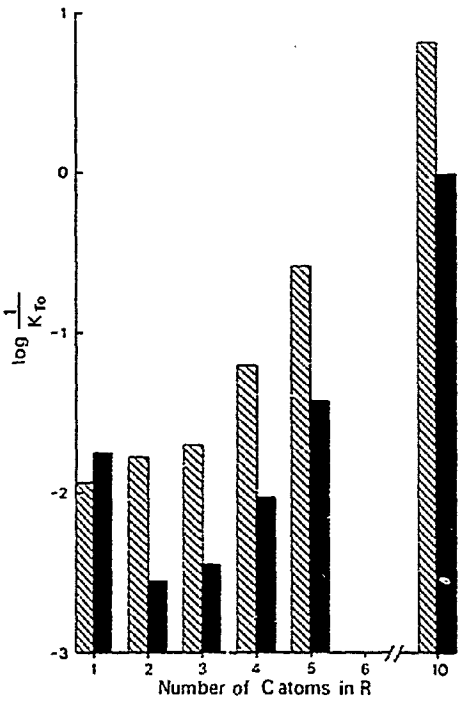

0
-10
-20
-30
log 1/K_To
10
08
06
04
02
V_To/V_Cl10
C2H5
R-+N-CH2-CH2-OH
C2H5
2
4
6
8
10
Number of C atoms in R

FIGURE 7.3.

Bindingstrengths of dimethyl- and diethyl-n-alkyl (2-hydroxyethyl) ammonium ions, as a function of the length of the alkyl chain. Binding constants, K_{T_o}, are calculated from apparent affinity constants, $\overline{K}_{T_o}$, on the basis of Eq.7.3 and calculated f_3/f_{-1} ratios (Table 7.3), with the assumption that $f_1 = f_{-1}$. For $n > 3$, relative values are independant of these assumptions (see text). Hatched bars, dimethyl series; solid bars, diethyl series.


1
0
-1
-2
-3
log 1/K_fo
1
2
3
4
5
6
10
Number of C atoms in R

7.4. The glucose system is the more sensitive to detergents but is not significantly inhibited by the choline analogs at concentrations which strongly inhibit choline transport.

IV. Failure of Large Choline Analogs to Penetrate into the Cells

According to the results given in Table (7.3), many of the choline analogs, though bound to the carrier, do not undergo transport at a significant rate. This result was subjected to an independant proof in the case of two such inhibitors. Cells (17% hematocrit) were incubated for 12 hours at 37° C with either 34.1 μM dimethyl-n-pentyl (2-hydroxyethyl) ammonium or 17.7 μM diethyl-n-decyl(2-hydroxyethyl) ammonium ions (4.7 or 8.8 times the $\overline{K}_{To}$ value, respectively). Cells were then harvested, chilled and washed as in experiments on choline efflux. Uptake of ^{14}C-choline into cells preincubated with the inhibitor was found to be as rapid as in the control. Relative rates with and without inhibitor were 1.12 ± 0.02 and 0.93 ± 0.08, respectively. Since an inhibitor present inside the cells at these concentrations should have caused strong inhibition, it may be concluded that neither the diethyl-n-decyl nor dimethyl-n-pentyl analog enters the cell, either by transport via the carrier, or by passive diffusion through the membrane.

TABLE 7.4.

Effects of detergents and choline analogs on the glucose and the choline transport system of human erythrocytes. Assays of transport rates: uptake of ^{14}C-choline (3.7 μM, 24 m Ci/mmol) measured by the method described in the text; exit of D-glucose (110 mM) determined by the light-scattering method (53).

Inhibitor	Concentration	%Inhibiton	
		Glucose System	Choline System
Triton X-100	1.33 mg/100 ml	83.3	12
Cetyldimethyl benzyl ammonium Cl^-	7×10^{-3} mM	47	19.8
di-n-butylamino ethanol	1.4 mM	2	56
diethyl-n-decyl (2-hydroxyethyl) ammonium Br^-	4.45×10^{-2} mM	2	95.8
dimethyl-n-pentyl (2-hydroxyethyl) ammonium Br^-	1.16×10^{-2} mM	0	61

V. Comparison of a Cationic Substrate and its Neutral Isostere

The possible requirement of a positive charge for substrate binding was investigated by comparing the effects of isoamyl alcohol and dimethyl amino ethanol on the uptake of 0.65 μM ^{14}C-choline. Cells were preincubated with the neutral alcohol or the tertiary amine at a concentration of 100 μM for 10 min, after which uptake rates were measured. The amine strongly depressed transport, while isoamyl alcohol had no effect. The rate relative to the control was 0.03 ± 0.007 for dimethylaminoethanol, and 1.06 ± 0.05 for isoamyl alcohol.

A possible reason for lack of inhibition by isoamyl alcohol could be its non-specific adsorption by the membrane or other components of the cell, with the result that the concentration left in solution would be very low. This possibility was tested for a closely related alcohol, n-butanol, which was available in radioactive form. This alcohol (n-butanol (1-^{14}C), 6.4 m Ci/mmol) was incubated with cells at 37° C under identical conditions of concentration (100 μM) and hematocrit; the cells were then sedimented, and the remaining radioactivity in the suspending medium determined. The concentration in the medium was found to be reduced by only 10% following this treatment. When a butanol concentration of 10 μM was used, it was still found that 10% was removed by adsorption in the cells.

VI. Effect of Iodide Ion on Choline Transport

Since some of the analogs were prepared as the iodide, the possible effect of this ion on transport was determined. Rates of choline exit were measured at varying concentrations of NaI in a medium containing a total concentration of NaI and NaCl of 154 mM together with 5 mM sodium phosphate buffer. Iodide ion was without effect, as the following figures show: rates in the presence of either 0, 1, 3, 10, or 30 mM NaI were 2.81 $\pm$ 0.04, 2.84 $\pm$ 0.18, 2.78 $\pm$ 0.05, 2.87 $\pm$ 0.05 and 3.08 $\pm$ 0.16, in units of 10^{-2} µmol/litre of cells/min.

DISCUSSION

I. The Relationship Between Apparent and True Values of Affinity and Rate Constants

The interpretation of trends in affinities for series of related substrates and inhibitors is complicated by discrepancies between true and apparent values, discrepancies which are greater the higher the rate at which the substrate is transported. This is evident upon inspection of eqn. (7.3). With rapidly transported substrates such as choline, for which $f_3 >> f_{-1}$, saturation may occur at much lower concentrations than is expected from the value of the true dissociation constant K_{To}, for in this

case $\bar{K}_{To} << K_{To}$ (assuming that $f_1 \approx f_{-1}$).* In the case of inhibitors (where the transport rate, and f_3, are equal to zero) true and apparent affinities are related by the factor $(1 + f_1/f_{-1})$, which depends only on the partition of the carrier in the membrane in the absence of substrates or inhibitors, and is therefore a constant. For this reason, the relative affinities of different inhibitors are directly seen.

It is also true that the rates of reorientation of the carrier-substrate complex for good substrates are not directly apparent in zero trans flux experiments, because of the rate-limiting return of the unloaded carrier. Fortunately, the more useful ratio of f_3/f_{-1} may be estimated from (v_∞/v_o) values if the ratio f_1/f_{-1} is known. Later, evidence will be presented to show that this ratio is approximately unity, and accordingly, from eqn. 7.2.,

$$(v_\infty/v_o)_{To} = 2/(1 + f_3/f_{-1}) \qquad (7.4).$$

Once values for f_3/f_{-1} have been assigned, the apparent affinities may be corrected. The calculated values of both

*Footnote

On an intuitive level, the increase in apparent affinity of a good substrate may be understood as a consequence of the cyclic carrier movement involved in transport. With such a substrate, $(f_3 >> f_{-1})$, the loaded carrier moves through the membrane faster than the free carrier returns, and carrier on the trans side of the membrane accumulates. The latter is unavailable to bind substrate and therefore adds to the total concentration which, in effect, has formed a complex with the substrate. The substrate concentration required to "saturate" the carrier is therefore lowered. (Cont'd on next page).

f_3/f_{-1} and K_{T_o} are included in Table 7.3, where it is seen that uncertainty about the value of f_3/f_{-1} is large only in the case of the best substrate, choline itself, where the ratio f_3/f_{-1} is at least 17 and could be much larger. Correspondingly, the true affinity of choline must be smaller than the observed value by a factor of 10 or more. Owing to the uncertainty in this measurement, we shall avoid basing any conclusions on these particular values of the constants for choline.

II. Evidence for Specific Interaction Between Choline Analogs and the Carrier

The largest choline analogs, such as dimethyl- and diethyl-n-decyl(2-hydroxyethyl)ammonium, may superficially resemble detergents. Their potency against the choline system, however, cannot depend on any property of this kind, as the following observations prove. (1) The glucose transport system of the same cells is highly sensitive to inhibition by anionic, cationic and neutral detergents, as

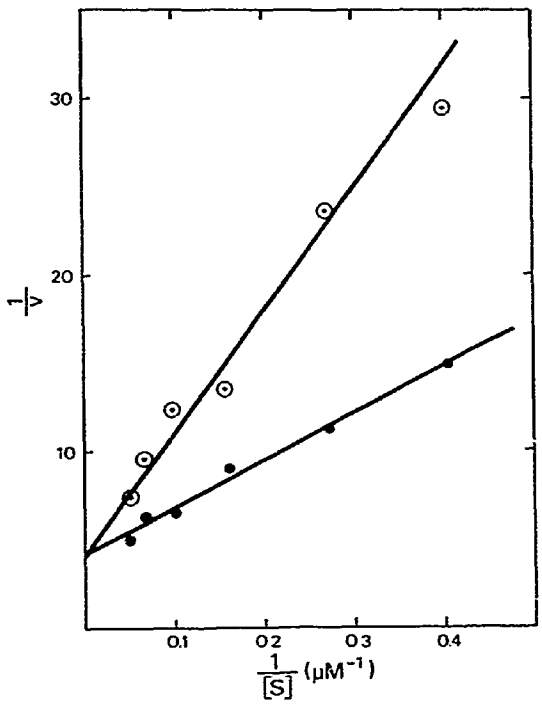
*Footnote (cont'd from previous page)

In the case of inhibitors, saturation occurs when the carrier is entirely in the form of a complex. However, since the inhibitor is restricted to the outside medium, only a fraction of the carrier can form a complex directly; the rest must migrate across the membrane, and only then can it be trapped by the inhibitor, whose concentration must therefore be higher than otherwise. In a sense, the inhibitor competes with the trans side of the membrane for the carrier.

well as non-polar substances generally (60), but is not inhibited by the choline analogs at concentrations that greatly depress choline transport (Table 7.4). (2) The choline system is relatively insensitive to detergents. Even the cationic detergent cetyldimethylbenzyl ammonium chloride, which resembles the larger choline analogs, is more effective against the glucose than the choline system (Table 7.4). (3) The concentration dependance of inhibition of choline transport conforms to a simple saturation process, in which one inhibitor molecule binds to one carrier site. Detergents should probably exhibit a higher than first order dependance on concentration, without apparent saturation. (4) The dimethylpentyl analog was shown to inhibit choline uptake in a purely competitive fashion (Fig. 7.4), whereas unspecific detergent effects might be expected to be non-competitive. (5) There is a constant difference between the affinities of corresponding dimethyl and diethyl analogs (having the same substituent R), which extends throughout the series and includes both substrates and inhibitors (Fig. 7.3). This constancy is indicative of a highly specific binding site, and shows that all the analogs become bound at this site. (6) Affinities are not simply determined by the hydrophobic nature of the analogs. Thus, inhibitors containing about the same number of methylene groups may have widely different affinities: e.g. the dimethylpentyl,

FIGURE 7.4

The inhibition of ^{14}C-choline entry by dimethyl-n-pentyl (2-hydroxyethyl)ammonium iodide (12.9$_{\mu}$ M). Points on the upper and lower lines represent rates in the presence and absence of the inhibitor respectively. Units of influx rates: $_{\mu}$ moles per litre of packed cells per min.


30
20
10
$\frac{1}{v}$
0.1
0.2
0.3
0.4
$\frac{1}{[S]}$ (μM^{-1})

diethyl butyl and tripropyl analogs have binding constants of 3.7,105, and 630 μM respectively (Table 7.3) . (7) In experiments to be reported later, we show that when present in the external solution, inhibitors with both long and short chains protect the system against inactivation by N-ethyl-maleimide. They do so by holding the carrier in the form in which the binding site is exposed on the external surface of the membrane. By contrast, Triton X-100, even at concentrations that visibly alter membrane morphology, fails to alter the distribution of the carrier in the membrane and so has no effect on inactivation by the reagent.

III. Structure-Activity Relations

A. Affinity

Our observations are readily understood if we consider the cation binding site to have subsites for the three methyl substituents on the quaternary nitrogen atom in choline. At two of these positions the restrictions upon size are severe, at the third mild. Presumably when a choline analog enters the substrate site, its hydroxyethyl moiety and positive charge become fixed in position, but the trialkyl ammonium group is free to rotate so that the two smallest substituents occupy the most restricted methyl sites, while the largest occupies the third position and may project upwards, out of the binding site. In all members of the dimethyl series, the two methyls would therefore

occupy the restricted sites, while the alkyl chain extends upwards; in all members of the diethyl series with the exception of the first (the diethylmethyl analog), two ethyl groups should occupy these sites and the third, the alkyl chain, would project upwards. With the diethylmethyl analog, a methyl and an ethyl group are likely to occupy the restricted sites, rather than two ethyls. The affinity of this compound is therefore higher than would be expected from its position in the diethyl series.

That enlargement at two of the methyl subsites is severely limited is shown by the observations that one methyl and one ethyl group are tolerated (compare the dimethylethyl and diethylmethyl analogs, for which K_{To} values are 59 and 57 μM respectively), whereas two ethyl groups are squeezed out (the dimethylethyl and diethylmethyl analogs, above, are to be compared with the triethyl, for which K_{To} is 367 μM).

The relative constancy of the calculated K_{To} values in the first members of the dimethyl and diethyl series (excepting, as noted above, the diethylmethyl analog) suggests that the third, and least restrictive, methyl site accommodates an alkyl chain as long as propyl without crowding and without any positive contribution to binding. The introduction of a branched chain at this site does,

however, result in crowding, judging by the fall in affinity of the dimethylisopropyl analog.

Lengthening of the alkyl chain beyond propyl leads to steadily increasing affinities in both the dimethyl and diethyl series, and this suggests the existence of non-polar structures of considerable size in the region immediately adjacent to the choline binding site, in agreement with observations made by Martin on a series of trimethyl-n-alkyl ammonium ions (61).

When corresponding analogs (with the same R substituent) in the dimethyl and diethyl series are compared, it is found that in every case (except R = 1) the diethyl analog has the lower affinity by a constant factor of 6.5, as is illustrated in Fig. 7.3. The constancy of this effect is of interest for several reasons. First, it depends on the increment in binding strength with each added methylene group being very similar in both series. The increment is in fact relatively constant throughout, even with the addition of 12 C atoms (Fig. 7.1). The conclusion from this must be that a rather extensive region is available immediately adjacent to the transport site, where non-specific, probably hydrophobic bonding, occurs. Second, it confirms the conclusion reached above regarding severe crowding at the point in the substrate site where two of the methyl groups are bound. Third, the unusual degree of specificity

for the dimethyl (2 hydroxyethyl) ammonium ion indicates that in all members of both series the quaternary group is held at the same place in the carrier, which must be the choline binding site. The competitive inhibition seen with the dimethylpentyl analog (Fig. 7.4) serves to confirm this conclusion. Fourth, the simplicity of the relationship suggests that estimates based on calculated K_{To} values rather than experimental constants, $\overline{K}_{To}$, are essentially correct. If based on $\overline{K}_{To}$, constancy is still seen for those members of the series that are inhibitors but not for substrates, since $\overline{K}_{To}$ and K_{To} values for inhibitors differ by a constant factor, $(1 + f_1/f_{-1})$.

B. The Rate of Transport

Even the slightest enlargement in the structure of the substrate results in a severe reduction in transport activity. This is to be contrasted with the effect on binding strength, since increased size may either have little effect on affinity, may weaken binding, or more often may have the opposite effect and elevate affinity, as we have seen. The smallest additions to the choline structure, which fail to affect affinity, sharply diminish the carrier-reorientation rate. For example, with the dimethyl-propyl and diethylmethyl analogs, the rate of the carrier reorientation step f_3 is at least 4 times smaller than that for the dimethyl analog or choline. A reduction in affinity, which is a reflection of crowding at the binding site, is always accompanied by a severe reduction in the transport rate. For example, in the transition from the diethylmethyl to the

triethyl analog, affinity falls by a factor of 6 to 7, but the rate of the carrier reorientation step is depressed by a factor of at least 30, virtually abolishing transport. Positive contributions to binding by the long alkyl substituents are also accompanied by a loss in transport activity, as is seen in the comparison of the n-butyl and n-propyl derivatives in the dimethyl series: the affinity is seen to rise by a factor of 4, while f_3 falls by a factor of 30, again reducing the transport rate to a barely detectable level.

Whereas enlargement of the substrate molecule lowers the rate of transport, diminishing its size has no measurable effect on the rate, even where affinity declines. This is shown by the example of tetramethyl ammonium, bound 30 times less strongly than choline, but transported at approximately the same rate.

It is concluded from this that the requirements for binding at the carrier site on the surface of the membrane and for the subsequent step of translocation through the membrane are distinctly different.

Earlier, a series of trimethyl-n-alkyl ammonium ions was investigated by Martin (61), and it was found that as the alkyl chain was lengthened an abrupt transition occurred from a substrate transported approximately as well as choline (trimethyl-n-propyl ammonium ion) to an inhibitor which was

not transported at all (trimethyl-n-butyl ammonium ion). At the transition point, a drop occurred in apparent affinity, rather than the regular increments seen elsewhere in the series.

In view of our present understanding of interactions in the binding site, this transition may be understood as a displacement of the alkyl chain from the 2-hydroxyethyl site to the non-polar region outside the cation binding locus. So long as the alkyl chain lies in the former site, transport is permitted, but when it protrudes outwards to form an attachment in the adjoining non-polar region, movement is prohibited. Clearly, severe spatial limitations must be encountered around both the quaternary nitrogen atom and the alcoholic hydroxyl group of choline.

IV. The Mechanism of Transport

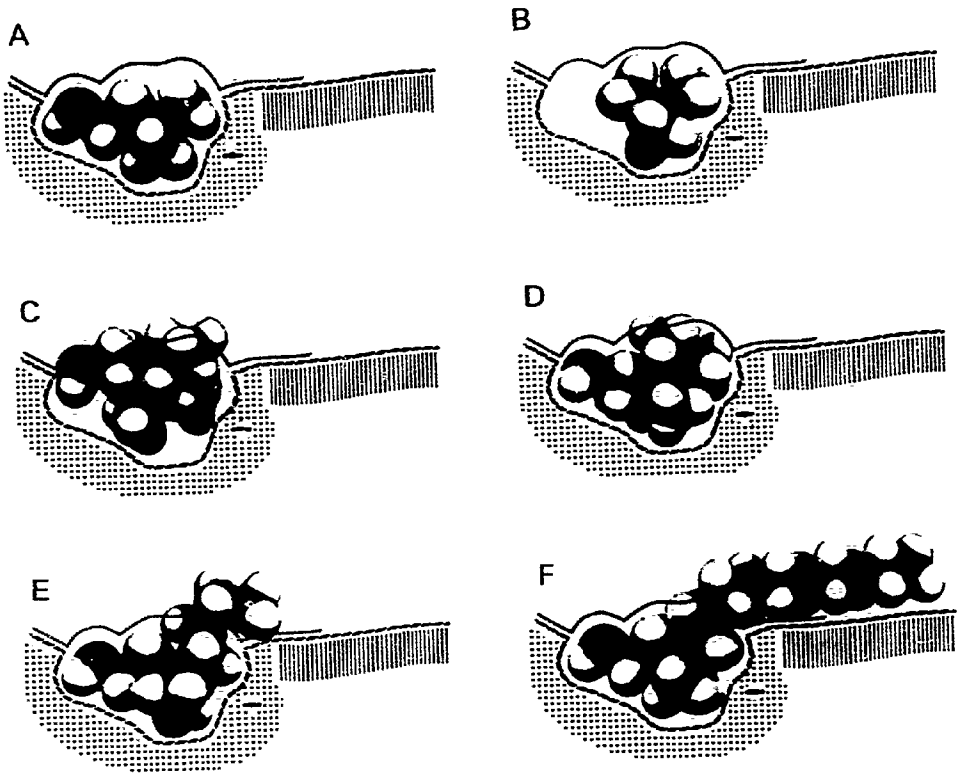
As seen above, the speed of the translocation step is sharply reduced by even the smallest increment in the substrate size, whereas binding becomes stronger when the substrate molecule is large. It appears to follow that restraints upon occupancy of the carrier site become far more severe when the site leaves the surface of the membrane and penetrates into the interior. This is understandable if we envisage the substrate bound at the surface site as being only partly enclosed by the carrier and free to protrude out of the site; and the substrate within the membrane, as being completely surrounded by structures in the carrier that

match its shape at every point. Two half sites complementary to the substrate may therefore cooperate in the process of transport, one presented to the aqueous medium, the other capping the exposed surface of the substrate in the course of the carrier reorientation step. These ideas are illustrated in Fig. 7.5.

That the binding of substrate could induce a conformational change in the carrier, facilitating the reorientation steps in transport, may be suggested by the observation that the complex with choline traverses the membrane far more rapidly than free carrier ($f_3/f_{-1} > 17$). This hypothesis, however, fails to account for the rapid transport of tetramethylammonium ion, which is equal to that of choline, despite the fact that its affinity is 30 times lower. Clearly, the hydroxymethylene group makes an important contribution to choline binding, but does not facilitate the translocation step.

FIGURE 7.5

A diagrammatic representation of the proposed carrier site with various substrate analogs bound. The lower section shows a profile of the site exposed on the external surface of the cell membrane. This site is closely complementary to one half of the choline molecule, and is open above. It contains a negative charge, as indicated in the drawing, adjacent to which is an extensive non-polar region capable of adsorbing long alkyl chains, as in F. The dotted profile above this external site represents a complementary site which, as a prerequisite for carrier reorientation must move into place, thus completely enclosing the substrate molecule. Substrates too large to fit into the space created by the apposition of the two half-sites fail to undergo translocation in the membrane but may be strongly bound to the lower half-site (F). Substrates that are bulkier than choline at all three N-methyl positions fail to make close contact with the lower site and are therefore both weakly bound and non-transported (C). Substrates small enough to be enclosed in the double site without crowding are well transported, even though weakly bound (B). Substrate analogs are as follows: A, choline; B, tetramethyl ammonium; C, triethyl(2-hydroxyethyl) ammonium; D, dimethyl-isopropyl(2-hydroxyethyl)ammonium; E, dimethyl-n-butyl (2-hydroxyethyl)ammonium; F, dimethyl-n-decyl(2-hydroxyethyl) ammonium ion.


A
B
C
D
E
F

SUMMARY

The relationships between structure, affinity and transport activity in the choline transport system of erythrocytes have been investigated in order (i) to explore the nature of the carrier site and its surroundings, and (ii) to determine the dependance of the carrier reorientation process on binding energies and steric restraints due to the substrate molecule. Affinity constants and maximum transport rates for a series of trialkyl derivatives of ethanolamine, were obtained by a method that involves measuring the trans effect of unlabeled analogs upon the movement of radioactive choline. The main conclusions are as follows: (1) An analysis of transport kinetics shows that the affinity constants determined experimentally differ from the actual dissociation constants in a predictable way. The better the substrate, the higher the apparent affinity relative to the true value, whereas the affinity of non-transported inhibitors is underestimated by a constant factor. (2) The carrier-choline complex undergoes far more rapid reorientation (translocation) than the free carrier. (3) The carrier imposes a strict upper limit upon the size of a substrate molecule that can participate in the carrier reorientation process; this limit corresponds to the choline structure. A smaller substrate such

as tetramethylammonium, despite relatively weak binding forces, is unhindered in its translocation, suggesting that a carrier conformational change dependant upon substrate binding energy is not required for transport. (4) A positive charge in the substrate molecule is required for binding. (5) Small increases in the size of the quarternary ammonium head, as in triethylcholine, sharply lower affinity, consistent with a high degree of specificity for the trimethylammonium group. (6) Lengthening the alkyl substituent in derivatives of dimethyl- and diethylaminoethanol causes a regular increase in affinity, suggestive of unspecific hydrophobic bonding in a region very near the substrate site.

CHAPTER 8

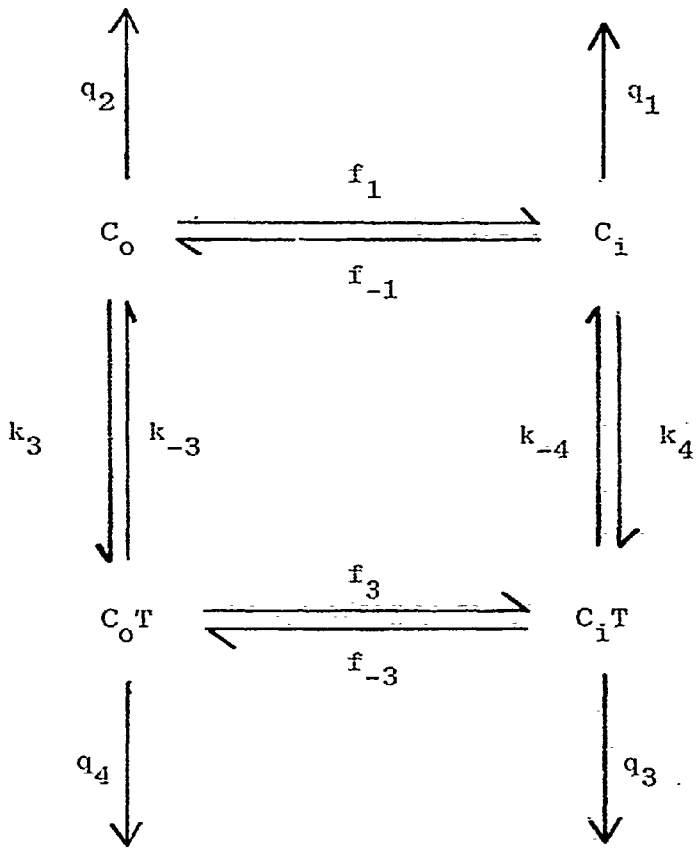
TESTING CARRIER MECHANISMS WITH IRREVERSIBLE INHIBITORS

INTRODUCTION

Among the many possible approaches to the problem of biological transport, the use of irreversible inhibitors, particularly in conjunction with kinetic studies of substrate movement, appears to hold the promise of advancing our understanding of the molecular events in the translocation process. By means of this method, observations of considerable interest have been made; for example, evidence has been obtained leading to the suggestion that the inward- and outward-facing forms of the choline carrier react with an irreversible inhibitor at different rates and therefore have different conformations (27, 62). However, it has been asserted, on the basis of a theoretical analysis, that there are severe limitations inherent in this approach and inescapable ambiguity in the interpretation. Lieb and Stein (18), the authors of this criticism, demonstrated that the classical carrier model (Fig. 8.1) demands a definite consistency among experimental parameters for rates of transport and inactivation; they concluded, however, that different formulations of the classical model itself cannot be distinguished, for example forms in which there are either one, two or three carrier-substrate complexes. In consequence, it should be impossible to decidewhether inward- and outward- facing forms of the carrier react with

FIGURE 8.1.

Scheme representing transport by a carrier system in which a carrier-substrate complex forms on the inner and outer surfaces of the membrane (C_iT and C_oT respectively). Each carrier species may react with an irreversible inhibitor at a rate given by the constants q_1 to q_4. Rate constants for carrier-reorientation are designated by the letter f, and those for complex formation by the letter k.

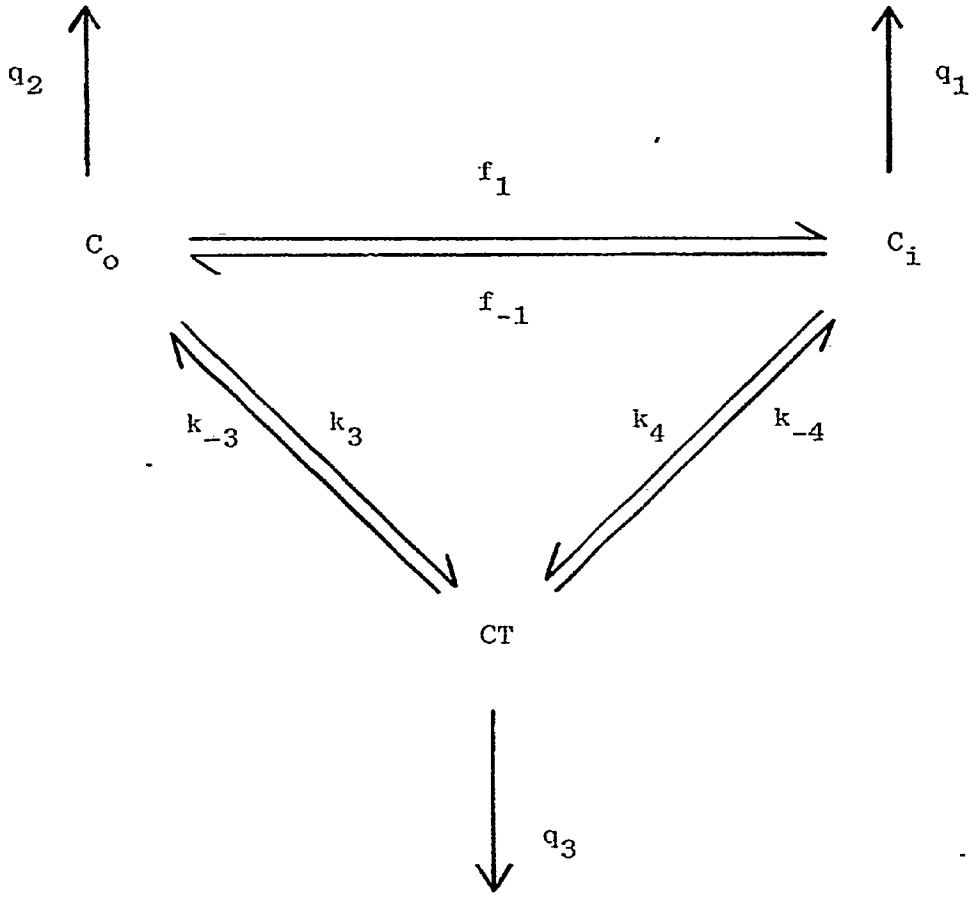

q2
q1
f1
Co
Ci
f-1
k3
k-3
k-4
k4
f3
CoT
CiT
f-3
q4
q3

an inhibitor at different rates, or whether any intermediate forms of the carrier that may exist are inactivated at a rate different from other forms. In an earlier analysis of transport kinetics (24) the same authors had reached an equally discouraging conclusion, to the effect that very limited information could be obtained about the carrier mechanism from studies of substrate movement, and in particular that it was not yet profitable to consider whether reorientation of the carrier complex or breakdown of this complex is rate-limiting. For the sake of simplicity therefore, these workers (18,24,32,63), found it necessary to merge both these steps in an analytical description of transport, producing a variant of the carrier model in which there is a single carrier substrate complex, rather than a complex with substrate on either side of the membrane (Fig. 8.2).

If correct, this analysis has far-reaching significance for both the design and the interpretation of experiments. It will be shown, however, that the conclusions are in error. In particular, it is shown that it is theoretically possible to distinguish the one-complex and the two-complex formations of the carrier model and to decide whether or not breakdown of the complex is rate limiting. In addition, observations on rates of transport and on rates of irreversible inhibition are described which eliminate the one-complex model, and which also eliminate

FIGURE 8.2.

Transport and inactivation scheme for the one-complex carrier formulation of Lieb and Stein ([18]), corresponding to the two-complex scheme in Fig.8.1 (q.v).


q_2
q_1
f_1
C_o
C_i
f_{-1}
k_{-3}
k_3
k_4
k_{-4}
CT
q_3

dissociation of the carrier-substrate complex as the slow step in transport. It is concluded that the classical formulation of the carrier model, with two species of complex, and with carrier reorientation as the rate-limiting step, is still an adequate representation of membrane transport systems.

THE ANALYSIS OF TRANSPORT AND INACTIVATION KINETICS

The conclusion that various formulations of the carrier model cannot be distinguished, rested upon the demonstration that a relationship exists among a number of selected experimental parameters for transport and inactivation, which is identical whether the analysis is based on the one-complex, the two-complex or a three-complex model, the latter involving an intermediate form. The relationship which was established is as follows:

$$\frac{Q_{To}}{\bar{V}_{To}} + \frac{Q_{Ti}}{\bar{V}_{Ti}} = \frac{Q_{ToTi}}{\bar{\bar{V}}_{T}} + Q_o \left(\frac{1}{\bar{V}_{To}} + \frac{1}{\bar{V}_{Ti}} - \frac{1}{\bar{\bar{V}}_{T}} \right) \qquad (8.1).$$

The naming of the parameters in eqn. (8.1) follows the conventions described in Appendix 1. (see Eqn.A.8). The values of the parameters in terms of the individual rate constants appearing in the kinetic scheme in Figures 8.1 and 8.2 are given in Appendix 1 and 2(Tables A.4,A.6).Since the three models give rise to the same relationship and since this equation includes many of the experimental transport and inactivation parameters, the conclusion was drawn that no

relationship could exist which could differentiate among various formulations. Such an extrapolation from this particular case to the general one would require a separate proof, and the principle must therefore be questioned. Thus, although a relationship of the form of eqn. (8.1) does not distinguish among the models, it does not follow that other relationships cannot do so. In dealing with observations on the inactivation of choline transport by N-ethyl maleimide, it is specifically shown (below) that certain relationships do exist which have the desired property. The experimental values of these constants are found to be inconsistent with the one-complex formulation of the carrier but consistent with the two-complex formulation.(See Table 8.1)

CHOLINE TRANSPORT AS A TEST SYSTEM

The choline transport system is irreversibly inhibited by the thiol reagent N-ethylmaleimide. The effects of two substrates, choline and tetra methylammonium ion and of the reversible competitive inhibitor, decamethonium, led Martin (62) and Edwards (27) to propose the very interesting and potentially useful hypothesis, that the reagent acted upon the inward-facing form of the carrier, but did not react with the outward-facing form. As we have seen, Lieb and Stein (18) decided that it would not be permissible to reach such a conclusion on the basis of steady state data. This criticism would depend implicitly on two assumptions ;

TABLE 8.1.

Equations used in testing the one-complex and the two-complex formulations of the carrier mechanism.

Experimental Parameter	Two-Complex Scheme	One-Complex Scheme
$\frac{Q_{To}/Q_o}{(v_{oo}/v_o)_{To}}$	$\frac{\frac{q_1}{f_{-1}} + \frac{q_3}{k_{-4}} + \frac{1}{f_3}\left(1 + \frac{f_{-3}}{k_{-4}}\right)q_4}{q_1/f_{-1} + q_2/f_1}$	$\frac{q_1/f_1 + q_3/k_{-4}}{q_1/f_{-1} + q_2/f_1}$
Q_{ToTi}	$\frac{q_3/f_{-3} + q_4/f_3}{1/f_3 + 1/f_{-3}}$	q_3

(i) that reversible inhibitors represent a special case whose complex with the carrier is not directly comparable to the substrate complex, and (ii) that no prior knowledge is possible about the rate-limiting steps in transport. In the present study the hypothesis will be subjected to a quantitative test with the aid of a series of substrate analogs in which there is a gradual transition from a good substrate to a non-transported inhibitor.

This problem is of great interest because the detection of conformational differences between the inward and outward facing carrier forms would be important for our understanding of the molecular nature of transport, and partly because the hypothesis, if proven, would enable us to determine the distribution of the carrier in the membrane. Having a probe for carrier distribution would allow us to study, for example, the interactions between the carrier and surrounding structures, including the aqueous medium. It would also allow us, for the first time, to determine the ratios of substrate dissociation constants on the inner and outer membrane surfaces; for it must be borne in mind that apparent affinity constants determined in flux studies may differ widely from the true values, since their magnitudes are strongly dependant upon ratios of rates for carrier re-orientation steps (Chapter 7).

TESTING THE HYPOTHESIS ON N-ETHYLMALEIMIDE

In attempting to devise a stringent test of the hypothesis that only the inner carrier form reacts with N-ethyl maleimide , it was noted that any pressure which increases the proportion of carrier on the inner surface should, by hypothesis, increase the rate of inhibition, whereas any pressure diminishing this form should reduce it. A substrate analog on one side of the membrane can exert such a force, and the strength and direction of this force depends upon its rate of transport: a series of substrate analogs with varying transport rates therefore provides us with a tool for altering the carrier distribution to varying degrees. A very poor substrate present externally would tend to hold the carrier in a complex on the outer surface, and therefore protect against inactivation, while a very good substrate, for which reorientation of the complex is more rapid than reorientation of the free carrier form, would tend to shift the carrier on to the inner surface of the membrane and therefore accelerate inhibition.

If the model and hypothesis are correct, predictable relationships should exist between the parameters of inactivation and any other process that is also directly proportional to the internal carrier form. Unidirectional efflux is just such a process, as can be seen in equation 8.2. below. It follows that if inactivation and efflux

rates are both directly proportional to $[C_i]$, then any force altering the carrier distribution must have an identical effect on these two rates. Since a substrate outside the cell exerts such a force, the hypothesis requires that the ratio of inactivation rates in the presence and absence of a substrate analog should be equal to the ratio of efflux rates under the same conditions.

METHODS

I. The Rate of Inactivation by N-ethylmaleimide

A. Treatment of Cells:

Cells (2.5% suspension) were treated with 1 mM N-ethylmaleimide in the presence of a choline analog at a saturating concentration outside the cells or in its absence. The suspending medium, held at 37°C, contained 154 mM NaCl and 5 mM sodium phosphate buffer at pH 6.8. Solutions of N-ethylmaleimide and the analog were added to the cell suspension at zero time. Samples of the suspension (40 ml) were removed at intervals and placed in 50 ml tubes containing mercaptoethanol (final concentration, 10.6 mM). The tubes were immediately spun in a clinical centrifuge for 3 min, after which the supernatant was aspirated and the cells washed 3 times with clean buffer. The treated cells were then transferred to

conical tubes, packed by centrifugation (10 min) and assayed for choline uptake.

B. Uptake Assay

The procedure for determining uptake rates was given in Chapter 5. The concentration of ^{14}C-choline (30 or 53 mCi/mmol) in which the cells were placed was 3.0 μM.

C. Determination of Inactivation Rates:

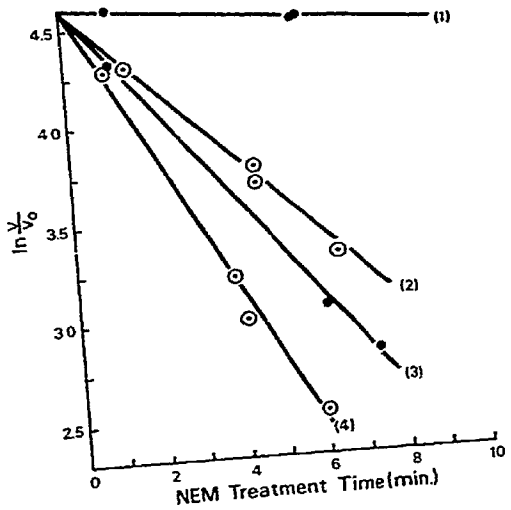
Plots were constructed of the natural logarithm of the rate of choline entry, as determined in the uptake assay, against the time of treatment with N-ethylmaleimide. The pseudo-first order inactivation rate constant was obtained from the slope of the line, by a least squares analysis. Results under four different conditions are shown in Fig. 8.3. In every case a linear relationship is observed.

II. The Rate of Exit

The rate of exit of ^{14}C-choline was determined in the presence of a substrate analog at a saturating concentration outside the cell or in its absence, exactly as when inactivation rates were determined. The internal concentration of ^{14}C-choline was approximately 3 μM. The theoretical basis of the parameters obtained and the details of the experimental method have already been described in Chapter 6.

FIGURE 8.3

The reduction in choline transport rates as a function of time of treatment with 1 mM N-ethyl maleimide, pH 6.8, 37° C, in the presence of choline analogs.
(1) dimethyl-n-decyl (2-hydroxyethyl) ammonium; (2) diethylmethyl (2-hydroxyethyl) ammonium; (3) control; (4) choline.Concentrations and results are listed in Table 8.2.


(1)
(2)
(3)
(4)
4.5
4.0
3.5
3.0
2.5
0
2
4
6
8
10
NEM Treatment Time(min.)

III. Materials

Synthesis of the choline analogs was described earlier (Chapter 7).

RESULTS

I. Inactivation and Transport Rates

Experimental values of Q_{To}/Q_o and of $(v_\infty/v_o)_{To}$, with standard deviations, are recorded in Table 8.2 and are plotted in Fig. 8.4. Values are also given for the ratio of inactivation rates in the presence of an analog and in the presence of choline (Q_{To}/Q_{cho}); the corresponding ratio of rates (v_{To}/v_{cho}) is also given, namely the rate of exit of ^{14}C-choline with an analog or with unlabeled choline outside the cell. In these experiments, both inactivation and transport rates were determined in the same sample of cells, and the figures are therefore directly comparable. The line drawn through the experimental points in Fig. 8.4. has a unit slope and passes through the origin, indicating that for every substrate the ratios $(v_{oo}/v_o)_{To}$ and Q_{To}/Q_o are equal; in addition v_{To}/v_{cho} is equal to Q_{To}/Q_{cho}. In other experiments carried out at intervals over a period of time, determinations of inactivation and transport rates were sometimes made with different samples of cells, and these are less reliably compared with one another. The

TABLE 8.2.

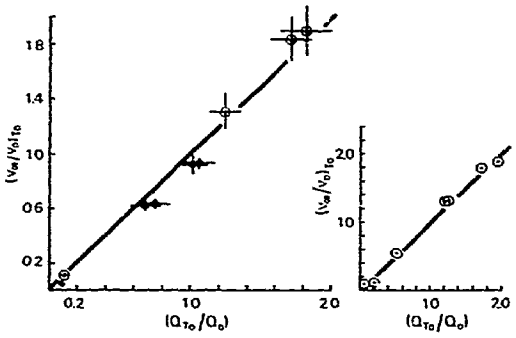
Effects of choline analogs at saturating concentrations outside the cells on the rates of inactivation by 1 mM N-ethylmaleimide and on the rates of ^{14}C-choline efflux (2 μM). $\overline{K}_{To}$ is the experimental half-saturation constant for an analog determined in a zero trans entry experiment. (Chapter 7). Q_{To} and Q_o are inactivation rates in the presence and absence of an analog, respectively, and Q_{cho} is the rate in the presence of a saturating concentration of choline outside the cells. Similarly $(v_{oo}/v_o)_{To}$ is the ratio of choline exit rates in the presence and absence of an analog; $(v_{oo})_{To}$ is the choline exit rate with a saturating concentration of a given analog outside the cell, and $(v_{oo})_{cho}$ the ^{14}C-choline exit rate in the presence of unlabeled choline at a saturating concentration outside the cell. Experiments were done with three different cell samples, designated as 1, 2 and 3 (fourth column).

TABLE 8.2

Analog	Concentration (μM)	$\bar{K}_{To}$ (μM)	Cell Sample	$\frac{Q_{To}}{Q_o} \cdot \left(\frac{v_\infty}{v_o}\right)_{To}$	$\frac{Q_{To}}{Q_{cho}}$	$\frac{(v_\infty)_{To}}{(v_\infty)_{cho}}$
Choline	50	6.3 ± 0.6	1	1.81 ± 0.19	1.90 ± 0.10	
Dimethylethyl(2-hydroxyethyl) -ammonium I^-	80	12.4± 0.6	1	1.71 ± 0.15	1.84 ± 0.09	
			2		1.06 ± 0.11	0.93 ± 0.04
			3		1.02 ± 0.10	0.92 ± 0.07
Diethylmethyl(2-hydroxyethyl) ammonium I^-	385	35.2± 7.7	1	1.24 ± 0.12	1.31 ± 0.10	
			2		0.75 ± 0.09	0.63 ± 0.04
			3		0.68 ± 0.09	0.62 ± 0.03
Diethyl-n-decyl(2-hydroxyethyl) ammonium Br^-	18	1.95± 0.05	1	0.098± 0.015	0.11 ± 0.02	
			3		0.089± 0.007	0.06 ± 0.014

FIGURE 8.4

Plots of inactivation and transport data which are given in Tables 8.2, 8.3 and 8.4. The plot on the left shows the data in Table 8.2, and the inset on the right shows those in Tables 8.3 and 8.4. In the main figure (left), the bars indicate the standard errors of the measurements. The open points are for rates in relation to control values, as indicated on the axes. The solid points are for rates relative to determinations in the presence of choline (Q_{To}/Q_{cho} and v_{To}/v_{cho})


1.8
1.4
1.0
0.6
0.2
0.2
1.0
2.0
$(V_{ss}/V_{so})_{T_o}$
(Q_{T_o}/Q_o)
2.0
1.0
1.0
2.0
$(V_{ss}/V_{so})_{T_o}$
(Q_{T_o}/Q_o)

results of a number of experiments are brought together in Tables(8.3,8.4). When the means of these values of Q_{To}/Q_o are plotted against the mean values of$(v_\infty/v_o)_{To}$, a straight line of unit slope is produced which passes through the origin, as before (Fig. 8.4, inset).

We now know the probable reason for this variation in different cell samples: Q_o varies because the distribution of the free carrier in the membrane varies (i.e. the f_1/f_{-1} ratio is not constant; see Table A.4 in the Appendix and Chapter 9). Since both Q_{To}/Q_o and$(v_\infty/v_o)_{To}$ depend on this distribution, comparisons should preferably be made with a single sample of cells. In order to minimize deviations which have this cause, it seemed justifiable to discard experimental determinations of Q_{To}/Q_o in which f_1/f_{-1} is greater than 60% or less than 40%. In a total of 9 experimental determinations only 2 were eliminated on this basis. Thus in the great majority of cases, the variation was not great, but sufficient to affect the precision of the estimates. In the case of $(v_\infty/v_o)_{To}$ ratios, none of the values could be discarded, since v_o by itself does not provide an estimate of f_1/f_{-1}.

II. Specific Nature of the Analog Effects

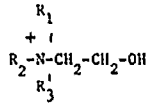
A number of independant pieces of evidence were

TABLE 8.3

Effects of choline analogs at saturating concentrations outside the cells on rates of inactivation by 1 mM N-ethyl maleimide. The experiments and symbols are as described before (Table 8.2). Experiments were carried out on 7 different samples of cells, the results for one of which(#1) are also reported in Table 8.2.

TABLE 8.3.

Structure



Q_{To}/Q_o

Cell Sample

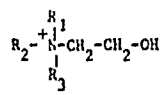
R_1	R_2	R_3	$\bar{K}_{To}$ (μM)	Concentration (μM)	1	6	7	8	9	10	11	Mean
Me	Me	Me	6.3	52	1.83							
				25		1.85	2.16					1.95 ± 0.185
Me	Me	Et	12.4	80	1.72							
				42			1.70			1.68		1.72 ± 0.02
Me	Et	Et	35.2	385	1.25							
				195		1.34	1.20	1.17				1.24 ± 0.07
Me	Me	n-Prop	33.2	190				1.02		1.40		1.21 ± 0.27
Me	Me	iso-Prop	303	1370				0.53				0.53
Me	Me	n-But	30.1	114					0.28		0.17	0.23 ± 0.08
Et	Et	n-Decyl	1.95	18	0.08							
				12			0.07	0.04			0.10	0.07 ± 0.03

TABLE 8.4

Effects of choline analogs at saturating concentrations outside the cell on rates of efflux of ^{14}C-choline, as described in Table 8.2. Experiments are reported on 5 different samples of cells, only one of which is included in the Q_{To}/Q_o measurements given in Table 8.3.Extrapolated values are calculated from a linear plot of rates at varying concentrations of external substrate, T_o(eqn. 6.2)

TABLE 8.4

Structure



$(v_\infty / v_o)_{To}$

Cell Sample

R_1	R_2	R_3	$\bar{K}_{To}$ (μM)	Concentration (μM)	1	2	3	4	5	Extrapolated value	Mean
Me	Me	Me	6.3	52	1.90	1.85	2.07	1.82	1.88	1.93	1.91 ± 0.09
Me	Me	Et	12.4	80	1.84	1.74	1.9	1.82	-	1.79	1.81 ± 0.06
Me	Et	Et	35.2	385	1.31	1.26	1.29	1.31	-	1.38	1.31 ± 0.05
Me	Me	n-Prop	33.2	-	-	-	-	-	-	1.32	1.32
Me	Me	iso-Prop	303	2400	0.61	-	0.55	0.60	-	0.48	0.56 ± 0.06
Me	Me	n-But	30.1	-	-	-	-	-	-	0.12	0.12
Et	Et	n-Dec	1.95	18	0.10	-	0.12	0.09	-	0.02	0.08 ± 0.04

previously cited to show that the acceleration or inhibition of choline transport produced by various analogs is solely the result of binding at the carrier site and that detergent-like effects are not involved (Chapter 7). Further, Edwards (27) observed simple saturation curves for both choline and the competitive inhibitor decamethonium in their effects on N-ethylmaleimide inactivation rates, and the observed half-saturation constants for the substrate and the reversible inhibitor agreed with those obtained in transport. The gradual progression which we have observed from increased inactivation rates to protection in going from well transported substrates to non-transported inhibitors, as well as the strictly quantitative relationship between rates of transport and rates of inactivation in the presence of analogs, powerfully argues against generalized effects of the analogs. It may also be noted that the concentrations of analogs in the inactivation experiments which were required at near saturating concentrations were determined from apparent binding constants in transport measurements. Were the effects on transport and inactivation mediated by different mechanisms, the estimated concentrations could have been inappropriate and in consequence the quantitative relationship between transport and inactivation rates in Fig. 8.4 would not have been found. Another important observation is that Triton X-100, which visibly alters

the morphology of the membrane (64), does not affect the inactivation rate (Deves, R. and Krupka, R.M., unpublished observations). Taken together, these many observations conclusively demonstrate the specific nature of the effects of the analogs.

RELATION BETWEEN INACTIVATION AND TRANSPORT RATES

The experiments have established that for a whole series of substrate analogs ranging from the well transported choline to non-transported inhibitors, the following relationship is obeyed

$$\left(\frac{v_\infty}{v_o}\right)_{T_o} = \frac{Q_{To}}{Q_o}$$

It is easy to visualize the conditions that are required to explain this observation, even without reference to detailed kinetic equations. This we shall now try to do.

Both v_∞ and v_o, the rates of exit of choline into a medium containing a substrate analog, or into pure buffer solution, can be described by the equation below:

$$v = f_{-2}\,[C_i]\,[S_i] \qquad (8.2).$$

where f-2 and $[S_i]$ are constants, but where $[C_i]$, the concentration of free carrier on the inner surface of the membrane, differs under the two conditions. These two rates are therefore related by an expression of the following form:

$$\left(\frac{v_\infty}{v_o}\right)_{To} = \frac{[C_i]_{To \to \infty}}{[C_i]_{To = o}} \qquad (8.3).$$

It is to be noted also that since the internal concentration of choline, $[S_i]$, is very low in relation to its half-saturating value, the internal choline should not itself significantly alter the carrier distribution.

Similarly, inactivation rates may be expressed in terms of individual carrier species (see Appendix 1, eqn.A.7):

$$Q_{To} = \left([C_i]\ q_1 + [C_iS]q_3 + [C_oS]q_4\right) / Ct \qquad (8.4).$$

The free carrier in the external form, C_o, does not appear in this equation because with a saturating concentration of analog outside the cells, all the external carrier is present as the substrate complex, C_oS. In the absence of the substrate analog, the inactivation rate is given by

$$Q_o = \left(q_1\ [C_i] + q_2\ [C_o]\right) / Ct \qquad (8.5).$$

It is easily understood that the following equality

$$\left(\frac{v_\infty}{v_o}\right)_{To} = \frac{[C_i]_{To \to \infty}}{[C_i]_{To=o}} = \frac{Q_{To}}{Q_o} \qquad (8.6).$$

will hold only if the terms $q_3[C_iS]$, $q_4[C_oS]$ and $q_2[C_o]$ are negligible. We shall see that the first becomes negligible if dissociation from the complex is rapid, making the steady state concentration of C_iS disappearingly low, and that the others drop out because the external carrier forms do not react with N-ethylmaleimide ($q_2 = q_4 = 0$).

The equality that is observed in ratios of inactivation and transport rates, with external choline as the reference condition ($Q_{To}/Q_{cho} = v_{To}/v_{cho}$), may be understood in a similar way, but we shall consider the interpretation of this observation in a later section.

DETAILED ANALYSIS OF THE EXPERIMENTAL RESULTS

I. Q_{To} and $(v_\infty / v_o)_{To}$

The analysis involves determining the conditions under which $(Q_{To}/Q_o)/(v_\infty / v_o)_{To} = 1$ for all substrate analogs, and also the conditions or models that are inconsistent with the experimental observations. The complete expressions for this ratio derived on the basis of either the one-complex or the two-complex schemes are given in Table 8.1.

In order to formally demonstrate the correctness of the theory, we shall take two other experimental results into account. The first is that in the presence of choline at a saturating concentration, and at equilibrium inside and outside the cells, the inactivation rate (Q_{ToTi}) is comparable to that in the absence of any substrate or inhibitor (Chapter 10). The second observation is that with a non-transported substrate analog bound on the inner surface of the membrane, the inactivation rate is greater than in the control in the absence of substrates or inhibitors (Chapters 9 and 10). The general expressions for Q_{ToTi} are given in Table 8.1.

The analysis will be carried out in two parts; in the first, it will be assumed that the inactivation constants q_3 and q_4 are characteristics of individual carrier states and do not depend upon the nature of the substrate analog that is bound; and in the second, that q_3 and q_4 may vary with different substrates.

I. The one-complex model (Fig. 8.1)

A. Invariant q_3 and q_4

In this scheme* it is seen (Table 8.1) that $Q_{To}/Q_o = (v_\infty/v_o)_{To}$ if the following equality holds:

$$q_3/k_{-4} - q_2/f_1 = 0 \qquad (8.8)$$

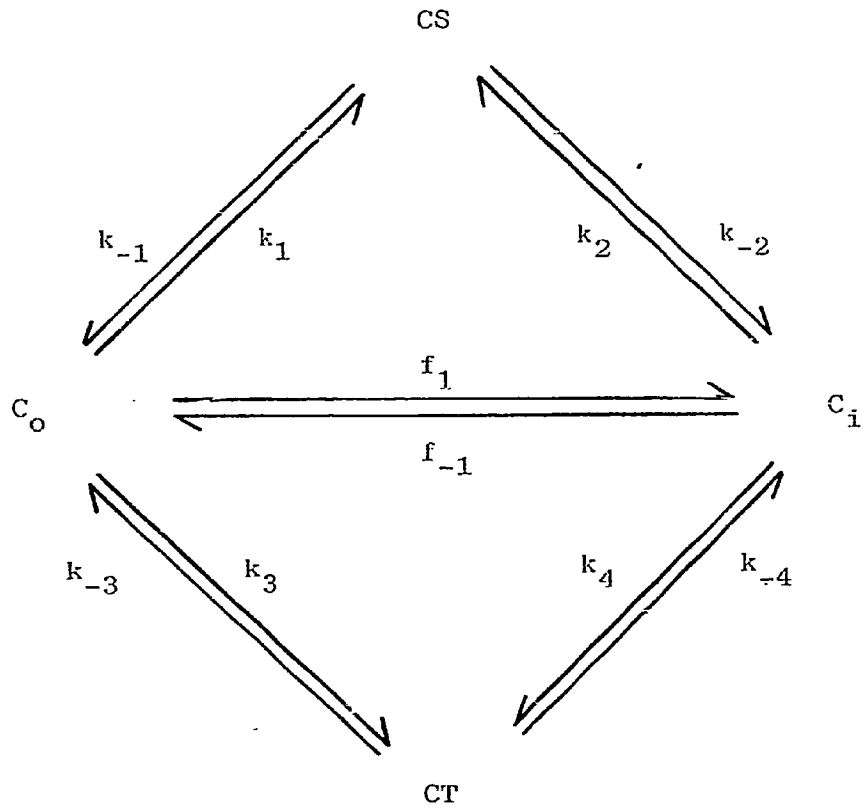
Since k_{-4} must vary for different substrates, the term $\frac{q_3}{k_{-4}}$ must vary correspondingly. On the other hand, the term $\frac{q_2}{f_1}$ is characteristic of the carrier system alone, and is independent of the substrate. In order that eqn. (8.8) may remain equal to zero with a wide range of substrates, therefore, it is necessary that both q_2 and q_3 be equal to zero. However, q_3 (Table 8.1) is equal to Q_{ToTi}, and experimentally this is found to be non-zero, so that this interpretation must be rejected. An attempt might be made to save the theory by assuming that the term q_3/k_{-4} in eqn. (8.8) is negligible compared to q_1/f_1, and this could be true with a good substrate, for which $k_{-4} >> f_1$, and for which, therefore, reorientation of the free carrier is the rate-limiting step in transport. However, with a substrate whose maximum transport rate is low compared to choline's, k_{-4} must be smaller than f_1, and consequently the term in q_3 could not be negligible if q_3 is non-zero.

In conclusion, the one-complex model is inconsistent with the experimental observations, if q_3 is invariant.

*Footnote: Rate equations for the inactivation scheme in Fig. 8.2 and for the two-substrate scheme in Fig. 8.5 are given in Appendix 2.

FIGURE 8.5

Kinetic scheme for the one-complex carrier model, for transport in the presence of two substrates, S and T.


CS
k_{-1}
k_1
k_2
k_{-2}
f_1
C_o
C_i
f_{-1}
k_{-3}
k_3
k_4
k_{-4}
CT

B. Variable q_3 and q_4

If the magnitude of q_3 depends on the nature of the bound substrate molecule, it would in theory be possible for variations in this constant to balance those in the transport constant, k_{-4}, thus preserving the equality in eqn. (8.8); i.e., q_3/k_{-4} would be a constant equal to q_2/f_i. With poor substrates, as we have seen, k_{-4} must become rate-limiting, and in a series of substrates it must decline as the transport rate drops. With a non-transported analog, $k_{-4} = 0$. The inactivation constant, q_3, must fall correspondingly, and with the non-transported analog it too must become zero. The prediction from this is that such an analog, when bound on either the internal or external surface of the membrane, should completely protect the system against inactivation by N-ethylmaleimide. We have found, however, that certain bulky tertiary alkyl derivatives of ethanolamine, which do not undergo transport, and which are bound to the carrier on the inner surface of the cell membrane, fail to protect the system, and in fact increase the inactivation rate (Chapter 9), in agreement with the observations of Edwards (27) on metanephrine.

We conclude therefore that the one-complex formulation of the simple carrier must be abandoned; for regardless of the assumptions made, it is inconsistent with the observed behaviour of the transport system.

II. The two-complex model

A. Invariant q_3 and q_4

In the two-complex scheme it is seen from Table 8.1 that $Q_{To}/Q_o = (v_\infty/v_o)_{To}$ if

$$\frac{q_3}{k_{-4}} + \frac{q_4}{f_3}\left(1 + \frac{f_{-3}}{k_{-4}}\right) - \frac{q_2}{f_1} = 0 \qquad (8.9).$$

The terms in q_3 and q_4, if non-zero, must vary for different substrates (since f_3, f_{-3} and k_{-4} are involved in determining both the affinity and transport rate of a substrate), while the term in q_2 must be constant, since f_1 is a rate constant for the free carrier. With a single substrate the expressions could fortuitously equal zero even though q_2, q_3 and q_4 are non-zero, owing to a chance relationship between these constants and k_{-4}. However, the expression could not remain equal to zero for all the analogs which we have studied, since they vary widely in both affinity and maximum transport rate. We conclude, therefore, that all three terms in eqn. (8.9) must be insignificant in comparison with q_1/f_{-1} (Table 8.1).

There is a difficulty, however, arising from this conclusion. Q_{ToTi}, which from the expression in Table 8.1 is equal to $(f_3q_3 + f_{-3}q_4)/(f_3 + f_{-3})$, is found to be non-zero experimentally, and therefore, it must be assumed that either q_3 or q_4 is non-zero. However, as stated above, both these terms must be negligible in eqn. 8.9. There is a simple solution to this difficulty; for the importance of the term

$\frac{q_3}{k_{-4}}$ in eq (8.9) is crucially dependent on the relative magnitude of the carrier reorientation rate and the rate of substrate dissociation (f_{-1} and k_{-4} respectively). If $k_{-4} >> f_{-1}$ the $\frac{q_3}{k_{-4}}$ term will be negligible compared to the term $\frac{q_2}{f_1}$, even though q_3 and q_2 may be of similar magnitude. The term in q_4, on the other hand, is insensitive to the ratio of these rates, and for this reason the conclusion cannot be avoided that q_4 is equal to zero.

In summary, the only conditions under which the two-complex model can satisfy the data, if q_3 and q_4 are substrate - independent constants, are:

(i) $q_2 = q_4 = 0$

(ii) $q_1 \neq 0$; $q_3 \neq 0$

(iii) $k_{-4} >> f_{-1}$

B. Variable q_3 and q_4

If q_3 and q_4 depend on the nature of the bound substrate molecule, their values could be proportional to some function of the transport constants, f_3, f_{-3} and k_{-4}, in a way that satisfies the equality in eqn 8.9. The term $\frac{q_2}{f_1}$, on the other hand, must be constant, as we have seen. In examining this possibility we shall consider three different cases individually.

a) $q_3 \neq 0$; $q_4 \neq 0$.

The term q_3 in eqn 8.9 is seen to depend on k_{-4}, and

that in q_4 on f_3, f_{-3} and k_{-4}. From our observations on different substrates we know that the maximum transport rate and the affinity are completely unrelated constants (Chapter 7). For example, with a poor substrate, for which the translocation constants, f_3 and f_{-3}, are close to zero, the affinity may either be very high relative to choline's or very low (eg. triethyl (2-hydroxyethyl) ammonium and dimethyl-m-decyl (2 hydroxyethyl) ammonium ions have half-saturation constants of 700 and 0.1 μM respectively). Again,with very good substrates, having high values of f_3, affinities may vary by a large factor,as in the example of choline and tetramethyl ammonium, whose affinity constants are 6 and 200 μM respectively, but which have comparable maximum transport rates (Chapter 7). Quite certainly, therefore, no invariable relationship exists between factors which determine affinity, the dissociation constant k_{-4} being one, and factors that determine carrier reorientation rates, namely f_3 and f_{-3}. Consequently, it would be impossible for the terms in q_3 and q_4 to vary in a compensating manner that could preserve the equality in eqn (8.9), with all substrates. We therefore reject the hypothesis that both q_3 and q_4 are non-zero.

b) $q_3 = 0$; $q_4 \neq 0$

A sufficient objection to this hypothesis is that poorly transported substrate analogs bound on the inner surface of the membrane increase the rate of inactivation

(Chapter 9), whereas if $q_3 = 0$, they should protect the system.

We might still attempt to rescue the hypothesis by the ad hoc assumption that q_4, which is large for a good substrate, is zero for a non-transported substrate analog, while the contrary is true for q_3: this constant is taken to be large for an inhibitor and zero for a good substrate. It follows that for intermediate substrates, q_3 and q_4 would both be non-zero, a suggestion which we have already ruled out (see (i) above). The hypothesis must, therefore, be abandoned.

c). $q_4 = 0$; $q_3 \neq 0$

It is now required that $q_3 / k_{-4} = q_2 / f_1$. The objection to this hypothesis is that the inactivation rate with an internally bound inhibitor should be directly related to the affinity(for in this case $K_{Ii} = \frac{k_{-4}}{k_4} 1 + f_{-1}/f_1$). Leaving aside the implausible nature of this requirement, we have observed that several different inhibitors bound only on the inner surface of the membrane with different affinities have very similar effects in increasing the rates of inactivation (Chapters 9 and 10). These experiments are totally inconsistent with the present proposal, which is therefore rejected.

II. Q_{To} / Q_{Cho} and V_{To} / V_{Cho}

A. The one-complex scheme

By writting equations for Q_{To} and v_{To} for two different substrates we can determine the conditions required to satisfy the equality found in the experiments:

$$\frac{(Q'_{To})}{(Q_{To})} = \frac{(v'_{To})}{(v_{To})} \qquad (8.11).$$

where the primed parameters represent those for a second substrate. The necessary condition for equality of these experimental ratios is found to be

$$\frac{q_3}{k_{-4}} = \frac{q_3'}{k'_{-4}} \qquad (8.10).$$

To satisfy eqn. 8.10 it is necessary that if k_{-4} approaches zero, as in the case of a very poor substrate, then q_3 must approach zero. Exactly as in the earlier analysis, this requirement is ruled out by the observation that a non-transported analog bound internally should reduce the rate of inactivation to zero, whereas experimentally the opposite, that is an increase, is found. It must, therefore, be assumed that $q_3 = 0$. Again this is ruled out since experimentally Q_{ToT}, which equals q_3 (Table 8.1), is found to be non-zero. The model, therefore, fails to account for the observations.

B. The two-complex scheme

The condition required for the equality observed

experimentally is now found to be (8.12).

$$\frac{f_{-1}}{k_{-4}} q_3 - \frac{f_{-1}}{k'_{-4}} q_3' + q_4 \frac{f_{-1}}{f_3} \left(1 + \frac{f_{-3}}{k_{-4}}\right) - q'_4 \frac{f_{-1}}{f'_3} \left(1 + \frac{f'_{-3}}{k'_{-4}}\right) = 0$$

Since the dissociation and reorientation rate constants (k and f respectively) are unrelated for a series of substrates (as seen above), the q_4 terms would vary in an unpredictable way with different substrates and must, therefore, be zero. As we have seen, however, either q_3 or q_4 must be non-zero since Q_{ToTi} is non-zero. The terms in q_3 become negligeble in eqn (8.12) if $k_{-4} >> f_{-1}$, as was already seen, even though q_3 is non-zero.

THE ONE-COMPLEX FORMULATION OF THE SIMPLE CARRIER

In addition to the evidence against the one-complex scheme which was considered above, the scheme suffers from a logical inconsistency when called upon to explain reversible inhibition (Fig. 8.2). Because this model postulates that a separate complex cannot be distinguished on either side of the membrane, it must be assumed that a non-transported inhibitor capable of binding on both sides could not possibly exist, since any substance capable of reaching the intermediate complex from both sides would necessarily be a substrate The only possible explanation for a truly non-transported inhibitor are (a) the inhibitor binds exclusively on one side of the membrane and not both, or (b) an inhibitor and a substrate produce radically different types of complex, so

that while a substrate forms a single distinguishable complex an inhibitor forms two, one on each surface of the membrane.

On the first hypothesis absolutely asymmetrical binding of inhibitors should be the rule. However, this is not found; for example phloretin, a competitive inhibitor of glucose movement in erythrocytes, is equally bound on both sides of the membrane (55). On the second hypothesis, it is difficult to account for the fact that the decline in substrate activity in a series of analogs occurs gradually, starting with a rapidly transported substrate and ending in analogs that fail to undergo transport (Chapter 7). The sudden emergence of a unique property in inhibitors is in no way evident.

Despite the appeal of its mathematical simplicity, therefore, the one-complex model should be abandoned, particularly as its use would tend to obscure evidence on the very steps which we seek to illuminate.

SUMMARY

The potential usefulness of irriversible inhibitors in testing transport models is examined in relation to the underlying kinetic theory, and earlier theoretical criticisms of this approach are shown to be ill-founded. We now find that the combination of transport and inactivation data can provide clear rejection criteria not only for models other than the classical carrier, but for different formulations of the classical carrier itself. These principles are applied in an experimental study of the irreversible inhibition of choline transport by N-ethyl maleimide in the presence of a series of choline analogs, and a linear relationship is established between the transport rate for an analog at a saturating concentration outside the cells and its effect under the same conditions upon the rate of carrier inactivation. On the basis of these and other observations, the following conclusions about the carrier mechanism can be drawn. (1) The classical carrier model can account for all the observations. (2) There are two different forms of the carrier-substrate complex, the form in which the substrate site is exposed to the internal solution reacts with N-ethyl maleimide, whereas the form in which the site is available externally does not react. (3) The carrier reorientation step, and not substrate dissociation, is the rate-limiting step in transport. (4) A formulation of classical carrier having only one

carrier-substrate complex cannot account for the observations and is therefore rejected. ($_5$) The external carrier complex is unreactive with N-ethyl maleimide whether or not the bound substrate analog is transported.

CHAPTER 9

RATES OF INACTIVATION OF INTERNAL CARRIER FORMS BY N-ETHYL MALEIMIDE: EVIDENCE FOR A CARRIER CONFORMATIONAL CHANGE DURING COMPLEX FORMATION

INTRODUCTION

Evidence for a carrier conformational change which occurs during the process of transport was obtained in previous studies of the irreversible inhibition of the choline system (Chapter 8). The carrier was found to exist in two distinguishable forms, one in which the substrate binding site is exposed to the solution outside the cell and the other in which the site is exposed to the internal solution. Only the inward-facing forms react with N-ethyl maleimide: the outward-facing forms, both the free carrier and the complex with substrate, are unreactive. In his earlier study, Edwards (27) had concluded that the inward-facing carrier reacted at the same rate whether it was free of substrate, or present as a complex. This conclusion rested upon semi-quantitative observations, however, and was not tested in the previous chapter. Experiments are now reported in which inactivation rate constants for the free carrier and the complex are determined individually, and are shown to differ.

Aside from providing evidence for a conformational change in the carrier induced by the substrate, it will be seen that this enables us to determine the distribution

of the carrier in the membrane in either the presence or absence of substrate, and this information is useful in itself. First, from a knowledge of the carrier distribution in the presence and absence of a saturating concentration of a substrate analog at equilibrium inside and outside the cell, the ratio of true substrate affinities on the two sides of the membrane may be determined. Hence substrate specificity at both the internal and external sites may be probed, with the aim of deciding if these sites interact in an identical way with substrates. The apparent affinities derived from transport measurements do not provide this information, since they strongly depend on rates of translocation through the membrane, and not on binding strength alone. Further, the distribution of the free carrier in the membrane, when determined under conditions that perturb the membrane itself, gives us an insight not otherwise available into the physical relationship between the carrier conformation and membrane structure.

THE EXPERIMENTAL APPROACH

In order to determine the rate of reaction of the internal carrier substrate-complex with N-ethyl maleimide, choline analogs were used which have a vastly greater affinity for the internal than for the external carrier site and, as a result, sequester the carrier as an internal complex. These analogs enter the cell by a route

independent of the choline carrier by virtue of the fact that they are tertiary rather than quaternary amines.

The reaction of the free carrier on the inner side of the membrane was studied in three different ways. In one, the cells are placed in a solution containing a saturating concentration of choline, and because choline undergoes very rapid transport, this should cause the carrier to accumulate almost entirely as the free carrier on the inner membrane surface. In the second method, inactivation and transport rates are measured at varying internal concentrations of choline, and the rate of reaction of the free carrier inside is then calculated by a linear extrapolation of the data.

Both these methods depend on choline being a rapidly transported substrate, for which reorientation of the carrier-substrate complex is more rapid than reorientation of free carrier; otherwise the two estimates are expected to differ. The third method specifically tests the adequacy of choline for use in these experiments.

The new evidence shows that the complex is more rapidly inactivated than the free carrier, which is not easily explained unless the substrate induces a conformational change in the carrier. Hence three different conformational forms of the carrier are now distinguished: (i) the two external forms, C_o and C_oS; (ii) the free internal carrier form, C_i; and (iii) the internal substrate complex, C_iS.

METHODS

I. Inactivation rates

A. External choline

Washed cells were treated with 1 mM N-ethyl maleimide at 37° for varying lengths of time, as described in the previous chapter. The solution bathing the cells (isotonic phosphate buffer, 5 mM, pH 6.8) contained choline at a saturating concentration (5 to 10 times its half-saturation constant, $\overline{K}_{To}$). The residual transport activity was estimated by measuring the rate of uptake of ^{14}C-choline (2.5 μM). This assay is described in detail in Chapter 5.

B. Internal choline

Washed cells were incubated for 18 hrs. in solutions containing various concentrations of unlabelled choline, after which the external choline was removed by washing the cells in the cold. The loaded cells were treated with 1 mM N-ethyl maleimide and the remaining transport activity determined as described in section A, above.

C. Control

Cells were treated with N-ethyl maleimide in the absence of substrate. Otherwise the procedure is as above.

II. Transport rates

The rate of transport in cells containing varying concentrations of unlabelled choline was determined from the zero-time intercepts in plots of the logarithm of the

transport rate vs. the time of treatment in N-ethyl maleimide. The slope of this plot is equal to the inactivation rate (Chapter 8), and its intercept at zero time is equal to the transport rate in the intact cells.

III. Materials

Tertiary amines were distilled at reduced pressure before use. All chemicals were reagent grade as in previous Chapters. ^{14}C-choline (30 mCi/mnol) was obtained from New England Nuclear, and the purity tested as described in Chapter 5.

RESULTS

I. Reactivity of the internal carrier-substrate complex

In order to attain the condition in which a substrate is bound only on the inner surface of the membrane, we would wish to have available an analog which does not undergo transport on the carrier but penetrates the cell membrane by another route. At the same time however, the substrate must be retained inside the cell long enough for the inactivation experiment to be completed. Such a substrate analog did not appear to be available. Edwards (27) had employed metanephrine for this purpose, but its exit was so rapid that very short reaction periods with N-ethyl maleimide, and hence unusual reaction conditions, were required.

We therefore considered the possibility that permeable choline analogs might be found which are asymmetrically

bound to the carrier, as in the example of certain sugar derivatives with the glucose transport system (4,5). Asymmetrical binding is easily demonstrated in studies of the inhibition of transport, in particular in zero trans entry and exit experiments (Chapter 2). In brief, the underlying theory is as follows. When the inhibitor binds on both sides of the membrane, both uptake and efflux will be non-competitively inhibited. If, however, the inhibitor is bound on only one side, even though present on both, the inhibition will be competitive in one direction and non-competitive in the other. For example, if the inhibitor adds only to the inner carrier form it competes with internal but not with external substrate, and therefore competitively inhibits exit, while non-competitively inhibiting entry.

The tertiary choline analog, dibutylaminoethanol, was found to behave in precisely this manner (Figs. 9.1 and 9.2),indicating that it is preferentially bound at the carrier site on the inner surface of the membrane. The structure-activity relationships for this system (Chapter 7) lead us to expect that this amine, like dimethylbutyl (2-hydroxy ethyl) ammonium ion and other bulky choline analogs, should bind to the carrier site but should fail to undergo transport. This expectation is confirmed by the observations, for it was shown that a good substrate never produces non-competitive kinetics, regardless of which compartment it occupies (Chapter 2).

Figure 9.1 Inhibition of ^{14}C-choline exit by di-n-butylaminoethanol, 2.4 mM. Upper line, rates with inhibitor, lower line, control. Inhibition constant, 1.13 ± 0.18mM. The ratio of the maximum velocities, in the presence and absence of inhibitor, calculated from a least squares analysis of the points, was 0.83 ± 0.14. The inhibition is therefore almost purely competetive.

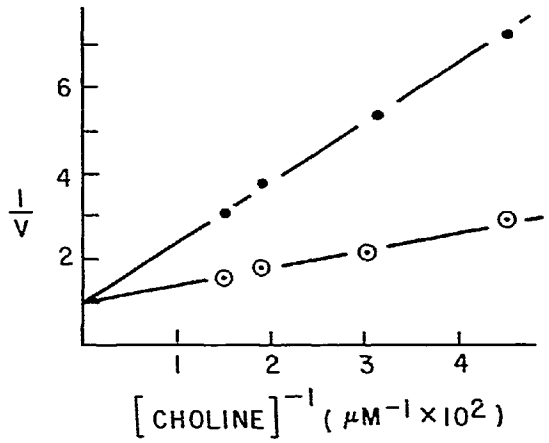
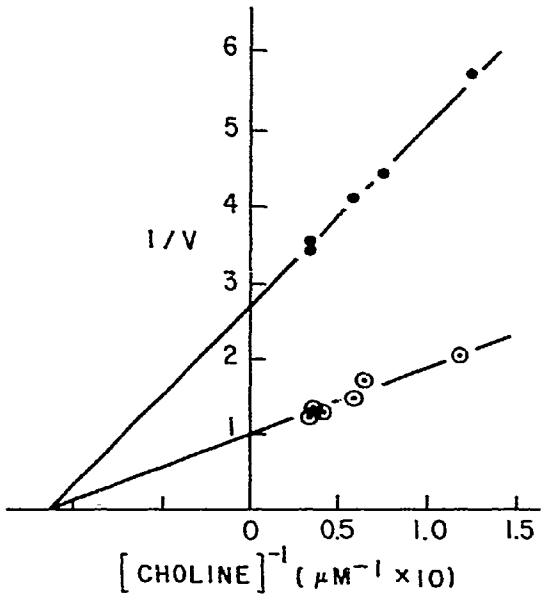

6
4
2
1/V
1
2
3
4
[CHOLINE]$^{-1}$ ($\mu M^{-1} \times 10^2$)

Figure 9.2 Inhibition of ^{14}C-choline entry by di-n-butylaminoethanol, 2.4 mM. Upper line, rates with inhibitor; lower line, control. Inhibition constants: from the slopes, 1.29 ± 0.36 mM; from the intercepts, 1.40 ± 0.27 mM. As these two constants are approximately equal inhibition is of the purely non-competitive type.


6
5
4
1/V
3
2
1
0
0.5
1.0
1.5
[CHOLINE]$^{-1}$ (μM^{-1} x10)

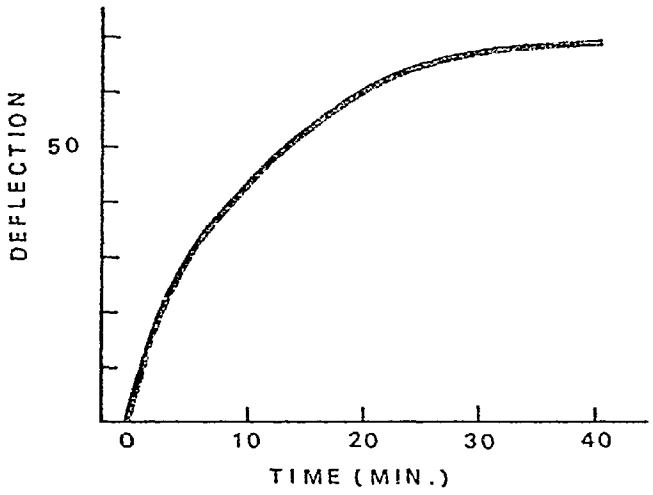
Furthermore, an analog bound on only one side of the membrane is unlikely to be a substrate, since transport must be reversible.

Hence, in the presence of dibutylaminoethanol the carrier should be trapped in the form of an internal complex, and the inactivation rate should be equal to the rate at which this complex reacts with N-ethyl maleimide.

In the above experiments, the cells were preincubated with dibutylaminoethanol for periods of approximately 1 hr. before choline transport rates were determined. The inhibition patterns show, as seen above, that the inhibitor is present inside the cells after the preincubation period. This conclusion was confirmed by observing entry and exit rates of 100 mM dibutylaminoethanol, using a light scattering technique which responds to osmotic shrinking and swelling of the cells (65). Entry of the amine was found to be complete within 30 min., as shown in the tracing in Fig. 9.3.

The inactivation rate was then examined in the presence of dibutylaminoethanol at near-saturating concentrations. As expected, the inactivation rate was greater than the control rate in the absence of substrates or inhibitors ($0.57\ min^{-1}$ compared to $0.19\ min^{-1}$). This behaviour is to be contrasted with that seen with non-penetrating inhibitors, such as dimethyl-n-butyl (2 hydroxyethyl)

Figure 9.3 Tracing from light-scattering assay showing the osmotic swelling of the cells which accompanies the entry of di-n-butylaminoethanol present in the external solution at a concentration of 100 mM. Equilibrium across the membrane is reached after approximately 30 min. Temp. 37°C.


DEFLECTION
50
0
10
20
30
40
TIME (MIN.)

ammonium ion, which trap the carrier on the external surface of the membrane and therefore protect the system against inactivation (Chapter 8).

Two other tertiary amines, dimethyl- and diethyl-aminoethanol, were tested for their effects on the rate of inactivation, and the results are summarized in Table 9.1. That these amines also equilibrate across the cell membrane during the preincubation period was demonstrated by means of the light scattering technique, as described above for the case of dibutylaminoethanol. Diethylaminoethanol had much the same effect as dibutyl-aminoethanol, indicating that it too adds preferentially on the inner side of the membrane and what is more important for the present study, that the reactivity of CS_i is the same for both. If diethylaminoethanol is bound on only the inner surface of the membrane competitive inhibition of choline exit is expected, and was found (Fig. 9.4).

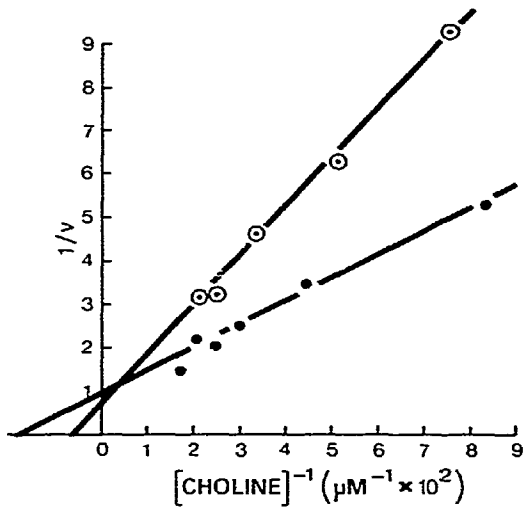
It is an important part of the argument, also, that increased inactivation rates are not produced by all tertiary amines. Entirely in line with reasonable expectation, dimethylaminoethanol, which is no bulkier than choline, has little effect on inactivation, indicating that it adds to the carrier on both sides of the membrane and therefore does not particularly disturb the carrier distribution.

Table 9.1

Inactivation rates with 1 mM N-ethyl maleimide in the presence of tertiary amines. Determinations are shown for 6 different samples of cells. Cells were preincubated with the amines for approximately 1 hr. before treatment with N-ethyl maleimide at 37°, pH 6.8. $K_{(app)}$ is the half-saturation constant for inhibition of choline transport.

			Cell Sample					
Analog	K(app) μM	Concentration μM	1	2	3	4	5	6
Dimethylamino-ethanol	10 (approx.)	32		0.20 ± 0.009				
		91		0.19 ± 0.002				
		100			0.23 ± 0.010			
		250	0.25 ± 0.010					
		500	0.24 ± 0.008					
Diethylamino-ethanol	200	1000	0.57 ± 0.027					
		2000			0.48 ± 0.030		0.53 ± 0.015	
Dibutylamino-ethanol	1100	5000				0.54 ± 0.008		
		10000				0.66 ± 0.006	0.54 ± 0.010	0.55 ± 0.018
Control			0.16 ± 0.014	0.19 ± 0.020		0.22 ± 0.008		0.17 ± 0.06

Figure 9.4 Inhibition of ^{14}C-choline exit by diethylaminoethanol, 0.2 mM. Upper line, rates with inhibitor; lower line, control. Inhibition constant 0.18 ± 0.027 mM.


9
8
7
6
5
4
3
2
1
1/v
0 1 2 3 4 5 6 7 8 9
[CHOLINE]$^{-1}$ ($\mu M^{-1} \times 10^2$)

The specific nature of these effects is demonstrated by the agreement between the half saturation constant for dibutylaminoethanol as determined from its effect on N-ethyl maleimide inactivation rates (1.15 ± 0.03 mM) and on the inhibition of choline transport (1.13 ± 0.18 mM). The experiment with N-ethyl maleimide was analyzed by a method described previously (66).

II. Reactivity of the internal free carrier

A. The effect of saturating external choline

The simplest solution to the problem of measuring the reactivity of C_i would be to throw the carrier into this form by means of a very good substrate outside the cell at a saturating concentration, as suggested by Edwards (27) and Martin (62). Such a substrate would have to meet the following conditions: (i) the carrier-substrate complex must move inward across the membrane more rapidly than the free carrier returns, and (ii) the substrate complex must dissociate very rapidly on the inner membrane surface. These requirements may be demonstrated mathematically by writing down an expression for the steady-state concentration of the free carrier on the inner surface, as a fraction of the total carrier, when the external substrate concentration is saturating:

$$\frac{[C_i]}{C_t} = \frac{1}{1 + \frac{f_{-1}}{f_3}\left(1 + \frac{f_3 + f_{-3}}{k_{-4}}\right)} \qquad (9.1)$$

Equation (9.1) was derived from a general kinetic analysis of the two complex model in Fig. 8.1 (Chapter 8), following the King-Altman schematic method. If dissociation is very rapid compared to carrier reorientation ($k_{-4} >> f_3, f_{-3}$), and if in addition reorientation of the complex is much more rapid than that of free carrier ($f_3 >> f_{-1}$) this ratio approaches unity, meaning that all the carrier is in the form of C_i.

A number of observations support the idea that choline is indeed such a substrate. First, the following evidence shows that break-down of the substrate complex is very rapid. (i) Neither non-competitive inhibition by a trans inhibitor (Fig. 9.2) nor trans-acceleration (Chapter 7) occurs if this process is rate-limiting (Chapter 3). (ii) The experimental relationship between transport rates and N-ethyl maleimide inactivation rates was shown to be inconsistent with the carrier model unless the assumption was made that dissociation rates are far more rapid than the reorientation process (Chapter 8). Second, the following observations have shown that the substrate complex moves far more rapidly than the free carrier. (i) The occurrence of trans-acceleration requires this assumption (Chapter 6). (ii) When choline is placed inside the cells at a saturating concentration, the system is given almost complete protection against N-ethyl maleimide (27), indicating that the carrier exists almost

entirely in the external form.

Experiments were therefore carried out with the use of choline. Observed rates of inactivation of cells placed in a solution containing N-ethyl maleimide and choline at a saturating concentration are recorded in Table 9.2. The mean value of Q_{To} is found to be 0.33 ± 0.019.

B. The effect of varying internal choline concentrations

The second approach to the problem of measuring the rate at which N-ethyl maleimide reacts with C_i is less obvious than that above, and involves an extrapolation from rates measured at varying internal choline concentrations. If choline has the properties which we have argued it may have, then if it is present within the cells the carrier should be almost entirely in the form of C_o and C_i; and the greater the concentration of choline. the greater the proportion of C_o and the smaller the proportion of C_i. As N-ethyl maleimide, according to previous evidence, does not react with C_o, the rate of reaction will be proportional to the concentration of C_i. On the other hand, if radioactive choline is placed outside the cells at a very low concentration, which does not significantly affect the carrier distribution, it may be shown that the rate of its entry is proportional to the concentration of C_o. We therefore expect a definite relationship to exist between the influx rate (v) under

Table 9.2

Inactivation rates with 1 mM N-ethyl maleimide in the presence of choline in the external solution. The reaction was started by the addition of a solution of choline and N-ethyl maleimide to a cell suspension. Reactin conditions: 37^{o}, pH6.8. Each determination was carried out with a different sample of cells. The mean value of Q_{To} is 0.333 ± 0.019.

Choline Concentration*	Q_{To}
25μM	0.348 ± 0.012
25μM	0.300 ± 0.003
25μM	0.341 ± 0.240
25μM	0.332 ± 0.035
50μM	0.357 ± 0.026
50μM	0.348 ± 0.063
50μM	0.316 ± 0.018
50μM	0.322 ± 0.015

* The binding constant for choline influx, $\overline{K}_{To}$, is 6.3 ± 0.6μM.

conditions of varying internal choline concentration (unlabelled) and the rate of carrier inactivation by N-ethyl maleimide (Q). From a kinetic analysis of the classical carrier scheme (Fig. 8.1) the following general relationship is actually found:

$$\frac{1}{Q} = I + M\frac{v}{Q} \qquad (9.2)$$

I and M are constants whose values are as follows:

$$I = (f_{-1} - f_{-3}) / (f_{-1}\, q_3 - f_{-3}\, q_1) \qquad (9.3)$$

$$M = \frac{k_{-3}}{k_3 f_3 C_t} \quad \frac{(f_1 + f_{-3})\, q_1 - (f_1 + f_{-1})\, q_3}{f_{-3} q_1 + f_{-1}\, \bar{q}_3} \qquad (9.4)$$

Equation (9.2) has been derived on the assumption that breakdown of the carrier-substrate complex is far more rapid than other steps in transport. We now make the further assumption, as above, that transfer of the carrier-substrate complex in the membrane is much more rapid than that of the free carrier ($f_{-3} \gg f_1, f_{-1}$). The constants now reduce to the following expressions:

$$I = 1/q_1 \qquad (9.5)$$

$$M = \frac{k_{-3}}{k_3 f_3 C_t} = \frac{K_{So}}{f_3 C_t} \qquad (9.6)$$

We therefore predict that if the reciprocal of the inactivation rate, 1/Q, is plotted against the ratio, v/Q, of the unidirectional rate of entry of labelled choline

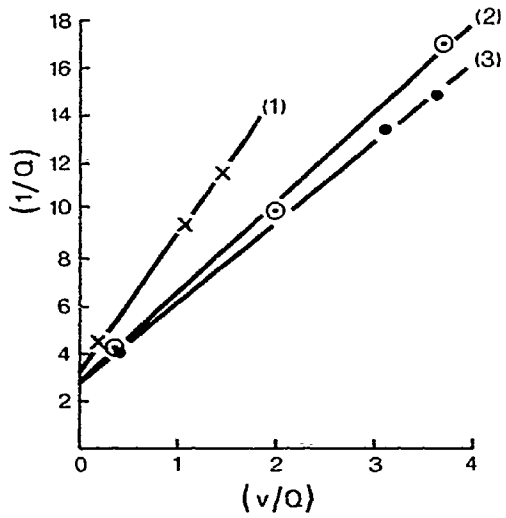
and the inactivation rate, a straight line should result whose intercept is equal to $1/q_1$. It is evident from the full expressions for M and I above, that though a straight line should be found regardless of the relative values of carrier reorientation rates, the intercept may equal some combination of $1/q_1$ and $1/q_3$. If $q_1 = q_3$ there is no problem whatever and the same result is found with every substrate. As the results above indicate that q_1 and q_3 differ, agreement between the values of q_1 obtained by the two methods depends on the correctness of the assumptions about the transport of choline and about the lack of reactivity of the external carrier forms.

The experiments were carried out as suggested by the above analysis. Cells containing various concentrations of unlabelled choline were treated with N-ethyl maleimide and rates of transport in the treated cells were then determined. The rates of transport in cells loaded with unlabelled choline were also estimated. The procedures used in these experiments are described under Methods. The results of three experiments are plotted according to eqn.(9.2) in Fig. (9.5). The mean value for q_1 was found to be 0.342 ± 0.032.

C . Constancy of the effect of external choline on different cell samples

A third and independent estimate of q_1 may be obtained by another route. In the course of many experiments to

Figure 9.5 Plot of inactivation rates (Q) determined in the presence of varying concentrations of choline inside the cells, and of rates of ^{14}C-choline entry (v) under the same conditions. According to theory (eqn. 9.2 and 9.5) the intercept on the ordinate is equal tc $1/q_1$. The results of three different experiments are shown in which the choline concentrations with which the cells were incubated were as follows (1) 0, 20.4, 40.8 μM; (2) 0, 20.4, 61.1 μM (3) 0, 40.8, 70.2 μM. The values of q_1 in the three experiments, obtained by a least squares treatment of the data are (1) 0.306 ± 0.002, (2) 0.369 ± 0.048 and (3) 0.352 ± 0.033.


18
16
14
12
10
8
6
4
2
(1/Q)
0
1
2
3
4
(v/Q)
(1)
(2)
(3)

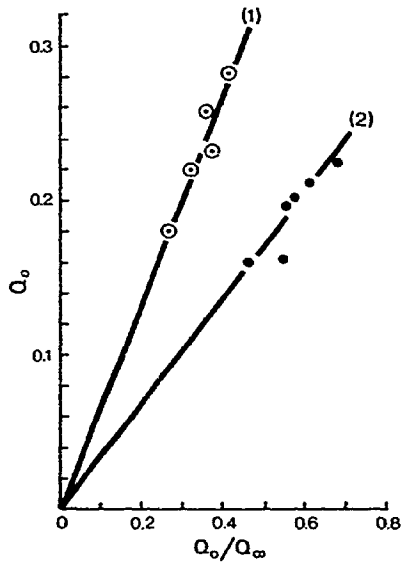
determine q_1 or q_3 by the methods described above, the values were found to be rather constant, even though the cells had a different origin in each experiment. However, the value of Q_o, the inactivation rate in the absence of inhibitors and substrates was more variable. Similar findings were previously reported by Edwards (27). Since Q_o depends on the constants q_1 and q_2, where $q_2 \approx 0$ and where q_1 does not vary, variations in Q_o are likely to reflect an altered partition of the carrier in the membrane with different cell samples. In terms of the constants in Fig. 8.1 this partition is given by the f_1/f_{-1} ratio. From an inspection of the mathematical expressions for Q_o and Q_{To} it is seen that if the assumption is made that $f_3 \gg f_1$ or f_{-1}, which is justified in the case of choline transport, then variations in the f_1/f_{-1} ratio should cause Q_o to vary while Q_{To} should remain constant:

$$Q_o = \frac{f_1 q_1}{f_1 + f_{-1}} \tag{9.7}$$

$$\frac{Q_o}{Q_{To}} = \frac{(f_{-1} + f_3)\, f_1}{(f_1 + f_{-1})\, f_3} \tag{9.8}$$

If $f_3 \gg f_{-1}$, the second expression reduces to $f_1/(f_1 + f_{-1})$, the same factor that multiplies q_1 in the first expression. It follows that Q_o/Q_{To} should be related to Q_o by the constant q_1. A plot is shown in Fig. (9.6) of the values

Figure 9.6 Inactivation rates with choline at a saturating concentration (>25μM) in the external solution (Q_{To}), and in the absence of substrates or inhibitors (Q_o) as measured with different samples of cells. The variation in Q_o is greater than the probable error of individual measurements, whereas Q_{To} is relatively constant. Upper line, data of Edwards (27); lower line, data from the present experiments. Points are plotted in accordance with the theoretical relationship $Q_o = q_1 Q_o/Q_{To}$ (eqn 9.2). The lines drawn in the figures are calculated from the estimates of q_1, which was 0.7 in the experiments of Edwards (27) and 0.34 in the present experiment


(1)
(2)
0.3
0.2
0.1
Q_o
0
0.2
0.4
0.6
0.8
Q_o/Q_∞

obtained in different experiments according to the relationship.

$$\frac{Q_o}{Q_{To}} = q_1 Q_o \qquad (9.9)$$

If the hypothesis is correct the experimental points should lie on a straight line passing through the origin and having a slope equal to q_1. The data are seen to be in agreement with the value of q_1 obtained in our other experiments. These observations therefore add to the confidence which we may place in the experiments and in the analysis, and, in addition, indicates that the distribution of the free carrier actually does vary in different cell samples.

DISCUSSION

The main conclusion of the present work is that the internal carrier-substrate complex, C_{Si}, reacts more rapidly with N-ethyl maleimide than does the free carrier on the inner surface, Ci. The trust to be placed in this conclusion must now be examined more closely.

It was shown above that if $q_1 = q_3$, the estimate of q_1, obtained by the second method is the same for all substrates, and is equal to q_3 (eqn. 9.2). The fact that the estimate differs significantly from q_3, therefore, can only mean that the constants are actually different, and that q_1 must be smaller than q_3. The precise value of q_1 may still be questioned however. If choline were

not translocated much more rapidly than the free carrier ($f_3 \approx f_1$) the estimate of q_1 by the first method above would be too low, because some of the carrier would remain in the external form, which does not react. The value 0.33 must therefore be a lower limit for q_1.

It may be concluded, for the following reasons, that the present estimate of q_1 is likely to be in close agreement with the true value. First, as we have seen, it cannot be smaller than 0.33, but must be smaller than 0.6. Second, internal choline almost completely protects the carrier against N-ethyl maleimide, showing that because of its rapid translocation, the carrier is present almost entirely on the trans side of the membrane. Third, the constancy of the inactivation rate in the presence of external choline, when the control rate varies, is expected only if $f_3 \gg f_{-1}$. Since the carrier-substrate complex reacts more rapidly than the free carrier, it is probable that the carrier changes its conformation on forming a complex. This alteration in form may be an initial step in the reorientation process in which the substrate site, initially facing inwards, turns and faces outwards (though it should be added that the use of the word "turn" does not imply any particular mechanism).

A question which unfortunately has not been definitly answered is whether the carrier complex reacts at the same

rate with N-ethyl maleimide whether an inhibitor or a good substrate is bound. The fact that the inactivation rate for the external complex is the same (that is, very low) for both substrates and inhibitors, suggests however that the inactivation rate, governed by q_3, may be independent of the carrier reorientation rate.

SUMMARY

Previous studies showed that the choline carrier assumes different conformations on the internal and external surfaces of the cell membrane, which are clearly distinguished by their rates of reaction with N-ethyl maleimide: only the inner form reacts, producing inactivation of transport. We now report experiments to determine the reaction rates individually for the two inner forms, the free carrier and the substrate complex. The pseudo first order rate constants for these species at pH 6.8 and 37°C, with 1mM N-ethyl maleimide, are 0.33 ± 0.02 and 0.57 ± 0.06 min^{-1}, respectively. Hence three conformational forms of the carrier are distinguished: (1) the inwardly facing free carrier; (2) the inwardly facing carrier-substrate complex; and (3) the outwardly facing carrier.

CHAPTER 10

SUBSTRATE SPECIFICITIES OF THE INNER AND OUTER CARRIER SITES

INTRODUCTION

In every carrier system, regardless of its detailed mechanism, the substrate binding site or sites must become exposed on the inner and outer surfaces of the cell membrane; for if transport is to occur, the substrate molecule must be picked up on one side of the membrane and later released on the other side. An important question to be asked concerns the relationship between the inner and outer sites: is a single physical site alternately made accesible to substrate molecules in the internal and external solutions, or are two separate sites actually involved? From this point of view, it would be of great interest to establish the substrate specificities of the inner and outer sites separately. Finding the true substrate affinities on either side of the membrane, however, is not an easy matter, because experimental half-saturation constants depend on both the affinity of a substrate and its rate of transport. The more rapid the transport, the higher the apparent affinity relative to the true value; and it is possible therefore for differences in measured binding strength on the two sides of the membrane to be a consequence of differences in the rates of inward and outward carrier movement.

Thus, in the transport scheme in Fig. 8.1 the substrate dissociation constants on the outer and inner surfaces are equal to k_{-3}/k_3 and k_{-4}/k_4 respectively, but the apparent values are:

$$\bar{K}_{To} = \frac{(f_1 + f_{-1})(k_{-3}f_{-3}+k_{-3}k_{-4}+k_{-4}f_3)}{k_3\{k_{-4}(f_{-1} + f_3) + f_{-1}(f_3 + f_{-3})\}} \qquad (10.1)$$

$$\bar{K}_{Ti} = \frac{(f_1 + f_{-1})(k_{-3}f_{-3} + k_{-3}k_{-4} + k_{-4}f_3)}{k_4\{k_{-3}(f_1 + f_{-3}) + f_1(f_3 + f_{-3})\}} \qquad (10.2)$$

The ratio of these apparent constants is:

$$\frac{\bar{K}_{To}}{\bar{K}_{Ti}} = \frac{k_4\{k_{-3}(f_1 + f_{-3}) + f_1(f_3 + f_{-3})\}}{k_3\{k_{-4}(f_{-1} + f_3) + f_1(f_3 + f_{-3})\}} \qquad (10.3)$$

If carrier reorientation is the rate-limiting step in transport, as indicated by the evidence given above, then this expression reduces to

$$\frac{\bar{K}_{To}}{\bar{K}_{Ti}} = \frac{k_3k_4}{k_3k_4}\frac{(f_1 + f_3)}{(f_1 + f_3)} \qquad (10.4)$$

The individual rate constants in an equibrating system have the following relationship with one another: $f_1f_{-3}k_{-3}k_4 = f_{-1}f_3k_3k_{-4}$ (Eqn. A.3); and when this equality is introduced into eqn. 10.4 the ratio of affinities is found to be

$$\frac{\bar{K}_{To}}{\bar{K}_{Ti}} = \frac{1/f_1 + 1/f_3}{1/f_1 + 1/f_3} \qquad (10.5)$$

Hence even if the substrate dissociation constants are equal ($K_{To} = K_{Ti}$, where $K_{To} = k_{-3}/k_3$ and $K_{Ti} = k_{-4}/k_4$), the ratio of apparent affinities will be unequal if f_3/f_{-3} is different from f_1/f_{-1}.

If, on the other hand, substrate dissociation were rate-limiting, which is contrary to our findings, the ratio of experimental affinity constants would still not be equivalent to the ratio of true affinities, as may be seen by inspection of eqn. 10.3, with $k_{-3} \leq f_1$ and $k_{-4} \leq f_{-1}$

Studies on the choline transport system of human erythrocytes are now described, in which the ratio of the true affinities is measured for a number of choline analogs. N-ethyl maleimide was shown earlier to act as a probe for the distribution of the carrier across the membrane, by reason of the fact that it reacts only with the inner carrier forms. In consequence, the rate of inactivation of the system in the presence or absence of substrate analogs at equilibrium across the membrane provides a simple measure of the carrier distribution under these circumstances. The relationship between experimental inactivation rates and the partition of the carrier in the membrane at equilibrium will now be considered.

In the absence of substrates or inhibitors, the measured inactivation rate is governed by the expression for Q_o (Table A.4):

$$Q_o = \frac{f_2 q_1 + f_{-1} q_2}{f_1 + f_{-1}} \qquad (10.6)$$

It has been shown experimentally that $q_2 = 0$ (Chapters 8 and 9), and with substitution of this value into eqn. 10.6, it is found that

$$\frac{f_{-1}}{f_1} = \frac{q_1}{Q_o} - 1 \qquad (10.7)$$

From the schemes in Fig. 8.1 it is also seen that in the absence of substrates or inhibitors,

$$\frac{f_{-1}}{f_1} = \frac{[C_o]}{[C_i]} \qquad (10.8)$$

In the presence of a substrate analog at equilibrium inside and outside the cell and at a saturating concentration, the inactivation rate, Q_{ToTi}, is given by (Table A.4):

$$Q_{ToTi} = \frac{f_3 q_3 + f_{-3} q_4}{f_3 + f_{-3}} \qquad (10.9)$$

Since experimentally $q_4 = 0$, it is found from eqn. 10.9 and the transport scheme in Fig. 8.1 that

$$\frac{f_{-3}}{f_3} = \frac{f_{-1} K_{Ti}}{f_1 K_{To}} = \frac{q_3}{Q_{ToTi}} - 1 \qquad (10.10)$$

It may also be seen from this transport scheme that

$$\frac{f_{-3}}{f_3} = \frac{f_{-1} K_{Ti}}{f_1 K_{To}} = \frac{[C_o S]}{[C_i S]} \qquad (10.11)$$

When the substrate analog is a competitive inhibitor that does not undergo transport ($f_3 = f_{-3} = 0$), it may be shown that the second equalities in eqn. (10.10) and (10.11) are still valid.

In the previous chapter, the values of the inactivation constants, q_1 and q_3, were determined. With these values, therefore, the partition ratios for the free carrier and the complex may be determined, and from these the relative values of K_{Ti} and K_{To} for various choline analogs may be calculated (eqn. 10.7 and 10.10).

RESULTS

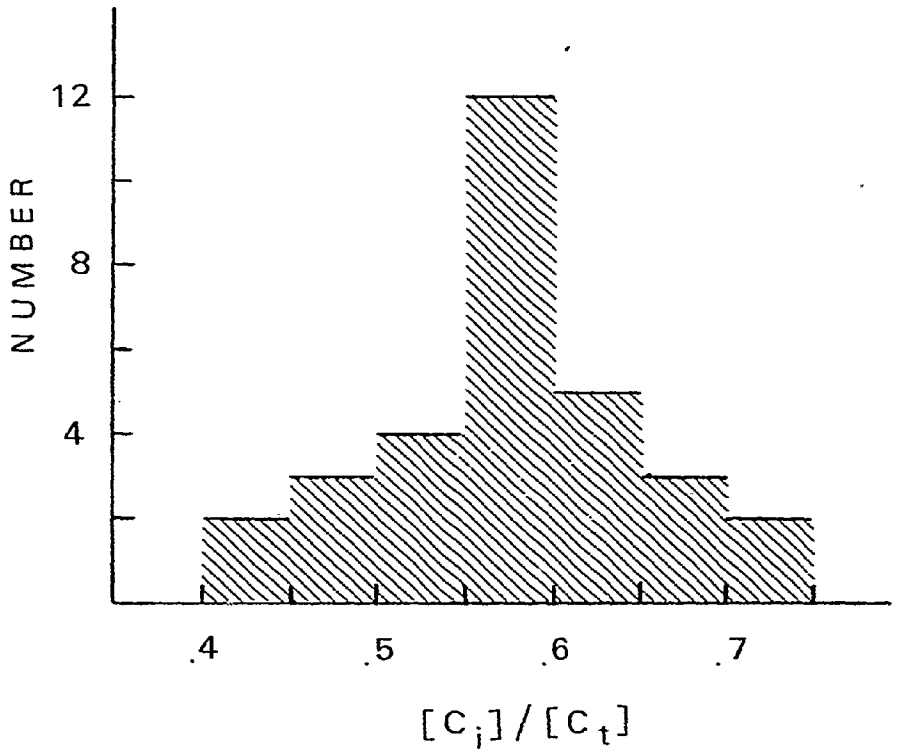
I. Distribution of the free carrier

Evidence was presented in the last chapter to show that the distribution of the free carrier between the internal and external forms varies in different samples of cells, and this is manifested in variations in the values of Q_o. It should be stressed that the differences in Q_o values for different samples are far larger than the probable errors of individual determinations. This error is usually 5-10%, while Q_o has been found to vary between 0.15 min^{-1} and 0.24 min^{-1}, which represents a deviation from the mean of 23%. It was also demonstrated that Q_{ToTi} does not vary in this way, which is in accord with theoretical expectations. Hence the variation in Q_o cannot be ascribed to experimental error.

Observations on many samples of cells have been gathered in the histogram in Fig. 10.1, which shows that

FIGURE 10.1

The distribution of the free carrier found in different samples of cells.The number of cases with a given distribution is recorded in the ordinate and the proportion of carrier in the internal form in the abscissa.


12
8
4
NUMBER
.4
.5
.6
.7
[C_i] / [C_t]

in most cases (70%) the carrier has a slight preference for the internal surface. ($[C_i]/C_t$ = .50-.65), while in a few cases this fraction is either below 0.45 or above 0.70 (13% of the cases).

Edwards (27) has suggested that this variation in carrier distribution may depend on the internal potassium concentration, which falls as the cells age. Potassium, though weakly bound to the carrier, is believed to act as a good substrate, facilitating the relatively rapid reorientation of the carrier in the membrane, and therefore increasing the abundance of the external carrier form. In proportion to potassium concentration, therefore, the carrier concentration on the external surface of the membrane rises. This hypothesis requires that in the absence of potassium, the natural equilibrium would greatly favor the inner carrier form, implying that a strong asymmetry is built into the carrier on the membrane.

II. Inactivation rates in the presence of equilibrated substrates and inhibitors

With the use of the procedures described in the previous Chapter, values of Q_{ToTi} were determined in the presence of choline and a number of analogs, all of which are tertiary amines. Every analog was shown,

by means of the light scattering technique already described, to come to equilibrium inside the cells during the pre-incubation period. In each experiment the substrate or inhibitor concentration was sufficient to give nearly complete inhibition of ^{14}C-choline flux. The analogs were distilled at reduced pressure before use. The results are given in Table 10.1.

DISCUSSION

The trends in the ratios of affinities on the two membrane surfaces for this series of choline analogs are unmistakable. Dibutylaminoethanol acts as a point of reference, since its binding was demonstrated to be predominantly on the inner membrane surface; the evidence for this was the observation of competitive inhibition of entry. It is therefore clear that di-n-propylaminoethanol, di-isopropylaminoethanol and diethylaminoethanol are also preferentially bound on the inner surface. By contrast, dimethylaminoethanol and choline are bound on both surfaces, though with a slight preference for the outer surface. Bis-dimethylamino-2-propanol, dimethylcyclohexylamine and dimethylbenzylamine have a decided preference for the outer surface.

Table 10.1.

Inactivation rates, Q_{ToTi}, in the presence of equilibrated substrate analogs at saturating concentrations. N-ethyl maleimide concentration, 1 mM, pH 6.8, 37°C. The carrier parameters f_{-3}/f_3 and K_{Ti}/K_{To} were estimated on the basis of a value of 0.57 min^{-1} for q_3, 0.33 min^{-1} for q_1 and 0.19 min^{-1} for Q_o.

Substrate Analog	Concentration (mM)	K(app) (mM)	Q_{ToTi}	Estimated Parameters $\frac{f_{-3}}{f_3}$	Estimated Parameters $\frac{K_{Ti}}{K_{To}}$
Di-n-butyl-aminoethanol	5 10	1.13	0.54 ± 0.008 0.66 ± 0.006, 0.54 ± 0.010, 0.54 ± 0.018	-	~ 0
Di-n-propyl-aminoethanol	5 10	~ 1	0.63 ± 0.023 0.63 ± 0.049	-	~ 0
Di-isopropyl-aminoethanol	10	~ 0.2	0.48 ± 0.017	-	0.2
Diethyl-aminoethanol	1 2	0.18	0.57 ± 0.027 0.48 ± 0.03, 0.53 ± 0.015	-	0.1
Dimethyl-aminoethanol	0.1 0.25 0.5	0.010	0.23 ± 0.01 0.25 ± 0.01 0.24 ± 0.008	1.4	1.9
Choline	0.05	0.006	0.25 ± 0.02, 0.21 ± 0.003, 0.21 ± 0.007 0.25 ± 0.007	1.6	2.1
Bis-1,3 dimethyl-amino-2-propanol	100*	-	0.15 ± 0.004	2.8	3.8
Dimethyl-cyclohexylamine	10*	-	0.12 ± 0.008	3.8	5.1
Dimethyl-benzylamine	10*	-	0.11 ± 0.005	4.2	5.7

* These concentrations produce at least 80% inhibition of choline influx.

It is apparent from these results that the specificities of the internal and external binding sites are different. The earlier study demonstrated that the external site severely limits the size of the quaternary ammonium group that may be adsorbed (Chapter 7); for example, even the triethyl analog of choline has difficulty squeezing into the site. All the amines in the present series with substituents larger than methyl on the nitrogen atom are therefore weakly bound at the outer site. If the inner cation binding site should be more spacious than the outer one, then the amines would be expected to bind preferentially there. In the series dimethyl- diethyl-, and dibutyl-aminoethanol, progressively weaker binding is seen; and it therefore follows that the inner site as well as the outer site tends to exclude the bulkier amines. The severity of the exclusion, however, is mitigated at the internal site, and this, rather than preferential binding of bulkier analogs, accounts for the stronger binding of diethyl, di-n-propyl, di-isopropyl, and dibutylaminoethanol at the substrate site in the inward-facing form of the carrier. The different specificities of the cation binding loci in the inner and outer sites cannot be attributed to auxiliary structures, such as the non-polar region adjacent to the external site. Instead, regions of the site appear to be involved that directly contact the quaternary group of choline, for any enlargement of

this group leads to an immediate reduction in binding strength. The difference in specificity must therefore result from a difference in the essential structures of the external and internal sites, a conclusion that rather strongly suggests the involvement of two physical entities. If this is true, the evidence presented earlier (Chapter 5) requires that only one of these sites be exposed at the membrane surface at any given time.

At certain other points in the binding sites, it appears that the tendency of the inner site to exclude bulky structures is greater than that of the outer. Thus the second ammonium group of bis-dimethylamino-2-propanol, and the benzyl and cyclohexyl substituents in dimethylamine derivatives, are more excluded from the inner than from the outer site. It is possible that these structures occupy the region where the hydroxyethyl chain of choline is normally adsorbed.

Finally, the reasons may be noted for believing that the effects of the tertiary amines, like those of the quaternary analogs, are specific.

First, a normal saturation curve is seen in the effect of dibutylaminoethanol on the inactivation rate, and the half-saturation constant calculated from this curve is in close agreement with the value obtained from the inhibition of transport (Chapter 9). In addition, the

mechanism of dibutylaminoethanol inhibition was shown to be purely competitive, indicating that it becomes bound at the substrate site (Chapter 9). Second, the full range of effects is seen: some tertiary amines accelerate the inactivation rate; one, dimethylaminoethanol, has almost no effect; while others reduce the rate inactivation. The different effects do not depend on the hydrophobic binding capacity of the substituents on the nitrogen atom, but are understandable if they depend upon addition to a highly specific binding site in the carrier. The precipitous fall in affinity that accompanies even a slight enlargement of the ammonium group can only be explained in this way.

SUMMARY

The substrate specificity of the carrier site exposed on the outer surface of the cell membrane is shown to be significantly different from that of the site on the inner surface. This is demonstrated from the effects, on N-ethyl maleimide inactivation rates, of choline analogs that readily penetrate the cell by a route other than the choline carrier. The difference in specificity appears to involve parts of the site that directly interact with choline, rather than auxiliary sites, and it may therefore be suggested that physically distinct sites on the two membrane surfaces are involved.

CHAPTER 11

GENERAL CONCLUSIONS

It is a satisfying task in this last part of the thesis to review the evidence obtained from the various projects and to consider the work as a whole. The original aim was to explore the use of the kinetic approach in transport studies and then to apply this approach in the study of a particular membrane transport system. We shall begin by considering the progress that has been made in the first of these areas.

1) It had become apparent to us during the course of our studies that a lack of general understanding existed with respect to the kinetics of reversible inhibition of transport. This being such an elementary subject, it seemed imperative to correct the deficiency. The main contributions of the new analysis are as follows. First, it allows us to correctly interpret experiments in which either competitive or non-competitive inhibition is found. In the absence of such a treatment, inhibition patterns may be misleading; for depending on the relative distributions of substrate and inhibitor, inhibition by a substrate analog which is bound at the carrier site may appear to be either competitive or non-competitive. Second, the treatment provides a simple means of detecting asymmetric inhibition. The latter could play a physiological role in the regulation of solute movement. In a study to be

presented elsewhere, we have demonstrated that where an inhibitor acts on only one side of the membrane, the transport system is transformed into a valve; since under non-equilibrium conditions, the inward and outward fluxes with a given substrate concentration on either one side of the membrane or the other can be very different. In an active transport system this arrangement could prevent the loss of accumulated solute in the absence of an energy supply, without interfering with normal solute uptake. Third, reversible competitive inhibitors provide a new test for rate-limiting steps in transport.

2) The formulation of a variety of kinetically simple and distinguishable models that could account for the essential features of mediated transport also seemed to call for attention. This has led to the formulation of 5 different models and of several simple but incisive tests that distinguish among them. One such test, which may be very valuable, involves the use of reversible competitive inhibitors to determine whether one carrier site is exposed at any instant, or whether 2 sites, one on each membrane surface, are exposed at the same time.

3) In another part of the work, the potential use of irreversible inhibitors in the study of transport systems is explored. The situation here was different from that with reversible inhibitors, since a recent paper had

strongly argued that little could be learned about the details of a mechanism in this way. It is now shown that irreversible inhibitors actually constitute a powerful tool in transport studies.

4) An easily understood system of notation has been proposed in the present work. In this system the many possible transport parameters derived from different kinds of experiments are designated in a clear and simple manner. In addition, general rate equations are derived, which are written entirely in terms of experimental parameters. This simplifies considerably the appearance of the equations, making them more accesible and easier to use. These equations are presented in the Appendices.

5) An accomplishment of immediate practical consequence is the development of an experimental method for determining the transport parameters of unlabelled substrates. The method involves monitoring the effects of the latter on the transport of a single labelled substrate.

We shall now move on to consider the second general aim of the study. This was to subject a particular transport system to experimental tests, in order to discover its underlying mechanism. A facilitated system was chosen for study, for such a mechanism exhibits the essential features of all mediated transport systems, without the added complication of energy-coupling devices.

The most general statement that can be made regarding the choline transport system, after it has been subjected to a great many experimental tests, is that in every respect it conforms to the predictions of a model of the classical carrier type. Let us consider the evidence for this conclusion.

1) Inward-facing and outward-facing carrier forms for both the free and complexed carrier are clearly recognized. This finding excludes a number of other possible mechanisms. It rules out, for example, a variant of the carrier model strongly recommended by Lieb and Stein (18) in which, for purposes of analysis, the internal and external carrier complexes are merged into a single species. The observations also rule out models in which there is only one form of the free carrier, as in Models 1, 2, 4 and 5 in Chapter 4.

2) Independent evidence, from studies with reversible inhibitors, shows that the carrier site is alternately exposed on either side of the membrane, and that substrate sites are not simultaneously accesible on both sides of the membrane. This rules out the "oligomeric internal transfer model" of Lieb and Stein (8), the introverting hemipore model of LeFevre (9), and the membrane lattice model of Naftalin (47).

It is seen, therefore, that these two independent pieces of evidence combine to rule out all models in

which there is a single form of the free carrier, or in which two sites are simultaneously accessible to substrate on the inner and outer surfaces of the membrane.

3) The occurrence of non-competitive inhibition by a competitive inhibitor on the trans side of the membrane (with respect to the substrate) is additional evidence in support of a model of the classical carrier type, and is inconsistent with several other models in which a competitive inhibitor must produce competitive kinetics, as in the cases of Models 1 and 4 in Chapter 4.

4) The inner and outer carrier sites have different substrate specificities. The outer site is particularly sensitive to the size of the quaternary ammonium group in the substrate, while the inner site is less sensitive. Physically distinct sites therefore seem likely. A mechanism could operate having the kinetic characteristics of the simple carrier, but involving interacting subunits on the inner and outer surfaces of the membrane, each of which contains a substrate binding site.

5) Evidence has also been obtained for the involvement of conformational changes, both in the process of substrate translocation, and in the course of carrier-substrate complex formation. The binding of the hydroxymethylene group of the substrate does not facilitate the conformational change involved in translocation, whereas the binding of the quaternary ammonium group does.

6) The relative rates of the three main steps in the transport process have been determined. The most rapid is dissociation of the substrate from the carrier, which does not limit the rate of transport. The slowest of the three steps is reorientation of the free carrier. The reorientation of the carrier-choline complex is more rapid than reorientation of free carrier, and in zero trans flux does not limit the rate.

One last comment may be made about this system. It is a remarkable fact that though the original conception of the classical carrier may seem naive, considering what is now known about the structure of the membrane, its kinetic behavior is nevertheless consistent with all observations on the behavior of transport systems, while certain mechanisms that seem sophisticated in their conception, fail to account for the observations. The original idea involved a "ferryboat" migrating back and forth through the membrane, but it now seems most unlikely that any protein sufficiently complex to contain a highly specific binding site for a molecule like glucose or choline, could move in the membrane in this way. These facts may be reconciled if the actual mechanism depends on some combination of movable binding sites and movable barriers in a pore, such that relatively small displacements of these structures are required by transport.

APPENDIX 1

TRANSPORT AND INACTIVATION RATE EQUATIONS FOR THE CLASSICAL CARRIER MODEL

TRANSPORT RATE EQUATIONS

I. General rate equations for the transport of two substrates:

With the aid of the King Altman schematic method (67), a general equation for transport (eqn. A.1) was derived on the basis of the scheme in Fig. 6.1 (Chapter 6), without any assumptions as to the rate-limiting steps in the process. Eqn. (A.1) defines the rate of transport of substrate S.

$$\frac{-d[S_i]}{dt} = \frac{\dfrac{\bar{V}_{Si}}{\bar{K}_{Si}}\left([S_i] - \alpha\,[S_o]\right) + \dfrac{\tilde{V}^T_{Si}}{\tilde{K}^S_{To}\,\bar{K}_{Si}}\left([S_i][T_o] - \dfrac{\alpha}{\beta}[S_o][T_i]\right)}{1 + \dfrac{[S_o]}{\bar{K}_{So}} + \dfrac{[S_i]}{\bar{K}_{Si}} + \dfrac{[T_o]}{\bar{K}_{To}} + \dfrac{[T_i]}{\bar{K}_{Ti}} + \dfrac{[S_o][S_i]}{\tilde{K}^S_{So}\,\bar{K}_{Si}}} \qquad (A.1)$$

$$+ \frac{[T_o][T_i]}{\tilde{K}^T_{To}\,\bar{K}_{Ti}} + \frac{[S_o][T_i]}{\tilde{K}^T_{So}\,\bar{K}_{Ti}} + \frac{[T_o][S_i]}{\tilde{K}^S_{To}\,\bar{K}_{Si}}$$

The full expressions for these and other commonly encountered parameters in terms of microscopic rate constants are listed in Tables A.1 and A.2, and their definitions are given below. The constants α and β are the ratios of the final concentrations of substrate achieved inside and outside the cell:

$$\alpha = \left(\frac{[S_i]}{[S_o]}\right)_{final} = \frac{\bar{V}_{So}/\bar{K}_{So}}{\bar{V}_{Si}/\bar{K}_{Si}} = \frac{f_{-1}f_2k_1k_{-2}}{f_1f_{-2}k_{-1}k_2} \qquad (A.2)$$

$$\beta = \left(\frac{[T_i]}{[T_o]}\right)_{final} = \frac{\bar{V}_{To}/\bar{K}_{To}}{\bar{V}_{Ti}/\bar{K}_{Ti}} = \frac{f_{-1}f_3k_3k_{-4}}{f_1f_{-3}k_{-3}k_4} \qquad (A.3)$$

Table A.1 Experimental parameters for substrate affinity, expressed in terms of individual rate constants for the transport scheme in Fig. 6.1. Equilibrium constants for carrier-substrate complex formation are defined as follows: $K_{So} = k_{-1}/k_1$; $K_{Si} = k_{-2}/k_2$; $K_{To} = k_{-3}/k_3$; $K_{Ti} = k_{-4}/k_4$. The symbols G and H stand for the following combinations of constants:

$G = k_{-3} f_{-3} + k_{-3} k_{-4} + k_{-4} f_3 \qquad H = k_{-1} f_{-2} + k_{-1} k_{-2} + k_{-2} f_2$

Experimental parameter	Experiment	Labeled substrate (cis)	Unlabeled analog (trans)	Unlabeled analog (cis)	General expression	Rapid dissociation
$\bar{K}_{So}$	Zero trans entry	S_o	-	-	$\frac{(f_1 + f_{-1})H}{k_1\{k_{-2}(f_{-1}+f_2) + f_{-1}(f_2+f_{-2})\}}$	$\frac{K_{So}(f_1 + f_{-1})}{(f_{-1} + f_2)}$
$\bar{K}_{To}$	"	T_o	-	-	$\frac{(f_1 + f_{-1})G}{k_3\{k_{-4}(f_{-1}+f_3) + f_{-1}(f_3+f_{-3})\}}$	$\frac{K_{To}(f_1 + f_{-1})}{(f_{-1} + f_3)}$
$\bar{K}_{Io}$	"	S_o	-	I_o	$K_{Io}(1 + f_1/f_{-1})$	$K_{Io}(1 + f_1/f_{-1})$
$\bar{K}_{Si}$	Zero trans exit	S_i	-	-	$\frac{(f_1 + f_{-1})H}{k_2\{k_{-1}(f_1+f_{-2}) + f_1(f_2+f_{-2})\}}$	$\frac{K_{Si}(f_1 + f_{-1})}{(f_1 + f_{-2})}$
$\bar{K}_{Ti}$	"	T_i	-	-	$\frac{(f_1 + f_{-1})G}{k_4\{k_{-3}(f_1+f_{-3}) + f_1(f_3+f_{-3})\}}$	$\frac{K_{Ti}(f_1 + f_{-1})}{(f_1 + f_{-3})}$
$\bar{K}_{Ii}$	"	S_i		I_i	$K_{Ii}(1 + f_1/f_{-1})$	$K_{Ii}(1 + f_1/f_{-1})$
$\tilde{K}^S_{So}$	Infinite trans entry	S_o	S_i	-	$\frac{k_{-1}(f_1+f_{-2}) + f_1(f_2+f_{-2})}{k_1(f_2 + f_{-2})}$	$\frac{K_{So}(f_1 + f_{-2})}{f_2 + f_{-2})}$
$\tilde{K}^T_{To}$	"	T_o	T_i	-	$\frac{k_{-3}(f_1+f_{-3}) + f_1(f_3+f_{-3})}{k_3(f_3 + f_{-3})}$	$\frac{K_{To}(f_1 + f_{-3})}{f_3 + f_{-3})}$
$\tilde{K}^T_{So}$	"	S_o	T_i	-	$\frac{k_{-3}(f_1+f_{-3}) + f_1(f_3+f_{-3})\ H}{k_1\{k_{-3}f_{-3}(k_{-2}+f_2+f_{-2}) + k_{-2}f_2(k_{-3}+f_3+f_{-3})\}}$	$\frac{K_{So}(f_1 + f_{-3})}{(f_2 + f_{-3})}$
$\tilde{K}^S_{To}$	Infinite trans entry	T_o	S_i	-	$\frac{k_{-1}(f_1+f_{-2}) + f_1(f_2+f_{-2})\ G}{k_3\{k_{-1}f_{-2}(k_{-4} + f_3 + f_{-3}) + k_{-4}f_3(k_{-1}+f_2+f_{-2})\}}$	$\frac{K_{To}(f_1 + f_{-2})}{(f_{-2} + f_3)}$
$\tilde{K}^S_{Io}$	exit	S_i	I_o	-	$K_{Io}\left\{\frac{k_{-1}(f_1+f_{-2}) + f_1(f_2+f_{-2})}{k_{-1}f_{-2}}\right\}$	$K_{Io}(1 + f_1/f_{-2})$
$\tilde{K}^S_{Ii}$	entry	S_o	I_i	-	$K_{Ii}\left\{\frac{k_{-2}(f_{-1}+f_2) + f_{-1}(f_2+f_{-2})}{k_{-2}f_2}\right\}$	$K_{Ii}(1 + f_{-1}/f_2)$

Table A.2 Experimental parameters for rates of transport in terms of rate constants in the scheme in Fig. 6.1. C_t is defined as the total concentration of the carrier in all forms.

Experimental parameter	Experiment	Labeled substrate (cis)	Unlabeled analog (trans)	General expression	Rapid dissociation
$\bar{V}_{Si}$	zero trans exit	S_i	-	$\frac{k_{-1}f_1f_{-2}C_t}{k_{-1}(f_1 f_{-2}) + f_1(f_2+f_{-2})}$	$\frac{f_1f_{-2}C_t}{(f_1 + f_{-2})}$
$\bar{V}^T_{Si}$	infinite trans exit	S_i	T_o	$\frac{k_{-1}k_{-4}f_{-2}f_3C_t}{f_3k_{-4}(f_{-2}+k_{-1}+f_2) + f_{-2}k_{-1}(k_{-4}+f_3+f_{-3})}$	$\frac{f_{-2}f_3C_t}{(f_3 + f_{-2})}$
$\bar{V}_{To}$	zero trans	T_o	-	$\frac{k_{-4}f_{-1}f_3C_t}{k_{-4}(f_{-1}+f_3) + f_{-1}(f_3+f_{-3})}$	$\frac{f_{-1}f_3C_t}{(f_{-1} + f_3)}$
$\left(\frac{v_\infty}{v_o}\right)_{To}$	exit	S_i	T_o	$\frac{f_3k_{-4}(1 + f_{-1}/f_1)}{k_{-4}(f_{-1}+f_3) + f_{-1}(f_3+f_{-3})}$	$\frac{1 + f_{-1}/f_1}{1 + f_{-1}/f_3}$
$\left(\frac{v_\infty}{v_o}\right)_{Ti}$	entry	S_o	T_i	$\frac{f_{-3}k_{-3}(1 + f_1/f_{-1})}{k_{-3}(f_1+f_{-3}) + f_1(f_3+f_{-3})}$	$\frac{1 + f_1/f_{-1}}{1 + f_1/f_{-3}}$

In an equilibrating system α and β equal unity, while in active transport they may be very large.

The corresponding rate equation for the transport of substrate T is as follows:

$$\frac{-d[T_o]}{dt} = \frac{\frac{\bar{V}_{To}}{\bar{K}_{To}}\left([T_o]-[T_i]/\beta\right)+\frac{\tilde{V}^S_{To}}{\tilde{K}^T_{Si}\bar{K}_{To}}\left([T_o][S_i]-\alpha[T_i][S_o]/\beta\right)}{1+\frac{[S_o]}{\bar{K}_{So}}+\frac{[S_i]}{\bar{K}_{Si}}+\frac{[T_o]}{\bar{K}_{To}}+\frac{[T_i]}{\bar{K}_{Ti}}+\frac{[S_o]}{\tilde{K}^S_{So}}\frac{[S_i]}{\bar{K}_{Si}}+\frac{[T_o]}{\tilde{K}^T_{To}}\frac{[T_i]}{\bar{K}_{Ti}}+\frac{[S_o]}{\tilde{K}^T_{So}}\frac{[T_i]}{\bar{K}_{Ti}}+\frac{[T_o]}{\tilde{K}^S_{To}}\frac{[S_i]}{\bar{K}_{Si}}} \qquad (A.4)$$

It is to be noted that the denominators of the two equations (A.1 and A.4) are identical.

The assignment of constants in eqns. A.1 and A.4 may be verified by writing rate expressions for individual experiments, including only those terms that are pertinent in a given situation (see columns 3, 4 and 5 in Table A.1 and definitions of the constants below). For example, $\bar{K}_{To}$ is determined from the rates of entry of substrate T into cells free of substrate (zero trans entry). The rate equation may be written down directly from eqns. A.3 and A.4:

$$\frac{d[T_i]}{dt} = \frac{\bar{V}_{To}[T_o]/\bar{K}_{To}}{1+[T_o]/\bar{K}_{To}} \qquad (A.5)$$

$\bar{K}_{To}$ is clearly the apparent affinity constant in this experiment.

II. General rate equation for the transport of a substrate in the presence of a competitive inhibitor:

Assuming that T does not undergo transport ($f_3 = f_{-3} = 0$), equation (A.1) is readily converted to the general rate

equation for transport of a substrate in the presence of a competitive inhibitor. The equation is now re-written, with substitution of the symbol I (inhibitor) for T:

$$\frac{-d[S_i]}{dt} = \frac{\frac{\overline{V}_{Si}}{\overline{K}_{Si}}\left([S_i] - \alpha[S_o]\right)}{1 + \frac{[S_o]}{\overline{K}_{So}} + \frac{[S_i]}{\overline{K}_{Si}} + \frac{[I_o]}{\overline{K}_{Io}} + \frac{[I_i]}{\overline{K}_{Ii}} + \frac{[S_o][S_i]}{\tilde{K}^{S}_{So}\,\overline{K}_{Si}} + \frac{[S_o][I_i]}{\tilde{K}^{S}_{Ii}\,\overline{K}_{So}} + \frac{[S_i][I_o]}{\tilde{K}^{S}_{Io}\,\overline{K}_{Si}}} \qquad (A.6)$$

The expressions for the apparent inactivation constants are listed in Table A.1, and their definitions are given below.

III. Nomenclature for transport constants

In the absence of any general agreement on the nomenclature for the parameters of a transport system, we have adopted a convention here which appears to have several advantages. First it is sufficiently flexible to allow numerous constants to be named, and second, the significance of each constant is directly understandable. The subscript attached to the affinity constant K designates the particular substrate (S or T) whose affinity is measured and the side of the membrane (inside the cell, i, or outside, o), where it becomes bound.

A bar drawn above the constant (-) indicates that the affinity has been determined in a zero trans experiment (no substrate is present on the trans side); a squiggle (~) indicates that the affinity has been determined in an infinite trans experiment (an unlabeled substrate is present on the trans side at a saturating concentration). The superscript associated with the squiggle designates the substrate on the trans side. A double bar (=) indicates equilibrium

exchange or exchange in the final steady state. In the absence of any symbol above the constant, K represents the true dissociation constant: the ratio of dissociation and association constants for complex formation. A similar convention is adopted for the maximum velocity, V.

In naming individual rate constants in a kinetic scheme representing the process of transport, we have adopted a convention which differentiates constants for carrier reorientation (f) from those for association and dissociation of substrate (k). The advantage in doing so is that when general expressions are written for experimental parameters as in Table A.1, the consequences of having either fast or slow dissociation steps are immediately apparent.

Experimental constants which are commonly determined in transport studies are defined below:

$\overline{K}_{So}$, $\overline{K}_{To}$: Constants determined in zero trans entry experiments for S and T respectively.

$\overline{K}_{Si}$, $\overline{K}_{Ti}$: Constants determined in zero trans exit experiments for S and T respectively.

$\overline{K}_{Ii}$, $\overline{K}_{Io}$: Competitive inhibition constants with inhibitor restricted to one side of the membrane (inside or outside). When determined in zero trans exit and entry experiments respectively, where the substrate and inhibitor are present on the same side of the membrane, the inhibition is competitive. Where substrates and inhibitors are on opposite

sides, inhibition is non-competitive. In either case, whether inhibition is competitive or non-competitive, these constants are determined from the substrate-dependent component of the inhibition (in a reciprocal plot, from the slopes).

$\tilde{K}^{T}_{So}$, $\tilde{K}^{S}_{To}$: Constants determined in infinite trans entry experiments for S into saturating T and for T into saturating S, respectively.

$\tilde{K}^{S}_{So}$, $\tilde{K}^{T}_{To}$: Constants determined in infinite trans entry experiments for S and T, respectively. In each case the same substrate whose entry is followed is present on the trans side of the membrane at a saturating concentration.

$\tilde{K}^{S}_{Ii}$, $\tilde{K}^{S}_{Io}$: Non-competitive inhibition constants with inhibitor restricted to one side of the membrane (inside or outside), and with the substrate and inhibitor on opposite sides of the membrane. The constant is determined from the substrate-independent component of the inhibition (in a reciprocal plot, from the intercepts).

$\bar{\bar{K}}_{S}$, $\bar{\bar{K}}_{T}$: Constants determined in equilibrium exchange experiments for substrates S and T.

$\bar{V}_{Si}$, $\bar{V}_{Ti}$: Maximum rates of zero trans efflux of substrates S and T respectively.

$\bar{V}_{So}$, $\bar{V}_{To}$: Maximum rates of zero trans influx of substrates S and T respectively.

$\tilde{V}^{T}_{Si}$: Maximum rate of infinite trans efflux of substrate S into a solution of substrate T at a saturating concentration.

$\left(\frac{V_\infty}{V_o}\right)_{T_o}$: Effect of an unlabeled analog (T) at a saturating concentration outside the cells, on the rate of exit of a substrate(S) present inside at a low concentration compared to $\bar{K}_{Si}$.

$\left(\frac{V_\infty}{V_o}\right)_{T_i}$: Effect of an unlabeled analog (T) at a saturating concentration inside the cells, on the rate of entry of a substrate(S) present outside at a low concentration compared to $\bar{K}_{So}$.

IV. Relationships among experimental parameters

The parameters involved in the above rate equations are not independent; in many cases, relationships exist which may be exploited in calculating unknown constants from those found by experiment or in testing the consistency of data. These relationships are summarized in Table A.3.

Table A.3. Summary of obligatory relationships among experimental parameters.

$$\tilde{K}^{S}_{So}\,\bar{K}_{Si} = \tilde{K}^{S}_{Si}\,\bar{K}_{So}$$

$$\tilde{K}^{T}_{So}\,\bar{K}_{Ti} = \tilde{K}^{S}_{Ti}\,\bar{K}_{So} \qquad \tilde{K}^{S}_{To}\,\bar{K}_{Si} = \tilde{K}^{T}_{Si}\,\bar{K}_{To}$$

$$\bar{\bar{K}}_{S} = \frac{1}{\alpha}\,\bar{\bar{K}}_{S}$$

$$\tilde{V}^{S}_{So} = \tilde{V}^{S}_{Si} = \bar{\bar{V}}_{S}$$

$$\tilde{V}^{T}_{So} = \tilde{V}^{S}_{Ti} \qquad \tilde{V}^{S}_{To} = \tilde{V}^{T}_{Si}$$

$$\frac{\bar{V}_{So}}{\bar{K}_{So}} = \frac{\alpha\bar{V}_{Si}}{\bar{K}_{Si}} \qquad \frac{\bar{V}_{To}}{\bar{K}_{To}} = \frac{\beta\bar{V}_{Ti}}{\bar{K}_{Ti}}$$

$$(v_{\infty})_{To} = \frac{\tilde{V}^{T}_{Si}}{\tilde{K}^{T}_{Si}} \qquad \frac{v_{\infty}}{v_{o}}_{To} = \frac{\tilde{V}^{T}_{Si}/\tilde{K}^{T}_{Si}}{\bar{V}_{Si}/\bar{K}_{Si}}$$

$$\bar{\bar{K}}_{S} = \tilde{K}^{S}_{So}\left(\frac{v_{\infty}}{v_{o}}\right)_{Si} \qquad \bar{\bar{K}}_{S} = \tilde{K}^{S}_{Si}\left(\frac{v_{\infty}}{v_{o}}\right)_{So}$$

$$\frac{(v_{\infty}/v_{o})_{To}}{(v_{\infty}/v_{o})_{Ti}} = \frac{\beta\bar{K}_{To}}{\bar{K}_{Ti}} = \frac{\bar{V}_{To}}{\bar{V}_{Ti}}$$

$$\frac{((v_{\infty}/v_{o})_{To})_{1}}{((v_{\infty}/v_{o})_{To})_{2}} = \frac{(\bar{V}_{To})_{1}}{(\bar{V}_{To})_{2}}$$

INACTIVATION RATE EQUATIONS

I. Rates in the presence of a substrate analog

A general equation for the inactivation rate may be written on the basis of the transport scheme in Fig. 8.1

$$Q = \left(q_1[C_i] + q_2\,[C_o] + q_3\,[C_{Ti}] + q4\,[C_{To}]\right)/C_t \quad (A.7)$$

where Q is the measured inactivation rate constant and q_1, q_2, q_3 and q_4 are inactivation rate constants for the various carrier forms and where C_t is the total carrier concentration. Expressions for the individual concentration terms, $[C_i]$ etc., may be determined by means of the King-Altman schematic method, and when these are substituted into eqn. (A.7), following expression for the inactivation rate is found:

$$Q = \frac{Q_o + Q_{To}\,\dfrac{[T_o]}{\bar{K}_{To}} + Q_{Ti}\,\dfrac{[T_i]}{\bar{K}_{Ti}} + Q_{ToTi}\,\dfrac{[T_o][T_i]}{\bar{K}_{Ti}\,\bar{K}^{S}_{To}}}{1 + \dfrac{[T_o]}{\bar{K}_{To}} + \dfrac{[T_i]}{\bar{K}_{Ti}} + \dfrac{[T_o][T_i]}{\bar{K}_{Ti}\,\bar{K}_{To}}} \quad (A.8)$$

The expressions for the experimental parameters are listed in Table (A.4), and their definitions are given below:

II. Definitions of experimental inactivation constants:

Q_o : Inactivation rate constant in the absence of substrates or inhibitors for a given concentration of N-ethylmaleimide.

Q_{To} : Inactivation rate constant in the presence of a saturating concentration of substrate T outside the cells.

Q_{Ti} : Inactivation rate constant in the presence of a saturating concentration of substrate T inside the

Table A.4

Experimental inactivation rate constants expressed in terms of individual constants for the scheme in Fig. 8.1.

Experimental Inactivation Rate Constant	Analog location (saturating) inside	outside	General	Rapid dissociation
Q_o	-	-	$\frac{f_1q_1 + f_{-1}q_2}{f_1 + f_{-1}}$	$\frac{f_1q_1 + f_{-1}q_2}{f_1 + f_{-1}}$
Q_{To}	-	+	$\frac{k_{-4}f_3q_1 + f_{-1}f_3q_3 + f_{-1}(k_{-4} + f_{-3})q_4}{k_{-4}(f_{-1} + f_3) + f_{-1}(f_3 + f_{-3})}$	$\frac{f_3q_1 + f_{-1}q_4}{f_{-1} + f_3}$
Q_{Ti}	+	-	$\frac{k_{-3}f_{-3}q_2 + f_1f_{-3}q_4 + f_1(k_{-3} + f_3)q_3}{k_{-3}(f_1 + f_{-3}) + f_1(f_3 + f_{-3})}$	$\frac{f_{-3}q_2 + f_1q_3}{f_1 + f_{-3}}$
Q_{ToTi}	+	+	$\frac{f_3q_3 + f_{-3}q_4}{f_3 + f_{-3}}$	$\frac{f_3q_3 + f_{-3}q_4}{f_3 + f_{-3}}$

cells.

Q_{ToTi} : Inactivation rate constant with saturating, and equal, concentrations of substrate T inside and outside the cells.

APPENDIX 2

TRANSPORT AND INACTIVATION RATE EQUATIONS FOR THE ONE-COMPLEX FORMULATION OF THE CARRIER MODEL

TRANSPORT RATE EQUATIONS

The general rate equation for transport for the one-complex scheme in Fig. 8.5 is of exactly the same form as in the scheme containing two forms of the carrier-substrate complex (Fig. 6.1), as given in eqns. A1 and A4.

For the purpose of analyzing the experiments presented in Chapter 8 we wish to have an expression for $(v_\infty/v_o)_{To}$. This parameter represents the effect of an unlabeled analog at a saturating concentration on the rate of exit of a substrate present inside the cells at a low concentration compared to its affinity constant. The required ratio is found from the rate equation for the efflux of substrate S in the presence of an analog (T) outside the cells, which is found to be:

$$v = \frac{\frac{\overline{V}_{Si}[S_i]}{\overline{K}_{Si}} + \frac{\tilde{V}^T_{Si}}{\tilde{K}^T_{Si}\overline{K}_{To}}[S_i][T_o]}{1 + \frac{[S_i]}{\overline{K}_{Si}} + \frac{[T_o]}{\overline{K}_{To}} + \frac{[S_i][T_o]}{\tilde{K}^S_{Si}\overline{K}_{To}}} \qquad (A.9)$$

The detailed expressions for the parameters in equation (A.9) are shown in Table (A.5). Defining v_∞ as the rate of exit of substrate S when $[S_i] \to o$ and $[T_o] \to \infty$, and v_o as the rate when $[S_i] \to o$ and $[T_o] = o$. Their ratio is found from eqn. (A.9) to be:

Table A.5 Experimental transport parameters for the one-complex formulation of the simple carrier model (Fig. 8.2).

Experimental parameter	Experiment	Labeled substrate (cis)	Unlabeled analog (trans)	Equivalent expression
$\overline{K}_{Si}$	zero trans exit	S_i	-	$\frac{(k_{-1} + k_{-2})(f_1 + f_{-1})}{k_2 (f_1 + k_{-1})}$
$\overline{K}_{To}$	zero trans entry	T_o	-	$\frac{(k_{-4} + k_{-3})(f_1 + f_{-1})}{k_3 (f_{-1} + k_{-4})}$
$\tilde{K}^S_{Si}$	infinite trans exit	S_i	S_o	$\frac{k_{-2} + f_{-1}}{k_2}$
$\tilde{K}^T_{Si}$	infinite trans exit	S_i	T_o	$\frac{(k_{-1} + k_{-2})(k_{-4} + f_{-1})}{k_2 (k_{-1} + k_{-4})}$
$\overline{V}_{Si}$	zero trans exit	S_i	-	$\frac{k_{-1} f_1}{k_{-1} + f_1}$
$\tilde{V}^T_{Si}$	infinite trans exit	S_i	T_o	$\frac{k_{-1} k_{-4}}{k_{-1} + k_{-4}}$

$$\left(\frac{v_\infty}{v_o}\right)_{To} = \frac{\tilde{V}^T_{Si}}{\tilde{K}^T_{Si}} \cdot \frac{\bar{K}_{Si}}{\bar{V}_{Si}} = \left(\frac{k_{-4}\, f_{-1}}{k_{-4} + f_{-1}}\right)\left(\frac{1}{f_1} + \frac{1}{f_{-1}}\right) = \bar{V}_{To}\left(\frac{1}{f_1} + \frac{1}{f_{-1}}\right)$$

INACTIVATION RATE EQUATIONS

The derivation of the rate equation follows the same method as in the two-complex case (Appendix 1). The inactivation rate is now given by (see Fig. 8.2):

$$Q = \left(q_1[C_i] + q_2[C_o] + q_3[C_T]\right) / \; C_t \qquad \text{(A.10)}$$

$$= \frac{Q_o + Q_{To}\,\dfrac{[T_o]}{\bar{K}_{To}} + Q_{Ti}\,\dfrac{[T_i]}{\bar{K}_{Ti}} + Q_{ToTi}\,\dfrac{[T_o][T_i]}{\bar{K}_{Ti}\,\tilde{K}^T_{To}}}{1 + \dfrac{[T_o]}{\bar{K}_{To}} + \dfrac{[T_i]}{\bar{K}_{Ti}} + \dfrac{[T_o][T_i]}{\bar{K}_{Ti}\,\tilde{K}^T_{To}}} \qquad \text{(A.11)}$$

The experimental inactivation parameters are defined in the same way as in the case of the two-complex model (Appendix 1) and the equivalent expressions are listed in Table A.6.

Table A.6 Experimental inactivation parameters for the one-complex formulation of the simple carrier model (Fig. 8.2).

Experimental parameters	Location of the analog inside	outside	Equivalent expression
Q_o	-	-	$\frac{f_1 q_1 + f_{-1} q_2}{f_1 + f_{-1}}$
Q_{To}	-	+	$\frac{k_{-4} q_1 + f_{-1} q_3}{k_{-4} + f_{-1}}$
Q_{Ti}	+	-	$\frac{k_{-3} q_2 + f_i q_3}{f_1 + k_{-3}}$
Q_{ToTi}	+	+	q_3

APPENDIX 3

COMPUTER PROGRAM FOR CALCULATION OF TRANSPORT PARAMETERS OF UNLABELED SUBSTRATE ANALOGS

Data required:

Exit rates of labeled substrate in the presence of unlabeled analog (trans) at varying concentrations.

A correction for the appearance of label due to cell lysis is calculated from the counting efficiency. Experimental constants are obtained with their standard errors.

Computer language:

"Extended Basic".

```
0002 DIM G$[10]
0004 PRINT "AVOID CALCULATION OF CONCENTRATION IN OVERN. INCUBATION?"
0006 INPUT G$
0008 PRINT
0010 IF G$="YES" THEN GOTO 0090
0012 PRINT
0014 PRINT "SUPERNATANT CONCENTRATION"
0016 PRINT "CPM/2ML,SUPERNATANT=";
0018 INPUT C1
0020 PRINT "RATIO,SUPERNATANT=";
0022 INPUT R1
0024 PRINT "DILUTION FACTOR,SUPERNATANT=";
0026 INPUT F1
0028 PRINT "SPECIFIC ACTIVITY=";
0030 INPUT A9
0032 LET D1=C1*F1/(.4375+.2*R1)
0034 PRINT "DPM/ML, SUPERNATANT";D1
0036 LET M1=D1/A9
0038 PRINT "MICROMOLES/ML,SUPERNATANT";M1
0040 PRINT
0042 PRINT "PACKED CELL CONCENTRATION"
0044 PRINT "CPM/2ML,CELL=";
0046 INPUT C2
0048 PRINT "RATIO,CELL=";
0050 INPUT R2
0052 PRINT "DILUTION FACTOR,CELL=";
0054 INPUT F2
0056 LET D2=C2*F2/(.4375+.2*R2)
0058 PRINT "DPM/ML,CELL";D2
0060 LET M2=D2/A9
0062 PRINT "MICROMOLES/ML,CELL";M2
0064 PRINT
0066 PRINT "TOTAL RADIOACTIVITY"
0068 PRINT "CPM/2ML,TOTAL=";
0070 INPUT C3
0072 PRINT "RATIO,TOTAL=";
0074 INPUT R3
0076 PRINT "DILUTION FACTOR,TOTAL=";
0078 INPUT F3
0080 LET D3=C3*F3/(.4375+.2*R3)
0082 PRINT "DPM/ML,TOTAL";D3
0084 LET M3=D3/A9
0086 PRINT "MICROMOLES/ML,TOTAL";M3
0088 GOTO 0094
0090 PRINT "SPECIFIC ACTIVITY"
0092 INPUT A9
0094 PRINT
0096 PRINT "INTERNAL CONCENTRATION"
0098 PRINT "NUMBER OF DETERMINATIONS=";
0100 INPUT N3
0102 LET P=0
0104 PRINT "CPM/ML"; TAB(10);"RATIO"; TAB(20);"DILUTION FACTOR"
0106 FOR H=1 TO N3
0108   INPUT C4;
0110   PRINT  TAB(10);
0112   INPUT R4;
0114   PRINT  TAB(20);
0116   INPUT F4
0118   LET D4=C4*F4/(.4375+.2*R4)
0120   LET P=P+D4
0122 NEXT H
0124 LET P4=P/N3
0126 PRINT "INTERNAL CONCENTRATION(DPM/ML SUSPEN.)=";P4
0128 PRINT
0130 PRINT "AFFINITY DETERMINATION"
0132 PRINT
```

```
0134 PRINT "TIME CORRECTION FACTOR (MIN)=";
0136 INPUT M
0138 PRINT "NUMBER OF CONCENTRATIONS OF ANALOG, EXTERNAL=";
0140 INPUT N1
0142 PRINT "0-L.,100% HAEMOLYSIS=";
0144 INPUT 09
0146 LET A6=0
0148 LET A8=0
0150 LET N7=0
0152 DIM L[20],E[20],U[40],V[40],W[40],Z[20]
0154 FOR J=1 TO N1
0156   PRINT
0158   PRINT "CONCENTRATION OF ANALOG"
0160   INPUT L[J]
0162   PRINT
0164   PRINT "NUMBER OF TIMES FOR THIS CONCENTRATION=";
0166   INPUT N2
0168   PRINT
0170   GOSUB 0390
0172   PRINT
0174   PRINT "CPM/2ML"; TAB(10);"RATIO"; TAB(20)"TIME"; TAB(30);
0176   PRINT "DPM/ML"; TAB(40);"LOG CT/CT=0"
0178   FOR I=1 TO N2
0180     INPUT U[I];
0182     PRINT  TAB(10);
0184     INPUT V[I];
0186     PRINT  TAB(20);
0188     INPUT W[I];
0190     PRINT  TAB(30);
0192     LET D5=U[I]*.5/(.4375+.2*V[I])-P4*(1.028-.504*V[I])/09
0194     PRINT D5;
0196     PRINT  TAB(40);
0198     LET Y=LOG((10*P4-9*D5)/(10*P4))
0200     PRINT Y
0202     LET X=W[I]+M
0204     GOSUB 0406
0206   NEXT I
0208   PRINT "HAVE THIS DATA BEEN CORRECTLY ENTERED?"
0210   INPUT G$
0212   PRINT
0214   IF G$="YES" THEN GOTO 0220
0216   PRINT "THE CORRECT DATA ARE AS FOLLOWS:"
0218   GOTO 0158
0220   GOSUB 0422
0222   LET E[J]=B
0224   PRINT
0226   PRINT "CONSTANTS CALCULATED FOR BEST FIT OF SLOPE &INTERCEPT"
0228   PRINT "RATE CONSTANT(1/MIN)=";E[J]
0230   PRINT "RATE, MICROMOL/ML CELL/MIN=";E[J]*P4*10/A9
0232   IF N=2 THEN GOTO 0236
0234   PRINT "S.D.(RATE)=";S7*P4*10/A9
0236   PRINT "INTERCEPT=";A
0238   IF N=2 THEN GOTO 0242
0240   PRINT "S.D.(INTERC)=";S8
0242   GOSUB 0462
0244   LET Z[J]=Z
0246   PRINT
0248   PRINT "CONSTANTS CALCULATED FOR BEST FIT OF SLOPE &FIXED INTERC
0250   PRINT "RATE CONSTANT(1/MIN)=";Z[J]
0252   PRINT "RATE,MICROMOLES/ML CELL/MIN=";Z[J]*P4*10/A9
0254   IF N=1 THEN GOTO 0258
0256   PRINT "S.D.(RATE)=";R9*P4*10/A9
0258   IF L[J]>0 THEN GOTO 0266
0260   LET A6=A6+E[J]
0262   LET A8=A8+Z[J]
0264   LET N7=N7+1
```

```
0266 NEXT J
0268 IF N1-N7<2 THEN GOTO 0384
0270 LET P7=0
0272 LET P8=0
0274 LET P6=0
0276 LET P9=0
0278 GOSUB 0396
0280 FOR J=1 TO N1
0282   IF L[J]=0 THEN GOTO 0298
0284   LET X=ABS((1-P[J]*N7/A6)/L[J])
0286   LET Y=P[J]*N7/A6
0288   GOSUB 0406
0290   IF ABS(((1-P[J]*N7/A6)/L[J]))<P7 THEN GOTO 0294
0292   LET P7=ABS((1-P[J]*N7/A6)/L[J])
0294   IF P[J]*N7/A6<P6 THEN GOTO 0298
0296   LET P6=P[J]*N7/A6
0298 NEXT J
0300 PRINT
0302 PRINT "CONSTANTS CALCULATED FOR BEST FIT OF SLOPE AND INTERC"
0304 PRINT "ANALOG CONC","V/V0","(V/V0-1)/S0"
0305 FOR J=1 TO N1
0306   IF L[J]=0 THEN GOTO 0316
0310   PRINT L[J]; TAB(15);
0312   PRINT P[J]*N7/A6; TAB(30);
0314   PRINT ABS((1-P[J]*N7/A6)/L[J])
0316 NEXT J
0318 GOSUB 0426
0320 PRINT
0322 PRINT "1/L(MICR)=";B; TAB(22);"KSO'=";B/A; TAB(40);
0324 PRINT "INTERCEPT=";A
0326 IF N1-N7<3 THEN GOTO 0332
0328 PRINT "S.D.(1/L)=";S7; TAB(20);"S.D.(KSO')=";S3; TAB(40);
0330 PRINT "S.D.(INTER)=";S8
0332 GOSUB 0390
0334 FOR J=1 TO N1
0336   IF L[J]=0 THEN GOTO 0352
0338   LET X=ABS((1-Z[J]*N7/A8)/L[J])
0340   LET Y=Z[J]*N7/A8
0342   GOSUB 0406
0344   IF ABS(((1-Z[J]*N7/A8)/L[J]))<P6 THEN GOTO 0348
0346   LET P6=ABS((1-Z[J]*N7/A8)/L[J])
0348   IF Z[J]*N7/A8<P9 THEN GOTO 0352
0350   LET P9=Z[J]*N7/A8
0352 NEXT J
0354 PRINT
0356 PRINT "CONSTANTS CALCULATED FOR BEST FIT OS SLOPE & FIXED INTER"
0358 PRINT "ANALOG CONC","V/V0","(V/V0-1)/S0"
0360 FOR J=1 TO N1
0362   IF L[J]=0 THEN GOTO 0370
0364   PRINT L[J]; TAB(15);
0366   PRINT Z[J]*N7/A8; TAB(30);
0368   PRINT ABS((1-Z[J]*N7/A8)/L[J])
0370 NEXT J
0372 GOSUB 0426
0374 PRINT "1/L(MICR)=";B; TAB(22);"KSO'=";B/A; TAB(40);
0376 PRINT "INTERCEPT=";A
0378 IF N1-N7<3 THEN GOTO 0384
0380 PRINT "S.D.(1/L)=";S7; TAB(20);"S.D.(KSO')=";S3; TAB(40);
0382 PRINT "S.D.(INTER)=";S8
0384 END
0386 PRINT  TAB(78);"#";
0388 PRINT  TAB(78);"#";
0390 LET S1=0
0392 LET S2=0
0394 LET S3=0
0396 LET S4=0
```

```
0398 LET S5=0
 400 LET R7=0
0402 LET R8=0
0404 RETURN
0406 LET S1=S1+X
0408 LET S2=S2+Y
0410 LET S3=S3+X*X
0412 LET S4=S4+Y*Y
0414 LET S5=S5+X*Y
0416 LET R7=R7+Y*Y/(X*X)
0418 LET R8=R8+Y/X
0420 RETURN
0422 LET N=N2
0424 GOTO 0428
0426 LET N=N1-N7
0428 LET A1=S1/N
0430 LET A2=S2/N
0432 LET B=(S5-N*A1*A2)/(S3-N*A1*A1)
0434 LET A=A2-B*A1
0436 IF N=2 THEN GOTO 0460
0438 LET S6=(S4-S2*S2/N-((S5-S1*S2/N)^2)/(S3-S1*S1/N))/(N-2)
0440 LET S6=ABS(S6)
0442 LET S7=SQR(S6/(S3-N*A1*A1))
0444 LET S8=SQR(S6)*SQR(S3/(N*S3-S1*S1))
0446 LET R=(S5-S1*S2)/(SQR(S4-S2*S2)*SQR(S3-S1*S1))
0448 LET P1=S8/B
0450 LET P2=S7/B
0452 LET P3=P1*P1+P2*P2-2*R*P1*P2
0454 LET Q1=SQR(P3)*B/B
0456 LET Q3=SQR(P3)*B/A
0458 LET Q2=R*S7*S8
0460 RETURN
0462 LET Z=S5/S3
0464 IF N=1 THEN GOTO 0468
0466 LET R9=SQR((R7-(R8^2)/N)/(N-1))
0468 RETURN
0470 IF N=2 THEN GOTO 0242
```

APPENDIX 4

Source of Chemicals

The sources of chemicals used in these studies were as follows:

Aquasol	New England Nuclear
1,3-Bis-dimethylamino-2-propanol	Aldrich Chemical Company
Chloramphenicol	Sigma Chemical Company, Inc.
^{14}C-Choline Cl^-	New England Nuclear
Choline Cl^-	Eastman Kodak Company
Cytochalasin B	Aldrich Chemical Company
2-Di-n-butylaminoethanol	Pfaltz and Bauer, Inc.
2-Diethylaminoethanol	Aldrich Chemical Company
2-Di-isopropylaminoethanol	Aldrich Chemical Company
2-Dimethylaminoethanol	Aldrich Chemical Company
N,N-Dimethylbenzylamine	Eastman Kodak Company
N,N-Dimethylcyclohexylamine	K & K Laboratories, Inc.
2-Di-n-propylaminoethanol	Aldrich Chemical Company
D-Glucose	Fisher Scientific Co. Ltd.
2-Mercaptoethanol	Eastman Kodak Company
N-Ethyl maleimide	Sigma Chemical Company, Inc.
Phloretin	BDH Chemicals

In every case the chemical was of the highest grade available commercially.

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