Membrane Structure and Function

EUGENE P. KENNEDY, C. FRED FOX, and JAMES R. CARTER

From the Department of Biological Chemistry, Harvard Medical School, Boston

ABSTRACT An understanding of the biochemical basis of membrane function is an important goal of present day biology. In this paper, a biochemical approach to the problem of the specific transport of sugars across the membrane of *Escherichia coli* is discussed. A new biochemical model for lactose transport system in this organism is presented, in which a specific membrane protein (M protein) plays the role of the sugar carrier. Experiments which have led to the discovery of such a protein, its specific labeling, and partial purification are briefly reviewed.

Problems of membrane structure and function are of central interest in many important fields of biology today. The mechanism of transmission of the nerve impulse, the mode of interaction of cells with each other in the developing embryo, the mechanism of action of hormones such as insulin—all these apparently diverse fields of biology have in common the need for a much more complete understanding of membrane function for continued progress. The explication of membrane phenomena in biochemical terms, however, has proved to be an exceedingly refractory problem. For example, it has now been more than 40 years since the discovery of insulin. In this period, as a result of the work of hundreds of laboratories throughout the world, the chemistry of the hormone has been completely elucidated, and a great deal has been learned about the physiological framework in which the hormone acts. However, the fundamental biochemical effects of insulin on tissues remain still to be elucidated. As is well known, one of the principal theories of the mechanism of action of insulin is that it promotes in some undisclosed manner the passage of sugars across membranes. The present status of this theory has recently been reviewed by Levine (1), who was one of the first to bring it to attention. It is clear that a complete verification or rejection of this hypothesis depends upon an understanding of the fundamental mode in which sugars cross the cells of living membranes. It is indeed this aspect of membrane function that has engaged the attention of our own laboratory, although we have chosen to study the problem in bacterial, rather than in mammalian systems.

The membranes of living cells throughout Nature appear to be made of similar building blocks—lipid and protein. The lipid moieties of metabolically

active membranes may vary in composition quite widely from species to species, but the presence of glycerophosphatides as important lipid units is a universal feature of membrane structure. Our laboratory has been for some time engaged in the study of the biosynthesis of these lipid molecules. This topic has been the subject of several reviews (2–4) and will not be considered in detail here. With the thought that bacterial systems offer obvious advantages for the study of specific transport across membranes, we turned a few years ago to the examination of the glycerophosphatide metabolism of *Escherichia coli*. Although much work remains to be done, at least the outline of the biosynthetic processes involved in the formation of glycerophosphatides in this organism is now known (5, 6). It, therefore, appeared to be timely to attempt an investigation of specific aspects of membrane function, and we have chosen for study the system responsible for the transport of lactose and other galactosides into *Escherichia coli*.

The brilliant contributions of workers at the Pasteur Institute (7–9) have made it clear that lactose and other sugars do not enter the cells of *E. coli* by a process of simple diffusion, but rather their uptake is mediated by specific, genetically controlled transport systems. A great deal has been learned about the kinetics and specificity of the lactose system as studied in the intact cell. However, in his 1964 review, Kepes (9) pointed out that despite persistent effort, virtually nothing had been learned as yet about the biochemical basis of this transport system. For these reasons lactose transport in *E. coli* appears to present to the biochemist a system offering at once the greatest challenge and the greatest opportunity.

Previous models of the β-galactoside transport system in *E. coli* put forward by Cohen and Monod (7), and in a modified form by Kepes (10) have postulated the existence of an enzyme called *permease* thought to catalyze a reaction involving a hypothetical energy-rich compound and the substrate to be transported. As a result of this enzyme-catalyzed reaction, the substrate is postulated to cross the cell membrane. However, despite diligent search in many laboratories, no enzyme having properties required for the hypothetical permease has been demonstrated. On the other hand, there is decisive evidence to indicate that a specific protein of some kind is required for the transport process.

With the hope that a reevaluation of the information presently available about the lactose transport system might suggest new experimental approaches, we have attempted to formulate a simplified model of the system in which the genetically controlled receptor molecule need not have the properties of an enzyme. Such a model is shown in Fig. 1. It is perhaps worthwhile to remind ourselves at this point that neither this nor any similar model gives an accurate representation of reality, but has value only in so far as it may suggest fruitful lines of experimentation.

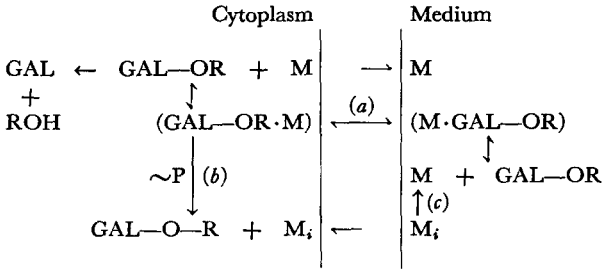

FIGURE 1. Simplified working model of the β-galactoside transport system.

In the model shown, a molecule of β-galactoside in the medium combines with a specific component of the membrane, a protein for which the non-committal designation, membrane protein or M protein, is used. The association of the galactoside with the protein is of the simple Michaelis-Menten type. The complex then passes through the membrane in the process described as reaction (*a*). It is postulated that the rate of diffusion of the free sugar through the membrane is negligible in comparison with the carrier-mediated process. On the inner surface of the membrane, the complex may simply dissociate. If the enzyme β-galactosidase is present in the cell, and if the β-galactoside is a substrate which can be hydrolyzed by this enzyme, a continuous removal of galactoside by the enzyme-catalyzed hydrolysis may lead to a substantial flux of galactoside from the medium into the cell. This process does not require coupled metabolic energy and is to be sharply distinguished from the *accumulation* of unaltered galactoside within the cell against a thermodynamic gradient.

Alternatively, in the accumulation process, the (M-β-galactoside) complex may undergo the transformation shown as reaction (*b*) in the model. Reaction (*b*) requires some source of metabolic energy since accumulation is completely abolished by inhibitors such as azide or dinitrophenol. We postulate that the transformation involved in reaction (*b*) may be that of the protein carrier, rather than of the substrate. In this reaction, the M protein is converted to M_i, a form that has a greatly reduced affinity for β-galactoside. M_i may then move back through the membrane to the exterior surface where it is reconverted to the active form M in a reaction not requiring coupled metabolic energy (process *c*).

There are three features of this model that deserve comment in relation to the experimental attack on the problem of membrane function. First, the model sharply distinguishes between the entry process and the accumulation process. Second, it postulates that the expenditure of metabolic energy in the accumulation process leads to the formation of a derivative of the carrier that has greatly reduced affinity for the substrate. Thus, metabolic energy is used to unload the carrier, rather than to activate it. According to this model, the

maintenance of a concentration gradient would require the continuous expenditure of energy, a prediction consistent with the experimental findings of previous workers. Finally, it should be noted that this model suggests that the receptor protein for galactosides in the membrane of these cells may not be an enzyme. The importance of this last point in the design of experiments is obvious, since it leads to the conclusion that the recognition of this essential component should be based on stoichiometric methods, rather than on presumed catalytic properties of the protein.

The recognition by stoichiometric methods of proteins present only in trace amounts in cells is a rather formidable undertaking. Early estimates (11, 12) suggested that the entity described as "permease" is present in cells at the discouragingly low level of about 300/cell. However, the important studies of Zabin (15) have shown that while β-galactosidase (the product of the *z* gene) constitutes about 5% of the total protein of fully induced cells of *E. coli*, thiogalactoside transacetylase, (the other known protein of the *lac* operon) makes makes up about 0.3% of cellular protein. On the assumption that M protein is coded by the *y* gene located between the genes for β-galactosidase and thiogalactoside transacetylase, it would seem reasonable to suppose that the amount of M protein in cells would probably not be less than that of thiogalactoside transacetylase, i.e., about 0.3%, a considerably higher level than that suggested by the earlier estimates. These considerations encouraged us to attempt the isolation of the M protein by a method based on stoichiometric labeling.

The general plan of these experiments was to find an inhibitor of reaction (*a*) of the kind known to act by irreversible combination with proteins. If one measures the rate of hydrolysis of a substrate such as *o*-nitrophenyl-β-galactoside (ONPG) by intact, fully induced cells of *E. coli* poisoned with azide, then the rate-limiting step is the entry of the galactoside into the cell, since β-galactosidase is present in large excess, as shown by previous workers, notably Koch (13). Reaction (*b*) is prevented by azide. The rate of hydrolysis of ONPG by intact cells poisoned with azide is therefore in effect an assay for functional M protein. Earlier studies by Kepes (10) had shown that some element of the transport system is sensitive to sulfhydryl inhibitors such as *p*-chloromercuribenzoate. Carrier systems in animal tissues also display a sensitivity to sulfhydryl reagents. Therefore, amongst the reagents which we tested were included a number of sulfhydryl inhibitors of which *N*-ethylmaleimide (NEM) has proved to be the most convenient.

With the use of this reagent, it was shown (14) that the β-galactoside transport system contains a component sensitive to *N*-ethylmaleimide. This component is neither β-galactosidase nor thiogalactoside transacetylase, since the activity of these enzymes in cells treated with *N*-ethylmaleimide was not signif-

icantly depressed under conditions in which transport was completely blocked. Furthermore, it was shown that the addition of thiodigalactoside, a substrate for which the transport system has a very high affinity, protected the *N*-ethylmaleimide–sensitive component from reaction with the inhibitor. The finding of this protective effect was of decisive importance for two reasons. In the first place, it revealed that the *N*-ethylmaleimide–sensitive component is indeed a receptor molecule for galactosides. Second, it offered us a great technical advantage since it opened a way for the specific labeling of the M protein in an experiment of the kind outlined in Fig. 2.

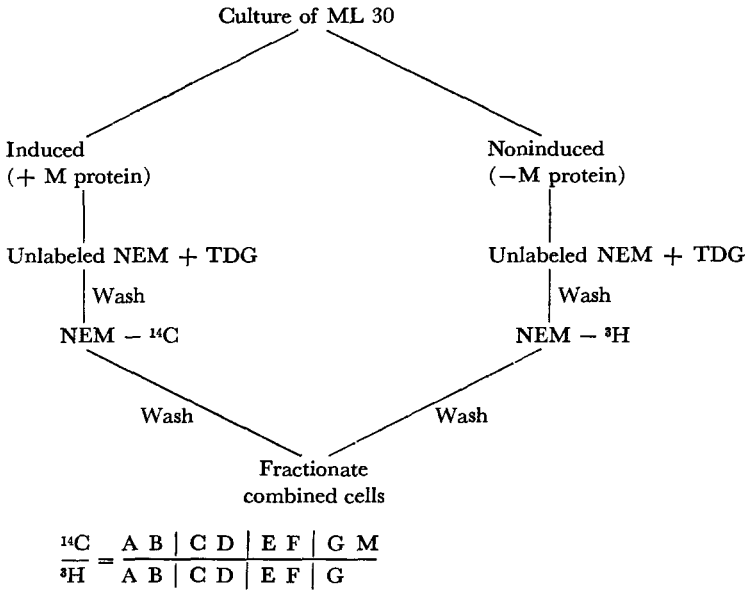

FIGURE 2. Plan of the "double label" experiment.

In this experiment cells of *E. coli* strain ML 30 (first isolated in Monod's laboratory) are used. These cells have the genetic constitution $i^+z^+y^+x^+$. These cells are, therefore, inducible and do not contain significant amounts of the products of the structural genes of the *lac* operon unless they have been induced. The culture is divided into two portions, one of which is induced, using isopropylthiogalactoside, a gratuitous inducer. The other portion is not induced. Both portions, however, are treated in otherwise identical fashion with unlabeled *N*-ethylmaleimide in the presence of thiodigalactoside. In this step the sulfhydryl-containing proteins of both the induced and the uninduced cells soak up the unlabeled *N*-ethylmaleimide *except for the M protein, which is protected by its combination with thiodigalactoside from reacting with the N-ethylmaleimide.* The cells now are washed free of the thiodigalactoside and unlabeled *N*-

ethylmaleimide and reincubated with labeled *N*-ethylmaleimide in the absence of the protective sugar. ^{14}C-labeled NEM is used for the induced cells, whereas tritiated NEM is used with the uninduced cells. Once again the NEM is washed out from the cells, which are now combined and fractionated.

Since the reaction with unlabeled NEM in the first incubation is never complete, the mixture contains a variety of labeled proteins (A,B,C. . .). Those derived from the uninduced cells are labeled with tritium. These proteins are also present in the induced cells, labeled with ^{14}C. Therefore, fractions containing these proteins will have a $^{14}C/^{3}H$ ratio of unity. The mixture, however, also contains one protein (the M protein) labeled with ^{14}C but not with tritium, since it is not present in significant amounts in the uninduced cells. The presence of the M protein in fractions derived from the mixture of cells will thus be revealed by an increase in the ratio of ^{14}C/tritium.

This experiment is described in some detail because it offers a general approach to the study of specific components of transport systems. Such an experiment derives its crunch from three elements. First, the genetic control over the presence of the transport system offers great technical advantages. Second, the introduction of the protection step in which the cells are incubated with unlabeled inhibitor in the presence of the protective sugar greatly amplifies the effects later observed upon fractionation of the mixture. Finally, the use of two isotopes has the advantage that isotope ratios can be measured with high precision in fractions derived from the mixture of cells. In effect, every sample counted carries with it its own control in the form of isotope derived from the uninduced cells.

By the use of this fundamental approach, it has proved possible to establish directly and unequivocally the presence in induced cells of *E. coli* of a protein having the characteristics required of the M protein in our model.

It is not possible in this brief presentation to describe the details of these studies. Some of the results to date may be briefly summarized.

1. The M protein is localized in the membrane-containing fraction of *E. coli* to which it is held by lipid protein bonds. The radioactive *N*-ethylmaleimide derivative of the protein can be extracted by buffers containing surfactant, and can be fractionated by conventional methods.
2. It is a protein of the *lac* system, distinct from β-galactosidase and from thiogalactoside transacetylase.
3. It is coded by the *y* gene.
4. The native M protein can be detected in cell-free fractions of *E. coli* and can be shown to retain its specific affinity for galactosides. The affinity of the protein for thiodigalactoside, measured in the cell-free fractions, is approximately the same as the apparent Michaelis constant for the transport of this sugar in the living cell.

Although it is apparent that only a beginning has been made in the study

of this particular transport system, one may hope that modern chemical approaches to the study of proteins and of lipid protein interactions combined with the powerful tools of the bacterial geneticist should be capable of resolving at least certain features of the problem of the specific structure and function of membranes. The technical advantages of working with bacteria are for this reason very obvious. However, if a detailed understanding of the process of sugar transport in bacteria is obtained, some features of such a scheme may be subject to experimental verification in animal systems.

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Discussion

Dr. Luria: Are there any questions for Dr. Kennedy at this point? If not, I may add a factual comment of my own.

You will remember that Dr. Kennedy has postulated that the M protein is essential for galactoside transport, but that the energy-requiring reaction may not be necessary for transport but only for accumulation of substrates. Stimulated by this suggestion, Miss Fields, Dr. Ruby, and I have done experiments using some very powerful inhibitors of energy production in *E. coli*, that is, two colicins, E1 and K, which from all indications stop completely the supply of ATP. We find a very gratifying confirmation of the hypothesis presented by Dr. Kennedy: these colicins are practically without any effect on the utilization of substrates of β-galactosidase by the intact cells, that is, on the actual transport, but they completely suppress all accumulation of those galactosides which are substrates of the enzyme.

I am glad that the Chairman in this case has a little bit to add to the wonderful session that the speakers have contributed. Thank you and good night.