THE CONCEPT OF CARRIER TRANSPORT AND ITS COROLLARIES IN PHARMACOLOGY

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I. NOTATION

v	Transport rate
v_{max}	Maximal transport rate
D'	Permeability constant
S, R	Substrate concentration
C	Free carrier concentration
C_t	Total carrier concentration
CS	Concentration of carrier-substrate complex
I	Concentration of an inhibitor
K_m	Dissociation constant of carrier-substrate complex
K_s	Dissociation constant of enzyme-substrate complex
S'	Relative substrate concentration ($\equiv S/K_m$)
S''	Relative substrate concentration ($\equiv S/K_s$)
F	Transport resistance ($\equiv (S_1 - S_2)/v$)
E	Enzyme concentration
n	Velocity constant of enzymatic reaction
a, b	Velocity constants of non-enzymatic substrate-carrier reactions
M	Unidirectional flux

Indices $_1$ and $_2$ refer to the two membrane sides. Asterisk (*) indicates labelled.

II. INTRODUCTION

The cell membrane was once considered a static structure, protecting the cell against loss of essential constituents by diffusion and allowing molecules and

ions to exchange according to their lipoid solubility (66, 306, 307), molecular size (64, 65), and electric charge (276). In the past decades it has proved necessary to revise this view. It has been found that the cell membrane is an active part of the cell machinery, that in many cases the transfer of substances into and out of cells is due to pump-like devices in the membrane, and that such pumps can be utilized by the cell for different specific cell functions. It has become obvious that in these transport systems the mode of passage across the membrane could not be restricted to simple diffusion through aqueous channels ("pores") or through continuous lipoid layers. In many cases it proved necessary to assume a closer association with the constituents of the membrane, allowing higher specificity, higher efficacy, and wider possibilities of modification and regulation. The assumption that transmembranous passage may involve temporary binding to parts of the membrane, suggested early by Osterhout (303, 304) and by Lundegårdh (260), has become more and more popular. For binding structures which are not rigidly fixed but can in some way move across the membrane the term "carrier" was introduced.

The existence of carriers is still hypothetical. There is now, however, a considerable body of evidence supporting this assumption, and no adequate alternatives have been offered in the literature in order to explain the quantitative and qualitative relations of the known examples of transport. Moreover the closer theoretical study of carrier systems has led to a number of results of a strictly quantitative nature, which can be used in the analysis of experiments to characterize special types of systems and of conditions. In this way, a new field has begun to develop that may be termed "carrier physiology" and "carrier pharmacology."

It is the aim of the present review to provide a survey of this development. In view of this purpose the observations to be discussed were chosen according to their bearing on carrier processes. Problems like the details of energy-yielding metabolism, important as they obviously are, have been treated only insofar as they are related to the carrier mechanism proper. It was felt that a stage which warrants a general treatment has now been reached. Therefore, the attempt was made to combine description and discussion of general principles in a first general part, and observations in a second special part, and to link the two parts by cross references.

A. Characterization of the concept and its main elements

The essential elements of the carrier mechanism are the *reaction* of the transport substrate with a membrane component to form a complex, and the *movement* of the complex from one side of the membrane to the other, where the substrate is released to the aqueous medium.

For the *binding reaction*, covalent, ionic, and hydrogen bonds have been suggested in various cases. *Enzymatically catalyzed reactions* are suggested by a number of characteristic transport features, including high structural specificity, particularly stereochemical specificity, high turnover rates, and sensitivity toward enzyme inhibitors and protein reagents, and, in the case of permeases, by ease of induction.

The *movement* of the complex has been visualized by most authors as diffusion of the free substrate-carrier complex molecule across the membrane. Another possibility which has been proposed is that of rotating molecules, as suggested by Lundegårdh (261) and complemented by Danielli's (91) distinction between "rotating molecular carriers" and "rotating molecular segment carriers." Danielli further discussed the possibility of a "propelled shuttle or carrier" in which an adsorption center on a contractile protein is on one side of the membrane when the protein is contracted, and on the other side when it is extended. All these variations at the present stage are highly speculative and so far no experimental means are available to distinguish them. They should be considered as special forms of carrier mechanisms, expected to display similar kinetics and, under suitable conditions, the phenomenon of counter-transport.

In contradistinction to these mechanisms, transmembranous passage involving binding to fixed membrane constituents, as has been suggested occasionally (411), will not be included in the present review for reasons which will emerge in the general part.

It is believed that the biological importance of carrier mechanisms is bound to their operation across membranes rather than across the cytoplasm within cells, as has occasionally been suggested for transcellular transport (236, 376, 377). Some essential differences between membranous and cytoplasmic carrier systems were discussed by Wilbrandt (446). The membranous localization of the mechanisms of transcellular transport appears to be one of the reasons for the close relationship between transcellular transport (*i.e.*, across cells) and transmembranous transport (*i.e.*, into and out of cells). As will be shown in the special part, there are parallelisms in kinetics, structural specificity, dependence on metabolism, and action of inhibitors. Accordingly, there is an increasing number of observations indicating that in some cases intracellular accumulation precedes transcellular uphill transport. Examples now include glucose absorption from the intestine (82), reabsorption in the kidney of galactose (232) and of sulfate (105), anion secretion in the mammary gland (136) and in the stomach (257), possibly renal secretion of *p*-aminohippurate (84, 103), and amino acid absorption from the intestine (3).

B. Phenomena and concepts related to the principle of carrier transport

1. Membrane structure and factors affecting passive permeability. The solutes for which transport mechanisms have been observed are in general not molecular species of small size or high lipoid solubility, as are those which penetrate cell membranes easily (98, 306, 307, 453). The majority of transported solutes, as will be shown, are highly hydrophilic substances, including electrically charged ions, and some are compounds of considerable molecular size. Moreover, in a number of cases agents capable of blocking the specific mechanism are able not only to lower the rate of transmembranous passage but to prevent penetration more or less completely. The mechanisms thus would appear to enable solutes to pass membranes which structurally are impermeable to their molecules. With respect to the type of reactions to be expected in transport systems it may be

postulated, therefore, that they should be capable of increasing the lipophilic properties of the substrate molecule (344).

2. Concepts of mechanisms and definitions. It appears useful to delineate the concept of carrier transport in relation to a number of related concepts and phenomena.

The term "*active transport*" is frequently used to characterize specific systems. Many attempts have been made to define this term in a manner that would be both precise and useful. Unfortunately the definitions offered so far differ widely, reflecting mainly two trends. Some authors feel that any functional participation of the cell in transport (including substrate-carrier reactions) should be considered as a kind of cellular activity; therefore, they denote carrier transport as "active" (245). Others prefer the thermodynamic distinction between such translocations that do require energy, and those that do not (344, 345, 421, 423). Definitions using concepts of irreversible thermodynamics have been offered by Scheer (366), by Jardetzky and Snell (203), and by Kedem (211). Since no general agreement has been reached as to the meaning of "active transport," the term will not be used in the present review. This decision is not motivated by a lack of appreciation for the value of definitions, but rather by the consideration that at a stage at which a precise and generally accepted definition is not available, the use of descriptive terms involves less risk of misunderstanding.

The term "*facilitated diffusion*" was introduced by Danielli (91). It includes a number of transfer mechanisms which have in common translocations which are not brought about by simple diffusion but still lead to equilibration, rather than to accumulation, of the substrate. Some of them meet the definition of carrier transport in a broad sense. Others, however, like the system of the "expanded lattice," in which diffusion is modified by a stretching action on a porous system, or the hydrogen bonding pore ("polar pore") as suggested by Stein and Danielli (411), do not involve a reaction of the substrate with a mobile carrier. Facilitated diffusion, therefore, is not identical with carrier transport, although the two terms overlap to a certain degree.

In a discussion of calculations of ion-transport efficiencies from experiments with isotopes, Ussing (419) introduced the concept of "*exchange diffusion.*" He pointed out that such calculations may lead to erroneous results if part of the movement of the ions across the membrane occurs by an exchange mechanism in which a given ion can cross the membrane only in exchange for another ion of the same species. He illustrated this possibility by the model of spheres of permutite floating in an oil layer between two aqueous layers, and at the interfaces exchanging bound ions for ions from the solutions. Ussing's discussion constitutes a valuable contribution to the analysis of isotope experiments. It appears useful, however, to point out that exchange diffusion does not require a system in which the carrier is necessarily capable of moving only in the substrate-complex form. In any carrier system, under conditions of appreciable saturation of the carrier, part of the movement of the substrate molecules will occur in exchange for others. Thus the participation of exchange diffusion in carrier transport is by no means limited to particular types of carriers or to

specific conditions under which free carrier molecules cannot move. Exchange diffusion should rather be considered as a necessary consequence of carrier transport mechanisms in general, depending quantitatively on the degree of saturation.

A phenomenon that has attracted increasing attention in recent years is that of *pinocytosis* (uptake of external solution by vesicles formed at the cell surface). It was first described by Lewis (255) and extensively studied by Holter (190, 191) and by Bennett (13). Its close relation to phagocytosis has been characterized schematically by the statement that cells "drink" external material in pinocytosis, while they "eat" it in phagocytosis. Pinocytosis has been considered by some authors as a special form of a carrier mechanism, and has also been termed "active transport" (13, 14). Although obviously, in a sense, a "carrier" function of the vesicles cannot be denied, the quantitative study of the phenomenon at the present stage would not yet appear to have made sufficient progress to assess its potentialities and its contributions to transfers observed experimentally in different cells. Therefore, pinocytosis will not be treated in the present review. The term "carrier transport" will be used with the meaning of a mechanism on the molecular rather than on the microscopic level.

The terms to be used in this review to characterize transport apart from the carrier aspect will be few and of a descriptive nature. A transport will be termed "uphill" if the substrate is moved from a lower to a higher chemical or electrochemical potential. A system capable of achieving an uphill transport (although under some conditions it may not actually do so), will for the sake of simplicity be characterized by the short term "pump." The terms "accumulating" and "equilibrating" will likewise be used for the characterization of systems capable or not capable, respectively, of uphill transport.

3. Carriers of plurienzyme reactions. The term "carrier" in enzymology has been used for the description of co-enzymes of transferases like ATP or pyridine nucleotides. If, for example, pyridine nucleotide as co-enzyme of a dehydrogenase transfers hydrogen from a substrate to, say flavine nucleotide, it accepts hydrogen from the substrate in a reaction on a first protein and subsequently carries it to another protein on which it gives it up to flavine nucleotide. In this way, it acts as a carrier for hydrogen. If, as discussed above, the reaction between substrate and carrier is catalyzed enzymatically, the similarity between transport carriers and enzyme carriers is not a superficial one. As was pointed out previously (348) a transport carrier may well be a special form of a co-enzyme, characterized by the property of lipoid solubility, and may thus move between two enzyme molecules located on the two sides of a lipoid membrane. Mitchell, in a number of publications, has stressed the similarity and possible identity of transport carriers and enzyme carriers (279, 280, 281), arguing that in this manner it should be possible "to describe membrane transport in orthodox biochemical terms." In analogy to the term "group transfer," he introduced the term "group translocation."

III. GENERAL PART. THEORY OF CARRIER TRANSPORT

A. Carrier kinetics in systems not capable of uphill transport

The fundamental equation common to all types of carrier transport relates the transport rate (v) to the movement of the substrate-carrier complex:

$$v = D'(CS_1 - CS_2) \tag{1}$$

According to this equation the transport is a reversible first order process with respect to concentrations or amounts of CS. This may indicate diffusion of the complex or other kinds of molecular movement with similar kinetic characteristics such as those mentioned in the introduction. When in the following kinetic discussion the term "diffusion" is used, it includes the modes of movement which follow diffusion kinetics. Since CS_1 and CS_2 are unknown variables, the problem is to replace them by the substrate concentrations and the parameters of the system.

A carrier system is characterized by three consecutive steps: the binding reaction between substrate and carrier, the diffusion of the complex formed, and the splitting reaction on the other side of the membrane. In the general case, the kinetics of such a transport will depend on the velocity constants of these three steps. The complexity of the relations to be expected will increase if the reactions are catalyzed by enzymes. However, the relations become much simpler if one of the three steps is rate-limiting. In most calculations, as, for instance in the treatment by Widdas (442), the assumption was made that the diffusion constant is smaller than the rate constants of the reactions, and that the substrate therefore may be assumed to be in equilibrium with the carrier. This case will be treated first in some detail. The main additional features, under conditions of greater complexity, will be discussed subsequently.

1. The equilibrium system. a. The general rate equation. If the free carrier (C) and the substrate-carrier complex (CS) have numerically equal diffusion constants, *i.e.*, $D_C' = D_{CS}' = D'$, and only under these conditions, the following relationship is valid (349, 442, 443, 444):

$$v = D'C_t\left(\frac{S_1}{S_1 + K_m} - \frac{S_2}{S_2 + K_m}\right) \tag{2}$$

in which the transport rate is determined by two terms, a capacity term ($D'C_t$) and a saturation term (the difference between two degrees of saturation). Since the maximal value for the saturation term is 1 (for the saturation degrees 1 and 0, respectively), $D'C_t$ is the maximum possible transport rate. It cannot be raised further by any increase of the substrate concentrations. Equation 2 thus represents a type of saturation kinetics. Replacing $D'C_t$ by v_{max} yields the following frequently used form of the equation:

$$v = v_{max}\left(\frac{S_1}{S_1 + K_m} - \frac{S_2}{S_2 + K_m}\right) \tag{3}$$

It now appears that the transport is determined by the two parameters, V_{max} and K_m, which have been aptly termed capacity factor and affinity factor (62). Introducing the "relative" substrate concentration $S' \equiv \frac{S}{K_m}$, yields the form:

$$v = v_{max}\left(\frac{S_1'}{S_1' + 1} - \frac{S_2'}{S_2' + 1}\right), \tag{3a}$$

which is particularly useful in the description of competition and inhibition (compare below equations 9, 10, 11, and 23).

A special case of equation 3 results when the second saturation term approaches zero. The transport kinetics then will resemble closely that of an enzymatic reaction in the form given by Michaelis and Menten (277). Frequently this type of kinetics is demonstrated by a linear plot according to Lineweaver and Burk (256), $\frac{1}{v}$ *vs.* $\frac{1}{S_1}$, or $\frac{S_1}{v}$ *vs.* S_1). If either of these plots yields a straight line with a positive ordinate intercept, this indicates Michaelis-Menten kinetics. From the intercepts, the two parameters of the system, v_{max} and K_m, can easily be determined.

As will become evident in the experimental part, observations of transport following Michaelis-Menten kinetics are numerous. If such cases are to be interpreted in terms of carrier mechanisms (as they mostly are), one possible interpretation according to equation 3 is that, under the conditions of the experiment, $S_2 < K_m$. Three experimental conditions leading to this relation may be mentioned: 1) Measurement of initial rates of penetration. 2) High values of K_m on side 2, due to metabolic reactions affecting the affinity. This will be discussed later as a possible mechanism of uphill transport. 3) Permanently low values of S_2, due to metabolic elimination of the substrate.

The carrier interpretation used here for systems following equation 3 or the Michaelis-Menten equation would imply that only K_m will be expected to differ in a pronounced manner for different substrates, but not v_{max}. The observation of marked differences for v_{max} indicates that in some way the conditions differ from the assumptions used here, and thus requires re-interpretation.

b. Characteristics of the system under limiting conditions (447, 448, 450, 452, 460). Limiting conditions will prevail if the substrate concentrations S_1 and S_2 are either much smaller or much larger than K_m. For the discussion of the system under either of these conditions, a more suitable form of equation 3 is the following:

$$v = v_{max}\frac{(S_1 - S_2)K_m}{(S_1 + K_m)(S_2 + K_m)} \tag{4}$$

From this equation the kinetic consequences of limiting conditions are most readily recognized.

α. S_1, $S_2 \ll K_m$: low saturation. Under these conditions the denominator in equation 4 reduces to K_m^2. The transport then can easily be seen to follow dif-

fusion kinetics (the rate being proportional to the difference between the concentrations), with a permeability constant proportional to v_{max} and inversely proportional to K_m. This implies that the experimental finding of an example of transport which follows diffusion kinetics can never exclude the participation of a carrier mechanism, with a high value of K_m. The type of "diffusion kinetics" has been termed D-kinetics.

β. S_1, $S_2 \gg K_m$: high saturation. This condition has interesting consequences, differing in a pronounced manner from what would be expected in a simple Michaelis-Menten system. The deviations concern mainly two points: the transport kinetics and the relation between transport rate and affinity.

The *transport kinetics*, as can again be seen from equation 4, will be given by the following relation:

$$v = v_{max} \cdot K_m \left(\frac{1}{S_2} - \frac{1}{S_1} \right) \tag{5}$$

The kinetic type represented by equation 5 has been termed E-kinetics. It may be characterized by the statement that the rate of transport is proportional to the difference between the reciprocals of the two substrate concentrations. Another possible interpretation, also following from equation 5, is as a type of saturation kinetics with the special feature of a saturation level inversely related to S_2.

With respect to the *relation between transport rate and affinity*, equation 5 shows that here, contrary to low saturation conditions, the rate is directly proportional to K_m. This would lead to the consequence that two substrates differing in their affinities to a carrier system will show the following behavior, somewhat surprising at first sight: at high concentrations the substrate with the higher affinity will penetrate more slowly, at low concentrations faster than the other. Observations to be discussed in the experimental part agree with this prediction (447).

c. Carrier vs. adsorption mechanism (351). Equation 4 has interest from an additional point of view. It has frequently been pointed out that the kinetics of binding to a carrier and of adsorption to a fixed site are formally identical. The question therefore has been raised as to whether transport processes obeying equation 4 could involve adsorption rather than binding to a carrier. This appears possible only if the adsorption area extends throughout the membrane from one side to the other, so that the jumps between two neighboring elements 1 and 2 are rate limiting. In this case the rate of transport from 1 to 2 will be proportional to the fraction of occupied sites in the element 1, and the fraction of free sites in the element 2, resulting in a relation formally identical with equation 4. The transport kinetics in such a system therefore will be identical with those of a carrier transport. The agreement, however, holds only for the overall rate relation given in equation 4, not for the unidirectional fluxes, as will be discussed later.

2. Steady state systems (349). a. The transport resistance. If the carrier is not in equilibrium with the substrate the rates of the substrate-carrier reactions on

the two sides of the membrane have to be taken into account. The kinetics can then be calculated using the condition that in the steady state the two reaction rates must equal the diffusion rate. While the results of such calculations can again be presented by rate equations resembling equation 2, in this case, for reasons seen immediately, a different way of presentation proves more suitable, *i.e.*, to use the transport resistance, $F \equiv \frac{S_1 - S_2}{v}$, rather than the transport rate, v_7 to characterize the transport. For simple diffusion, F equals D′, *i.e.*, it is constant. For the transport system under consideration, the resulting relation depends on whether or not the reaction is enzymatic.

b. Non-enzymatic systems. With non-enzymatic reactions the governing relation is:

$$F = \frac{1}{C_t}\left[\frac{1}{b}(S_1' + 1) + \frac{1}{a}(S_2' + 1) + \frac{K_m}{D'}(S_1' + 1)(S_2' + 1)\right] \qquad (6)$$

The total resistance appears to be composed of three additive terms indicating partial resistances involving the rate constants of the three steps of the system. The full recognition of the usefulness of the transport resistance term will become evident in the discussion of the kinetics of inhibition, where resistance increments can be used for the characterization of inhibitory effects.

If in equation 6 $\frac{1}{a}$ and $\frac{1}{b}$ are very small as compared to $\frac{1}{D'}$—in other words if the rate constants of the reactions are high as compared to the diffusion constant—equation 6 is identical with equation 5. Only under these conditions will E-kinetics be observed in the range of saturating concentrations (if S_1', $S_2' \gg 1$). In non-enzymatic systems the experimental finding of E-kinetics will indicate, therefore, that the diffusion term is limiting.

LeFevre's treatment (248) assumes conditions like those underlying equation 6, with b as the limiting factor (second and third term negligible).

D-kinetics, in terms of transport resistance, means constant values for F (independent of S_1 and S_2). Equation 6 shows that for S_1', $S_2' \ll 1$ the transport resistance will be constant. Thus, under low saturation conditions, the transport again cannot be distinguished from simple diffusion by its kinetics.

c. Enzymatic systems (349). If the reactions concerned are enzymatic, certain approximations may be introduced in order to treat the process quantitatively. They are: 1) different sites for S and C or CS; 2) equilibrium between E and S and between E and C or CS; 3) The two equilibrium constants do not depend on whether or not the other site is occupied. 4) The migration of S (on the enzyme) to C (on the enzyme) contributes to the rate determination (in the steady state), which introduces considerable changes in the kinetics. The transport resistance then will be given by the more complex relation:

$$F = K_m \left[\frac{1}{E_1 n_1} (1 + S_1'')(1 + S_2') \left(1 + \frac{K_c}{C_t}\right) + \frac{1}{E_2 n_2} (1 + S_1')(1 + S_2'') \left(1 + \frac{K_c}{C_t}\right) + \frac{1}{D'C_t} (1 + S_1')(1 + S_2') \right] \quad (7)$$

The most obvious difference between equations 6 and 7 appears to be that in the enzymatic system characterized by equation 7, with saturating concentrations E-kinetics will always emerge, regardless of whether the diffusion term or one of the reaction terms is limiting. For S_1', S_1'', S_2', $S_2'' \ll 1$, F is again constant, indicating D-kinetics under low saturation conditions.

B. Systems capable of uphill transport

The systems discussed so far lead only to equilibration, not to accumulation. The diffusion of the carrier-substrate complex CS across the membrane will proceed as long as $CS_1 > CS_2$. As soon as equilibration has been reached, the gradient for CS vanishes, and no more net movement occurs.

Conditions for the possibility of uphill movements can be discussed in thermodynamic or in kinetic terms. Thermodynamic analysis of membrane transport processes (344) has been of special usefulness in cases where uphill transport is considered. Such transport must be energetically compensated for by spontaneous processes. It further appears from different quasi-thermodynamic approaches that, when a molecular flow of matter through a membrane is driven by chemical reactions, the coupling is mediated by another flow primarily produced by the reactions. Since the scalar forces of chemical affinity are not able to produce a unidirectional flow, the possibility of coupling requires a particular spatial arrangement, where some of the reactants of the energy-yielding process are separated by the membrane (Rosenberg, 1948 (344), Rosenberg and Wilbrandt, 1955 (349)). In the treatment of Jardetzky (203) this is expressed by the condition that there must be a source and a sink, which condition is considered to be a special case of a general theorem by Curie.

Weak interaction between the simultaneous flow of different quantities may be considered as normal, and the force exerted by one flow on another may be in either direction in such cases. However, uphill transport of considerable extent must be based on much stronger interaction, and will in general be due to chemical affinity between different components transported together. Thus, from the observation of an uphill transport of a component, S, it may be concluded that there must also be a simultaneous flow of another component, C, and that the two components are transported in the same direction, bound together more or less firmly in a complex, CS. The continuous supply and removal on either side of the membrane, then, is effected by chemical reactions, which may be of metabolic origin or may be due to the addition of another component, R, competing with S for C. The last-mentioned phenomenon has been called "counter-transport."

Kinetically, the requirements for uphill transport may be outlined as follows:

The fundamental rate equation for all carrier transport as given in equation 1, in the case of equilibrium between carrier and substrate, may be written in the form:

$$v = D'(CS_1 - CS_2) = D'\left(\frac{C_1S_1}{K_m} - \frac{C_2S_2}{K_m}\right) \tag{8}$$

In terms of this equation, the condition for a carrier transport system to be capable of operating uphill is that for $S_1 = S_2$ the transport rate v shall not be zero. Obviously this is possible either by changing the ratio $C_1:C_2$ or by the introduction of different values for K_m on the two sides of the membrane. An example of the verification of the first possibility is counter-transport. The second possibility may be realized by linking transport and metabolism through reactions changing the numerical value of K_m.

1. Counter-transport. Counter-transport is an uphill transport which is independent of metabolic energy. The necessary energy is derived from the downhill movement of a second substrate using the same carrier. Rosenberg (1948), in a thermodynamic treatment, has argued that the necessary condition for uphill transport is the maintenance of a gradient in the membrane for the carrier, either by communication with the surroundings or by chemical reactions on both sides of the membrane (344). Widdas (1952) has derived kinetic equations for the special case in which the chemical reactions concerned occur between carrier and a second, competing substrate (442). Rosenberg and Wilbrandt (1957) arrived at similar relations (351). Furthermore, they have shown that such uphill transport is not to be expected when the competition of the two substrates concerns fixed sites in the membrane (351).

In a qualitative way the phenomenon of counter-transport can be derived from a consideration of the gradients in the membrane. Suppose a first substrate, R, is equilibrated on the two sides of a carrier membrane. A second substrate, S, then is added to the external medium. This creates an inwardly directed gradient for the complex, CS, resulting in an outwardly directed gradient for the free carrier, C. The substrate R, originally equilibrated, then will meet unequal concentrations of free carrier, C, on the two sides of the membrane. Therefore, more of the complex CR will be formed on the inside and less on the outside of the membrane. A gradient of CR will thus arise, producing an outward movement of CR and the release of R on the outside, thus establishing an uphill transport for R.

a. The kinetics of counter-transport. Kinetic equations have been derived which characterize counter-transport in a quantitative manner. In the system discussed, after some substrate S has crossed the membrane and the concentrations S_1 and S_2 have been established, the rate of transport of R will be given by the following equation:

$$v_R = v_{max}\left(\frac{R'}{S_1' + R' + 1} - \frac{R'}{S_2' + R' - 1}\right) \tag{9}$$

$$= v_{max} \frac{R'(S_2' - S_1')}{(S_1' + R' + 1)(S_2' + R' + 1)} \tag{9a}$$

The equation shows that the condition for v_R to become zero is $S_1 = S_2$. In other words, as long as the gradient for S remains there will be a transport of R across the membrane. The transport then will occur uphill. It was shown that the uphill movement of R will continue until the following relation holds:

$$\frac{R_1}{R_2} = \frac{S_1' + 1}{S_2' + 1}, \tag{10}$$

which defines the maximal accumulation ratio that can be reached. The equation shows that only for $S_1' > 1$ is appreciable accumulation to be expected, *i.e.*, that high saturation on one side of the membrane is required for the counter-transport.

Counter-transport could be effected by gradients of metabolites or by gradients produced by transport of some other substance. It would, for example, be possible that a substrate A, after having been pumped up to a high concentration on one side of the membrane, moves down its own gradient, and thereby raises a second substrate B against *its* gradient. In this way one uphill transport (substrate B) would be driven indirectly by the energy used directly for the transport of another substrate, A. Primary and secondary transports could thus arise. Examples of counter-transport will be discussed in the treatment of sugar and amino acid transport.

b. Bearing on the conclusiveness of the carrier assumption and for the analysis of carrier systems. An important difference between carrier transport and adsorption transfers as discussed above lies in the fact that counter-transport can be elicited only in carrier systems, but not in adsorption systems. This can be shown by a comparison of equation 9 with the corresponding relation in the case of the adsorption transfer (under equivalent conditions):

$$v_R = v_{max} \left[\frac{R'}{S_1' + R' + 1} \cdot \frac{1}{S_2' + R' + 1} - \frac{R'}{S_2' + R' + 1} \cdot \frac{1}{S_1' + R' + 1} \right] \tag{11}$$

From this equation it can be seen that for all possible values of S_1 and S_2, v_R will be zero, in other words, no counter-transport can ensue.

In a more general way, counter-transport should be expected in all systems in which the binding site is movable (including the various modifications mentioned in the introduction), but not in systems involving fixed binding sites. Thus, it yields a criterion for the distinction of these two types of transport which is particularly valuable, since, as shown above, their overall transport kinetics do not differ. Since it was the movable binding site which was stressed in the introduction as the essential element of carrier transport, counter-transport holds an important position as a reliable criterion for the operation of carrier mechanisms in transport. The same is true with respect to the decision whether or not two transport substrates use the same mechanism. While, as will be

shown later, competition may well occur between substrates sharing only certain elements of a transport (*e.g.*, affinity to enzymes involved) without both being necessarily capable of forming a diffusible complex, the counter-transport phenomenon may be considered as proof that two substrates share affinity for the carrier.

c. Isotope counter-transport and its relation to Ussing's flux ratio criterion. A special case deserving a short discussion is that of R being an isotope of S. In cases where two different substrates for a transport system are not available, the possibility of performing a counter-transport test in this form may be welcome. The procedure, then, is to equilibrate the cell under investigation with a low concentration of a labelled substrate, to add a higher concentration of unlabelled substrate to the external medium, and to test for a transitory movement of labelled substrate from the cell into the external medium. If such a movement is observed, the transport may be concluded to involve a carrier mechanism.

This test has close connections with the well-known and widely used flux ratio criterion introduced by Ussing. In Ussing's test the unidirectional fluxes are determined by isotope methods. If their ratio is identical with that of the substrate activities on the two sides of the membrane (or, in the case of ions, with the ratio of what has been defined by Ussing as electrochemical activities), a transfer is concluded to be due to "passive penetration."

The quantitative relation between counter-transport and flux ratio can be demonstrated by introducing flux resistances,

$$F_1 \equiv \frac{S_1}{M_1} \quad \text{and} \quad F_2 \equiv \frac{S_2}{M_2}, \tag{12}$$

analogous to the previously defined transport resistance. Ussing's criterion then states that in passive penetration the opposite flux resistances at all substrate concentrations are equal:

$$F_1 = F_2. \tag{13}$$

In a carrier system the unidirectional fluxes of the over-all transport are given by the (independent) fluxes of the diffusion step. According to equation 3, then, the flux resistances are

$$F_1 = \frac{S_1 + K_m}{V_{max}} \quad \text{and} \quad F_2 = \frac{S_2 + K_m}{V_{max}}, \tag{14}$$

and consequently, Ussing's criterion is valid only for the case in which $S_1 = S_2$. If now in such a system, labelled substrate is equilibrated before the introduction of S, its two flux resistances will be equal:

$$F_1^* = F_2^* = \frac{S^* + K_m}{V_{max}}. \tag{14a}$$

The introduction of unlabelled substrate in the (higher) concentrations, S_1 and S_2, then will, according to equation 11, change the resistances to:

$$F_1^* = \frac{S^* + S_1 + K_m}{V_{max}} \quad \text{and} \quad F_2^* = \frac{S^* + S_2 + K_m}{V_{max}}, \tag{15}$$

or, since $S^* \ll S_1, S_2$,

$$F_1^* = \frac{S_1 + K_m}{V_{max}} \quad \text{and} \quad F_2^* = \frac{S_2 + K_m}{V_{max}}. \tag{15a}$$

The inequality of these resistances then will a) cause counter-transport (since the driving forces in the two directions are equal), and b) constitute a deviation from equation 13 involving the same factor as that found in equation 14. Corresponding conditions and equations apply if, instead of a labelled isotope, another substrate is used for the same carrier.

In an adsorption system, on the other hand, the flux resistances derived from equation 11 are equal. Consequently, the penetration here is passive according to Ussing's criterion, and no counter-transport can be induced.

This close correspondence between the two tests appears to be in good agreement with the conclusion arrived at by Ussing (420) in his well-known paper, "The distinction of diffusion and active transport by means of tracers," in the summary of which he writes: "Deviations from the equation indicate that the ion does not diffuse in the free state only, but, in part at least, as a component of some other moving particle in the membrane." According to this summary, what is indicated by a discrepancy between flux ratio and activity ratio corresponds very closely to the essentials of carrier transport.

2. Metabolic reactions involving changes in the carrier-substrate affinity. a. A simplified rate equation. The second possibility for uphill movement in a carrier system emerging from equation 8 is a difference in the values of K_m on the two sides of the membrane:

$$v = V_{max}\left(\frac{S_1}{S_1 + K_1} - \frac{S_2}{S_2 + K_2}\right). \tag{16}$$

If $K_2 \gg K_1$, S will move uphill until a steady state is reached, ($v = 0$), in which:

$$\frac{S_1}{S_2} = \frac{K_1}{K_2}. \tag{17}$$

Thus equation 17 gives the maximal accumulation ratio. For thermodynamic reasons, however, a numerical difference between K_1 and K_2 cannot exist without a coupling to other energy-yielding systems. One possibility of such a coupling which has been frequently assumed is that of metabolic reactions acting on the carrier in a manner which is asymmetric with respect to the membrane.

b. A more complete treatment. A possible scheme for the connection between an energy-yielding reaction and the carrier mechanism proper is the following.

Suppose the carrier C reacts at the inside of the membrane with a metabolite A from the internal medium, to form both the compound B, and the modified "carrier," Z, and Z is no longer capable of reacting with the substrate but is still capable of crossing the membrane: $C + A \rightleftharpoons Z + B$. If the metabolism, by providing A and removing B, keeps the concentration of these two metabolites constant, and if the assumed reaction is rapid so that equilibrium may be assumed, the following relation will hold:

$$Z_i = C_i \frac{A}{B} K_i = C_i K_x , \tag{18}$$

in which K_x is a constant. On the other side of the membrane, either a spontaneous back-reaction may be assumed by which Z is transformed back into C, or, more likely, this reaction will in a similar way be linked with metabolism: N reacting with Z and forming a second metabolite M, simultaneously with C. The concentration of Z on the external side (Z_0) then will be given, under conditions resembling those assumed above for equation 18, by the following relation:

$$Z_0 = C_0 \frac{M}{N} K_0 = C_0 K_y . \tag{19}$$

From equations 18 and 19 and the rate equation 8 (holding for equilibrium between C and S), the relation emerges

$$v = v_{max} \left[\frac{S_1}{S_1 + K_m(1 + K_x)} - \frac{S_2}{S_2 + K_m(1 + K_y)} \right], \tag{20}$$

which is identical with equation 16 if K_1 is replaced by $K_m \cdot (1 + K_x)$, and K_2 by $K_m \cdot (1 + K_y)$. If the conditions for constant and different values of K_1 and K_2 indicated by these relations are kept in mind, it is possible to use the simpler form of relation in equation 16 for the following discussion.

3. Leak-and-pump systems. There are now numerous observations on record showing intracellular accumulation of various transport substrates, particularly amino acids, ions, and sugars. In a number of these cases the accumulation has been interpreted by various authors in terms of two opposing fluxes of substrate, an inwardly directed flux following Michaelis-Menten kinetics and an outwardly directed flux with linear kinetics. The net rate of entrance of substrate then will be given by the equation:

$$v = A \frac{S_1}{S_1 + K_m} - BS_2 . \tag{21}$$

Under these conditions a steady state finally will be reached in which the two fluxes are equal and the internal concentration remains constant:

$$S_2 = \frac{A}{B} \frac{S_1}{S_1 + K_m} . \tag{22}$$

The equation shows that the relation between the internal and external concentrations is formally identical with that between a reaction rate and the substrate concentration according to Michaelis and Menten. A plot of the reciprocals of external *vs.* internal concentration, then, will yield a straight line.

The inward flux in such an accumulation system necessarily must be a pump, in agreement with the general interpretation of these observations. With respect to the outward flux, in view of the linear kinetics assumed, a simple diffusion process might be—and in some cases has been—assumed. It should be noted, however, that as repeatedly stressed in the previous discussion, linear kinetics may well be observed in a carrier system if the saturation is low. Quantitative agreement with equation 21 or 22, therefore, cannot be considered as conclusive evidence that the leak is a process of simple diffusion. In fact, in the experimental part observations will be discussed indicating that, in some cases at least, the outward flux cannot be simple diffusion.

A characteristic feature of "leak-and-pump" systems, reported in several observations, is the following. If, after a first substrate, S, has reached a certain steady state level of accumulation, a second substrate or a competitive inhibitor is added to the external medium, the steady state level of accumulation falls, substrate S, accumulated previously, moves out of the cell, and a new level is established. In terms of equation 22, the effect of the second substrate then may be interpreted as due either to a decrease of A or an increase of K_m, or to the introduction of an inhibitor term in the manner indicated by equation 9 or 23. It would appear, however, that the assumption of two different pathways for exit and entrance is not obligatory for the explanation of the kinetics expressed by equations 21 and 22, or of the displacement phenomenon.

The formal resemblance of equation 22 to an adsorption isotherm and the observation that the effect of a competing substrate (lowering of the steady state level of accumulation) quantitatively resembles the replacement in an adsorption system, might invite the interpretation that the accumulating uptake of the substrate is a process of adsorptive binding rather than an accumulation by a pump. Arguments furnishing conclusive evidence against this interpretation in the case of bacterial permeases and of amino acid accumulation in tumor cells will be discussed in the experimental part. Other observations related to the phenomenon will be mentioned in the discussions of cation transport in red cells, and anion transport in various types of glandular cells.

C. Competition and inhibition

An obvious corollary of all transport systems involving binding of the transport substrate to specific sites is the possibility of competition and competitive inhibition. In principle, it is common to adsorption systems and to carrier systems, both in a broad sense of the word. Because of the basic differences between these two types, however, parallelisms are restricted, and with certain types of inhibitors, the kinetics differ appreciably. Before entering into the discussion of inhibition kinetics it will be useful to discuss the different types of inhibition that may be expected.

1. Possible types of inhibitors (*352*, *453*). In accumulating systems a first distinction would appear to be between inhibitors blocking the supply of energy and others interfering directly with the transport mechanism proper.

It is obvious that an uphill transport requires the supply of energy in some form. Two possibilities have been discussed: counter-transport, elicited by a counter-gradient for a substrate of the same system, and a link to metabolic reactions affecting the affinity. A large variety of inhibitors of metabolism therefore will be expected to interfere with transport, either by blocking the supply of a metabolite causing counter-transport or by blocking energy-yielding reactions like those discussed above in connection with equations 18 and 19. They include compounds like cyanide, carbon monoxide, dinitrophenol, azide, monoiodoacetic acid, and others for the inhibitory effects of which on transport a wealth of observations exists. In the chain of reactions finally leading to uphill transport, the sites of the inhibitory reaction in most of these cases will be more or less distant from the transport reactions proper. In general, therefore, they will contribute little to the understanding of the transport mechanisms and will not be treated extensively in this review. The term *secondary inhibitors* has been proposed for this group. On the other hand, transport itself and the immediately linked chemical reaction offer a number of possibilities for inhibitory reactions; substances acting here may be termed *primary inhibitors*. It is this group which appears to offer most interest for the purposes of the present review. Inhibitors of equilibrating transports, in general, belong to it.

Within the group of primary inhibitors, again, a number of different types may be expected depending on the complexity of the system visualized. If the reaction between carrier and substrate is non-enzymatic there are two possibilities corresponding to competitive and non-competitive inhibition of enzymes: a reaction with the carrier at, or close to, the site of reaction with the substrate (competitive inhibition), or a reaction at a different site, interfering with the capacity of the carrier to react, which is independent of the substrate concentration (non-competitive inhibition). In cases where, for reasons discussed above, simple Michaelis-Menten kinetics hold, the distinction is made easy by the graphic method introduced by Lineweaver and Burk: the straight line presenting the system in the absence and presence of the inhibitors will have a common intercept with the ordinate axis in the case of competitive inhibition, and different intercepts in the case of non-competitive inhibition. Obviously, in the case of enzymatically catalyzed reactions an additional group of possible inhibitors will be enzyme inhibitors. Competition in this case will be possible both with the substrate and with the carrier.

This brief consideration demonstrates the possible existence of five inhibitory types: inhibitors reacting non-competitively with the enzyme or with the carrier, substrate-competitive or carrier-competitive inhibitors reacting with the enzyme, and finally substrate-competitive inhibitors reacting with the carrier. It has been proposed to term these types, in the order mentioned: I_{EN}, I_{CN}, I_{ES}, I_{EC}, and I_{CS}, the first index indicating the site, the second the type of inhibitory reaction.

In the interpretation of experimental results these distinctions in general

have not been made. In most cases substrate-competitive inhibitions have been referred to a reaction with the carrier. Actually kinetic analysis, as will be seen, while yielding clear-cut criteria for the distinction between substrate-competitive and non-competitive types, does not allow an easy differentiation between substrate-competitive inhibition on the enzyme and on the carrier.

With respect to the inhibitor type I_{cs}, a further distinction is essential between compounds fixing the carrier in the interface (for instance, due to a pronounced hydrophilic molecular structure), and others resembling the substrate more closely which are capable of penetrating the membrane in the form of carrier-complexes. The kinetics for the latter type of inhibition differ fundamentally from that of the first due to the complicating interference of counter-transport. For the non-penetrating and the penetrating types the terms I_{cs} (np) and I_{cs} (p) have been proposed.

2. Inhibition kinetics. Since the inhibitor type I_{cs} (p) has special importance and since, as discussed, it differs from other types in a pronounced manner, it appears advisable to treat this special type first. The other types subsequently can be included in a common discussion.

a. Inhibitor I_{cs} (p). If an inhibitor of this type is present in a carrier system in the same concentration on the two sides of the membrane, the kinetics of the transport of the substrate S from the concentration S_1 to the concentration S_2 will follow a relation essentially similar to equation 9:

$$v = v_{max}\left[\frac{S_1'}{S_1' + I' + 1} - \frac{S_2'}{S_2' + I' + 1}\right] \tag{23}$$

The peculiar situation arising from the fact that this type of inhibitor acts on the transport not only by inhibition, but in addition by counter-transport, is seen immediately from the fact that, depending on the conditions, I_{cs}(p) may either inhibit or accelerate the transport. This is shown by the following consideration. If, in order to assess the effect of the inhibitor, I, the difference Δv between the rates in the absence and in the presence of I is obtained (by subtracting equation 23 from equation 3a), this difference is found to contain the factor $(I' + 1 - S_1'S_2')$. This means that only if $I' + 1 > S_1'S_2'$ has Δv a positive sign (implying inhibition of the transport), whereas for $S_1'S_2' > I' + 1$ the sign is negative. In other words, while high inhibitor concentrations slow the transport, low concentrations accelerate it. Under the latter condition the force introduced by the counter-transport overbalances the increase in resistance produced by the competitive inhibition.

If, in equation 23, the second saturation term is negligibly small as compared to the first (such possibilities have been discussed above), again the relations are much simpler. In this case the rate will be given by the product of the capacity term and the first saturation term in equation 21. This then is the case in which a Lineweaver-Burk plot will give a straight line of the type characteristic for competitive inhibition in enzyme reactions. Numerous observations of this kind have been reported.

For competing substrates, values of K_m determined from their inhibitory power (using either Lineweaver-Burk plots or equation 23) and from their penetration rates (using equation 3 unmodified or in a simplified form) should agree. Discrepancies invite a re-examination of the assumptions used.

b. The resistance increment as a quantitative measure of inhibition. The degree of inhibition in general is indicated by the fractional decrease of a rate (fractional inhibition or percentage inhibition). With increasing complexity of the system, however, the expressions representing fractional inhibition become too involved for useful application. As mentioned previously, the concept of transport resistance permits characterization of an inhibitory effect by an additional resistance term, thus yielding simpler and therefore more useful equations.

c. Other inhibitor types. Non-penetrating inhibitors. The equations relating the transport resistance increment to the substrate concentration for the various types of inhibitors will not be given here, for the sake of brevity. Three different types of relations are obtained for a) I_{ES} and $I_{CS}(np)$, b) I_{EN} and I_{EC}, and c) $I_{CS}(p)$. A kinetic analysis thus allows distinction between a) and b) or c), but not between I_{ES} and I_{CS}, or between I_{EN} and I_{EC}. The use of non-penetrating inhibitors ($I_1 \neq 0$, $I_2 = 0$) yields particularly useful criteria: for a) ΔF rises linearly with increasing S_2 independently of S_1, while for b) ΔF depends both on S_1 and S_2.

Because of the singular position of the type $I_{CS}(p)$ (*cf. supra*), only for this type does ΔF decrease with increasing substrate concentration and may (for high substrate concentrations) assume negative values. This is in accord with the conclusion drawn from equation 23 that this type of inhibitor may, under certain conditions, accelerate rather than inhibit; this serves as another illustration of the fact that here the inhibitor has a double action: a) it affects resistance, and b) it introduces new forces (counter-transport).

3. The asymmetry of inhibition in equilibrating carrier systems. Inhibitor type $I_{EC\text{-}S}$ (352, 453). Since any type of substrate-competitive inhibition must, by definition, depend on the substrate concentration, it is to be expected that under certain conditions the effect of an inhibitor on entrance of a substrate into a cell and on exit from the cell may be different. For external non-penetrating inhibitors of the types I_{ES} and I_{CS}, it was shown that the transport resistance increment depends only on the inside substrate concentration, independent of the external concentration. The asymmetry of inhibition to be expected in this case therefore should be characterized in the following way. Since in general in entrance experiments the internal substrate concentration will be lower than in exit experiments, this will lead to higher ΔF values, *i.e.*, stronger inhibition for exit than for entrance. In a ΔF *vs.* S_i plot the points obtained in entrance and exit experiments, however, should lie on the same straight line. This type of asymmetry, as expected for I_{ES} or I_{CS}, may be termed *asymmetry of first order.*

As was shown, an asymmetry of higher order must be expected in the case of an inhibitor of a type not discussed so far, termed $I_{EC\text{-}S}$. It is characterized by a twofold substrate-competitive action, one at the binding site of the substrate on the enzyme, and the other at the enzyme-bound (but not on the free) carrier. This type of action might also be considered as an enzymatic reaction of I

with C. The equation relating ΔF to S_1 and S_2, in this case, contains v, implying that ΔF will depend on the sign of v, *i.e.*, on the direction of the transport. This is not so for the types discussed above. It is due to the fact that asymmetry will be induced in this case not only by the difference between S_1 and S_2, but in addition by that between C_2 and C_1. Observations pointing to the existence of this inhibitor type (by the fact that the ΔF *vs.* S_2 plots do not coincide for entrance and exit) will be discussed in the special part.

D. Criteria for carrier mechanisms

What conclusions can be drawn from the phenomena and relations discussed here, with respect to the weight of various arguments for or against the assumption of a carrier mechanism in a given case? Which criteria are conclusive, which suggestive, and which uncertain, for or against the operation of carriers?

Only a very short discussion of some pertinent points will be attempted here. It should first be stressed that there are no unequivocal criteria to prove that a transfer cannot be a carrier transport. Under low saturation conditions the kinetics in all systems discussed above are indistinguishable from those of simple diffusion; furthermore, competition and counter-transport cannot be observed (provided that low saturation holds for both substrates involved), and the flux ratio will be practically identical with the activity ratio. The only indication, under these conditions, that some binding reaction may be involved, is an unexpectedly high structural specificity, particularly stereochemical specificity, which, however, may be observed regardless of whether the binding site is fixed or movable.

Among the positive criteria, both saturation kinetics and competition indicate binding, but not necessarily to a movable site. The demonstration of counter-transport or isotope counter-transport, then, will be welcomed as rendering existing evidence conclusive. The demonstration of uphill transport is highly suggestive of a carrier mechanism. Although, according to Rosenberg (344), there are a number of possibilities for the flow of a thermodynamic quantity from a higher to a lower potential, capable of raising a substrate from a lower to a higher chemical potential, practically speaking it will in most cases be the flow of chemical matter. The necessary link between the two flows, then, will be binding of the two substrates to each other or to a common movable site.

IV. SPECIAL PART. EXPERIMENTAL OBSERVATIONS

A. Sugars and related compounds

Although in some cases intracellular accumulation of sugars has been established, in the majority of cells only equilibration of these substrates is observed. This has offered a valuable opportunity to study the characteristics of the transport mechanism proper, independent of its connection with energy-yielding metabolic reactions. The lack of accumulation might lead—and actually in some cases has led—to the conclusion that sugar penetration occurs by simple diffusion. However, while in a considerable number of cell types special transport

mechanisms for sugars have been demonstrated, the presence of such mechanisms has not been excluded in any system.

In a number of cells sugar metabolism is rapid as compared to penetration, so that the transport in general will not reach equilibrium. Various means have been employed to overcome this difficulty, including metabolic inhibitors, low temperatures, non-metabolized substrates, and finally the use of the steady state internal sugar concentration as a criterion. This concentration will be determined by the rates both of penetration and of utilization. Provided that the utilization rate is known, changes in the penetration rate can be evaluated.

Increasing evidence has accumulated during the past years of an effect of insulin on the transport system. While only a few years ago the discussion as to whether the primary site of action of insulin is on metabolism or on cell penetration was still intense and opinions were divided, it now appears established that at least part of the insulin action concerns the entrance of sugars into the cell. Moreover, it has become possible to characterize the effect of insulin in a quantitative manner in terms of the prime parameters of simple transport systems, capacity and affinity.

1. Observations in various cells. a. Mammalian cells and cell layers not reported to respond to insulin (red cells, lymphocytes, tumor cells, L-cells, placenta). The *red cell* has the advantage of a metabolic rate sufficiently low to allow sugars to equilibrate, consequently extensive studies of this cell have been conducted.

The question of specificity and of the structure-activity relations in the transport system involved has been dealt with by LeFevre in this Journal (246) and will not be discussed here.

With respect to penetration kinetics, early observations pointed to the inadequacy of an interpretation based on simple diffusion. In 1920 Ege *et al.* (114), finding the penetration of glucose into human red cells to be much faster from lower than from higher sugar concentrations, concluded that adsorption at the cell surface precedes penetration. Similar deviations from diffusion characteristics were later found in the attempt to determine permeability constants for glucose (7, 456). A more recent analysis (443, 455, 460) accounted very satisfactorily for these peculiarities as well as for others (272). It revealed close agreement with the carrier kinetics as derived in the general part (p. 116): at low concentrations of glucose D-kinetics are approached (104, 448), whereas at high concentrations excellent agreement with E-kinetics was found (443, 455, 460). Sorbose (443) and fructose (446), even in high concentrations, followed D-kinetics. This is in accordance with the theory, since from competition experiments (see below) sorbose and fructose are known to have a low apparent affinity for the system, which, according to equation 4, favors D-kinetics.

As to the numerical values of the parameters in human cells, three different methods of evaluation of K_m yielded 9 mM (244), 10 mM (443) and 8 mM (452), and for v_{max} a value of 600 mmol/L cell H_2O per min was reported. In rabbit cells, Park *et al.* (313) found K_m = 43 mM and v_{max} = 125 mg/hr per 100 ml cells (equivalent to *ca.* 0.15 mmol/L cell H_2O per min), *i.e.*, about the same K_m, but v_{max} 4000 times lower than in human cells. For different sugars,

contrary to prediction for simple systems as represented in equation 2, numerical equality in v_{max} was not generally observed; the values found decreased in the order D-glucose = D-mannose = D-galactose = D-xylose > L-arabinose > D-ribose (454). This indicates some inadequacy in the assumptions underlying equation 2, possibly a slow rate of reaction (*i.e.*, no equilibrium with respect to the reaction between C and S).

Competition for the transport between different sugars has been demonstrated repeatedly (247, 313, 445, 448), and was most extensively studied by LeFevre and Davies (247). They found the following order of decreasing affinity: glucose, mannose, galactose, xylose, arabinose, sorbose, and fructose. For more details LeFevre's (246) review should be consulted. The prediction that the rate of transport will be inversely proportional to K_m only under low saturation conditions, whereas at high concentrations the order should be reversed (see p. 117), was verified in experiments with 5 sugars in human red cells (447). Counter-transport and isotope counter-transport were demonstrated in two studies (313, 351) using glucose and mannose gradients for inducing uphill transport of labeled glucose or of xylose in human and in rabbit cells, respectively. A large number of inhibitors was found to slow or block sugar transport (38, 243, 244). They include SH-inhibitors like *p*-chloromercuribenzoate (243), $HgCl_2$ (243, 459), gold chloride (459), various lachrymators (459), the phlorizin group (phlorizin (243, 445), its aglucone phloretin (250, 459), and "polyphloretin phosphate," a polymerized phloretin phosphate (38, 459)), various general anesthetics in high concentrations (459), corticosteroids like cortisone and desoxycorticosterone (cortexone) (350), and protein reactors like tannic acid and dinitrofluorobenzene (38).

The action of phlorizin and polyphloretin phosphate has been analyzed in several studies (38, 348, 352, 459). Kinetically they were found to display a substrate-competitive action. With the ΔF-test (*cf.* p. 128), a pronounced inhibition asymmetry of higher order was observed (352); it was interpreted to indicate the inhibitor type I_{EC-S} (*cf.* p. 128).

Summarizing the experiments with sugar transport in red cells, this system, with a rare completeness, displays the characteristic features of equilibrating carrier transport discussed in the general part.

In one study in which *lymph node cells* were investigated by Helmreich and Eisen (172) it was found that between 17 and 37°C penetration was too rapid to be followed. At lower temperatures the sugars investigated did not equilibrate because of rapid metabolism. Blocking metabolism by iodoacetic acid led to equilibration. From the steady state concentration and the rate of uptake, conclusions were drawn as to the rate of penetration. Phlorizin lowered the rate of uptake without affecting the steady state concentration; this was taken to mean that it inhibits penetration and utilization to the same extent. In a similar way competition was demonstrated for transport between glucose and mannose, as well as between glucose and fructose.

A number of reports deal with sugar penetration into ascites *tumor cells*. With cells of Gardner lymphosarcoma, Nirenberg and Hogg (298, 299) found

competition between galactose and fructose with respect to the rate of utilization by whole cells, but not by homogenates. It was concluded that the competition was for transport rather than for the enzyme system (in accordance with the observation that galactose does not affect hexokinase activity). In whole cells, fructose was utilized twenty times more slowly than glucose, while in the homogenate the rates were equal, again indicating differences in penetration. On a similar basis, transport inhibition by a number of steroids, including testosterone, desoxycorticosterone (cortexone), diethylstilbestrol, and progesterone, was demonstrated.

In a study of Ehrlich ascites tumor cells (80) a marked temperature dependence of sugar penetration was observed, with respect not only to the transport rate but also to the parameters v_{max} and K_m. Lineweaver-Burk plots, in a first approximation, appeared to be shifted in parallel upward at lower temperatures, indicating a marked decrease of v_{max} (for instance, 7-fold between 15°C and 10°C for 3-methyl glucose) with a corresponding change in K_m, the ratio $\frac{K_m}{v_{max}}$ remaining approximately constant. At 20°C the plot yielded a straight line through the origin, indicating D-kinetics. Furthermore, v_{max} for galactose and for 3-methyl glucose (both at 10°C) were considerably different. The authors commented: "One would hesitate to attribute such temperature coefficients to enzyme catalysed reactions or to a 'carrier' type of mechanism of sugar transport," but they would be "not too difficult to imagine . . . if they involved the lipid protein layers of the cell membrane." Structural specificity was pronounced: while 3-methyl glucose, D-galactose, L-arabinose, and D-xylose penetrated rapidly, L-sorbose was slower and D-ribose slowest. The values of K_m evaluated from the initial rates ranged between 0.032 and 1.6 M.

Tissue cultured *L-cells* (originating from mouse fibroblasts) were studied by Rickenberg and Maio (336) with respect to entrance of labelled galactose. The rate of galactose uptake appeared to follow Michaelis-Menten kinetics with competitive inhibition by glucose, by phlorizin and, much more strongly, by phloretin (as indicated by Lineweaver-Burk plots). The values obtained for K_m with respect to penetration were 1 mM for glucose and 0.5 mM for galactose. However, in experiments in which glucose inhibited galactose uptake and galactose inhibited glucose uptake, different K_m values were obtained for the same sugars from their competitive powers: 22 mM for galactose and 13 mM for glucose. As was pointed out previously, on the basis of relatively simple assumptions like those used in the derivation of equations 3a and 19, no difference between K_m values obtained from penetration and from inhibition should be expected. Such a difference, therefore, would appear to indicate again that the simple carrier scheme used for the derivation of these equations should only be considered as a first approximation and that the meaning of the parameters v_{max} and K_m, as determined from penetration and inhibition experiments, require further study.

With *placenta*, Widdas (442) found non-linear kinetics for glucose transport, interpreted by him as carrier kinetics.

b. Mammalian cells reported to respond to insulin (muscle, heart muscle, lens, ciliary body). α. Evidence for a saturable system. Helmreich and Cori (171) studied the penetration of pentoses into striated muscles of eviscerated animals and observed no competitive inhibition of the entrance of pentoses or of galactose (non-metabolized sugars) by either glucose or 3-methyl glucose. A Lineweaver-Burk plot for xylose entrance yielded a straight line going through the origin, indicating D-kinetics.

As early as 1939, however, findings of Lundsgaard (264) had pointed to a saturable mechanism. He found the rate of sugar uptake in perfused muscles not to be a linear function of the sugar concentration but to approach a saturation level at higher concentrations. Since no free glucose was found in the muscle cells, the non-linear kinetics could not be referred to the metabolism and pointed to a non-linear transport mechanism.

In recent years several observations on competition between sugars were reported. Kipnis and Cori, while finding no competitive inhibition of xylose entrance into the intact diaphragm by glucose (218), observed mutual inhibition between glucose, 2-desoxy-glucose, and mannose (219). The conclusion that non-metabolized and metabolized sugars use different pathways for entry into the muscle was recently questioned by Battaglia and Randle (10, 11). In the intact diaphragm preparation they found competition not only between metabolized sugars but also with a number of non-utilized compounds.

Thus, considerable evidence today points to a saturable sugar transport system in striated muscle.

With heart muscle, Fisher and Lindsay (125) likewise observed competition between glucose and galactose for the entry mechanism. Park *et al.* later (314) demonstrated counter-transport of 3-O-methylglucose induced by a glucose gradient in this organ.

β. The action of insulin. The suggestion of a hormonal regulation of sugar transport by the islet system was made as early as 1914 by Höber (in an addendum to a paper by Kozawa), 8 years prior to the discovery of insulin (231). It was prompted by unexpected specificities of sugar transfer in red cells, referred to above. For these particular cells the suggestion could not be substantiated later. Nevertheless, in retrospect this early prediction appears noteworthy. In 1939 Lundsgaard observed that the rate of sugar uptake in perfused striated muscle was increased in the presence of insulin (264). Since the intracellular glucose concentration was shown to be zero, the rate of entrance appeared to be limiting, indicating that the insulin effect must be on transport rather than on metabolism. The decisive stimulus came from the work of Levine *et al.* (253, 254) in 1949, who showed in eviscerated and nephrectomized dogs that the distribution volume of injected galactose, normally about 45% of the total volume, increased to 75% (practically identical with that of total water) in the presence of insulin. Galactose thus appeared to enter cells which normally are impermeable to it. The finding was confirmed by Drury and Wick (109) in 1952 in rabbits. In man, similar observations were reported by Segal *et al.* (369). Park's group (313) strengthened these observations, first by direct chemical

analysis of the sugar content in various muscles (heart, diaphragm, gastrocnemius) of eviscerated animals (312), and later by the *in vitro* demonstration of the insulin effect on isolated diaphragm, in this way excluding more stringently any possible interference by factors like release of epinephrine (adrenaline) (311). In eviscerated and nephrectomized rats, Helmreich and Cori reported essentially similar observations (171). Kipnis and Cori (218) found a 2- to 3-fold increase in the penetration rate of pentoses in the rat diaphragm, the distribution volume rising from 50 to 80% of the intracellular water. In a subsequent paper, the same authors (219) reported that insulin counteracts inhibition of glucose entry by intracellular 2-desoxy-D-glucose-6-phosphate.

In some of the observations mentioned so far, the effect of insulin was to increase the penetration rate, and in others the apparent distribution volume. The suggestion has been made repeatedly that the latter effect is due to opening of additional intracellular spaces for the entrance of sugar, implying a compartmentalization of the intracellular water space by penetration barriers. One consequence of this interpretation would be that insulin must be assumed to be capable of penetrating the external cell membrane, which, in view of the molecular weight of this compound, appears questionable. A possible interpretation not involving this consequence might be that distribution volumes higher than the extracellular but lower than the total water space for non-metabolized sugars are due to counter-transport phenomena. In general, because of the high rate of metabolism of glucose, the internal concentration of this sugar is practically zero in muscle and heart muscle cells, implying a steep glucose gradient. If the apparent distribution volume of non-metabolized sugars is decreased by counter-transport due to this gradient, the insulin effect on the glucose transport rate might, by way of a diminution of the glucose gradient, account for the increase of the apparent distribution volume of non-metabolized sugars. The increase in the numerical value of K_m in the presence of insulin (see below) might add to this effect, as can easily be derived from equation 10.

Other responses of sugar transport to insulin were reported for two cell types in the eye. Sugar penetration across the blood-aqueous barrier in the rabbit ciliary body was observed by Ross (353, 355) to be diminished in alloxan diabetic animals and to be inversely related to the blood sugar concentration. He further found (354) that the rate of sugar uptake by the isolated rabbit lens was increased in the presence of insulin by 350%, while the effect in the homogenate was only 33%; this was interpreted to indicate that insulin acts on the penetration of sugar across the cell membranes. These conclusions were recently questioned by Farkas and Patterson (121), who claimed that the effects of insulin and diabetes are indirect.

Fisher and Lindsay (125) found a valuable object for the demonstration and analysis of insulin action in the perfused rat heart, in which an effect on galactose distribution resembling those reported above was observed. In this test organ, recent work by the Park group has furnished most valuable contributions to the problem of insulin action. It was demonstrated with L-arabinose (314) that exit from a preloaded heart into an arabinose-free perfusion solution (practically

unidirectional and linear) was considerably faster in the presence of insulin. This finding shows that the insulin effect is independent of the direction of sugar movement, which excludes the introduction of pump-like elements as a possible component of the insulin effect.

More recently (284), in a noteworthy kinetic analysis it was shown that the steady state concentration of internal glucose can be satisfactorily attributed to simultaneous penetration into the muscle and removal by phosphorylation, if penetration is assumed to follow equation 3, and phosphorylation the Michaelis-Menten equation. Agreement with the observed rates of utilization and internal concentrations in the presence and absence of insulin could be shown if a 5-fold increase in K_m and a 13-fold increase in v_{max} in the presence of insulin was assumed. Insulin thus appears to act both on the capacity factor and on the affinity factor, increasing them to comparable extents. In view of this double action, Rosenberg (346) made the suggestion of a second carrier system, activated by insulin, arguing that in an action on the "normal" system a change in one parameter only would appear more likely. R. B. Fisher (124a), in a similar analysis of the effect of insulin on the transfer of D-xylose and L-arabinose, arrived at qualitatively corresponding results insofar as insulin increased v_m (30- and 400-fold, respectively). The capacity factor, v_{max}, however, was decreased by insulin (5.5- and 2-fold, respectively) in his experiments.

The effect of insulin on L-arabinose exit from heart muscle cells was more pronounced under aerobic than under anaerobic conditions, partly due to the fact that in the absence of insulin the transport rate was higher under the latter condition. Similar effects of anaerobiosis were reported by Randle and Smith (330, 331, 332) for the rat diaphragm. These authors further found that dinitrophenol, like anaerobiosis, accelerates sugar transport, as indicated by an increase of the apparent distribution volumes both of glucose and of xylose. They concluded that glucose entry is restrained by a process dependent on a supply of a substance generated during oxidative phosphorylation. In a later paper (333) they discussed the possibility that the sugar carrier may be phosphorylated by ATP or another high-energy phosphate compound, and, in the phosphorylated form, is unable to carry glucose. The effect of insulin, which, according to Stadie (406) does not interfere with oxidative phosphorylation, was interpreted as a specific action on phosphorylation of the carrier in the cell barrier.

c. Epithelial layers transporting uphill (intestine and kidney tubules). The fact that the kidney tubules are capable of *transporting sugars uphill* was finally established by the well-known micropuncture experiments reported by Wearn and Richards in 1924 (435), in which the normal glomerular filtrate was shown to contain glucose. If the glomerular filtrate contains glucose and the urine is free of it, it must have been reabsorbed uphill. Later, by tubular punctures, reabsorption from the frog proximal tubule was demonstrated directly and followed quantitatively by Walker and Hudson (434). In the mammalian kidney, on the basis of current methods of kidney physiology, uphill reabsorption is shown conclusively, when the concentration ratio, urine:plasma, for a substrate falls below 1.0.

That the intestinal epithelium is capable of uphill transport was demonstrated *in vivo* by Barany and Sperber (8), and *in vitro* by Fisher and Parsons (127). The first observations *on intestinal sugar absorption* which differed from expectations for diffusion systems concerned structural specificities. In 1902 Nagano (290) reported decreasing rates of absorption from the intestine of dogs in the order galactose, glucose, fructose, mannose, xylose, and arabinose. The same order was found in the rat by Cori (73), by Wilbrandt and Laszt (458), and by Westenbrink (438), and also in the pigeon (438), the frog (278), the guinea pig (156), and the rabbit (166). In man, Groen (154) obtained corresponding results for galactose, glucose, and fructose by the use of the Miller-Abbott tube. The order therefore appears to be quite general. Recently Wilson and Crane (463) and Wilson and Landau (464) tested a large series of sugar derivatives, mainly desoxy-sugars and methylated sugars, with respect to the ability to be absorbed uphill from the intestine. Of a total of 49 sugar compounds, only 14 were transported uphill. From the structural characteristics of the transported and non-transported sugars, the authors concluded that for uphill transport the hydroxyl group on carbon-2 is required, whereas the hydroxyl groups on the other carbons are dispensable. Crane (79) recently has surveyed the field of intestinal sugar absorption; for more details his excellent review should be consulted.

In the *kidney* of the frog the comparative rates of reabsorptions of a number of sugars were studied by Hamburger in 1922 (159) and by Höber in 1933 (175). Hamburger, interpreting his observations in terms of selective permeability of the glomerulus, was led to the conclusion that the glomerular membrane is impermeable to glucose but permeable to lactose and other di-saccharides. While this interpretation, which assumed unusual structural specificity, today is known to be erroneous for the glomerular membrane, essentially similar conclusions have recently been drawn from observations on bacterial permeases, to be discussed later (62). The order of decreasing reabsorption rates in the kidney was not identical with, but closely similar to that found in intestinal absorption. For various reasons an exact comparison appears impossible. In both cases, however, the higher absorption rate of hexoses as compared to pentoses, contrary to the expectation on the basis of molecular weights, is noteworthy.

The *kinetics* in both systems were observed to be non-linear. For absorption from the intestine, Cori's early observation of 1925 (73) that the rate of glucose absorption is independent of the sugar concentration was confirmed and extended in later studies *in vivo* by Verzár in 1935 (430) and by Vidal-Sivilla (433), using the ligated loop method. The absorption rates of glucose and galactose varied little with the concentration, whereas for xylose, sorbose, and mannose they were approximately proportional to the concentration. Fructose was intermediate. The linear kinetics for xylose, sorbose, and mannose, interpreted at the time as indicating diffusion, are consistent with a carrier transport of substrates with high K_m (see p. 117). The recent introduction of methods for the quantitative study of intestinal absorption *in vitro* (93, 126, 394, 462, 465, 466) allowed more accurate quantitative analysis of the kinetics. Fisher and Parsons in 1953 (128) found the rate of glucose absorption to follow the Michaelis-Menten equa-

tion with an apparent dissociation constant, K_m, of 8 to 9 mM. Riklis and Quastel in 1958 (339), with a similar method, obtained 7 mM. Additional values for other sugars are listed by Crane (79).

In the kidney the conditions for an accurate kinetic analysis are less favorable (389, 446). Glucose is completely reabsorbed at plasma concentrations within the normal range. When the plasma concentration is raised, reabsorption becomes incomplete and sugar appears in the urine. From a certain plasma concentration level on (around 300 mg%), the difference between the rate of glomerular filtration and urinary excretion of sugar becomes constant, indicating a constant rate of reabsorption. This maximal rate has been termed T_{mG}. It indicates saturation kinetics. However, the data obtained by current nephrological methods (for instance, reabsorption rate as a function of the filtered load) do not allow an evaluation of the rate-concentration relation, as discussed above, for penetration into cells and for absorption from the intestine, because the observed over-all rate of reabsorption is integrated over the whole range of decreasing tubular sugar concentrations. The relation between the integrated reabsorption rate and the parameters of an absorption system assumed to follow Michaelis-Menten kinetics has been discussed by Wilbrandt (446).

Other sugars studied with respect to reabsorption in the kidney include fructose, galactose, xylose, and sucrose. According to Steinitz (412) sucrose is not reabsorbed at all. Galactose, fructose, and xylose are reabsorbed (139, 374, 376, 379), the order of decreasing reabsorption rates, from published figures, being fructose, galactose, xylose. For all three sugars it was found that the clearance ratio increased with increasing plasma concentration, indicating nonlinear kinetics of reabsorption. For none of the sugars reported, however, could a well defined maximal reabsorption rate T_m be reached.

With respect to *competition* in intestinal absorption, an early observation was reported in 1926 by Cori (74), who found that from a mixture of glucose and galactose rats absorbed less of each sugar than from the individual solutions. More recent observations on *in vitro* preparations have confirmed this finding (78, 129, 242). Other competition effects reported from *in vitro* experiments have been listed by Crane (79). Among them, the inhibition of galactose absorption in the presence of mannose or glucosamine in the experiments of Riklis, Haber and Quastel (341) is of special interest, since these inhibiting sugars themselves are not accumulated. It illustrates (like many other observations) that competition does not necessarily imply common affinity to all components of a transport system. Negative findings on the other hand, like the lack of inhibition of 1,5-anhydro-D-glucitol absorption by 2-deoxy-D-galactose (78), are not conclusive evidence of lack of common affinity of the sugar pairs in question. If the affinities differ widely, the more strongly bound sugar will inhibit the less strongly bound, but not *vice versa*, and in concentrations below K_m competition vanishes for all sugars, as can easily be seen from equation 23.

In the kidney, the reabsorption of xylose appears to be completely blocked when the plasma glucose concentration is raised to a level saturating the transport system, as was early demonstrated by Shannon (376). In the case of fructose

reabsorption a similar effect was reported in the dog by Gammeltoft and Kjerulf-Jensen (139), but not by Hansen *et al.* (160).

Besides competitive sugars, agents interfering with intestinal absorption and tubular reabsorption include a long list of *enzyme inhibitors* like iodoacetic acid, fluoride (458), cyanide (93), mercuric ions (326), azide (93), chloretone (93), fluoroacetate (93), dinitrophenol (41, 137), and many others. Most of these agents interfere with either glycolysis, respiration, or oxidative phosphorylation, and may safely be interpreted as secondary inhibitors (*cf.* p. 126). They will not be discussed.

One inhibitor group, however, warrants a more detailed discussion: *phlorizin* and related substances. Phlorizin glycosuria was discovered as early as 1888 by von Mering (273). Final proof for the inhibition of sugar reabsorption was furnished by Walker and Hudson in 1937 (434) in experiments involving micropuncture of kidney tubules. In the intestine the inhibitory action of phlorizin was first noted by Nakazawa in 1922 (291), and later studied particularly by both Lundsgaard (263) and Wertheimer (437) in 1933. Since then a large number of publications have dealt with the effects on reabsorption in the kidney and on intestinal absorption (33, 106, 137, 205, 206, 292, 315, 379, 393). For further references and a more detailed discussion, see (79) and (389).

The question whether phlorizin is a primary or a secondary inhibitor has evoked some discussion. A number of reasons were given for the assumption that phlorizin interferes with the energy-yielding metabolism: the finding of Shapiro (380) that phlorizin is an inhibitor of certain dehydrogenases, the observation of Lotspeich and Keller (259) that it may reduce the rate of respiration in kidney slices, and the fact that it is capable of inhibiting the uphill transport of other substrates not closely related to sugars, and doubtlessly using different mechanisms. They include the tubular excretion, in several species, of phenol red (49, 319), of diodrast (49), of creatinine (373, 375, 391) and of creatine (318). No such effects, however, have been reported for reabsorption of substrates other than sugars (and closely related compounds like ascorbic acid). Phlorizin does not affect the reabsorption of chloride and bicarbonate (378), creatine (318), amino acids (320), or urea (371, 379).

Furthermore, there is increasingly accumulating evidence along other lines that the effects of phlorizin on sugar absorption are not only due to interference with the energy yielding metabolism. First, it may be recalled that phlorizin inhibits the equilibrating transport of a number of sugars into single cells, including tumor cells (80), heart muscle cells (283), striated muscle cells in eviscerated animals (212) and in the diaphragm (11), and erythrocytes (243, 445). In none of these cases is energy required for the transport. This is evident not only from the lack of accumulation but also from the fact that inhibitors known to block energy-yielding metabolic reactions, including dinitrophenol, iodoacetic acid, azide, and others, do not interfere with the transport (446, 456). Newey, Parsons and Smyth (292) have measured, in the isolated intestine, uptake and utilization of labelled glucose applied either from the mucosal or from the serosal side. Their experiments indicated that phlorizin inhibits the entrance into the epithelial cells from the mucosal, but not from the serosal side.

Furthermore, Smyth and his group (205, 315) have repeatedly pointed out that the concentrations required for inhibition of glucose absorption are considerably lower than for the aforementioned metabolic effects. They found absorption to be inhibited by concentrations as low as 10^{-6} M, whereas in the experiments of Shapiro and of Lotspeich and Keller inhibition of citrate and pyruvate metabolism required concentrations between 10^{-3} and 10^{-4} M. Matthews and Smyth (271) have shown recently that in the intestinal epithelium phlorizin inhibits not only the uphill transport but also the entrance of sugar into the cells, in contradistinction to dinitrophenol which interferes only with accumulation and not with entry. In kidney cortex slices of rabbits, Krane and Crane (232) reported the similar finding that an inhibitory effect of phlorizin on the entrance of glucose into the cells is observed, both under conditions of accumulation and in the presence of metabolic inhibitors blocking the uphill transport.

On the basis of these various findings it is now assumed by several authors (79, 271) that the uphill transport of sugars across the intestinal and tubular cells involves two steps, first the entrance of glucose into the cells, and second some additional process inducing accumulation, and that only the first step is affected by phlorizin. The inhibition by phlorizin of the tubular transport of a number of substrates other than sugar, then, might, in view of the prime importance of glucose as an energy-yielding substrate, be related to this blocking effect on sugar entry.

A peculiar discrepancy is observed between the relative efficacies of phlorizin and phloretin (its aglucone) in different cells. Phlorizin is much more active than phloretin in the intestine (205) and in the kidney (241), while in L-cells (336) and in red cells (250, 350, 459) the reverse is true. This might be related to the fact that the glucoside, because of the sugar moiety of its molecule, is accumulated in the epithelial cells like glucose itself. Ellinger and Lambrechts in 1937 (116), in a study on the action of colored azo derivatives of phlorizin, found that compounds capable of entering the cells produced glycosuria, while a non-penetrating derivative, containing a sulfonate group, was inactive. A somewhat similar observation showing glucose-like responses of glucosides was reported by Keyl and Dragstedt (215, 216): in the chick embryo, insulin was found to be necessary for the action of cardiac glycosides containing glucose.

As regards the assumed second factor, inducing sugar accumulation in the cells, recent observations point to a connection with ion shifts. As reported by Riklis and Quastel (339), the presence of sodium is indispensable for the uphill transport of sugar across the intestinal wall *in vitro*. Potassium, in lower concentrations, was found to stimulate it. The effect was maximal at 15 mM and fell off at higher concentrations. The indispensability of sodium was confirmed by Csáky (85, 86, 87) and by Crane (83).

Apparently, with respect to sugar accumulation, what matters is not the presence of these ions alone, but rather their uphill transport across membranes. This is indicated by the finding of Riklis and Quastel (340) that dinitrophenol inhibits the potassium-induced increase in sugar absorption rate. A related finding appears to be that of Csáky *et al.* (85) and of Crane (83) that the presence

of a cardiac glycoside (which inhibits the sodium pump) blocks sugar accumulation even if sodium is present. For the equilibrating entrance of sugar into the cells, however, only the presence of sodium appeared necessary, not the action of the sodium pump. Crane (83) discussed the possibility of a carrier common to sugar and sodium, transporting these two substrates into the cell, with the subsequent removal of sodium from the cell interior by the sodium pump, steepening the gradient for the transport complex and thus accomplishing accumulation of the sugar.

d. Yeast cells and bacteria; permeases. The problem of the mode of entrance both of monosaccharides and of di-saccharides into yeast cells has passed through various stages. Conway and Downey in 1950 (69), from determination of distribution volumes, concluded that yeast cells are impermeable to galactose and arabinose. This was confirmed and extended to sorbose by Rothstein in 1954 (357, 358). Rothstein (358) found no free glucose in the intracellular space. Of the two possible interpretations (entrance of free glucose but rapid removal by metabolism, or entrance mechanisms involving no passage of free glucose) Rothstein chose the latter interpretation. He assumed that glucose entrance into the cells is preceded by phosphorylation. For this reaction a hexokinase located on the external surface of the cell membrane was considered responsible.

Rothstein's arguments included the following. First, the three non-fermentable sugars, galactose, arabinose, and sorbose, also have in common the inability to react with yeast hexokinase. Second, if carriers were involved in the transport of free sugars, the induction of galactose fermentation by adaptation (which is possible in yeast) would imply the induced synthesis not only of hexokinase but also of the carrier.

The hexokinase theory became doubtful when Blakley and Boyer, in 1955 (32), reported that 6-deoxy-6-fluoroglucose competitively inhibits the fermentation of glucose and fructose, while it has only a slight inhibitory effect on yeast hexokinase. The authors suggested specific transport mechanisms prior to the hexokinase reactions. Similar conclusions had been drawn by Cramer and Woodward in 1952 (76) from their findings that 2-deoxy-D-glucose competitively inhibited the fermentation of glucose by living yeast cells, but not at all, or scarcely, in a cell-free extract of yeast. In a strain of sauternes yeast utilizing fructose preferentially to glucose, Sols (402) nevertheless found a smaller phosphorylation coefficient for fructose than for glucose, and likewise postulated specific transfer systems.

Recently, Burger *et al.* (42, 43) as well as Cirillo (59, 60) re-investigated the question of penetration of non-fermentable sugars into yeast cells, using somewhat different methods. D-Galactose, L-sorbose, D-xylose and *alpha*-methyl-D-glucose were found to penetrate into the cells. Glucose, however, inhibited the entrance and, when added after equilibration, displaced the non-fermentable sugars from the cells. Uranyl ions inhibited the entrance of sorbose. These observations strongly indicate a specific transport system with graded affinities. The displacement could reflect either counter-transport, or competition in a leak-and-pump system (*cf.* p. 125).

The inhibitory effect of glucose might be the reason for the failure of earlier experiments to demonstrate the penetration of non-fermentable sugars: the steep gradient of glucose due to its rapid utilization might have induced counter-transport of the non-utilized sugars tested, and thus might have kept their apparent distribution volume low.

Recent experiments by Kotyk (230) seem to indicate that glucose likewise penetrates the cell membrane in the non-phosphorylated form. After addition of glucose he observed a transitory peak in the internal free glucose concentration which lasted not more than 2 to 3 minutes. This peak appears to have escaped the attention of previous workers because of its short duration. From differences found in a respiration-deficient mutant between aerobic and anaerobic conditions (with respect both to oxidative phosphorylation and to the free glucose peak), the author assumed that the glucose carrier is a phosphorylated compound arising through oxidative phosphorylation.

Sols (403) has provided convincing evidence that not only mono-saccharides but also certain di-saccharides enter yeast cells by specific mechanisms. This appears to hold for maltose and for lactose. The evidence includes the observation of cryptic strains (which contain all enzymes necessary for maltose metabolism and yet do not ferment maltose), as well as the finding of Robertson and Halvorsen (342) that de-adaptation with respect to maltose can be used to induce this type of crypticism. It was found that maltose fermentation may be faster than glucose fermentation, which would not be possible if maltose were hydrolyzed by external enzymes prior to fermentation. Furthermore, for two different *alpha*-glucosides (turanose and maltose) the substrate specificity was different in homogenates and in intact cells. Different results were obtained for melibiose and *alpha*-galactosides, as well as for saccharose and *beta*-fructosides; all available evidence pointed to hydrolysis of these compounds by external enzymes prior to fermentation.

The observations in yeast cells just reported are closely related to recent findings in bacterial cells, which have led to the assumption of special accumulating agents termed "permeases" by Cohen and Monod (62). This term (the introduction of which has been criticized) was not meant to imply that the agents necessarily are enzymes, although this possibility was not excluded. Cohen and Monod appear to envision their function as that of carriers. The pertinent observations are as follows. It was found that bacterial cells (most studies have been carried out on *Escherichia coli*) are able to take up and accumulate, up to several hundred-fold, a number of substrates, mainly sugars, sugar derivatives and amino acids. Among the sugar derivatives galactosides, glucosides, glucuronides, and galactose especially have been studied. In the group of galactosides the possibility of replacing the oxygen at carbon-1 by sulfur and of producing in this way non-metabolized substrates has proved useful. The systems responsible for the accumulation are characterized in the first place by a very high degree of specificity. The action of galactoside permease, for instance, is confined to compounds with the unchanged galactose residue, and will not transport glycosides of other sugars or of galactose derivatives. A striking example of structural specificity reminis-

cent of the above-mentioned observations in yeast cells was reported by Doudoroff *et al.* (107): they described a mutant strain of *E. coli* which was cryptic with respect to glucose but not to maltose. This strain, then, could utilize maltose, hydrolyze the maltose molecule to form glucose, and metabolize glucose in the normal way. Glucose added externally, however, could not be utilized.

The high degree of structural specificity of the transport systems postulated in the permease interpretation of crypticism has, to a certain extent, delayed the general acceptance of the concept. Some earlier authors (including Monod, in 1952) preferred to assume that in cryptic strains some enzymes, while present in the cells, are in some way inactivated, but regain their full activity when the cells are broken up (251, 282).

Some permeases are constitutional, others inducible. From the observations on induction which revealed a number of characteristics known from protein synthesis, such as inhibition by chloramphenicol, it was concluded that permeases are protein molecules.

Kinetically the systems are characterized by relations between internal and external concentration in the steady state of accumulation, like those of equations 21 and 22. Correspondingly, the kinetics have been interpreted in terms of a unidirectional inward transport, following Michaelis-Menten kinetics, and a linear outward movement. As shown above, this type of kinetics will yield a relation between internal and external concentrations formally resembling that between the concentrations of free and bound substrate in a reaction with a complexing partner. The observations in the case of permease systems could, therefore, *a priori* be interpreted equally satisfactorily in terms of a "leak-and-pump" system, as discussed in connection with equations 21 and 22, or in terms of binding sites in the interior of the bacterial cells. The two interpretations were termed "catalytic" and "stoichiometric" by Cohen and Monod.

The arguments of these authors for the catalytic interpretation appear very strong. They point out that in some cases the amount of accumulated substrate is so large that it hardly appears possible to account for the necessary number of binding sites postulated for the stoichiometric interpretation. In the case of galactosides, for instance, in which the amount taken up may reach 5% of the dry weight, it would have to be assumed that every fraction of the protein molecules corresponding to a molecular weight of 2000 would have to take up one substrate molecule. Furthermore, in the adsorption mechanism the rate of entrance would be expected to depend on the fraction of free binding sites in the cell, and thus on the amount of substrate already accumulated. This would imply pronounced differences between initial rate of net accumulation (internal binding sites free) and rate of exchange of labelled substrate in the steady state (internal sites occupied), which is contrary to observation (*cf.* the similar situation in amino acid accumulation in tumor cells, see p. 148). Finally it has been shown by Sistrom (386a) that the accumulated material contributes to the osmotic pressure in a quantitative manner, in agreement with its being in free state.

If these arguments are accepted (and it appears difficult to escape them), the permease systems have many features in common with carrier systems, some of

which, in fact, may be taken as conclusive evidence for the participation of carriers. Uphill transport as such, as was pointed out above, indicates carriers to be operative. The displacement phenomenon certainly implies common affinity of substrate and competitor for a binding site in the transport system. Whether or not it constitutes counter-transport, as discussed in the previous section, would depend on whether the leak-and-pump interpretation is accepted as indicative of two different systems. In this case it might indicate merely competition for the entrance system, in accordance with the interpretation given by Cohen and Monod. In a recent observation by Kepes (214), however, that in a permeaseless strain displacement likewise occurs, this interpretation appears untenable, and the displacement has to be interpreted as a counter-transport phenomenon. Furthermore, in the same study Kepes showed that in the competition between TMG (methyl-thio-β-D-galactoside) and TDG (galactosyl-thio-β-D-galactoside) another feature is observed which strongly indicates a counter-transport phenomenon. It was found that TMG competes with TDG and, in the concentrations of 10^{-4} M and 5.10^{-4} M, slows the entrance of TDG into *E. coli*. In the low concentration of 2.10^{-5} M, however, TMG accelerated the entrance of TDG. As was pointed out in the general part (p. 127), this is characteristic of penetrating inhibitors competing for a common carrier. It was pointed out above that the acceleration will not be found with non-penetrating inhibitors of type $I_{CS}(np)$, and that it is a consequence of the counter-transport and therefore characteristic for type $I_{CS}(p)$.

The part of the catalytic interpretation which would appear least convincing, and which in fact in recent publications (193, 214) is beginning to be modified, is the assumption of two entirely independent systems for entrance and exit and of the exit occurring by simple diffusion. Apart from the general objection of the extreme wastefulness of such an arrangement in an otherwise highly elaborate system, there are now a number of observations indicating that not only the entrance, but also the exit system involves carriers. The kinetics cannot be considered as a valid argument in favor of diffusion. It has been pointed out repeatedly in the general part that linear kinetics by themselves can never disprove a carrier mechanism.

Kepes, in his study of competition quoted above, observed that the competitive inhibition by TMG affects both the entrance rate and the accumulation ratio in the steady state of TDG. In the catalytic interpretation these two values are, according to equations 21 and 22, linearly related. The factor of proportionality is B, the rate constant of exit. In Kepes' experiments, however, entrance rate and accumulation ratio of TDG were affected by TMG to different degrees, indicating that the proportionality factor was changed by the presence of TMG, or in other words that the competition concerns not only the entrance but also the exit of the substrate. He furthermore found that *para*-chloro-mercuribenzoate (PCMB), an SH-inhibitor interfering with the accumulation, appeared to affect not only the entrance but also the exit of the substrate, TDG. This was evident from the fact that TDG exit, after addition of PCMB (which would be expected to follow an exponential time course if the effect of PCMB were on the

entrance only), proceeded with constant rate. Furthermore it was much slower than expected from the exchange rate, as determined from the entrance rate of added labelled substrate. Kepes arrived at an interpretation of the system in terms of carrier mechanisms both for entrance and for exit, differing in that the entrance due to coupling to an energy-yielding reaction occurs uphill, while the exit corresponds to the simple equilibrating system as discussed above (equation 3).

Horecker *et al.* (193) produced further evidence for the interpretation that exit and entrance are not entirely independent. They found that in the presence of dinitrophenol (DNP) (which as mentioned above inhibits accumulation) both entrance and exit were affected. In this case, however, the exit rate was increased in the presence of the inhibitor. The experiments involved displacement of accumulated labelled substrate by the addition of a larger concentration of unlabelled substrate, with and without the presence of DNP in a concentration sufficient to diminish, but not to block accumulation. The rate constant of exit, B, was evaluated from equation 21 both for entrance ($A \neq 0$) and for exit ($A = 0$). These constants agreed very satisfactorily with each other and were both found to be increased by the presence of DNP. Blocking the supply of energy therefore appears to affect both systems, decreasing the rate of entrance and increasing the rate of exit. This implies a probable interdependence of the two systems.

Cohen and Monod distinguished between "permease *sensu stricto*," indicating the stereospecific inducible agent the lack of which causes crypticity, and the "permease system" which may, and probably will, include additional components. If a close correlation between permease systems and carrier systems is accepted, the question arises as to whether the permease *sensu stricto* is to be considered as the carrier or as an enzyme catalyzing the substrate-carrier reaction. The inducibility of the permease and its high structural specificity (contrasting with the wide spectrum of affinities found, for instance, in the erythrocyte system) *a priori* favors the assumption of an enzyme, coinciding with the conclusion reached by Kepes (*cf.* above). He considered the rate of the enzymatic reaction between substrate and carrier to determine the transport rate of the entrance system, corresponding to the enzymatic steady-state system presented above in equation 7, complemented, however, by a link coupling it to energy-yielding reactions as discussed above in connection with equations 17 to 20.

2. Suggestions as to the possible chemical nature of the carrier. General postulates as to the chemical nature of the assumed carrier molecules refer mainly to the basic element in the carrier concept that the reaction with the carrier enables the hydrophilic substrate, not capable of passing a lipoid membrane in the free state, to do so in a transport complex. They include, therefore, lipoid solubility and electrical neutrality of the complex, which in some measure would imply similar characteristics for the carrier itself.

As a model for considerations of this kind, experiments with a lipoid-soluble sugar complex, glucose benzoate, were carried out in red cells (347). Penetration occurred with much less species specificity than with glucose and was unaffected by phlorizin.

Park (310) observed that cell extracts can be used to render labelled glucose chloroform-soluble, and that this effect is inhibited by phlorizin.

Two more specific suggestions as to the nature of the carrier will be discussed, *i.e.*, the phosphorylation theory of sugar absorption put forth nearly 30 years ago, and the recent suggestion of multiple hydrogen bonding as a possible common element of many sugar transport systems.

a. The phosphorylation theory of sugar absorption. According to the phosphorylation theory which has been widely discussed in the past few decades, the carrier for sugar absorption from the intestine and from the tubular lumen in the kidney would be the phosphate radical, and the substrate carrier complex a sugar phosphate ester. A somewhat similar concept has been applied more recently to yeast cells (*cf.* above). The starting point for the phosphorylation theory was the early suggestion of Höber in 1899 (173), later taken up by Verzár (429), that the sugar molecules, after entering the epithelial cells, are transformed by some chemical reactions so as to maintain a steep gradient for free sugar. Elaborating on the suggestion made by Wilbrandt and Laszt (458) that this reaction might be phosphorylation, Lundsgaard (262) and Kalckar (210) assumed a phosphorylation of the sugar molecule at one end of the epithelial cell, followed by diffusion of the complex across the cell and dephosphorylation with release of free substrate at the other end. This suggestion, essentially that of a cytoplasmic carrier (*cf.* p. 112), was taken up by Krogh (236). Drabkin (108) later specified hexokinase as the phosphorylating, and phosphatase as the dephosphorylating agent.

Experimental support for the hypothesis was first seen in the demonstration by Lundsgaard (262) that phlorizin appeared to inhibit phosphorylation in extracts of intestine (which, however, is likely to have been due to inhibition of phosphorylase). Beck (12) interpreted the phlorizin effect as an interference with phosphatase. Later reports from two laboratories (31, 170) appeared to indicate that the specific order of absorption rates found for monosaccharides (*cf.* p. 136) coincided with the order of affinities for hexokinase. This, however, was not confirmed by Sols (400, 401).

An essential role of phosphatase in sugar absorption was concluded by a number of authors (37, 100, 148, 356) from histochemical findings indicating a close correlation between the occurrence of alkaline phosphatase and of pronounced ability to absorb sugar; *e.g.*, marked phosphatase activity is found in the brush border of the proximal kidney tubule and in the epithelia of the small intestine and rectum, but not in the non-absorbing epithelia of stomach and large intestine. The histological localization of phosphatase (at the luminal border of intestinal and renal epithelia) did not, however, agree with the mechanism depicted above. A transformation of the cytoplasmic carrier scheme into a membrane carrier mechanism, while eliminating this difficulty, would lead to the postulate that sugar phosphate esters penetrate cell membrane more easily than free sugar molecules. This corollary, improbable *a priori* on account of the hydrophilic nature and the electrical charge of the ester molecule, was disproved by the finding that phosphate esters are absorbed from the intestine only after hydrolysis (93, 360), and do not penetrate into red cells (347). The final and most

conclusive argument against the phosphorylation theory, however, was furnished by the work of Crane and Krane (81), who showed that both 1-deoxy-glucose and 6-deoxy-glucose are not only well absorbed but transported uphill in a manner characteristic for the specific sugar accumulation system in the intestine.

The phosphorylation theory, therefore, useful as it has been in stimulating a number of valuable studies, has now to be abandoned. The role of alkaline phosphatase remains a challenging problem.

b. The multiple hydrogen bond theory. In a recent survey, Rosenberg (346) has reviewed the extensive experimental data now available as to the structural requirements of the sugar molecule for the transport systems in different cells. With the exception of the finding of Wilson and Crane and Wilson and Landau (463, 464), that the hydroxyl group at carbon-2 is indispensable for accumulative transport across intestinal cells (not, however, for equilibrating penetration into the cells), the results obtained in a variety of cells including erythrocytes, tumor cells, muscle cells, and intestinal and renal epithelial cells, appeared to indicate that in general none of the hydroxyl groups at carbon atoms 1, 2, 3, 4, and 6 appears to be essential for transport, but that several of them contribute to the affinity to the system. Rosenberg suggested that the reaction with the carrier involves hydrogen bond formation between several hydroxyl groups of the sugar molecule and suitable groups of the carrier, and that each hydroxyl group contributes to the over-all affinity.

An exception was made for the hydroxyl group at carbon-2. In the red cell, the higher affinity of 2-deoxy-glucose as compared with glucose appears to indicate a disturbing effect of this group, possibly by steric hindrance.

B. Amino acids

1. Observations. The main difference between amino acid transport and sugar transport appears to be that only in a few single cell types has uphill transport of sugars been observed, while for amino acids accumulation appears to be the rule, and mere equilibration exceptional. In both cases, the absorbing epithelia perform uphill transports and in both cases many features point to carrier mechanisms.

a. Single cells. In 1913 Van Slyke and Meyer (428) observed that in most *cells of the animal organism* the amino acid concentration is higher than in the surroundings. These observations were confirmed for glutamic acid with the use of specific analytical methods (decarboxylases) by Krebs *et al.* (233). A recent study of the uptake of amino acids in *cultured human cells* by Piez and Eagle (317) revealed 5- to 11-fold accumulation ratios for the essential amino acids (excepting cystine which was not taken up at all, and lysine and arginine which were accumulated only 2- to 3-fold). The non-essential amino acids not supplied in the medium in general had much higher distribution ratios. This, however, would be due at least partly to their lower concentrations.

An extensive study of amino acid accumulation in various cells, mainly *tumor cells*, has been carried out by the groups of Christensen and of Heinz. A recent review by Christensen (52) may be consulted for details and for stimulating dis-

cussion. In 1952 Christensen and Riggs (55) found glycine, alanine, glutamic acid, and glutamine to be accumulated 7- to 15-fold in Ehrlich ascites tumor cells. The accumulation was inhibited by a considerable number of agents, including dinitrophenol, cyanide, arsenate, and iodoacetate. Nineteen amino acids were found to be transported, including some compounds not occurring naturally (57). The highest accumulation ratios were observed with diaminobutyric acid; glycine and alanine were intermediate, and leucine and valine reached the lowest ratios. The accumulation of glycine was inhibited by alanine, but enhanced by lysine. Other diamino-monocarboxylic acids likewise stimulated accumulation.

The accumulation of diaminobutyric acid was studied in more detail (58, 338). α,γ-Diaminobutyric acid was found to be accumulated more than α,β-diaminopropionic acid. Erythrocytes and diaphragm, in contradistinction to tumor cells, did not take up diaminobutyric acid *in vitro*. The compound injected *in vivo* was accumulated preferentially in the liver, but penetrated into tumor cells after saturation of the liver had been reached. Striated muscle and kidney accumulated it to some degree, but not as effectively as liver.

It was pointed out that the natural occurrence of diamino acids has been described so far only in antibiotics. Furthermore, α,γ-diaminobutyric acid is known to exert a strong action on the nervous system. It may well be that here, and possibly in other cases, accumulation and competitive potency with respect to penetration are related to pharmacological actions. Examples of other pharmacological effects of competitors in specific transport systems include 2-deoxyglucose, which has a pronounced effect on the heart (362), and methionine, a strong competitor for amino acid transport both in single cells and in the intestinal epithelium, which has been reported to interfere with growth (474).

The competitive action of methionine was compared by Wiseman and Ghadially (470) with that of four other amino acids in RD3 sarcoma cell suspensions *in vitro*. It was found that the strongest competitive action was not exerted by those amino acids which reach the highest accumulation ratio. On the contrary, of the amino acids which were compared, methionine was the most powerful competitor and was accumulated least. Since the accumulation ratio is parallel to the entrance rate (see below), this again appears related to the prediction made on the relation between transport rate and affinity (see p. 117).

The accumulation ratio (commonly denoted by r) depends not only on the amino acid tested but also on its concentration: the higher the concentration, the lower r. The value of r alone, therefore, is not sufficient to characterize the system. A quantitative interpretation of the relation between r and the substrate concentration was furnished by the kinetic analysis of transport given by Heinz (167). His treatment corresponds to that in equations 21 and 22, with the exception that it allows for an inward diffusion flux which in the case of high accumulation ratios will be negligibly small. Quantitatively this treatment agreed with the data given by Christensen and Riggs (55). Heinz's discussion closely resembles that of Cohen and Monod in the case of the permease systems (*cf.* above). Like these authors, he points out that the relationship between internal and external concentrations in the steady state would fit quantitatively the assumption of

adsorptive binding in the cell as well as the leak-and-pump scheme. His arguments against a binding mechanism include the finding (168) that, in contradistinction to whole cells, fragments of cells do not accumulate amino acids, and the amino acids taken up by the cells appear to have full osmotic activity as shown by Christensen *et al.* (58). Furthermore, Heinz found that preloading the cells with glycine increased the influx rate. In the case of binding to internal sites an opposite effect would be expected, since the rate then would depend on the number of free sites in the cell. The increased influx observed indicates participation of a counter-transport, and thus the involvement of carrier mechanisms.

Heinz and Mariani (169), in a study of the energy relations of the system, interpreted the transport in terms of a leak-and-pump system with strictly separate inward and outward currents using different pathways. A number of difficulties for this interpretation were encountered, however. In an attempt to block the pump completely by high concentrations of inhibitors (1 mM dinitrophenol +1 mM sodium iodoacetate), it was found impossible to reduce the influx rate to zero. If the remaining influx were to be interpreted as a diffusion transfer, it would be expected to vary linearly with the external concentration. This did not occur. The influx coefficient dropped sharply when the extracellular glycine concentration was raised and seemed to tend toward a constant minimal value. The authors commented: "Whether this means that the applied inhibition of metabolic energy supply is incomplete or that the transport carrier may react with glycine to some extent without energy supply is not decided." The situation parallels that in the case of permeases: in both cases the consequences of the strict application of the leak-and-pump scheme (leak implying simple diffusion) lead to difficulties.

The prediction, based on the leak-and-pump interpretation, that metabolic inhibitors should lower the influx rate but not affect the efflux rate was tested by Heinz (168). It was found, as expected, that the influx is diminished drastically in the presence of dinitrophenol, with or without added iodoacetate. The effect on the efflux rate was considered to be within the limits of experimental error.

An interesting observation concerns that connection between amino acid uptake and loss of potassium. There was a close parallelism between these two processes. Uptake of amino acids caused loss of potassium, varying in magnitude with the degree of accumulation. In the case of the powerfully accumulated α,γ-diaminobutyric acid (58), nearly the total cell potassium was lost. The interdependence appeared to be mutual: loss of potassium could be induced by amino acid uptake and addition of potassium in high concentration inhibited the amino acid accumulation. The question thus was raised whether one of the two processes could be interpreted as secondary. Christensen (53) considered the potassium loss as primary, since the cells never accumulated amino acids in the absence of potassium. Thus, this situation is in some ways reminiscent of that discussed above in the intestinal uphill transport of sugars, which was found to depend on ionic shifts.

In *bacteria*, as mentioned above, accumulation of amino acids has been studied

extensively. According to Cohen and Monod (62), permease systems are operative which resemble closely those discussed above, with respect to structural specificity, competition, displacement, metabolic dependence, and other features.

b. Absorbing epithelia in intestine and kidney. In the field of *intestinal absorption* one of the early observations showing that the amino acid transfer cannot be a process of simple diffusion was also made by Cori (75), who found that the rate of absorption from a mixture of two amino acids is not additive. Later, Höber and Höber in 1937 (176) studied the kinetics of intestinal absorption from ligated intestinal loops in rats and found the absolute rate to be independent of the concentration, in contradistinction to the absorption of solutes like urea and amides. They concluded that a specific transport mechanism must be involved.

In recent years, the intestinal absorption of amino acid has been extensively studied mainly by the group of Smyth and Wiseman in Sheffield and that of Hird and Sidhu in Melbourne. Gibson and Wiseman in 1951 (141) found that from loops of the small intestine in rats L-amino acids disappear faster than D-amino acids. These findings were confirmed in dogs with the Thiry-Vella pouch method by Clarke *et al.* (61), and finally substantiated by Matthews and Smyth (270), who found that this difference is also reflected by higher L-amino acid concentrations in the venous blood leaving the intestine during absorption in the dog. With an *in vitro* technique, Wiseman in 1953 (466) observed the same difference between L-amino acids and D-amino acids for a number of compounds: from racemic mixtures the L-isomers disappeared more rapidly than the D-isomers. Essentially similar findings were reported by Agar *et al.* (2), by Fridhandler and Quastel (138), and by Akedo *et al.* (5). Both Wiseman (466) and Agar *et al.* (2) found that monoamino-monocarboxylic acids may be transported uphill. In everted sacs of hamster intestine, Wiseman (468) demonstrated for 12 amino acids that the rate of transport and the maximal accumulation ratio reached followed the same order of decreasing activity: proline, threonine, alanine, glycine, serine, valine, histidine, hydroxyproline, phenylalanine, *iso*-leucine, leucine, and methionine. For the amino acids named the rate of transference varied about 4-fold, and the concentration gradient ranged between 1.18 and 2.08. In contradistinction to monoamino-monocarboxylic acids, neither diamino acids nor dicarboxylic acids appeared to be transported uphill (466, 467). However, the conclusion that the transport mechanism is confined strictly to neutral compounds was later modified (468) by the finding that histidine is transported uphill. The restriction to monoamino-monocarboxylic acids therefore was considered as a "general rule" only.

The uphill movement across the intestinal wall appeared to be preceded by an accumulation in the wall itself, as found by Agar *et al.* (3) who distinguished between uptake and absorption of amino acids. This is reminiscent of similar findings with respect to sugars in the work of Crane *et al.* (83) quoted above. The steady state of accumulation was reached for L-histidine in about 50 minutes. For the accumulative uptake in the intestinal wall, the same preference for L-amino acids was again observed as for the transintestinal transport and, as in

the case of the absorption transport (2, 138), cyanide and dinitrophenol were inhibitory. The uptake, however, was not completely abolished by these agents but reduced to the level observed with D-amino acids.

Competition between various amino acids has been observed repeatedly. Wiseman (467) found proline, glycine, histidine, and methionine to compete with each other. A characteristic feature in this competition was that the compounds absorbed at the highest rate and accumulated to the highest ratio were the weakest in competition, and *vice versa*. This finding prompted the similar study in tumor cells quoted above. It likewise recalls the prediction for equilibrating transport discussed in the first part in connection with equation 4. Closely related is the more recent finding of Finch and Hird (123) that the order of uptake rates in segments of rat intestine for 17 amino acids was reversed by changing the substrate concentration from 1 mM to 10 mM. At 10 mM the rates of the more lipophilic amino acids were lower, at 1 mM higher, than those of the more hydrophilic compounds. This is similar to the reversal of the order of sugar penetration rates in red cells quoted above (447). The direction of the difference appears to indicate, according to equation 4, that K_m (or K_1 in equation 17) is higher for hydrophilic, but lower for lipophilic compounds, which would seem reasonable for a lipoid carrier.

The observations with respect to competition at first seemed to indicate that for L-amino acids and D-amino acids partly or wholly different mechanisms exist. Agar *et al.* (4) found that the uptake of L-histidine is inhibited by L- but not by D-amino acids. In a later study, however, Jervis and Smyth (207) observed that D-isomers may be inhibited by L-isomers and *vice versa*: L-methionine inhibited the absorption of D-histidine markedly, and D-methionine that of L-histidine faintly but distinctly. The latter finding disagrees with that of Agar *et al.* (4) with respect to the same amino acids, both in absorption and transfer (disappearance from the luminal side and appearance on the mucosal side). Jervis and Smyth assume two stages in the transport, the first of which is common to D- and L-amino acids, the second stereospecific.

Newey and Smyth have recently (293, 294) studied the absorption of dipeptides. The classical interpretation of absorption as occurring exclusively after hydrolytic cleavage had been challenged by Fisher (124). Newey and Smyth found in experiments both *in vivo* and *in vitro* that dipeptides disappear from the mucosal side and amino acids appear on the serosal side. In addition, however, they found some unchanged dipeptides on the serosal side, particularly in the case of glycyl-glycine. The amounts were very small. These authors discussed the possibility of entrance of dipeptide into the cell followed by intracellular hydrolysis, which would agree with their result as well as the classical interpretation.

Finch and Hird recently (122, 123) performed a quantitative kinetic analysis of the uptake rates. They found satisfactory agreement with Michaelis-Menten kinetics and reported values for K_m ranging from 0.55 mM for lysine up to 80 mM for aspartic acid. Their findings contrast in some measure with those quoted above, particularly with respect to diamino acids. The fact that of 17 amino acids studied lysine had the lowest apparent dissociation constant was unexpected. It

also contrasted with the low inhibitory activity observed for lysine by the authors. Percentage inhibition between pairs of amino acids, as calculated from the observed values of K_m, in general agreed reasonably well with the observations within the group of monoamino-monocarboxylic acids. Marked discrepancies were found for lysine. It was concluded that L-lysine and probably L-ornithine and L-arginine "may not compete for a common mechanism with the other L-amino acids." For monoamino-dicarboxylic acids the low absorption rates reported above were, as expected, reflected in high values of K_m.

In the kidney the reabsorption of amino acids in general is nearly complete, in teleological accordance with their high nutritional value. In 1936 Kirk (221) found in man a clearance for total amino acids between 1 and 8 ml, indicating the reabsorption of about 95% of the filtered acids. His observation that administration of glycine raises the clearance up to 25 ml is an early demonstration of competition in reabsorption. Extensive studies were later carried out mainly by Pitts (320, 321). He found the rate of reabsorption of glycine in the dog to be related in a non-linear fashion to the filtered load, and to reach a maximal level at a filtration rate of about 35 mg amino nitrogen per minute. For DL-alanine, L-glutamic acid, and L-arginine, although non-linearity was likewise found, a well defined maximal rate of reabsorption was not reached. Other studies indicating non-linear reabsorption kinetics for amino acids were reported by Eaton *et al.* (112) and by Beyer *et al.* (27).

Following Kirk's early demonstration of competition, quoted above, similar observations have been made for individual amino acids. Pitts (320) found creatine reabsorption to be reduced to zero in the presence of glycine or alanine in saturating concentrations, and to be diminished by glutamic acid. Other observations on competition have led to the conclusion (390) that the tubule cells have two more distinct reabsorption mechanisms, a) for basic amino acids and b) for neutral amino acids other than glycine.

The question of stereospecificity was recently studied by Crampton and Smyth (77) who found that both alanine and methionine were reabsorbed more rapidly in the L-form than in the D-form.

An interesting observation is the marked accelerating effect of insulin on the transport of non-metabolized amino acids into the cells of the rat diaphragm, reported recently by Kipnis and Noall (220). It raises the question whether insulin acts on several systems or whether the observed effect is in some way related to its action on sugar transport discussed above.

2. Christensen's suggestion of pyridoxal as a possible amino acid and cation carrier. A suggestion made by Christensen in 1955 (51) as to the possible chemical nature of the amino acid carrier is interesting in a number of respects: a) it operates with compounds the existence of which has been established; b) it might possibly furnish a basis for the interpretation of the relation between potassium and amino acid transfer as described above, and c) it is supported by some interesting though not entirely conclusive experimental evidence.

One of Christensen's starting points was the role of pyridoxal in transamination. After Snell's demonstration in 1945 of non-enzymatic transamination by

pyridoxal (395) and of a catalytic role of metal ions in this reaction (275), and in connection with the finding that pyridoxal is a component of transaminase (367), Baddiley (6) studied chelate complexes of metals containing pyridoxal and ethylenediamine and suggested that similar complexes, with amino acids instead of ethylenediamine, are involved in transamination. This suggestion was accepted by Metzler *et al.* (274), and supported by spectrophotometric evidence of the postulated complexes in a study by Eichhorn and Dawes (115).

In Christensen's laboratory, Riggs *et al.* (337) found in 1953 the cellular accumulation of amino acids in tumor cells to be intensified by the addition of pyridoxal to the external medium. In a later publication (56), confirming this finding and extending it (with quantitatively less pronounced effects) to erythrocytes, the observation was added that tumor cells of pyridoxine-deficient animals had an inferior ability to concentrate glycine; however, they were stimulated by low levels of pyridoxal which were without effect on normal tumor cells. The authors concluded that there is a "normal function of pyridoxal in amino acid transfer." An inhibitory effect was also found with deoxypyridoxine.

From these findings, from the known coupling between transport of amino acids and cations (as discussed above), and from the established existence of pyridoxal amino acid complexes with metal ions, Christensen suggested in 1955 (51) that compounds of this type are the common transport complex for amino acids and cations.

An attractive model experiment involving the effect of pyridoxal on amino acid accumulation in tumor cells was reported by Christensen and Oxender in 1960 (54). It demonstrated the principle of utilizing the accumulation ability of single cells for transcellular transport by the introduction of an asymmetry factor. Tumor cells were embedded in a micropore filter which was used as a membrane between two solutions. After equilibration of the system with glycine, addition of pyridoxal to one of the solutions induced an uphill transport of the amino acid in the direction of the pyridoxal gradient. The maximal ratio of accumulation reached was low (1.17) but definite. Similar results could be obtained with α-amino*iso*butyric acid but not with N-dimethyl glycine or α-methyl glutamic acid, "in precise agreement with the specificity of the intracellular concentration process." In a similar manner a gradient for amino acids could be produced by a potassium gradient, and *vice versa*. The authors commented: "accordingly the idea gains strength that potassium migration may drive amino acid transport, and that the energy for both may be applied primarily to the maintainence of the potassium gradient."

A detailed mechanism was not suggested. One difficulty for an interpretation in terms of pyridoxal-amino acid chelates as common carrier complexes for amino acids and for potassium would appear to be that this type of complex might well account for coupled transports of the two substances in the same direction, but less readily in opposite directions.

In view of the many similarities between accumulation in single cells and epithelial transport of amino acids, the effect of pyridoxal on amino acid absorption from the intestine has been tested by several authors. In 1957 Wiseman (469) found no effect of added extracellular pyridoxal on the transport of four L-amino

acids by normal hamster small intestine. Higher concentrations produced inhibition. More recently, however, in experiments reported by Jacobs and Hillman (197) pyridoxal reversed the inhibition produced by deoxypyridine. These authors observed furthermore some protection against DNP inhibition with pyridoxal phosphate. Akedo *et al.* (5) reported diminished intestinal absorption rates for amino acids in B_6-deficient rats, and restoration to normal level by addition of B_6, but not of B_1 and B_2. The effect of B_6-deficiency was only on L-isomers, not on D-isomers of amino acids.

In a recent publication (53) Christensen's attitude toward his hypothesis has become somewhat more reserved. The reasons for this change in confidence include the fact that pyridoxal enters tumor cells rapidly whereas its stimulating action on transport continues for many hours, as Pal (309) observed. However, this finding hardly appears discouraging for the hypothesis, since according to equations 11 and 17 the asymmetry necessary for an uphill movement may be produced by other factors (for instance, as in Christensen and Oxender's experiment, by a potassium gradient), and the carrier concentration, then, will still affect the transport rate due to changes in v_{max} (since $D'C_t = v_{max}$, see equations 2 and 3).

C. Inorganic ions

In the case of ions, in addition to osmotic driving forces there may be that of an electrical potential difference. The decision whether or not a transport occurs uphill necessitates, therefore, the knowledge of the electrical potential difference across the membrane. In most cases the potential is not known accurately. This should always be kept in mind in discussing whether or not an ion transport is uphill.

The number of transported cationic and anionic electrical charges in a given transport must be equal. Therefore, if a transport mechanism exists for certain ions A of one sign, ions B of the opposite sign will likewise move, even without the operation of a special transport mechanism for B. They will, then, follow in a downhill movement the electrical potential difference produced by the specific transport of A. For which of the ion species transport mechanisms exist, therefore, can be decided only if the direction of the electrochemical potential difference is accurately known and allowed for. For example, the first observations on ion transport across the frog skin were interpreted by Krogh (235) in terms of a chloride transport. However, the later analysis by Ussing (227, 422, 423) clearly showed that under normal conditions only the sodium transport is uphill, and the chloride ions follow "passively" from higher to lower potential.

Simultaneous uphill transport of ions of both signs is not excluded. This has been demonstrated for the frog skin under conditions of stimulation of the skin glands by epinephrine (229), for the secretion of hydrochloric acid in the stomach, by Hogben (180), and for sodium chloride absorption from the rat ileum, by Curran and Solomon (88, 90). On the other hand, from the rat colon (89) and from the proximal tubules of the kidney in *Necturus* (441), only sodium appears to move uphill while chloride follows downhill.

A point characterizing some of the inorganic ion transports, particularly those

of K, Na, and Cl, lies in the fact that these ions are not metabolized, in the strict sense. The question therefore, whether the rate of entrance into a cell is limited by the rate of metabolism or by that of transport is not a complicating factor in these cases.

Nevertheless uphill transport is observed not only across absorbing epithelial layers but also across cell membranes into and out of individual cells. Evidence for such movements as well as their biological significance will be treated briefly for the alkali metal cations, sodium and potassium. An excellent and comprehensive review which may be consulted has recently been given by Ussing *et al.* (425).

1. The alkali metal cations. a. Observations. Present views on the permeability of *animal cells* to cations have evolved in steps. The well-known unequal distributions of sodium and potassium between cells and surroundings were first interpreted as due to impermeability of the cell membrane to cations (174). Bernstein's theory of excitation (20) replaced this view by the assumption of selective permeability to potassium. In 1941, Boyle and Conway (39) presented evidence for what was termed by Conway "standard permeability": permeability to potassium and chloride (and smaller ions of both signs), but not to sodium (and larger ions of both signs). This was supported by the finding that the cells behave as osmometers in experiments with varied concentrations of sodium chloride, but not of potassium chloride. This was first shown for muscle cells (39), and later for kidney cells (70). For muscle cells it was shown furthermore (72) that, as concluded from the rate of osmotic swelling in isotonic solutions, the order of decreasing rates of penetration for anions is chloride, bromide, and nitrate, while sulfate does not penetrate at all. Recent observations reported by Küsel and Netter (240) lend additional support to Conway's view.

The assumption of impermeability to sodium, however, soon became untenable, first because of the demonstration that labelled sodium readily exchanges across the membranes of different cells. One of the earliest findings of this kind was that of Cohn and Cohn (63) for red cells. Since then, isotope experiments have established sodium permeability for the vast majority of cells.

Isotope exchange alone would not necessarily be at variance with Conway's views. If it occurred by the mediation of a carrier capable of movement only when charged with sodium ("exchange diffusion"), the membrane could not be distinguished in osmotic experiments from a sodium-impermeable structure.

There are now, however, numerous observations indicating that under conditions unfavorable for a pump driven by metabolic energy (lack of oxygen, lack of substrate, presence of metabolic inhibitors, low temperature), net movements of sodium into cells occur, and that, after such a period, re-establishment of conditions favoring metabolism induces removal of sodium from the cells, from lower to higher electrochemical potentials; the latter indicates a pump. Examples include the early demonstrations by Harris (164, 165) and by Danowski (92) of sodium entrance into red cells in the cold, followed, at elevated temperature and in the presence of glucose, by extrusion. Essentially corresponding observations, with reversed directions, have been made for potassium: many cells lose this ion under conditions of low metabolism and regain it when the metabolism is

re-established. Observations of this kind are now on record for cells of a variety of organs, including retina (234, 417), brain (234, 417), tumor cells (265), kidney cortex (285, 286, 287, 440), liver (1), and others.

A special position among the inhibitors is held by the cardiac glycosides. Their action has recently been reviewed extensively by Hajdu and Leonard (157). For details this review may be consulted; only a short summary of the characteristics of the action of cardiac glycosides on alkali cation transport will be given here. Their inhibitory effect has been observed in many cell types, including erythrocytes (145, 208, 209, 363, 398), tumor cells (265), muscle cells (162, 269, 365), heart muscle cells (334, 368, 432), nerve fiber (47), frog skin (226, 461), and kidney tubules (195, 302, 414). It appears to be of wide occurrence. Although the main action is on uphill transport, and in many cases downhill movements are not affected (145, 162, 363, 368), the effect is not due to an interference with the energy-yielding metabolism. This was shown in the first report by Schatzmann on red cells with respect to the rate of glycolysis (363), and in later studies by Whittam (439) as well as by Kunz and Sulser (238) with respect to the formation of ATP. In nerve fibers, ouabain was shown by Caldwell and Keynes (47) to exert an inhibitory effect only on the external surface of the cell, but not when injected into the interior (in contrast to high-energy compounds, for the activating effect of which the reverse holds).

The current interpretation of the observations on sodium movements across cell membranes, first suggested by Dean (99) in 1941 and advocated vigorously in 1946 by Krogh (237), is that of a leak-and-pump system in which the sodium leaking into the cells is extruded continuously, at the same rate, by a metabolically driven pump, and potassium leaking out is pumped into the cells.

For potassium in red cells, the kinetics of the complete system were treated by Ponder (325), assuming that the pump works at constant rate and the leak linear kinetics. The result agreed well with experimental data. The dependence of potassium influx on the external potassium concentration was first studied by Raker *et al.* (329), by Sheppard and Martin (384), and by Solomon (396). They found constant influx rates independent of the potassium concentration. In the range of low potassium concentrations, Shaw (382) as well as Glynn (144) found a dependence following the Michaelis-Menten equation, superimposed in Glynn's observations on a linear component. An excellent survey on cation movements across red cells has been given by Glynn (146).

In a recent paper Solomon (397) assumed that the leak and the pump represent different pathways. His arguments include a comparison between pore diameters, as determined for red cells by two independent methods (147, 308), and the radii of the hydrated ions. The ionic radii given in this paper (Na 2.56 Å, K 1.98 Å) are slightly smaller than the estimated average pore radius (3.5 to 4.5 Å), indicating that some diffusion exchange of the ions across porous channels appears possible. For the pump, on the other hand, both kinetics and other arguments appear to indicate that it uses a different pathway. However, there is in this case again considerable evidence against the schematic interpretation that potassium entry and sodium exit occur through pumps and that, independently,

potassium leaves and sodium enters by diffusion through porous channels. Besides the fact that Glynn's kinetic analysis as quoted above points to a linear component in potassium influx (which might be due to diffusion and still be in harmony with the schematic leak-and-pump picture), the sodium influx was shown by Solomon (396) to exhibit non-linear kinetics and competition with lithium. Furthermore, Glynn (144) showed that potassium efflux likewise cannot be interpreted in terms of simple diffusion since the ratio of efflux to the linear component of influx does not follow Ussing's flux ratio criterion, efflux being higher than predicted from this criterion. The conclusion from these deviations appears to be that at least part of sodium influx and potassium efflux cannot be due to simple diffusion.

Kinetic studies of alkali cation uptake have also been carried out on *plant cells*. Olsen (300) found in a study of salt uptake by plants in water cultures that the rate of uptake of ions from the solution was independent of their concentration and not additive in the case of mixtures. In a later study on excised roots Epstein (118) was able to carry out accurate determinations. He found good agreement with the Michaelis-Menten equation for the uptake of rubidium, both in the absence and in the presence of sodium or potassium. These ions both were inhibitory. From a Lineweaver and Burk analysis it was concluded that the action of potassium and caesium is competitive, and that of sodium and of lithium non-competitive. The observations were interpreted in terms of carrier mechanisms.

The *mechanism of uphill movement* for potassium and sodium was interpreted by Solomon in an earlier paper in 1952 (396) in a manner closely resembling the system underlying equations 17 and 20. He assumed two separate carrier systems for sodium and for potassium, with different K_m values to account for the differences found in the relations between entrance rate and external concentration, and in addition internal metabolic reactions changing the affinity of the carriers to their substrates (lowering in the case of potassium and raising in the case of sodium). Both these systems would, according to his suggested scheme, transport not only uphill but downhill as well. In this point, Solomon's earlier suggestion differs from the later one quoted above (397). Likewise the ionic radii used in this earlier discussion (Na 7.9 and K 5.3 Å) are different.

The reason for Solomon's assumption of two carrier systems was that he found competition with sodium by lithium but not by potassium, and competition with potassium by rubidium but not by sodium. A scheme resembling that of Solomon in the assumption of an internal reaction affecting affinities, but differing in that only one carrier is assumed, was later suggested by Shaw (381) and used by Hodgkin and Keynes (178) as well as by Glynn (146). In this mechanism a carrier, X, with affinity for potassium transports this ion into the cell. There, by a metabolic reaction, X is changed to Y with affinity to sodium. Y transports Na to the external surface, where it is transformed back to X in a spontaneous reaction. It is again assumed that both carriers are capable of transporting in either direction, indicating the possibility of a "leak" through the pump.

The main merit of Shaw's scheme is its ability to account for a fact not men-

tioned so far: the *coupling between sodium and potassium transport.* The fact that both transports are inhibited by a number of common conditions enumerated above does not necessarily imply more than a common dependence on metabolism. However, the observation that in a number of cells lack of potassium in the external medium diminishes considerably sodium efflux, points to a direct coupling between the two transports. Observations of this kind have been reported in nerve by Hodgkin and Keynes (178), in muscle by Keynes (217), in red cells by Harris and Maizels (163) and by Glynn (144), and in frog skin by Ussing (424).

Recent work of Koefoed-Johnsen and Ussing (228) appears to indicate that in frog skin a coupled sodium-potassium pump is involved in the sodium transport. They pictured the epithelial cell in the skin as polarized with respect to ion permeability, the external membrane being selectively sodium-permeable, the internal potassium-permeable. Furthermore, in the internal membrane they visualized a linked pump for removal of sodium from, and uptake of potassium into the cell. The mechanism of sodium transport then is depicted as follows: sodium enters the cell from the external solution across the sodium-permeable membrane and is pumped out through the internal membrane in exchange for potassium; the accumulated potassium leaves the cell through the potassium-permeable internal membrane, the over-all result being a net transport of sodium from outside to inside. The experimental support for this interpretation includes the results of potential measurements across the skin between solutions with varied ion concentrations. They indicate that in the external medium, variation of the sodium concentration, but not of the potassium concentration, and in the internal medium variation of the potassium concentration, but not of the sodium concentration, affect the potential. In both cases the dependence of the potential on the concentrations approaches the expectation for reversible electrodes. Furthermore, as mentioned above, lack of potassium inhibits the sodium transport. Finally, measurements of osmotic swelling of the epithelial cells (carried out with a newly developed microscopic method), in accordance with predictions along the lines of the suggested scheme, showed swelling if in the internal solution sodium was replaced by potassium, but not if the same was done in the external solution.

In accordance with a number of parallelisms between frog skin and kidney tubules, indicating possible common elements in the transport systems, the analysis of membrane polarization in tubule cells carried out by Giebisch (142, 143) and by Whittembury (441) in *Necturus* has led to similar conclusions. This analysis was conducted by methods corresponding in principle to those used by Ussing, but involving the micropuncture technique. Again, the external membrane appeared to be potassium-permeable and essentially impermeable to sodium, and was interpreted to be the seat of a linked sodium-potassium pump. The internal membrane showed higher sodium permeability. The asymmetry, however, appeared less pronounced than in the frog skin, as also indicated by lower values of the transcellular electrical potential difference (20 mV as compared to 70 to 100 mV across the skin). A similar scheme, but placing the ex-

change pump in the internal membrane, was suggested for mammalian tubule cells by Pitts (322).

Transcellular transport thus would appear to utilize functional elements shared in principle by many different cell types, and adapted to the special functional purpose in transporting cells by a specific localization pattern of permeability and transport properties in the cell membranes.

As a second example of adaptation to specific cell function the ionic shifts across the membranes of excitable cells may be mentioned. In this case a specific pattern of transfer properties in the membrane is observed with respect to time rather than to space. The most pronounced and highly specific permeability changes for sodium and potassium during excitation, lasting very short times in the order of milliseconds, have led to interpretations in terms of specific carrier transport. They will not be discussed here since they have recently been treated very extensively in these reviews by Shanes (370). A recent review by Hodgkin (177) may also be mentioned. The study of the restorative processes during recovery, however, has yielded results having some bearing for the problem of the possible carrier which will be discussed in the following section.

b. Suggestions as to possible carriers. There appear to be sodium carriers and potassium carriers. One of the requirements for carriers of alkali cations, therefore, is that of specificity. Physico-chemical models for alkali cation carriers include two groups which, due to differences in the mode of binding, differ in the specificity to be expected. The first is the binding of hydrated ions to counter-ions, corresponding to salt formation in exchange resins. In this case, since small hydrated ions can approach the attracting counter-ions more closely, they are bound preferentially. The second possibility is binding in chelate rings. Here, since the ions are taken up in the unhydrated form, the order of decreasing binding affinity is heavy metals, earth alkali metals, sodium, and potassium. Chelation has been surveyed in these reviews by Chenoweth (50).

Among the suggestions as to possible ion carriers, the one made by Christensen that pyridoxal may be a common carrier for potassium and amino acids has been discussed above extensively. A second group of substances discussed by various authors is the phospholipids, particularly phosphatidic acid, and a third, the corticosteroids.

α. Phospholipids. In a study of sodium and potassium uptake by various lipoid fractions in the presence of tissue extracts, Solomon *et al.* (399) found that, of four fractions studied, phosphatidyl serine had the highest ability to discriminate between sodium and potassium. Phosphatidyl serine was further suggested as a possible sodium carrier by Kirschner (222).

Hokin and Hokin (185, 188) reported interesting evidence for the assumption that phosphatidic acid (a compound built of two fatty acids and one phosphoric acid radical bound to glycerol) can act as a sodium carrier. The authors suggested that at the inner surface of the cell membrane ATP phosphorylates diglyceride to form phosphatidic acid, catalyzed by an enzyme, diglyceride kinase. The phosphatidic acid then is assumed to take up two sodium ions and to carry

them across the membrane. At the external membrane surface it is split by the action of a phosphatidic acid phosphatase, with the release of sodium to the external medium, and of inorganic phosphate. The latter is assumed to move back into the cytoplasm to enter the phosphate pool, and finally to be used for oxidative phosphorylation again, thus closing the phosphate cycle. The diglyceride is supposed to return to the internal membrane surface where it is again phosphorylated by ATP, starting a new diglyceride cycle.

The evidence for this hypothesis rests mainly on the results of studies on the salt gland of the albatross, and on brain slices (182, 183, 186). The salt glands of marine birds have a cholinergic innervation and are stimulated by cholinomimetic agents. They secrete solutions of sodium chloride in concentrations as high as 0.84 M. In slices of this gland, the addition of acetylcholine (imitating activation by nervous impulses) led to a 10- to 20-fold increase in the turnover of phosphatidic acid (as measured by the incorporation of P^{32}). The acetylcholine concentrations necessary to produce the effect appeared to agree approximately with those required for stimulation of the gland *in vivo* and, as in the latter, the acetylcholine effect on lipoid turnover was abolished by atropine. The effect was specific in that the turnover of phosphatidyl choline and phosphatidyl ethanolamine was not (or only slightly) increased. The only other fraction affected was phospho-inositide, the turnover of which was increased 3-fold. Of the four cellular fractions studied (nucleus, mitochondria, microsomes, and solution), only the microsome fraction (which presumably contains the membrane material) showed a marked response. In slices of guinea pig cerebral cortex similar effects of acetylcholine were observed. Enzymatic activities corresponding to those predicted by the hypothesis were found in the microsome fractions of brain cortex. Soluble extracts from this fraction catalyzed the formation of P^{32} labelled phosphatidic acid from ATP^{32}, which was markedly increased by the addition of diglyceride. Deoxycholate extracts of brain microsomes liberated orthophosphate when incubated with phosphatidic acid.

Findings similar to the above were reported by Hokin and Hokin for other glands, including pancreas, salivary glands, thyroid, adenohypophysis, and adrenal medulla (184, 187, 189). Most of these studies, in fact, preceded that concerned with sodium transport. Hokin and Hokin's interpretations of these parallel findings in a variety of rather different exocrine and endocrine glandular structures are along the same lines ar depicted for sodium transport: they assume phosphatidic acid to be the carrier for a number of different substrates.

Impressive as the Hokins' evidence for the participation of phosphatidic acid in the sodium transport is, there are some points requiring further elucidation. The question may be raised as to whether the possibility has been satisfactorily excluded that the observations are related to common excitatory processes rather than to the secretory activities. Other points include the mode of return of inorganic phosphate to the cytoplasm and, in all cells with coupled sodium-potassium transport, the mechanism of the coupling.

In some measure recent findings on relations between adenosine triphosphatase and cation transport in various cells (mainly nerve fibers and red cells)

may be helpful here. The work on nerve ATPase was carried out by Skou (387, 388) and that on red cell ATPase simultaneously by Dunham and Glynn (111) and by Post *et al.* (328).

Skou was stimulated by the finding of Hodgkin and Keynes (178) that dinitrophenol, azide, and cyanide inhibit the sodium pump in nerve. The interpretation of these inhibitions as affecting oxidative phosphorylation was substantiated by the observation by Caldwell *et al.* (44, 45, 46) that in giant axons the inhibition by one of these agents can be counteracted by injection of energy-rich phosphate into the fiber (not, however, by its application to the external surface). Similar observations on red cells were reported by Straub (413) and Gardos (140) in which use was made of the permeability change during hemolysis for the introduction of ATP into the cells.

Skou studied an adenosine triphosphatase found in the submicroscopic particles of crab nerve. The enzyme was activated by magnesium and, in the presence of Mg, additionally by sodium. A further increase of activity could be produced by potassium. Skou interpreted the reaction mechanism as phosphorylation of an enzyme-sodium-potassium complex, with subsequent release of inorganic phosphate, sodium, and potassium. Ouabain was without effect on the enzyme in the absence of sodium and potassium, but inhibited the activation brought about by these ions. This effect, which appeared to indicate some relation to the ion transport system, was interpreted by Skou as an interference with the binding of the cations to the enzyme.

Dunham and Glynn reported findings in red cell ghosts largely resembling those noted by Skou, and showing additional parallelisms between ATPase activity and ion transport. The magnesium-dependent enzyme in this system, likewise, was activated by the simultaneous presence of sodium and potassium, but very little by either ion alone, reminiscent of the coupled transports of the two ions across the cell membrane. Cardiac glycosides inhibited the activation in concentrations of the same order as those necessary to block the sodium-potassium transport, and the inhibition was counteracted by high concentrations of potassium, as had been found previously for potassium influx in red cells (145). Both effects of cardiac glycosides showed the same structural dependence on the configuration at C-17 and on the saturation of the lactone ring.

From the fact that the enzyme defied all attempts of extraction into solution it was concluded that probably individual transport units in the membrane remained intact and continued to pump, and that "it may be that any or all of the observed alterations in ATPase activity resulted from a primary action on some other component of the transport system."

Post's results are qualitatively similar. They are impressive because of the quantitative parallelism between enzyme activity and transport parameters: the concentrations for half maximal activation by potassium were 3 mM for the enzyme and 2.1 mM for the transport, those for sodium activation were 24 mM and 20 mM, respectively, and the concentrations for half maximal inhibition by ouabain were 10^{-7} M for the enzyme and 3 to 7.10^{-8} M for the transport. An additional parallelism was found in the activation by ammonium,

with concentrations for half maximal effect of 8 mM for the enzyme and 7 to 10 mM for the transport.

Post's interpretation (327) follows the line of Shaw's scheme discussed above (381). He considered his "enzyme" preparation, which consisted of broken cells, as fragments of red cell membrane with qualitatively normal transport capacities. The sequence of events he visualized in the manner suggested by Shaw, assuming however, in accordance with Hokin and Hokin, activation of the carrier by phosphorylation, followed later by hydrolysis at a site not specified. The activation of the enzyme, then, was considered due to its being continuously resupplied with new substrate by the transport cycle; this would account satisfactorily for the cardiac glycoside effect on the activation.

As shown by Post's interpretation, the views of Hokin and Hokin and of the workers in the ATPase field are not mutually exclusive. They have in common an essential role of a phosphorylation brought about by ATP at the internal surface of the membrane, in accordance with the experimental results of Caldwell and of Straub and Gardos, and the assumption of a phosphorylated compound acting as the sodium carrier. Details of the views along these two lines may be considered as complementary.

A picture of a transport system thus emerges in which some elements are beginning to assume shape while other details, including the exact fate of inorganic phosphate and the possible nature of the potassium carrier, are still obscure.

β. Corticosteroids. The suggestion that corticosteroids might act as cation carriers by chelation in the side chain (415) was prompted by the resemblance between the structures of the supposed corticoid chelates and of genins of cardiac glycosides (the chelate ring corresponding to the lactone ring), by chemical studies of Bernauer and Fallab (19) indicating the possibility of chelation by corticosteroids, and by the fact that a number of antagonisms between cardiac glycosides and corticosteroids have been observed. Furthermore, there is increasing evidence indicating that corticosteroids are of importance for cation transport not only in the kidney but in other cell membranes as well (130, 155, 473). For example, the sodium pump in red cells was shown to be impaired in adrenalectomized rats (239). Other pertinent observations were reviewed by Wilbrandt (451).

The experimental evidence for the corticosteroid hypothesis so far is indirect and includes antagonisms between cardiac glycosides and corticosteroids in the kidney (414), in frog skin (461), and in smooth muscle (364), as well as increased toxicity of cardiac glycosides in adrenalectomized animals (153, 324) which is reversed by corticosteroids.

The kinetics of the antagonistic effect of high potassium concentrations on cardiac glycoside inhibition of potassium influx in red cells (145) were shown to agree with predictions derived from the chelation hypothesis (415, 449). A qualitative analogy to this effect may be seen in the well-known antagonism between potassium ions and cardiac glycosides with respect to certain cardiac effects.

Competitive antagonism between cardiac glycosides and corticosteroids could not be demonstrated *in vitro* in tumor cells (158), in red cells (196) (contrary to previous findings (415) as well as to a recent report (21)), or in the frog skin (461) (in contradistinction to the *in vivo* effect reported above); these failures may indicate that transport compounds of higher complexity are involved, and that the conditions *in vitro* are unfavorable for the supply of some of the components.

2. Divalent cations. a. Mammalian cells. Divalent cations have long been considered unable to penetrate cell membranes. Effects of divalent ions in general were considered to be due to external actions on the cell membrane. Here, likewise, the use of isotopes has changed current views. The exchange of bivalent cations across cell membranes is now receiving increasing attention.

In giant nerve fibers, Hodgkin and Keynes (179) found calcium movements across the membrane with a pattern resembling that of sodium: entrance during excitation, exit during recovery. The latter, as judged from the estimated internal calcium ion concentration, the external concentration, and the membrane potential, appeared to occur uphill against a difference in electrochemical potential of about 300 mV. In muscle, Bianchi and Shanes reported qualitatively similar findings, but differing quantitatively from those in nerve in the ratio between influx at rest and during excitation (28). In muscle, the additional influx per impulse appeared about 30 times higher than in nerve; this was interpreted by the authors as related to an essential function of internal calcium in the linkage between excitation and contraction.

In heart muscle, Niedergerke *et al.*, following views resembling those of Shanes with respect to the functional role of internal calcium, determined the rate of entrance of calcium (295) and of strontium (296) into heart muscle, and observed increased entrance rates under conditions both of low sodium and of low potassium. These effects reflected similar actions on the contractile strength (297), and appeared to depend on the ratio $(Ca)/(Na)^2$, confirming an earlier finding by Wilbrandt and Koller (457). The interpretation offered was competition of calcium and sodium for a common binding site, presumably in the cell surface. Whether this site is on a carrier was not discussed.

In absorption experiments on rat intestine, Solomon's group (110) recently found the flux ratio for calcium to fall below that predicted for independent diffusion from Ussing's criterion, which would be consistent with a carrier mechanism. There appeared to be a relationship to the sodium carrier system. Calcium in low concentrations enhanced, and in higher concentrations inhibited Na efflux, reminiscent of predictions from equation 23 for inhibitors of the type $I_{cs}(p)$ (see p. 127).

b. Plant cells and yeast. Along with his studies of rubidium uptake by barley roots, Epstein (119) also studied the uptake of bivalent ions. The results were essentially similar. Again, the kinetics of uptake appeared to follow the Michaelis-Menten equation and, again, different cations displayed mutual inhibition. For calcium the effect on strontium absorption was interpreted to be competitive, and for magnesium essentially non-competitive. From preloaded cells, labelled Sr was displaced by Ca, Mg, or unlabelled Sr.

Rothstein *et al.* (204, 359) studied the transport of magnesium and manganese into yeast cells. They found a transport system with considerable specificity, preferring Mg and Mn to other bivalent cations, depending on the metabolism of the cell, and displaying competitive inhibition. The uptake appeared to include two elements, one of surface binding and a second one of penetration, with different competition patterns; these findings were interpreted as indicating that the surface binding is not a necessary prerequisite to absorption.

The uptake was essentially unidirectional. Whether this was due to an uphill movement or to binding could not be established. The mechanism was assumed to involve a carrier of particular characteristics. The cation penetration depended on phosphate in a manner indicating that the process of phosphate penetration, rather than the presence of phosphate, is essential: preincubation with phosphate, followed by washing, enables the cells to take up Mn even in the absence of phosphate. This capacity then is maintained over periods of hours. The rate of its decay depends on the rate of metabolism. Sugar fermentation induces a rapid loss, while metabolism of ethyl alcohol and other substrates has little effect. These observations were interpreted to indicate that a carrier arises in a phosphorylation reaction during phosphate absorption, to be broken up by some link with the glycolytic metabolism.

3. Anions. It was pointed out above that there appear to be transport mechanisms for uphill movements of chloride. A number of observations, furthermore, point to the existence of systems capable of powerful accumulation of various other anions, particularly iodide, as first observed in the thyroid gland, and later in other glands including the mammary, salivary, and stomach.

In hypertensive patients treated with thiocyanate, Barker *et al.* (9) observed a goitrogenic action. The subsequent analysis revealed that its mechanism differed from that of antithyroid drugs which inhibit the synthesis of thyroxin. It was found by various authors (408, 416, 426, 427), both in animals and in man, that thyroids treated with propylthiouracil, although unable to synthesize thyroxin, still accumulate iodine. As concluded from electrometric determinations with silver iodide electrodes (427), I is apparently accumulated in the form of iodide ions. This accumulation is inhibited by thiocyanate (CNS). Other agents found to have an inhibitory action include dinitrophenol, inhibitors of respiration (135), and a number of anions, in the order of decreasing activity: ClO_4, IO_4, IO_3, ClO_3, $H(IO_3)_2$, ClO, NO_3, NO_2, and BrO_3 (407, 475, 476). These ions, like CNS, have two actions: they diminish both the rate and the steady state level of iodide accumulation. When added after the steady state has been established, they induce a release of iodide from the gland. The capacities of the various ions range in the same order with respect to these two actions (476), reminiscent of similar observations quoted above in the case of permeases in bacteria and of amino acid accumulation in tumor cells. A resemblance to these latter observations also exists with respect to the relationship between external and internal concentrations of iodide, S_o and S_i: raising S_o by injections of iodide in animals lowers the ratio S_i/S_o. A plot of this ratio against the logarithm of the administered iodide dose (which may be taken as proportional to S_o) yields a sigmoid curve resembling a dissociation curve (475). In the cases of bacterial per-

meases and of amino acid accumulation in tumor cells, as mentioned above, the relation between S_i and S_o was found to follow the Michaelis-Menten equation. It can readily be shown that these two relations are equivalent.

As in the observations on permeases and on amino acid accumulation in tumor cells (*cf.* pp. 142, 148), the question is raised as to whether the relation found implies binding to fixed sites in the cell or to a carrier. In the case of iodide accumulation in salivary glands (resembling closely that in the thyroid), Fletcher *et al.* (131) interpreted the observations in terms of "competitive adsorption." The strongest argument against this interpretation would appear to be that accumulation of iodide is observed not only in the cells of the salivary gland but likewise in the secreted saliva (192). Here, its concentration exceeded that in the plasma by a factor of 40, indicating an uphill transcellular transport of iodide. A further argument is the evidence mentioned above, indicating that the accumulated iodine is in the form of free iodide ions.

The same ions reported above as inhibitory for the intracellular accumulation of iodide in the thyroid also inhibit both the intracellular accumulation in slices of the salivary gland of the mouse (136) and the uphill transcellular transport of iodide across the gland (113). This parallelism between the transcellular transport and the apparent intracellular accumulation strongly suggests carrier mechanisms to be operative (*cf.* p. 119).

The nature of the inhibitory actions of anions appears to be competitive, as may be concluded from the parallel shift, in the presence of inhibitory anions, of the above-mentioned sigmoid curves relating to the accumulation ratio, S_i/S_o, and log S_o (475). While in the case of the thyroid the inhibitory anion thiocyanate was not found to be accumulated itself in the gland tissue (472), in the case of the salivary gland not only iodide but thiocyanate likewise was found to be concentrated in the secretion (113). Like that of iodide, the accumulation of SCN in the saliva was inhibited by ClO_4. From the fact that this inhibition may lower the concentration ratio, saliva:plasma, for ClO_4 below 1, it was concluded that SCN is not capable of passing the gland cells by free diffusion.

Observations resembling those on thyroid and salivary glands have also been reported for the mammary gland and the stomach. Both in the milk (40, 192) and in slices of mammary glands (136), iodide was found to be accumulated, and this effect was inhibited by the other anions mentioned and by metabolic inhibitors. Likewise iodide and SCN were accumulated in the gastric juice (192), and in the cells of the stomach surface epithelium and of the gastric pits (257).

The sum of these observations appears to point strongly to the existence of a carrier mechanism with graded affinities for a number of anions.

Rather close analogies were found in the iodide accumulation mechanism of marine algae. Here again the bulk of the accumulated iodine appeared to be in the form of free iodide ions (343). Accumulation ratios up to several thousand have been reported (383). Both metabolic inhibitors and anions, including ClO_4, SCN, and NO_3, were found to interfere with the accumulation (213, 224). The initial rate of uptake was shown to follow Michaelis-Menten kinetics (224), and the inhibition by the above-mentioned agents depended on the ratio of their concentration to that of iodide, pointing to a competitive mechanism.

Shaw (383) interpreted the transport mechanism to involve formation of I_2 from iodide by an enzyme, iodide oxidase, and to passage of molecular I_2 across the membrane. He pointed out that some of the inhibitors studied by him are either reducing agents ($S_2O_3^-$) or I_2-reactors (SCN^-). Clearly the anions enumerated above have in common an oxidizing action. If the suggestion of I_2 being the transport form is accepted, competitive inhibition by ClO_4, *etc.*, would be likely to occur *via* the enzyme rather than *via* the carrier. The general validity of this interpretation, however, would imply that in the stomach and in the salivary gland, where SCN is concentrated in the secreted solution, this ion is a transport substrate like I.

The recent report of Wolff and Maurey (471) that ouabain inhibits iodide accumulation in the thyroid and chloride transport in the stomach raises the question of a possible linkage to cation transport.

In *plant roots*, Epstein (117) studied the absorption of bromide. He found Michaelis-Menten kinetics and inhibition by other anions, which was competitive by chloride and non-competitive by nitrate.

Transport systems for *divalent anions* have been studied in a number of cell types. In the kidney, sulfate was found by Lotspeich to be reabsorbed with non-linear kinetics and a well defined T_m (258), and in the red cell phosphate entry was suggested by Gourley (150) to occur, at least partly, in the form of ATP formed at the outside. This suggestion was based on studies of the pronounced temperature dependence and on the effect of metabolic inhibitors (149, 151, 152). However, the difficulties in assessing the role of metabolic reactions inside the cell appear to be considerable. Vestergaard–Bogind and Hesselbo (431) concluded from experiments with high phosphate concentrations that "phosphate ions pass the erythrocyte membrane chiefly by a passive penetration process." Pfleger (316a) arrived at similar conclusions.

4. Hydrogen and hydroxyl ions. The reason for discussing jointly the transport of the positive hydrogen ions and the negative hydroxyl ions lies in their linkage as dissociation products of water, and in the fact that in most cases it is difficult to decide which of the ions is transported across a membrane, and indeed whether a transport of either actually occurs at all. If, for instance, acidification of the external medium is followed by an increase of the internal hydrogen ion concentration in a cell, this might be due to entrance of hydrogen ions, exit of hydroxyl ions, entrance of an undissociated acid, or exit of an undissociated base. Formally, the passage of undissociated acid molecules or base molecules across a membrane may be considered as a carrier mechanism for hydrogen ions or hydroxyl ions (with anions or cations, respectively, as carriers).

An illustration of the importance of penetration of undissociated bases is the absorption of weakly acid drugs from the stomach, studied recently by Hogben *et al.* (181), and the well-known accumulation of alkaloids and other weak bases in gastric juice (386). It is due to the penetration of the alkaloid base across the epithelium, followed in the lumen by a reaction between the base and hydrogen ions to form alkaloid cations. While the base concentrations will be equal on the serosal and mucosal sides, the alkaloid cations will be accumulated on the mucosal side. The net result, then, may be considered as an uphill transport of alkaloid cations, deriving the necessary energy ultimately from the transport sys-

tem for hydrogen ions. An example of an enzymatically catalyzed carrier system of this kind is the transport of hydroxyl ions across the red cell membrane in exchange for Cl^-, according to the early interpretation given by Jacobs (198, 199, 201). Jacobs showed that the transport is slow in the absence of bicarbonate or in the presence of carbonic anhydrase inhibitors. He suggested that OH^- reacts with CO_2 to form HCO_3^- (enzymatically catalyzed), and that HCO_3^- exchanges with Cl^- across the membrane and releases OH^- in the cell (again enzymatically catalyzed), the net result being an exchange of OH^- for Cl^-.

The outstanding cases of uphill transport of hydrogen ions in the mammalian organism are the secretion of hydrochloric acid in the stomach and the acidification of the urine in the distal tubule of the kidney. The interpretation of urine acidification as due to bicarbonate reabsorption has been shown to be inadequate by Pitts and Alexander, who demonstrated that the total amount of excreted acid may by far exceed the amount of bicarbonate filtered in the glomeruli (323). This led to the alternative suggestion of hydrogen ion secretion. A number of observations, both experimental and clinical, pointing to a competition between hydrogen and potassium ions for secretion (17, 18) has further prompted the assumption that the luminal membrane of the tubule cell is the seat of a carrier mechanism for these two ions. It was finally suggested that this mechanism is linked to the reabsorption of sodium in a manner similar to that found for movements of potassium and sodium in many other cells (*cf.* p. 154). Since in the tubular urine replacing sodium ions by hydrogen ions will lead to a decrease in the osmotic pressure (due to the buffering capacity of the urine), while replacing by potassium will not, a shift from hydrogen to potassium in the exchange mechanism will promote diuresis. This has been assumed to be the basic element in the diuretic effect of carbonic anhydrase inhibitors (322) and may, further, be partly responsible for the diuretic action of potassium salts (15).

In the secretion of hydrochloric acid in the stomach, the enormous electrochemical potential difference of some 300 to 350 mV involved would appear (*cf.* p. 119) to point to the operation of a carrier mechanism. The redox pump theory developed independently by Conway (67, 68) and by Davies (94, 97) assumes a carrier function of a reduced redox catalyst carrying H_2 outward across the membrane. Externally, H_2 is assumed to be oxidized to $2H^+$ by a metal catalyst which subsequently either enters the cell itself to be reoxidized or, in some way, transmits electrons across the membrane. The redox theory involves problems not immediately related to the carrier mechanism proper, including mainly the source of the hydrogen ions secreted (94) and the energetic relations to the chain of oxidative reactions and to oxidative phosphorylations (96). These considerations fall outside the scope of the present review. With respect to the nature of the carrier, it was pointed out by Davies (95) that lipoid-soluble redox catalysts are known, including ubiquinone and vitamin K, which may meet the requirements of the redox theory.

Conway has stressed the analogy between hydrochloric acid secretion in the stomach and in yeast cells, which under suitable conditions may produce a pH as low as 1.6 in the external medium (68). In these cells, Conway and Kernan (71)

demonstrated an effect of redox dyes on cation transport: dyes of high redox potential inhibited sodium exit from "sodium yeast" (cells enriched in sodium), while dyes with low potential interfered with the potassium-hydrogen exchange.

D. Other substrates

1. Substrates of secretion and reabsorption in kidney tubules. Secretion across tubular cells has been studied in a number of ways. In the mammalian kidney, conclusive evidence for *tubular secretion* requires demonstration of a rate of urinary excretion exceeding the rate of glomerular filtration. This implies an uphill transport. For dyes, microscopic observation either in kidney cortex slices or in isolated kidney tubules of fish (particularly flounder) (132, 134) or in tissue-cultured chicken mesonephros tubules (48) has been used. The kidney slice technique is useful also in the case of uncolored compounds the accumulation of which in the slice can be tested by chemical analysis, leaving unanswered, however, the question of localization. In birds, use has been made of the separate blood supply to the tubules through the renal portal vein (404). The field of tubular transport has been surveyed in this journal by Beyer (23).

The substrates of secretion comprise two main groups: *acids and bases*. They appear to use two different mechanisms, as is evident from competition experiments and from differences in the action of a number of inhibitors. The first *acid* for which secretion was established was phenol red. The early finding of Marshall and Crane in 1924 (267) that the excretion rate of this dye does not increase in proportion to the plasma concentration, as required for filtration, was followed in 1928 (268) by the demonstration of its excretion by the aglomerular kidney of the goosefish, and in 1933 (48) of its accumulation *in vitro* in chick mesonephros tubules. Shannon showed in 1935 (372) in the dog that the total rate of excretion is composed of a linear element, from filtration, and a saturation element with a well defined maximal rate of secretion, T_m. Besides phenol red a number of related dyestuffs are transported uphill. In competition experiments Forster *et al.* (133) found in flounder tubules that slowly accumulated dyes are more powerful inhibitors than those which are rapidly transported. This observation in experiments involving saturating substrate concentrations appears to be related to the dependence of the transport rate on the affinity to the carrier, as discussed above (see p. 117). Uncolored compounds secreted across tubule cells by the same mechanism (as concluded from mutual competition) include hippuric acid, *para*-aminohippuric acid (PAH), a number of iodinated compounds used in roentgenography, sulfonamides, penicillin, and others (390).

In experiments on kidney slices, Cross and Taggart (84) observed a marked enhancing effect of acetate on accumulation of PAH, paralleled *in vivo* by an increased value of T_{mPAH} (288). This interesting effect and its possible relation to the transport mechanism have been discussed extensively by Cross and Taggart (84). It is shared by other fatty acids, varying both with the chemical structure of the acid and with the animal species investigated. Shideman (385) observed in rabbit kidney slices a stimulating effect of hexanoate in concentrations below 1 mM, but inhibition above 3 mM. Similar observations were made with

octanoate. Butyrate stimulated in concentrations of 5 and 10 mM. In rat kidney slices, however, Despopoulos (101) found stimulation by 5 mM acetate and inhibition by 15 mM, while in dog slices 10 mM stimulated and 100 mM inhibited. The action of fatty acids, therefore, at least in a number of cases, appears to be diphasic: stimulating at low, inhibiting at high concentrations, the concentration of reversal depending both on the animal species and on the chemical structure of the acid. In the case of β-hydroxy butyric acid, Shideman found, in equal concentrations, D-isomers to be more stimulating, and L-isomers more inhibiting. This recalls the diphasic effect of competitive inhibitors of the type $I_{CS}(p)$, as discussed above (*cf.* p. 127), indicating the possibility that fatty acids are transport substrates with low affinity in general, and in particular with lower affinity in the short chain members and in the D-isomers.

The search for inhibitors which are not transported themselves was of practical importance in connection with the rapid excretion of penicillin, before salts of this acid with prolonged action became available (22). It led to the discovery of carinamide (23, 24) and probenecid (Benemid) (25) by Beyer. Other inhibitors interfering with PAH-accumulation in kidney slices without being accumulated themselves, have recently been studied by Despopoulos (102). He found uric acid, but not dioxypurines, to be inhibitory without affecting the rate of respiration in the slices. The inhibition therefore appears to be primary rather than secondary. It was stronger with methylated compounds, and this was interpreted as indicating an essential role of carbonyl groups. A similar action was found with a number of barbiturates (103), which again was more pronounced in N-methyl compounds. It was concluded that for this type of inhibition three carbonyl groups in a suitable spatial arrangement are required.

The structural requirement for the transport substrate itself was studied by several authors (103, 225). It was first found that the acid character as such is not sufficient, and that benzamido or benzimido structures appear particularly suitable (225). Despopoulos, from the study of 140 organic anions (103), recently arrived at the conclusion that the general structure required for tubular transport is: $R—C{:}O—NX—(CHR^{-})_n—COOH$, and that of the three oxygens, two (the carbonyl oxygens) form hydrogen bonds, while the third (OH) forms an ionic bond with the receptor. This three-point attachment was assumed to be reflected in the above-mentioned structural requirement for competitors.

The group of *basic compounds* sharing a common transport mechanism was first shown by Sperber (405) to include guanidine, N-methyl nicotinamide, piperidine, and methyl guanidine. Later, tolazoline (Priscoline) was added by Orloff *et al.* (301), and tetraethylammonium (TEA) by Rennick *et al.* (335). Like that of the acid substrates, the transport of the basic compounds is characterized by mutual inhibition within the group, which was shown by Farah to be competitive for TEA and Priscoline (120). The transport is depressed by a number of metabolic inhibitors including dinitrophenol, cyanide, azide, and iodoacetic acid. According to Farah, it differs from the acid system in the effect of inhibitors of the Krebs cycle (malonate, fluoroacetate, dihydroacetic acid), which affect the transport of acids but are without effect on the transports of bases.

This, however, might be due to the fact that these inhibitors are all acids, rather than to differences in the relation to the Krebs cycle. Such a possibility appears to be strengthened by the observation that on the other hand the basic dye cyanine 863 only acts on the accumulation of TEA, but not on that of PAH (316, 335).

With respect to *tubular reabsorption*, besides the sugars and amino acids discussed above, a number of other transport substrates have been described, including ascorbic acid, β-hydroxy butyric acid, lactic acid, and guanido-acetic acid. The first three have been shown to display non-linear kinetics with a maximal rate of transport. They are assumed not to share common transport mechanisms (390).

2. Uric acid and other purines. In the human kidney, uric acid is reabsorbed with non-linear kinetics and a well-established T_m (16). The inhibition by probenecid (29, 392) has been used in the treatment of gout. The field has been surveyed in these reviews by Bishop and Talbott (30).

An equilibrating transport system for uric acid has recently been described in red cells by Overgaard-Hansen and Lassen (305). The uptake of uric acid was found to be inhibited by hypoxanthine. These authors assumed the participation of a second mechanism unaffected by hypoxanthine. This interpretation has been used more recently by Christensen (53) in the analysis of effects of estradiol disulfate on uric acid movement across red cell membranes. Both inhibition and acceleration of uric acid uptake were observed with estradiol disulfate concentrations between 10^{-3} and 10^{-5} M. Since the inhibitory effect was seen in the absence, and the accelerating effect in the presence of hypoxanthine, they were ascribed to the assumed mediated and non-mediated component of the transport, respectively.

Another set of observations on transport of purines was reported in yeast cells by Roush *et al.* (361). Twenty-two compounds were studied among which adenine, guanine, hypoxanthine, xanthine, uric acid, 2,6-diamino purine, and *iso*guanine were rapidly removed from the medium to give high intracellular concentrations. The action of metabolic inhibitors was interpreted as indicating dependence of the system on glycolysis rather than on respiration. Competition was exerted not only by transport substrates but also by compounds not accumulated themselves.

3. Glycerol in red cells. In 1934 Jacobs and Corson (200) observed inhibition by traces of Cu^{++} of glycerol penetration in human red cells. The effect was specific, both with respect to the chemical structure of glycerol and to the animal species the cells of which were affected (199). The cell species sensitive to heavy metals likewise displayed a very pronounced dependence of the glycerol penetration rate on pH. In a later study (202), it was found that the concentration of copper ions necessary for 90% inhibition is as low as 6.10^{-7} M. At this concentration, at most 1% of the total cell surface could have been covered by copper. "Active patches" of small total surface area were assumed to be the site of a specific transport mechanism for glycerol penetration.

Stein (409, 410), in an analysis of this system, came to the conclusion that the

glycerol receptor is a peptide with an N-terminal histidine molecule, and that the terminal hydroxyl groups of glycerol form hydrogen bonds with the nitrogen atoms of histidine. Bivalent copper, according to Stein, blocks the nitrogen receptors. The pH dependence reflects the dissociation curve of histidine. Support was found in the competitive action of dihydroxypropane, in which the 1,3 dihydroxy compound showed higher activity than the 1,2 dihydroxy compound, and in the quantitative comparison of the effects of various complexing agents on Cu^{++} inhibition, which indicated an association constant of 10^{10}, close to that of the copper-histidine complex.

4. Serotonin. Recent observations on the uptake of serotonin by blood platelets (34, 35, 36, 161, 194, 418, 477) revealed accumulation with maximal apparent concentration ratios up to 1000 at 37°C. At 0 to 2°C, only equilibration was observed. The uptake at high temperature was inhibited by cyanide and iodoacetate, and its kinetics followed the Michaelis-Menten equation. However, the question whether serotonin within the cells was free could not be decided conclusively. The assumption of a specific transport system is strengthened by the recent finding of Weissbach *et al.* (436) that the uptake of serotonin in platelets is inhibited by cardiac glycosides and stimulated by K. Only the increment due to K is inhibited by ouabain. This recalls similar findings with respect to the uptake of glucose in intestinal cells. The possibility discussed for those cells of a linkage to ion transport is raised again here.

V. CONCLUDING REMARKS

The development of the field of carrier transport may be considered as having passed through several stages. The first period was characterized by difficulties in the interpretation of observations along "classical" lines. Unexpected specificities, deviations from diffusion behavior and uphill movements called for specific mechanisms and led to the qualitative picture of carrier mechanisms.

In the second stage, the quantitative consequences of the mechanism were formulated and compared with experimental data, so that a number of observed peculiarities could be accounted for in a quantitative manner. Along with some successes in this direction, however, new problems arose. Discrepancies in the numerical values of K_m obtained from inhibition and from penetration rates, and differences in V_{max} for different substrates may be mentioned as examples indicating that a re-interpretation of the relatively simple concepts used so far will be necessary. A number of observations, including inhibition of competitive transport by substrates not transported themselves, indicate that transport mechanisms may contain more than one binding site. Refinements of interpretations along these lines will form part of the tasks for future work.

The main problem, the identification of carriers and the demonstration of their action as isolated units, corresponding to the demonstration of enzyme reactions, meets with great experimental difficulties. Most of the approaches used so far are indirect, attempting to derive conclusions from a systematic variation of as many parameters of the systems as possible. Encouraging as some of the obtained results are, the desirability of a direct approach, analogous to the first isolation of enzyme molecules, is strongly felt by many workers in the field.

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