Biochemical Studies on Active Transport

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ABSTRACT The active transport process, so important in cell function, has been studied in the past with intact cells. Models which have arisen from this work all depend on: first, a specific protein to *recognize* the substrate; second, *translocation* of the substrate across the cell membrane; third, release of substrate within the cell and *restoration* of the system to its initial state. These steps are adequate for facilitated transport, but in active transport an *energy* input is required to maintain a concentration gradient. Parts of transport systems have been isolated recently. A protein which specifically recognizes β -galactosides has been partially purified. In another case, a protein that appears to be the recognition part of the sulfate transport system of *Salmonella typhimurium* has been crystallized, and many of its properties have been described. The role of this protein in recognition and in translocation is discussed. Also proteins that phosphorylate a variety of sugars as they enter the cell's interior provide a mechanism for concentrating sugars as their phosphates, against a gradient.

My method is to try to say what I cannot say, because I do not understand it.—NIELS BOHR

Biological transport mechanisms have been studied for many years owing to their importance in a wide variety of physiological processes. Often these studies have been aimed at devising mechanisms to explain transport. In spite of the development of models reasonably consistent with the data used to derive them, transport still is not clearly understood at the molecular level. An important question is whether transport systems are like enzymes in catalyzing covalent bond formation between substrate and a carrier molecule in the membrane, as at least implied by the name “permease” (1), or, alternatively, whether substrates are only adsorbed by the transport system by a protein of the sort for which the name “emphore” has been suggested (2), and of which hemoglobin is a well-known example. Questions such as these underline the need for new approaches and new ideas regarding the mechanism of transport. The object of this article is to describe some of these approaches briefly.

CATALYSIS AND TRANSPORT

The mechanism of biological transport presents no less difficult a problem than does the mechanism of enzyme catalysis. Indeed, these problems have many similarities, as has often been remarked. Enzymes change the chemical positions of groups of atoms, by moving them across energy barriers between covalently bonded states, at rates far greater than are found in enzyme-free systems. Transport systems change the spatial positions of molecules by helping them to cross spatial barriers, at rates far greater than observed otherwise. These similarities might well be more than a matter of words, but in neither case do we know enough to decide whether the mechanisms are similar at the molecular level.

More importantly, experimental approaches are similar in the two areas, whether or not the transport systems are enzymatic. This similarity can be a source of strength rather than of controversy. Enzymes can serve as models for investigation of transport, being by far the more advanced of the two as subjects of intensive study. It is amusing to consider that transport is now at the position where enzymes were nearly 40 years ago. Enzymes were reviewed in the *Annual Review of Biochemistry* for the first time in 1932, in the first volume (3). Transport was reviewed for the first time in this series in 1963 (4). Most of the 130 references in the review on enzymes reported the existence of catalyzed reactions, and examined the question of specificity, i.e. whether one enzyme catalyzes two rather similar reactions, such as the actions of sulfatases and phosphatases; in a few cases, the kinetics of substrate and inhibitor action was used to discuss mechanisms. The second major question, the chemical nature of enzymes, was a pressing one but was not discussed much in 1932. The contemporary reports of enzyme isolation and crystallization received rather little space and were treated with some skepticism. Since that time about 1000 enzymes have been identified, their degrees of specificity recognized, their kinetics developed in enormous detail, their purifications achieved completely in several hundred instances, and, most recently, their complete three-dimensional structures worked out in a few instances. In these last researches, combined with the concepts of physical and organic chemistry, we see the beginnings of understanding of the central act of catalysis.

Transport has recently moved at a rapid pace, since its trail has been blazed by the enzymologists and because there are now far more scientists in the world. "Permeationists" (we have not yet a word like enzymologist, nor encyclopedic works and journals on "permeology") have only recently started to write reviews and books, and to hold specialized meetings. They have identified a few dozen transport systems, done extensive kinetic studies on perhaps a half dozen, and have very recently isolated parts of several trans-

port systems in various degrees of purity. Needless to say, the central process of translocation is completely unknown.

The point of this comparison is that, in this writer's opinion, further solid progress on mechanisms of transport will be made only when we follow the lead of enzymologists and study isolated parts of transport systems.

MODELS FROM KINETIC STUDIES

Extensive investigations of rates of entry and exit in uninhibited and inhibited cells have established major points regarding transport mechanisms. For details, one can consult recent reviews (5-7). The paper that follows this one (8) provides an excellent example of kinetic investigations and the deductions can be drawn from them.

A complex problem is usefully divided into its parts, each of which hopefully is separately susceptible to attack. We can draw up a model of transport, based

FIGURE 1. A model for transport.

upon kinetics (Fig. 1). Here transport is divided into the first step, *recognition*, in which the substrate combines with a specific molecule; then *translocation*, a process by which the substrate is moved across the membrane; then comes a release of substrate inside the cell and return of the system to its original state (rather arbitrarily lumped together as *recovery*). Finally, an *energy*-providing step is included for cases of active transport.

The postulation of these steps immediately poses the problems of identifying the chemical components involved in each. Thus, one should be able to isolate recognition proteins, translocation substances whose properties will give a clue as to the nature of translocation, etc. Recently obtained results and concepts regarding these steps will now be discussed in sequence. Bacteria were used, unless otherwise stated.

RECOGNITION

Studies with intact cells have provided extensive evidence for a specific part of transport. Substrates are transported according to adsorption-saturation

curves. Specificity is generally much like that of an enzyme or antibody, being restricted to a structurally related group of molecules (for example, neutral amino acids in one system, or galactosides in another). The receptor sites must be protein, since only proteins have structures with the required degrees of discrimination. This conclusion is confirmed by recent studies in which

FIGURE 2. Crystals of sulfate-binding protein. The photograph was taken by Mr. L. H. Bernstein using a Zeiss polarizing microscope.

specific parts of transport systems have been isolated; in each case a protein is involved.

One of the most actively studied transport systems is the β -galactoside permease of *Escherichia coli*. A specific site has been identified with the use of the sulfhydryl reagent *N*-ethylmaleimide (9). This transport system and the protein are inducible, and are controlled by the gene *lacY*. The protein has been partly purified, and there are about 10,000 such "M proteins" per bacterium. This protein has also been observed by specific labeling and fractionation on DEAE-cellulose (10). Further studies await complete purification.

Proteins with affinities for such diverse transported molecules as inorganic sulfate (11), Ca^{++} (11*a*), amino acids (12), and galactose (13) have been identified. That these proteins are involved in their respective transport systems is supported by considerable evidence, including genetic and induction studies.

The sulfate-binding protein has been completely purified and crystallized (Fig. 2). It consists only of protein, of usual composition except for a low sulfur

TABLE I
COMPOSITION OF SULFATE BINDER*

Amino acid	Hydrolysis for		Estimated composition
	24 hr	72 hr	
	<i>residues/molecule</i>		
Lysine	18.4	18.8	19
Histidine	3.6	3.8	4
Arginine	8.1	7.7	8
Aspartic acid	28.6	29.9	30
Threonine	10.1	10.4	11 (10)†
Serine	11.7	10.7	13 (12)
Glutamic acid	21.0	21.0	21
Proline	8.9	9.2	9
Glycine	16.6	16.6	17
Alanine	23.2	23.4	23 (24)
Valine	12.6	16.3	17
Methionine	1.0	1.0	1
Isoleucine	8.7	11.7	12
Leucine	14.8	16.0	16
Tyrosine	8.5	8.7	9
Phenylalanine	8.3	8.7	9
Tryptophan (by optical density)			5
-NH ₂			~20

* A. Tsugita. Unpublished data.

† Numbers in parentheses are alternative values.

content (Table I). Tests for other components, including carbohydrates, lipids, phosphate, and metal ions, were negative. An earlier report of a small, variable amount of calcium ion can be attributed to contamination (perhaps from the calcium phosphate gel, hydroxylapatite, used for purification), since growth in the presence of radioactive calcium ion produced bacteria whose shock fluid contained less than 1 calcium ion/1000 molecules of binder protein.

Each protein molecule of mol wt 32,000 firmly binds one sulfate. Binding does not involve covalent bond formation or require an energy source. This is also the case for binding of amino acids or galactose to their respective proteins. In fact, no evidence exists for covalent bond formation during trans-

port. Sulfate is bound to its recognition site by ionic forces as shown by the tremendous ionic strength dependence, which is very much like that of an ion exchange resin although about 10^5 times stronger. Binding is abolished by amino group reagents such as acetic anhydride, suggesting that several positively charged groups might be placed so as to hold the negatively charged sulfate firmly.

The sulfate-binding protein is at or near the membrane surface, as indicated by ability of mutants to bind sulfate although they are unable to carry sulfate into the cell (14), and by the inactivation of binding by protein reagents, which cannot penetrate to the interior of the cell (15).

The sulfate binder as well as amino acid and galactose binders are released by osmotic shock, suggesting that they are located in or near the cell membrane and are held by weak forces. The M protein of β -galactoside transport is located in the membrane and requires detergents for its release, and so the affinity is stronger but is not a covalent linkage. By contrast, a proline transport system is firmly linked to membrane fractions of *E. coli* (16).

These results show that the recognition step of sulfate transport involves a noncovalent complexing of substrate to a specific protein located in or on the outside of the cell permeability barrier.

TRANSLOCATION

There is really no clear evidence as to how translocation, the central process of transport, operates. Kinetics suggests a cyclical process, as indicated in the model of Fig. 1. However, kinetics, by its nature, cannot prove either that the recognition protein functions as an enzyme which combines substrate with a low molecular weight carrier, the compound then diffusing across the cell, or that a physical change in the recognition protein carries the substrate across the membrane. For a time the former hypothesis was preferred, and phospholipids were suggested as likely carrier molecules. However, results on phospholipid turnover (17) make this seem unlikely.

Thus, if the binder protein is an enzyme, energy should be required to form a covalent bond between sulfate and a carrier. No catalysis of ATP breakdown in the presence of sulfate binder protein, bacterial lipid fractions, and sulfate has been observed. This result does not support an ATP-catalyzed combination of sulfate with some cell lipid component, although obviously all conditions and energy donors cannot be tested.

If the recognition protein itself directly translocates the substrate, the properties of this protein should tell us something about the mechanism of translocation. If the recognition protein is itself a carrier or emphore, it would translocate the substrate either by diffusion or by its conformational rearrangement. Diffusion would seem to require lipid solubility of the protein-sulfate complex. The complex is not appreciably soluble in a variety of lipid solvents,

nor is it able to carry sulfate into these solvents. The only evidence of affinity for lipids is the ability of the protein to dissolve small amounts of silicone oil (11). This appears to be a detergent-like coating action, and suggests that a lipophilic region of the protein exists. However, this evidence does not support a mechanism of translocation based on diffusion of protein-substrate complex across the membrane.

Mechanisms that depend on mechanical translocation would require that the binder be long enough to reach across the cell membrane, e.g. about 80 Å. The sulfate binder does satisfy this requirement. The shape can be approximated from the molecular weight and the diffusion coefficient or viscosity (11). These measurements indicate a highly asymmetrical molecule, with the longest dimension about 70–120 Å, depending on assumptions made as to whether it is a flattened disc or a spindle-shaped protein. The uncertainties of

FIGURE 3. Ultracentrifugal patterns of binder protein with and without added 1 mM sulfate. The run was made by Miss Barbara Bamman, using a sector cell in the Spinco model E ultracentrifuge. $s_{20} = 2.9S$ for both samples.

such physical chemical calculations are well known, but the results suggest that the molecule might well be long enough to stretch across the membrane. Attempts to see the molecule in the electron microscope or to measure its dimensions by other physical chemical methods are in progress.

If translocation is mechanical, one can visualize models of several sorts, such as rotational diffusion, small conformational changes which permit passage through the protein itself (18), or folding, contracting molecules in which one part of the protein is moved physically through the membrane while the other part remains fixed. The latter two models require conformational changes during translocation. Measurable changes in sedimentation or in mobility on gel electrophoresis have not been found when the protein combines with sulfate (Fig. 3). But some preliminary evidence exists for localized shape changes.¹ Also, fluorescence of the protein is changed when it combines

¹ A. Tsugita and A. B. Pardee. In preparation.

with sulfate (11), indicating a change in the environments of its aromatic amino acids. Further studies on the properties of this protein may yield clues regarding the mechanism of translocation.

RECOVERY

All models derived from kinetics involve a recovery step, in which the carrier releases the substrate inside the cell, and the system returns to its original state. So far no evidence regarding recovery is available from biochemical studies.

ENERGETICS

Transport is divided into facilitated and active processes, the former not requiring energy and the latter using energy to create a higher concentration inside the membrane than outside. Yet, in at least two cases, the energy input is supplementary to the basic process of translocation. Thus, in the case of transport of α -thioethylglucopyranoside into a yeast, the facilitated diffusion is always present, but active transport is only created under certain inducible conditions of growth and is thus a dispensable part of the entire transport system (19). Energy inhibitors prevent active transport of β -galactosides into *E. coli*, and yet do not interfere with the facilitated translocation of these compounds (20). Therefore, if one considers the fundamental event of transport as translocation through the cell membrane, the active part of the process is not central to the main problem, at least in these cases.

Biochemical studies on energetics have produced two particularly interesting results. The first system deals with the transport of a variety of carbohydrates. Mutants which have simultaneously lost their ability to transport a number of sugars are known (21). A mechanism for phosphorylation of these sugars has been reported; it requires three proteins: enzyme I, enzyme II, and Hpr protein (22). More recently, it has been shown that the mutants are defective in enzyme I or Hpr proteins (22–24). A scheme which seems consistent with most of the data presented is indicated in Fig. 4. One component of the phosphorylating system, enzyme II, which is membrane-bound, is actually involved in translocation. The protein Hpr is phosphorylated by enzyme I and phosphoenolpyruvate (PEP), and Hpr-phosphate phosphorylates the sugar bound to enzyme II at the interior of the cell. The total result is that sugar phosphate accumulates within the cell, and this allows active transport. In some cases, facilitated diffusion can exist in mutants which lack either enzyme I or Hpr. One can assume a noncatalytic separation of the sugar from enzyme II in these instances. In other cases, the sugar does not accumulate in the phosphorylated form; here it is assumed that a phosphatase action step can occur. This Hpr system thus seems to follow translocation,

and so might or might not be accessory to actual transport; the details of the relations among these events remain to be worked out.

Curiously, although a role of the Hpr protein was suggested for β -galactoside transport (25), mutants which are defective in enzyme I still are perfectly capable of transporting these compounds (26). Furthermore, the role of Hpr is to provide energy for transport, and yet facilitated diffusion can take place when inhibitors cut off the energy supply (20), indicating that Hpr-phosphate is not essential for lactose translocation or release (24). Perhaps, however, Hpr itself is required as an allosteric effector.

Another system in which the energy requirements for transport have been extensively studied is sodium-potassium transport by mammalian cells. The enormous amount of work on this system is summarized in the recent reviews. From a biochemical point of view, both an ATPase and protein-bound phosphate have been implicated. Recently, phosphate has been shown to attach to

FIGURE 4. A sugar transport scheme.

a γ -carboxyl group of glutamic acid of the protein (27). The ATPase has been shown to be in a membrane fraction, but its purification is difficult.

CONCLUSION

It is this writer's conviction that the process of transport will be understood when, as in enzyme catalysis, all of the parts of some transport system are isolated and their structures and interactions have been clarified. Kinetics is always a limited approach—it at best eliminates hypotheses, but by itself it can never provide final proof—owing to the complexity of biological systems with their numerous unknown parts. Recent biochemical studies of the sort outlined here give hope of rapid progress in the near future.

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Discussion

Dr. Kennedy: As Dr. Pardee mentioned, one of the laboratories that have been carrying out studies in this area has been that of Kaback and Stadtman at the National Institutes of Health. Dr. Kaback is here this afternoon, and I would like to ask him to begin the discussion.

Dr. H. R. Kaback: Unlike Dr. Pardee, it is my feeling from experience with membrane preparations that transport systems for various solutes may be radically different. This electron micrograph (Fig. 1, Discussion), taken by Dr. Vincent Marchesi of the National Cancer Institute, demonstrates the morphological properties of the preparation that I have been working with. As can be seen, the preparation consists of closed vesicles limited by a so-called "unit membrane." Furthermore, essentially no ribosomes or other internal structures can be seen.

As Dr. Pardee has already told you, preparations such as these, which lack most, if not all, of the cell's soluble proteins, nucleic acids, and cell wall, are able to concentrate proline against a gradient, as well as catalyze the rapid exchange of external proline or hydroxyproline with proline in the intramembranal pool. Membranes prepared from a mutant specifically defective in its ability to take up proline catalyze neither proline uptake nor exchange.

Similar membrane preparations also take up glycine and catalyze the exchange of external glycine, alanine, serine, or threonine with glycine in the intramembranal pool; however, in this case the uptake of glycine is mediated by a facilitated diffusion mechanism. In other words, glycine uptake into the intramembranal pool, although very rapid and highly specific, does not take place against a concentration gradient, nor does it require an actively metabolizing system. In analogy with the proline system, membranes prepared from a D-serine-resistant mutant which does not take up glycine do not catalyze glycine uptake or exchange.

Finally, as an example of a third and apparently different type of uptake mechanism, I would like to present some very recent data that have been obtained from studies of sugar uptake by isolated membrane preparations. I think these data will answer some of the questions raised by Dr. Pardee regarding the role of the P-enolpyruvate-P-transferase system recently described by Roseman, Kundig, and their coworkers in the uptake of sugars.

This slide (Fig. 2, Discussion) demonstrates the effect of increasing concentrations of P-enolpyruvate on the uptake of α -methyl glucoside by membrane preparations of *Escherichia coli* or GN-2. The data shown were obtained by allowing membranes to take up α -methyl glucoside in the presence of the concentrations of P-enolpyruvate shown, and rapidly filtering the incubation mixture after diluting it to terminate the reaction. After counting of the filters ("uptake"), each sample was extracted and assayed for free glycoside and glycoside-P (" α MG" and " α MGP," respectively). The uptake of α -methyl glucoside increases rapidly in response to increasing concentrations of P-enolpyruvate over the range of 0–10 mM; the rate continues to increase, but less rapidly, with further additions of P-enolpyruvate up to concentrations of 100 mM. It is also evident that most of the α -methyl glucoside taken up appears as α -methyl glucoside-P. Furthermore, with membranes prepared from GN-2 (defective in

FIGURE 1. Electron micrograph of membranes prepared from *E. coli* ML 308-225 ($i^{-}z^{-}y^{+}$). $\times 100,000$.

enzyme I of the phosphotransferase system), there is no stimulation of α -methyl glucoside uptake over the concentration range studied.

The effect of P-enolpyruvate is extremely specific. Of approximately 40 substrates tested, including many glycolytic pathway and Krebs cycle intermediates, as well as all of the nucleoside triphosphates, only P-enolpyruvate and, to a lesser extent, 2-P-glycerate stimulate α -methyl glucoside uptake and phosphorylation. Furthermore, the stimulatory effect of 2-P-glycerate is completely inhibited by NaF, indicating that it is converted to P-enolpyruvate before it can support α -methyl glucoside uptake and phosphorylation.

In order to investigate the precise role of the P-enolpyruvate-P-transferase system in sugar uptake, the following experiment was carried out. Membranes were pre-loaded with ^{14}C -glucose by incubating samples at 0°C in the presence of high concentrations of ^{14}C -glucose but without P-enolpyruvate for about 3 hr. The membranes

FIGURE 2. α -Methyl glucoside uptake by *E. coli* ML 308-225 and GN-2 membrane preparations as a function of P-enolpyruvate concentration.

were then centrifuged and the external ^{14}C -glucose removed. Finally, the membranes were rapidly resuspended in a solution containing ^3H -glucose and P-enolpyruvate and incubated for various periods of time. Glucose and glucose-P were then isolated from filtered samples and counted for both ^{14}C and ^3H . If glucose passes through an internal pool before it is phosphorylated, the ^{14}C -glucose already present in the internal pool should appear as ^{14}C -glucose-P before the external ^3H -glucose. Also, ^{14}C -glucose in the internal pool should decrease as it is phosphorylated and should be replaced by ^3H -glucose. In other words, the $^3\text{H}/^{14}\text{C}$ ratio of the free glucose pool should rise at a faster rate than the $^3\text{H}/^{14}\text{C}$ ratio of the phosphorylated derivative. Conversely, if glucose is phosphorylated directly without passing through the internal pool, the $^3\text{H}/^{14}\text{C}$ ratio of glucose-P should increase at a faster rate than that of the free glucose. As shown in the Fig. 3, Discussion, the latter alternative holds. The $^3\text{H}/^{14}\text{C}$ ratio of the isolated glucose-P increases at a much greater rate with time than the $^3\text{H}/^{14}\text{C}$ ratio of the free glucose. The control curves were obtained from an experiment identical with the one presented before, with the exception that no ^{14}C -glucose was present

during the preloading period and both ^3H - and ^{14}C -glucose were presented with P-enolpyruvate. As seen, the $^3\text{H}/^{14}\text{C}$ ratios for glucose-P and glucose are essentially the same and both approximate unity, thus ruling out the possibility of an artifactual isotope effect. This experiment provides very strong, if not conclusive, evidence that the P-enolpyruvate-P-transferase system is responsible for the entry of sugars as well as their concentration within the membranes as impermeable phosphorylated derivatives.

Other evidence consistent with these data is the following. First, the initial rates of appearance of free α -methyl glucoside in the intramembranal pool and the phosphorylation of α -methyl glucoside as a function of the external α -methyl glucoside concentration indicate that the uptake of free α -methyl glucoside into the free pool is linear over a 1,000,000-fold concentration range. On the other hand, the phos-

FIGURE 3. Uptake and phosphorylation of glucose-6- ^3H by membranes preloaded with glucose- ^{14}C .

phorylation of α -methyl glucoside follows saturation kinetics with an apparent K_m of approximately 4×10^{-4} M. This value is approximately 2 orders of magnitude lower than that obtained for whole cells. Second, although the membranes concentrate radioactivity from α -methyl glucoside against approximately a 200-fold gradient, the free α -methyl glucoside within the membranes is only about one-third of the external concentration. This means that the entry of α -methyl glucoside into the free pool is mediated by a mechanism which involves flow down a chemical gradient, i.e. passive diffusion. Thus, it appears that the uptake of α -methyl glucoside into the free pool has no relationship to the phosphorylation mechanism.

In summary, the three transport systems that have been studied in isolated membranes appear to be completely different: the proline uptake system seems to fulfill all of the criteria of a classic "active transport" system; the glycine uptake system is apparently a facilitated diffusion mechanism; and, finally, the sugar uptake mechanism involves group translocation, i.e. a covalent change in the transported species.

Dr. Kennedy: Thank you very much, Dr. Kaback. Dr. Kaback's results are in complete accordance with studies that have been carried out at several laboratories, including our own, which indicate that the uptake of glucose in *E. coli*, as measured by assays involving the accumulation of radioactivity from labeled substrate, is dependent upon the presence of the phosphoenolpyruvate (PEP)-linked enzyme system. That is not true, however, with lactose accumulation in *E. coli*. There is no evidence to support the view that the phosphorylation system is involved in lactose accumulation, and, in fact, mutants which completely lack enzyme I of the phosphorylation system have an unimpaired ability to accumulate lactose or related β -galactosides. So in the latter case we seem to have an example of what might be called active transport in the older sense of that term, that is, the uphill accumulation of an unaltered substrate, whereas in the case of the PEP-linked systems phosphorylation of the substrate is an essential part of the accumulation mechanism.

Dr. R. C. La Force: I'd like to make a comment about active transport. Excellent work is going on in finding proteins and molecules that seem to be specific for binding with them molecules that might look like carrier molecules to mediate transport. You can think up a simple system in which you can have transport of ions without having a carrier molecule that is specific for a particular ion. For example, you can think up a system in which there is a carrier molecule that binds any positive ion and yet is capable of producing oppositely directed, countergradient fluxes of sodium and potassium ions. Moreover, the values required for diffusion coefficients, etc., are consistent with what we know about the model membranes that have been discussed here, and also consistent with data on a real but rather messy membrane, frog skin. So, in doing the biochemical studies, it is not necessary to find proteins, for example, that are specific for holding potassium or sodium or lithium. I'll settle for a molecule that will bind any or all of these. Such a molecule could explain much of the data that we observe about the macroscopic behavior of active transport.

Dr. Kennedy: I might comment on that myself. I am sure that one could prepare any number of models which would be intellectually satisfying. The question is: Which are operating in nature? I think that the evidence strongly indicates the participation of specific proteins in bacterial transport systems. So, even though one could devise, and I am sure will continue to devise, many schemes which do not require the participation of proteins, it would seem that, in the systems that have been examined, proteins do in fact participate.

Dr. La Force: No, I am not saying that a protein is not necessary. I am just saying that it is not necessary that it be specific. You see, a carrier must exist. Of course, I am in complete agreement with that idea, but my position is that the carrier doesn't have to come in and grab just sodium. It can grab sodium, potassium, lithium, hydrogen indiscriminately, or various combinations of these, and still explain the observed behavior. The carrier can complex sodium 2:1 for the potassium or potassium 2:1 for sodium and still explain the experimental observations. There must be some kind of carrier molecule, I am in complete agreement with that, but when you go through a biochemical preparation and find no protein, find no fraction, for example, that has potassium in it, it doesn't mean that there isn't a carrier. That's the point I want to make.

Dr. Rand: This is a question based on ignorance of the experimental procedures that

you use for these studies. In the isolated membrane systems, what definite proof have you that in fact you are dealing with closed surfaces whereby molecules move across the membrane from one compartment to another, rather than with a complicated kinetic process of absorption and desorption of various substrates?

Dr. Kaback: This question brings up a very good point, and a very difficult one. There is no definitive evidence pertaining to the physical nature of the solute taken up by these membrane preparations; i.e. whether it is in free solution or adsorbed to the membrane. All I can say is that there is very good evidence that these membrane vesicles are osmotically intact and that proline uptake, at least, is inhibited by a number of general metabolic inhibitors. In any case, there is very little evidence with whole cells as to this question.

Dr. Kennedy: I would also remind you of the work of Siström in this regard. He measured the osmotic effect on spheroplasts of *E. coli* which had accumulated β -galactosides. The evidence indicated that the accumulated sugar was, in fact, osmotically active.

Dr. Gabriel J. Gasic: Could you follow the incorporation of sulfate groups through the bacterial cell wall by using electron stains such as colloidal iron at a low pH?

Dr. Andrew M. Goldner: Are any of these isolated permeases ion-dependent, similar to the sodium dependence of the amino acid or sugar carriers of the intestine?

Dr. R. H. Wasserman: In addition to those given by Dr. Korn, I would like to mention, if I may, another protein with possible transport function. The administration of vitamin D to a rachitic chick enhances calcium absorption and, as we have shown (WASSERMAN, R. H., and A. N. TAYLOR. 1966. *Science*. **152**:791. TAYLOR, A. N., and R. H. WASSERMAN. 1967. *Arch. Biochem. Biophys.* **119**:536), also leads to the formation of calcium-binding protein (CaBP) in intestinal mucosa. This protein has now been obtained in high purity and was found to have a molecular weight of $2.4\text{--}2.8 \times 10^4$, a formation constant for Ca of about $2.6 \times 10^5 \text{ M}^{-1}$, and the capacity to bind 1 Ca atom/molecule. Correlative physiological and nutritional studies suggested that CaBP may be intimately involved in Ca translocation across the intestinal epithelium.

Dr. Burton Pressman: It may not be necessary to find proteins with specific ion affinities to account for the ion selectivity of cation transport. I would like to point out that strong evidence has recently been published that the transport-mediating antibiotics have high intrinsic ion selectivity. These compounds are transport carriers. They have very small molecular weight, they have turnovers in the enzymatic range, several hundred a second, they are crystalline, they and their structural analogues can be synthesized; all these factors greatly facilitate the study of the molecular basis of their transport activity. Unfortunately, transport in model systems employing antibiotics is never active but exclusively downhill. I believe that continued study of these antibiotic systems will add tremendously to our knowledge in supplying prototypes of transport carriers at a very simple molecular level, which will enable us to predict what are the essential elements at the molecular level of a biological transport carrier.

Dr. Eric J. Simon: I want to ask Dr. Pardee to comment on the question of where energy is needed in transport, and particularly on the controversy whether the energy needed is for the uptake or for the prevention of exit when an accumulation occurs against a concentration gradient.

Dr. Pardee: I really can't say much about that in our own system. I don't know where energy enters. From what we have done it looks as if sulfate doesn't get into the cell

sufficiently to be measurably incorporated into protein in the absence of energy. So I think that energy is required in the translocation step, or at least in the release after it gets in. But in other systems—Roseman's system has been discussed extensively—there is passive passage of a sugar through the membrane, and release is probably an energy-requiring process. Perhaps, from those data, it is still possible that one could have some release after facilitated diffusion, in the absence of PEP, which would not be seen in Dr. Kaback's experiment. Energy decreases affinity for intracellular β -galactosides according to Winkler and Wilson (20).

I don't know where energy fits into most systems.

Dr. Mysels: Something that wasn't clear to the layman in this discussion is whether such isolated proteins can help the mechanism in a mutant that is naturally deficient in that protein.

Dr. Kennedy: The question really is whether the protein which is isolated can restore the transport process in the living cells of a mutant deficient in that transport restoration.

Dr. Pardee: In our system, it does not. There have been reports of such restoration or reconstitution from several laboratories. We have spent a lot of time looking for this, and so has Dr. Oxender. In my opinion, nobody has clearly demonstrated reconstitution; I do not believe the controls that most people use are adequate to make their point clearly. Perhaps someone would like to defend their results, but I find it very hard to believe that the published data prove reconstitution.