J. Membrane Biol. 4, 87–112 (1971)
© by Springer-Verlag New York Inc. 1971

The β-Galactoside Permease of *Escherichia coli*

Adam Kepes

Institut de Biologie Moléculaire,
Centre National de la Recherche Scientifique, Paris 5, France

Received 17 September 1970

Active transport across membranes is a typical membrane function, and it is felt that the elucidation of the detailed mechanism of this phenomenon might be highly relevant to the buildup of a realistic picture of membrane architecture and membrane function in general.

The very first general membrane function is to serve as barrier against escape from the cell of essential molecules and against irruption into the cell of unselected molecules from the environment.

This barrier function of the membrane has been studied thoroughly [31] since the end of the 19th century; its features helped establish the classical Davson-Danielli model of the membrane [7, 11, 35]. This model, in spite of growing dissent [40, 42], still dominates present thinking about membrane structure as testified by the recent success of the techniques of artificial lipid bilayers [1, 30]. In my personal opinion, the Davson-Danielli model, with some modification because of recent emphasis on hydrophobic interactions for the maintenance of native configuration of proteins in general [16], represents the quantitatively dominant membrane structure. This structure is inadequate to explain most phenomena of transport across membranes which often compel one to the view of proteins extending across the main hydrophobic layer of the membrane or of proteins moving across this layer, but it is possible to admit that functionally specialized minor spots interrupt the continuity of the impermeable layer of the membrane. The present controversy about membrane structure might be likened to a dispute between believers in the theory that a house is best described by a model of the walls and those who insist that the existence of doors and windows defeats the theory of the wall.

The history of transport is relevant to doors and windows within the membrane. No membrane limiting a cell can exist without these channels

of communication, and an adequate description of these channels is boun to throw light also on the "general" architecture of membranes a walls.

Two other typical membrane functions must be left aside in this stud) namely (1) the function of membranes to recognize the outer world (cell-tc cell interactions, membrane receptors to virus' phagocytable particles extraneous messages such as hormones or physical or chemical stimuli and (2) the membrane fabric as the building ground of structurally integrate multi-enzyme systems. The study of these two types of membrane functions in addition to their fundamental intrinsic interest, can be highly enlightenin with regard to the construction of membranes, but the variability of thes functions from one membrane to another, which corresponds to specializa tions of cells and subcellular compartments surrounded by membranes suggests that they have been evolved secondarily and embodied into th most appropriate structure in cells, i.e., the membranes. The recognitio systems are clearly secondary specializations; none of them is generall distributed and no one is indispensable as an integral part of the membran structure. For many enzymes, the membrane location is clearly an occasiona solution adopted by evolution among many alternate possibilities. Eve such highly structured systems as the energy-transducing multi-enzym chains, oxidative phosphorylation or photosynthesis, where the architectur of a membrane seems indispensable, do not form conversely an indispensabl part of the membrane.

The β-Galactoside Permease of Escherichia coli

The discovery of this transport system was based on the confrontatio of two observations: the specific crypticity [8] of certain mutants of *E. coi* toward lactose, and a high concentration in wild-type induced *E. coli* of th inducer TMG [1] [6]. The accumulation of TMG could rapidly be interprete as reflecting a transport mechanism which was essentially unconnecte with the mechanism of enzyme induction itself [34] (although, as it turne out later, it can contribute to the induction by ensuring a high concentratio of inducer in the cytoplasm [12]).

It was found that TMG also accumulated in the constitutive i^- strains whereas it was absent from the inducible cryptic strains. Similarly, th

1 Abbreviations used: TMG, methyl-β-D-thiogalacto-pyranoside; TDG, β-D-galacto 1-S-thio-β-D-galactoside; IPTG, isopropyl-β-D-thiogalactoside; TPG, phenyl-β-D-thio galactoside; ONPG, ortho-nitro-phenyl-β-D-galactoside; ATP, adenosine triphosphate ADP, adenosine diphosphate; CTP, cytidine triphosphate; PEP, phosphoenol pyruvate DNP, 2,4-dinitrophenol; PCMP, parachloromercuribenzoate; NEM, N-ethylmaleimide

genetic tool permitted immediate separation of the transport function from the enzyme β-galactosidase, since two structural genes with independent mutations could realize all four combinations: Z^+Y^+; Z^+Y^- (cryptic); Z^-Y^+ (Lac^- absolute); and Z^-Y^-. In this era, when the "one gene – one enzyme" concept dominated modern biology, it became clear that a gene product, and therefore a protein, was essential for a transport function, and since the effect was catalytic [26] (i.e., the protein was not used up during the reaction and could obviously act indefinitely), it received the name permease [5].

The name was a challenge to scientists who were mainly concerned at that time with the membrane property "permeability" more than with the membrane function "transport", and even those who studied transport would not think in terms of one transport-one protein according to the analogy one reaction-one enzyme. The name provoked more opposition than enthusiasm among transport people while it caused premature satisfaction among molecular biologists concerning the facility of the solution of the transport problem. The contrast is very illustrative between the suggestive and daring name permease given to this transport system, and the noncommittal name Na^+-K^+-dependent ATPase which appeared a few years later [41], and which gave an operationally inaccurate and irrelevant definition to the essential enzymatic part of the Na pump. Later, this was found to be a complex of a Na-dependent kinase activity and a K-dependent phosphohydrolase activity [33], but it was clear from the inception that its real purpose was cation transport.

The opposition to the name permease lasted through the subsequent 10 years; this is illustrated by the fact that after the success of their specific labeling procedure Fox and Kennedy [10] found it opportune to rename it the "M protein". M stands for membrane, and therefore the new name remained far behind the known functions of the protein.

The assumption that permease was a protein was more than abundantly documented by the early publications. Besides the identification and mapping of its structural gene *y*, it was shown that biosynthesis of permease required all the conditions of protein synthesis (carbon source, nitrogen source, and all essential amino acids) and was inhibited by chloramphenicol [6]. In inducible strains, synthesis of permease was strictly parallel to the synthesis of β-galactosidase, and this finding also established that the inducible protein was the only rate-limiting factor of transport in physiological conditions. As in many enzymes, the incorporation of parafluorophenylalanine in the place of the natural L-phenylalanine into permease gave an inactive product. It was shown that permease had an essential

sulfhydryl group which could be protected against inactivation by Sl reagents if an excess of substrate was present [21, 22].

Still in the period from 1956 to 1961, it was shown that transport c thiogalactosides was an active transport, and that accumulated TMG wa chemically unchanged and made full contribution to the intracellula osmotic pressure [39]. The active uptake of thiogalactosides is a saturabl process with Michaelis-type concentration dependence. K_m values have bee measured for TMG, TDG, IPTG, TPG, ONPG and lactose [21]. The tim course of active uptake ends with a plateau when the concentration ratio c intracellular thiogalactosides vs. their concentration in the medium i 50:1 to 500:1, and the plateau reflects continuing uptake balanced by equiv alent outflux [26]. The whole process can be characterized as a pump an leak mechanism. Rate of outflux was shown to be first order with regard t intracellular concentration, as well during the steady state [21] of accumu lation as during net efflux [27]. Uncoupling agents such as DNP or sodiun azide abolish the active uptake and cause the efflux of a previously accumu lated pool. The energy requirement of the process was also reflected by ai increased oxygen uptake [20] when carbon-starved cell suspensions ar exposed to the transport substrate.

In the first detailed paper on transport kinetics [21], a model was pro posed to account for all the facts known at that time. It was a mobile carrie model in which the carrier was distinct from the permease protein. Th latter was supposed to catalyze the formation of substrate-carrier comple from exogenous substrate and an energized derivative of the carrier. Th carrier was supposed to be independent of the lac operon, constitutive an less specific than the permease. This contention was based on the observatio of efflux of thiogalactosides upon addition of glucose, interpreted as a counter transport. This counter transport can also be observed in permease negative strains.

Koch adopted a similar model [27] except that the energy-dependen step was the dissociation of substrate from carrier on the inner face of th membrane instead of the complex formation at the outer face. This wa mainly based on the observation that permease-mediated downhill transport namely the hydrolysis of ONPG by intact cells, was not inhibited by un coupling agents to an extent comparable with uphill transport of non hydrolyzable thiogalactosides. With the work of Winkler and Wilson [43] it became clear that the mobile carrier was the permease protein itself According to these authors, the main effect of the energy coupling is to decrease the affinity of the carrier for the substrate on the intracellular face thus causing the otherwise equilibrating transport to become an active one

Later, I showed that, although permease was capable of supporting inward and outward fluxes of substrate in the energy-poisoned state, the leak from unpoisoned cells was occurring mostly through a channel unconnected with permease which was partly characterized [24] as a process inhibited by uncouplers of oxidative phosphorylation. This does not mean that the leak is an active process but probably it is a mediated passive transport where the aboundance of the unknown carrier depends on energy metabolism.

The models presented by Fox and Kennedy [10] and by Kennedy [17] are not basically different from the model of Winkler and Wilson except that they are much less explicative of a number of features of transport and less specific about the effect of the energy-coupled reaction which transforms the active form of the carrier M into the inactive form Mi. Likewise the model presented by Schachter and his co-workers [37, 38] where the energy coupling is inactivating the carrier by causing its dissociation from the substrate. Neither of these models is concerned with the reaction which restitutes the functional carrier from the inactive energized form. In a later section, a model will be presented which is not generally contradictory with the three last-mentioned models, but which embodies all identifiable reactions together with minimum postulates to close the cycle, and experimental results in support of each step will be quoted or described. But before discussing this model, the following section will be devoted to a critical survey of experimental results method by method, with the intention of showing which features will not be included in the general interpretation and why.

A Critical Survey of Methods

The Millipore filtration technique [3, 21] to separate cells from medium is used with slight variants in different laboratories. In this authors' laboratory, the incubated sample (usually at 25 °C) is first diluted into five to six volumes of ice-cold basal medium, the same medium as used for incubation without the additions, and then filtered and rinsed twice with the same ice-cold medium. In some other laboratories, dilution and rinse are done with the medium at room temperature; still others filter without dilution and rinse ice cold [43]. Others rinse with physiological saline [38] or distilled water. Periodically the cold dilution is accused of causing a cold shock with dramatic escape of the thiogalactosides [9]. It is well documented that efflux at or close to 0 °C is very much slower than at 25 °C but upon reexamination with strain K_{12} 3,300, it was found that cold dilution and rinse gave results 5 to 10% lower than the same technique at room tempera-

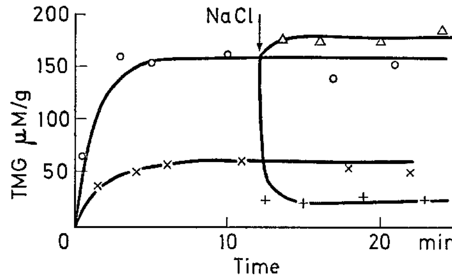


Fig. 1. Osmotic effects on the pool of TMG during filtration and rinse. A suspensic of *E. coli* 3,300 (220 μg dry weight per ml) in medium 63 glycerol chloramphenic was supplemented with 1 mM 14C TMG at time zero. Samples (1 ml) were filtered ar rinsed with the same medium (o — o). Alternate samples were filtered and rinsed with fivefold diluted medium (× — ×). At 12 min, NaCl was added to a part of the suspensic to make a final concentration of 0.15 M. From the high osmotic incubation mixtur 1-ml samples were filtered and rinsed with medium 63 (+ — +). Alternate samples wei filtered and rinsed with medium 63 containing 0.2 M NaCl (△ — △)

ture. This leaves all past results reasonably valid, since variations of 10 t 15 % in various conditions have always been considered as non-significan

In contrast, the accumulated pool is extremely sensitive to a differenc in osmotic pressure between the incubation medium and the rinse flui The effect as first observed in my laboratory was completely misleadin When 0.2 M NaCl or KCl was added to the incubation medium after TM had accumulated at the steady state, a dramatic drop of the intracellul concentration was observed. It was found much later that if the same sa concentration were also present in the rinsing fluid, the addition of the sa to the incubation mixture had no obvious effects (Fig 1). Therefore the contr experiment consisted of rinsing with iso-, hyper- and hypo-osmotic fluid in all cases, only the rinse with a hypo-osmotic fluid produced dramat losses of the pool. The efflux is very rapid; within 20 to 30 sec of filtratio and rinse, one-half to three-quarters of the pool is lost even at low temper ture. This loss is not a permease-mediated efflux since it also occurs wit PCMB-treated cells. In a recent paper, Schachter and Mindlin [38] reporte an inhibitory effect of KCl attributed to the uncoupling of the energ generating mechanism from the uphill transport mechanism. They observe that ONPG hydrolysis is not inhibited at the same KCl concentratio It is very likely that the observed effect is entirely caused by the osmot downshock during rinse. Therefore their argument about K inhibition will t disregarded. It might be useful to mention that the effect of osmotic dow shock is similar on some other sugar pools, e.g., the pool of galactose in galactokinase-deficient mutant of K_{12}.

ONPG hydrolysis is widely used as a measurement of permease activity irrespective of energy supply. Although this process clearly reflects penetration of ONPG rather than β-galactosidase activity, it is certainly prone to unexplained changes under a variety of circumstances. It is important to keep in mind that bacteria with a damaged diffusion barrier hydrolyze ONPG 8 to 15 times faster than normal bacteria. Therefore, even a small proportion of damaged bacteria can cause a large error of estimation of permease. Usually a blank control is subtracted from the total hydrolysis. Theoretically the best control would be the addition of TDG, a substrate with a high affinity to permease and a low affinity to β-galactosidase. TMG, IPTG and TPG are also strong inhibitors of β-galactosidase. The TDG control often gives blanks as high as 25 to 40% of the experimental sample. Although these blanks are fairly reproducible within a single experiment, they vary widely from one experiment to another, mainly when Tris-based or rich media are used for incubation. Koch [27] proposed a formaldehyde blank which is much lower than the TDG blank, but it can be feared that formaldehyde is not specific to galactoside permease and that it also inhibits ONPG penetration by the passive leak channel.

The main contribution to transport models of results obtained by the technique of ONPG hydrolysis was the so-called energy independence of downhill transport [27]. The experience shows that DNP at 1 to 2 mM or sodium azide at 20 to 40 mM, which both inhibit active uptake of TMG by 90 to 95%, slows down ONPG hydrolysis only by 15 to 30%. The hydrolysis remains inhibitable by TDG. It was found that the above concentration of uncoupling agents still permits uphill transport of TMG and TDG; the energy supply is not decreased to zero [25].

It was also found by continuous recording of ONPG hydrolysis that during a short initial period the inhibition by 60 mM azide was close to 80%, but it was followed by an acceleration [25] until the rate became similar to the control. It is easy to guess the reasons for a transitory period. ONPG is hydrolyzed to o-nitrophenol and galactose, and a galactose pool is built up. Galactose ultimately leaks out of the cells [36] (they were not induced to utilize galactose), and galactose is known to participate in counter-transport against TMG [24], thus presumably also against ONPG.

Therefore, the relative independence of ONPG hydrolysis can be explained by two statements: (1) energy is necessary to prime the pump, i.e., to build up an initial pool of galactose; and (2) in a later period, metabolic energy coupling becomes relatively unnecessary; the outflux of galactose under its own concentration gradient can replace energy coupling by flux coupling.

Actually, very strong energy inhibition such as that reported by Winkle and Wilson [43], caused by a combination of azide plus iodoacetate or b severe carbon starvation plus azide [28], dramatically inhibits downhi ONPG transport.

The second major contribution of the study of ONPG hydrolysis t permease mechanism was the observation of strong inhibition by α-meth glucoside [27, 44]. This was primitively observed with glucose-grow cultures and it was assumed to indicate a common part to the two transpo systems. Actually the PEP-hexose phosphotransferase system is present i similar amounts in bacteria grown on glucose or glycerol. Moreover, th inhibition by α-methyl glucoside was also found with bacteria grown o fructose, maltose, mannose, xylose, etc. [25], and even with bacteria grow on glycerol when thoroughly starved from carbon source (M. Jacquet & A. Kepes, *unpublished observations*). It is important to note that no dramati inhibition of uphill transport of TMG was observed in any of the above con ditions. If a common essential part of glucose and lactose transport can b ruled out, the explanation of these phenomena is still unclear. It is possibl that some feedback regulation by sugar phosphates on transport is involved but the correlations of various pools of sugar phosphates and the circum stances of inhibition of ONPG hydrolysis should be explored in detail

It must be concluded that the rate of ONPG hydrolysis as such is muc more influenced by ill-controlled factors (probably metabolic pools) than i the rate of uphill transport of nonhydrolyzable galactosides; therefore, whe findings with this technique confirm other results, they can be quoted a support, but conflicting results should be interpreted with utmost caution

Exit experiments have played an important role in the developmen of various permease models. The current error is to attribute any outflu of galactosides in a galactoside permease-positive organism to the operatio of galactoside permease. It was obvious from the beginning that minor lea mechanisms might exist since permease-deficient mutants can be induce with the usual thiogalactosides TMG and IPTG [12], and therefore these com- pounds must penetrate through some permease-independent mechanism.

If the inward leak can contribute 1% to the initial rate of active uptak under a concentration gradient of 1 mM, the outward leak under a con- centration gradient 100 times higher established by the active transport can possibly account for the steady state exchange.

In order to define permease-mediated efflux, a blank again must be subtracted, and the best possible definition of such a blank is the efflux insensitive to PCMB or NEM. By use of this definition, the efflux observed in a control exit experiment when it is caused by removal of exogenous

substrate goes entirely through the permease-independent channel. The flux through this channel is rather variable from one experiment to the other. Therefore, again, the temperature dependence of the rate of efflux [38] in these conditions is completely irrelevant to permease function.

One more warning should be made concerning exit experiments. An exit experiment usually begins with the dilution of a thick, preloaded cell suspension kept at 0 °C in some 100 volumes of warm medium. This dilution possibly causes a thermal shock and an osmotic shock, and therefore the slope between the point zero (sample before dilution) and the first experimental point might be largely different from the slope defined by the subsequent points. This "initial velocity" of efflux should be regarded with caution. When three or more experimental points fall on a straight line on a semilog efflux graph, a much more reliable measurement can be made than with initial velocity. This precaution was not observed in the efflux measurements of Winkler and Wilson [43], who found a saturable concentration dependence, whereas all other authors found first-order exit kinetics [24, 27, 28].

Counterflux experiments where one flux stimulates the opposite flux were among the most decisive in the formation of present permease models. Nevertheless, it must be kept in mind that, owing to the nonspecific leak channel, counter transport cannot be a one-to-one exchange. On the whole, only a major flux can substantially stimulate a minor flux in the opposite direction. Therefore, experiments where counterflux is measured as a stimulation of exit [21, 24, 28] and experiments where stimulation of uptake is observed are not done under identical conditions just by changing the side where the substrate is radioactively labeled.

In principle, counter transport must be reciprocal; disregard of this postulate led me to the erroneous concept of the nonspecific carrier [21]. There is only one condition where a single counter transport experiment is demonstrative, namely when the transport from downhill becomes uphill owing to the opposite flux. This was the case in the beautiful overshoot experiments of Winkler and Wilson [43].

Inactivation-protection experiments have made possible the specific labeling of permease [10], but their use in a quantitative way to derive information on the different configurations of permease protein is not warranted.

In many inactivation-protection experiments, where several galactosides have been compared as protective agents, the widely different affinities of these substrates for the transport system have been disregarded [18]. TMG has a K_m for transport 20 times higher than TDG; no wonder that in equal concentrations its protective effect is negligible. IPTG has a two-

times better affinity than TMG but still 10 times lower than TDG. Th apparent affinities might differ significantly from true dissociation constants but presently they are the only guide to predict the respective concentration which are expected to give similar protection. The same remarks hold oı binding competition experiments [19].

Rates of inactivation with SH reagents follow first-order kinetics unde well-controlled conditions, but absolute values are not accurately reproducibl from one experiment to the next. Fox and Kennedy [10] used 5×10^{-5} M NEM; in our laboratory, with strain K_{12} 3,300 in medium 63, this con centration fails to give obvious inactivation in 15 to 30 min. We currently utilize a 5 to 10 times higher concentration to obtain 90% inactivatior between 15 and 30 min. For these reasons, it is difficult to draw far-fetchec conclusions from protection experiments as described by Schachter *et al* [37] and Schachter and Mindlin [38] where statistics must be used to average effects of the order of 20% on the rate of inactivation in the presence oı absence of energy inhibitors. The results are not altogether unreasonable but they do not justify the designing of new models.

Experiments with *isolated membranes* conducted by Kaback [13, 14] led to the recent discovery that active transport of thiogalactosides was stimulated by D-lactate and to a minor extent by succinate and L-lactate [2]. This was interpreted as a direct involvement of redox energy in coupling to active transport. The stimulation by D-lactate was abolished by all relevant respiratory inhibitors but also by all inhibitors of oxidative phosphorylation. The intervention of phosphorylative energy was ruled out on the grounds that the preparation was unable to synthesize ATP and that ATP itself was unable to stimulate transport.

The rate of transport of thiogalactosides by maximally stimulated membrane vesicles was more than 10 times lower than transport by whole cells on a protein basis. Since membranes contain about one-tenth of the total cell protein, the transport activity measured was probably less than one-hundredth of that present in the cells from which the membranes were derived. Since cells do not use more than a small proportion of their energy supply for transport, the retention in the vesicles of one-thousandth of the original phosphorylative capacity would suffice to account for the observed transport. It seems difficult to rule out this possibility.

It can be argued that ADP was not present, but again a small contamination cannot be ruled out. The same vesicles are capable of synthesizing phosphatidyl glycerol without added CTP [29] and of incorporating glycine into phospholipid without further additions [15]. They also have a non-negligible endogenous phosphotransferase activity without added PEP

in the absence of fluoride routinely used in this type of experiment [14]. The absence of stimulation by added ATP is the most significant evidence against the involvement of phosphorylative energy. But the vesicles are almost completely impermeable to α-methyl glucoside 6-phosphate unless the temperature is raised above 40 °C [13], and, if transport is an asymmetric process, ATP (which is likely to be even less permeant) is probably required on the inner face of the membrane. Should an attempt be made to introduce ATP by prolonged preincubation, it must not be forgotten that membrane preparations exhibit a powerful ATPase activity and no ATP would survive unhydrolyzed through a prolonged incubation. I consider that the problem of the energy donor for active transport is not settled, and, until final elucidation, my preferred hypothesis is that energy is derived from the pathway of oxidative phosphorylation. If the immediate energy donor is not ATP, it is a high-energy precursor of it [23] or a molecule which derives its bond energy from ATP. This preference is based on inhibitor studies, and it is not exclusive of the findings of Pavlasova and Harold [32] who observed in anaerobic *E. coli* an inhibitory effect of uncouplers of oxidative phosphorylation without a concomitant change in the ATP level.

A Model of Active Transport by Galactoside Permease

The model represented below is inspired, in addition to the known experimental facts, by a small number of postulates. The first postulate is that the energy coupling involves a covalent reaction with the energy donor and permease protein (or a complex including permease protein). It is believed that the result of the reaction is the establishment of a covalent link between permease and one of the radicals of the energy donor according to the example of the phosphorylation of the Na^+-K^+-dependent ATPase. The derivative, symbolized by $P \sim A$, will hereafter be called the energized form of permease.

The second postulate is that energy coupling occurs in at least two steps, the second owing to the break of the bond postulated above, and this step will be called the energy dissipation step in contrast to the first which is the energy coupling step.

The third postulate is that permease protein can be present under different forms, e.g., free P complexed with the substrate PS or energized $P \sim A$, all of which can be potentially oriented with the active site outward or inward. The possibility of the energized form complexed with substrate is omitted from the model for reasons to be discussed later. The six configurations listed above must be able to transform in each other in such a way

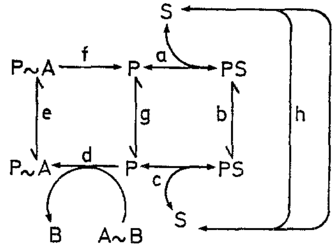


Fig. 2. Model of permease operation. Permease protein P located in the membrane can assume two orientations, active site outward and active site inward. The transition between these two species is designated reaction g or g'. Outward-oriented permease can combine to galactoside substrate S via reaction a to form a complex PS. The reverse reaction of dissociation is a'. PS can undergo a change in orientation inward via reaction b which is reversible as b'. Inward-oriented PS can dissociate via reaction c and release S in the cytoplasm. When the energy metabolism of the cell is intact, inward-oriented P can undergo a coupling reaction, d: $P + A \sim B \rightarrow P \sim A + B$, which is irreversible for thermodynamic reasons. This modified form of the permease protein can also undergo a change in orientation via reaction e and an energy-dissipating reaction f which results in outward-oriented P. Reaction f is also exergonic and irreversible. The fate of radical A is disregarded for lack of relevant experimental data. Galactoside substrates can cross the cell membrane by channels unrelated to permease h and i which can be distinguished operationally, i being inhibited by energy poisons such as sodium azide or 2,4-dinitrophenol. b, e, g, h, and i involve some kind of translocation; a, c, d and f are biochemical reactions

that the total rate of transformation of inward-oriented forms into outward-oriented ones be equal to the total rate of transformation in the opposite direction.

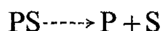
The model (Fig. 2) is a mobile carrier model where the permease protein P is the carrier. The transport occurs in three steps: a association of carrier with substrate, b change of orientation (translation, rotation or vibration or some more subtle and complex change in configuration), and c dissociation of substrate on the opposite side.

Active inward transport also includes reactions d, e, and f, namely, coupling, return of the energized carrier to the outer face and energy dissipation. The cycle $abcdef$ is written for inward active transport. Those reactions which are reversible will be written with a "prime"; thus, $c'b'a'$ is one possible way for efflux.

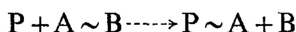
This cycle of active inward transport requires active permease P, external substrate Sex, and intact energy generating mechanisms furnishing $A \sim B$. Accordingly it can be inhibited by inactivators of P, NEM, PCMB, DNFB, etc., and by uncouplers of oxidative phosphorylation such as DNP and sodium azide as well as by respiratory inhibitors such as cyanide. It is an

asymmetric process, and one of the first features which insures unidirectional flux is that the energy donor $A \sim B$ is presumably confined in the intracellular space. Some other possible grounds for asymmetry will be mentioned later.

Reaction *a* during active inward transport is characterized by a Michaelis constant K_m which ranges from 2×10^{-5} for TDG to 5×10^{-4} for TMG. Maximal velocities have a narrower range, V_{max} for TMG is three to four times higher than for TDG. It is difficult to hypothesize which reaction is rate limiting and why V_{max} exhibits such variation. Diffusion step *b* might be different with different substrates attached to P; and TDG is bulkier and more hydrophilic than TMG but the difference of molecular weight is only about 100 – very small compared to the 30,000 molecular weight of P. A step more likely responsible for the differences in V_{max} would be reaction *c*, the dissociation of TDG inside. The distribution of P would favor the form PS when S is TDG, and reaction *c′* would compete with reaction *d* for free intracellular P. Another hypothesis to explain differences in V_{max} would be to suppose, instead of the reaction sequence $c - d$



$$PS \dashrightarrow P + S$$



$$P + A \sim B \dashrightarrow P \sim A + B$$

a single reaction $PS + A \sim B \dashrightarrow P \sim A + B + S$.

With configuration of PS different with different substrates, the enzymic reaction would have sufficient reason to occur with different velocities. This last hypothesis is similar to the reaction sequence *d* and *e* proposed by Schachter and Mindlin [38], which with our symbols would be $PS + A \sim B \dashrightarrow PS \sim A + B \dashrightarrow P + S + A + B$.

In their reaction *e*, dissociation and energy dissipation occur simultaneously, but in their model no provision is made for the recycling of free carrier, which would be difficult to reconcile with the facts which seem to indicate that the flux along path *g* is incapable of matching the maximal flux of substrate (see below).

$$g_{max} < b_{max}$$

One indication of the above is derived from the oxygen uptake experiments (Fig. 3). Oxygen uptake increases when TMG is added, and this is interpreted as an accelerated splitting of $A \sim B$ and consequently of ATP, resulting in a transition from state 3 to state 4 of the oxidative phosphorylation chain [4]. If ATP is not split before addition of TMG, it is presumably because of the lack of one of the reactants of reaction *d*, namely free P. Since in these conditions there is no PS, all free P is driven through reactions

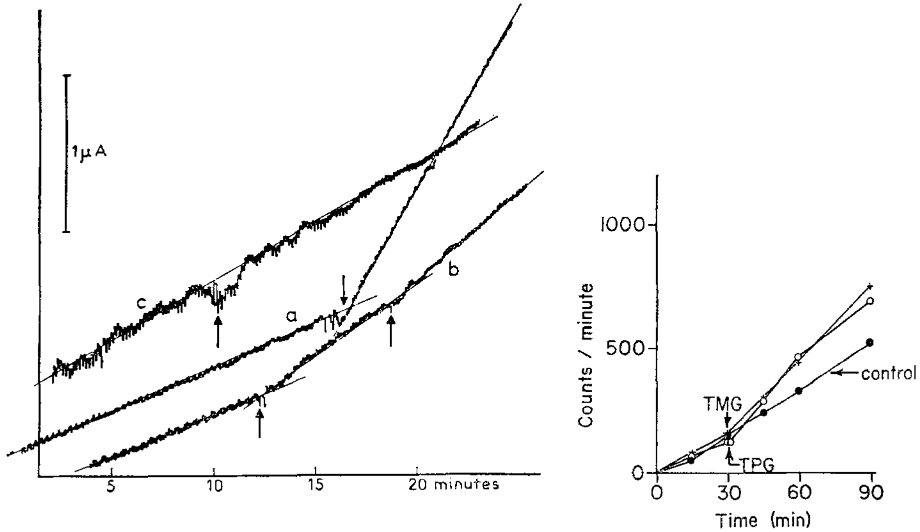


Fig. 3. Increase of oxygen consumption and CO_2 release during transport. *Left:* polarographic traces of oxygen consumption by *E. coli* in the absence of a carbon source. *a* ML 308, at the arrow 1 mM phenylthiogalactoside was added. *b* ML 30 previously induced by growth in the presence of IPTG. At the arrows, 1 mM TMG was added. *c* ML 3 (no permease). At the arrow, 1 mM TPG was added. *Right:* evolution of $^{14}CO_2$ from *E. coli* ML 30 grown on medium 63. ^{14}C fructose and IPTG resuspended without carbon source. To independent samples, 1 mM TMG and 1 mM TPG were added at 30 min

d, e, f to the outer face, and, if the flux along *g'* is slow, splitting of A$\sim$B will slow down accordingly. When S is added, reaction sequence *a, b, c* recycles P on the inner face, and reaction *d* together with increased respiration can start.

With the model of Schachter and Mindlin [38] also, reaction *d* would come to a halt in the absence of PS and would start with the addition of S. Actually the reasoning with oxygen uptake experiments suffers from the lack of knowledge about what fraction of the basal uptake might be caused by ATP consumption of permease at rest.

Efflux After Removal of S from the Medium. This efflux is essentially mediated by the nonspecific channels *i* and *h* [24]. These two channels are independent of the permease protein. Their mechanism is unknown, and actually there might be only one or more than two channels involved. In designing the two channels *i* and *h*, an operational distinction is made between the part of efflux which is inhibitable by uncoupling agents and the part which is not. When PCMB is added at the start or during the exit experiment, the rate of efflux does not significantly

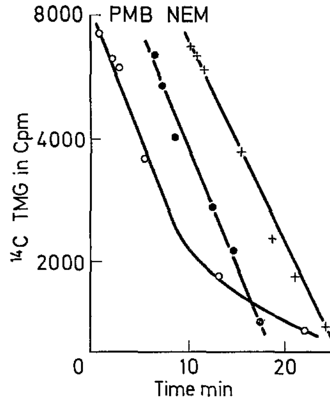
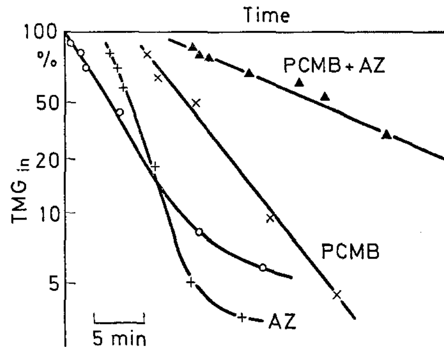


Fig. 4. Effects of SH reagents on the efflux of TMG. *E. coli* 3,300 was incubated with 1 mM ^{14}C TMG until steady state of accumulation, then chilled, centrifuged and resuspended in 1/200 original volume at 0 °C. Samples were diluted in 200 volumes of prewarmed medium 63 (∘—∘); the same medium contained 0.5 mM PMB (•—•) or 0.5 mM NEM (×—×). One-ml samples were filtered on Millipore filters, dried and counted



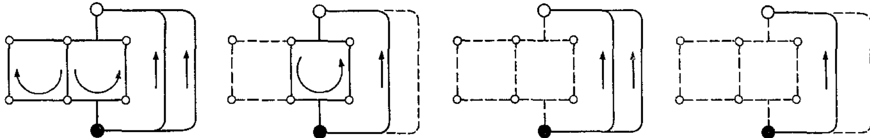


Fig. 5. Effects of uncoupling agents and thiol reagents on the efflux of TMG. *E. coli* 3,300 was incubated with 1 mM ^{14}C TMG until steady state of accumulation, then chilled, centrifuged and resuspended in 1/200 original volume at 0 °C. Samples were diluted in 200 volumes of prewarmed medium 63 (∘—∘) or the same medium containing 40 mM sodium azide (+—+), 0.5 mM PCMB (×—×), or both (▵—▵). One-ml samples were filtered on Millipore filters, dried and counted. *Inserts:* simplified flow diagrams according to model of Fig. 2. *See* explanations in text

deviate from the control (Fig. 4). Here, reaction sequence *a*, *b*, *c* is stopped by lack of S outside; therefore, for permease to contribute to the efflux, internal P should be furnished by reaction *g′*. Since action of PCMB slows the efflux

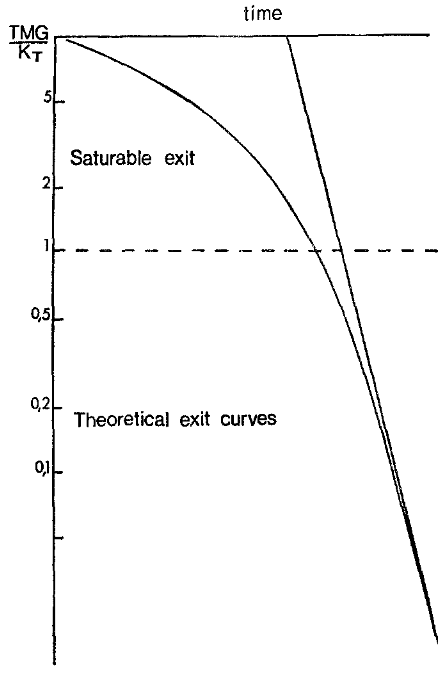


Fig. 6. Theoretical exit curves computed on the basis of two hypotheses. (*1*) Saturable exit kinetics was obtained by integrating $-\frac{ds}{dt}=V_{max}\frac{\text{Sin}}{\text{Sin}+K_m}$, with time taking arbitrarily the initial condition $\text{Sin}=10\times K_m$. (*2*) Nonsaturable exit curve was obtained by integrating $-\frac{ds}{dt}=k_{ex}\,\text{Sin}$ with the same initial conditions. Convexity of the initial part of the semilog plot is the criterion for saturable exit

insignificantly, the contribution of the reaction sequence g', c', b', a' to the efflux is negligible. This can be due to either negligible flux along path g' or to the fact that whatever free P can be recycled inside by reaction g' is captured by reaction *d* and made unavailable for reaction c'.

The latter hypothesis is actually confirmed by the addition of uncoupling agents which inhibit reaction *d*; the efflux is accelerated about twofold. Since at the same time channel *h* is inhibited, which supports a flux about twice that going through the non-inhibitable channel *i*, reaction g' is now supporting the major part of the outflux, which can be estimated to be two to three times slower than maximal flux of active uptake (Fig. 5).

The kinetics of efflux in this energy-inhibited state are first order (Fig. 6), nonsaturable and basically very different from the influx through the sequence *a*, *b*, *c*, which is saturable. This again raises the problem of the asymmetry of the system. It is conceivable that P oriented inward has a different configuration and therefore a much higher K_m than P turned out-

ward. This explanation was proposed tentatively in 1968 [24]. But it is equally possible, and even likely in the light of the argument above, that Michaelis kinetics are not observed simply because reaction c' is not the rate-limiting one, the rate-limiting reaction being g'.

When both PCMB and azide are added to the exit fluid, the rate of exit is dramatically inhibited (Fig.5). With TMG as the substrate, the exit rate is decreased by three-quarters as compared to the rate observed with azide alone, and by about half if compared to the control or the PCMB-poisoned state. This fits with the model by assuming that PCMB inhibited the permease-mediated exit where g' is the rate-limiting step and azide inhibited the nonspecific leak channel h, so that only channel i is operating. This channel might be simply free diffusion, and its total capacity of transport with maximal pool of TMG inside is about one-tenth of the maximal velocity of active uptake.

Exchange Diffusion (Fig. 7). Another way of abolishing net efflux (except for the minor non-inhibitable channel i) is to accumulate a substrate with saturating concentration in the medium, and then add an uncoupling agent [24]. This will inhibit reaction d and efflux through channel h. Other than through channel i, the only way for intracellular substrate to escape is to combine with free P entering through reaction g'. But free permease is extremely rare; since the substrate is at saturating concentrations on both sides of the membrane, all P is under the form PS. In these conditions, one-to-one exchange through the reaction cycle a, b, c, c', b', a' is going on at maximal velocity, but net efflux remains negligible in spite of a 20-fold concentration gradient between inside and outside, and no energy source is available to maintain it.

Uptake and Exchange in the Energy-Poisoned Conditions (Fig. 8). Winkler and Wilson [43] stated that in energy-poisoned bacteria the initial rate of uptake was comparable to that observed in control bacteria, except that it stops when intracellular concentration has reached the concentration present in the medium. With usual concentrations of substrate, equilibrium would be reached in about 1 sec, and no accurate measurement of flux is possible in such a short time. By increasing the substrate concentration by a factor of 20 to 50, more time is left to measure initial rates.

Under this circumstance, net uptake must go through the cycle a, b, c, g.

In contrast, once equilibrium is established, exchange can go on through the cycle of reactions a, b, c, c', b', a'.

The comparison of the rate of uptake and the rate of turnover shows that the latter is two to four times faster than the former (Fig. 8).

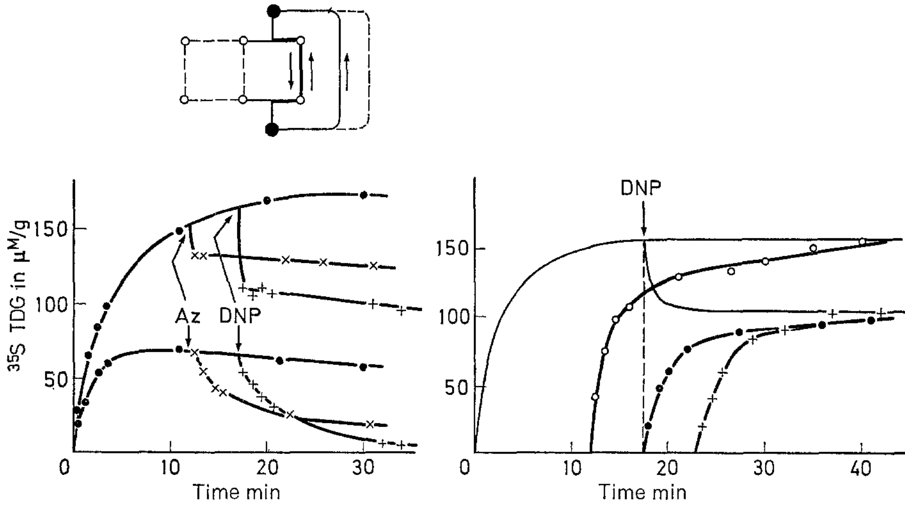


Fig. 7. Exchange diffusion mediated by permease. At the steady state of uptake (^{35}S TDG (• — •) with TDG 2.5×10^{-5} M, approx. 1 K_m (*lower curve*), and TDG 5×10^{-4} M approx. $20 \times K_m$ (*upper curve*), sodium azide 40 mM (× — ×) or 2,4-dinitrophenol (DN] 2 mM was added to separate samples of the incubation mixtures. While at low saturatio] the uncoupling agents cause complete outflux of TDG; at high saturation, a rapid parti: efflux is followed by a long phase of retention of TDG. This retention is not caused by block of transport, as shown by the active exchange (*lower figure*). During an uptak experiment with 5×10^{-4} M nonradioactive TDG, 2 mM DNP was added to the incubatio mixture. Trace amounts of ^{35}S TDG of high specific activity were added to sampl(before, at the same time, and after the addition of DNP. Thin lines represent the assume behavior of the pool of nonradioactive TDG based on separate experiments.—*Inser*. simplified flow diagram during exchange diffusion, based on the model of Fig. 2; P : predominantly under the form PS on both sides of the membrane. A net flux of substrat via permease would require an opposite net flux of free P, but this is in negligibl proportion

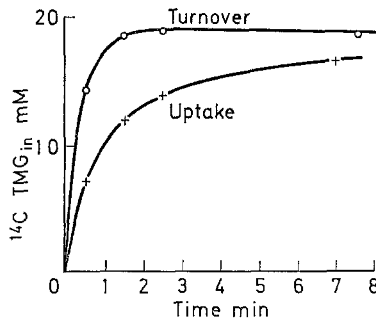


Fig. 8. Equilibrating transport by *E. coli* preincubated with an uncoupling agent. A suspension of *E. coli* 3,300 containing 2.5 mg dry weight per ml was preincubate(40 min with 40 mM sodium azide. ^{14}C TMG (50 mM) was added to a part of the suspension and uptake was followed by filtering 0.1-ml samples. Another part was allowed to accu mulate 60 mM nonradioactive TMG for 20 min, and then trace amounts of ^{14}C TM(of high specific activity were added to follow turnover. The difference in rates is attribute(to a limitation during net flux by process *g*

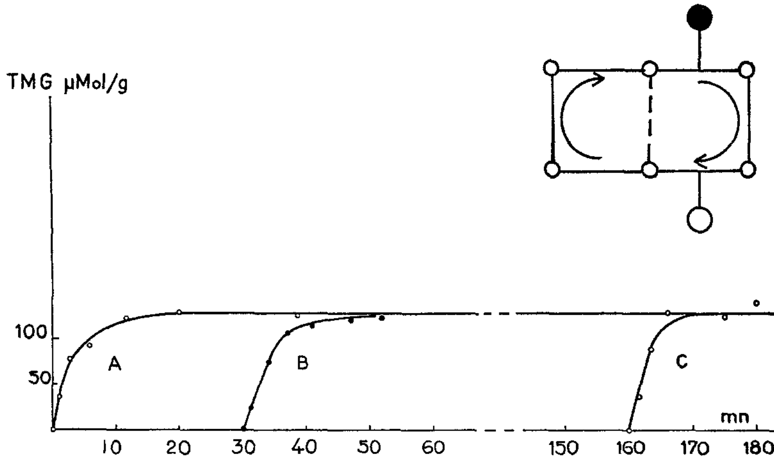


Fig. 9. Uptake and turnover of TMG during active transport. *E. coli* ML 308 in a suspension containing 250 µg dry weight per ml was supplemented with 1 mM ^{35}S TMG or 1 mM nonradioactive TMG. The nonradioactive portions received trace amounts of ^{35}S TMG of high specific activity at 30 min and 160 min. Then 1-ml samples were filtered on Millipore filters, dried and counted. Note the equality of rates in uptake and exchange. The rate-limiting reaction sequence in both cases is *a, b, c,* according to the model. Restitution of free permease to the outer face goes via *d e f* during net uptake, and partly through the same pathway and partly through c' b' a' during exchange

The steady state of active accumulation, in contrast, shows a rate of turnover equal to the rate of uptake (Fig.9). As shown above, uptake goes through the sequence *a, b, c, d, e, f.* At the turnover of steady state, substrate still enters through the pathway *a, b, c* and leaves partly through reaction sequence c', b', a' and partly through channels *i* and *h*. The flux of P from the inner to the outer face also follows two pathways, c', b', a' and *d, e, f.* Therefore, the flux through *d, e, f* matches the substrate flux through *i* and *h*. It was observed that the increased oxygen uptake which starts during active net uptake goes on with no major decrease during the subsequent steady state of substrate exchange. Therefore, at the steady state, flux through *d, e, f* and consequently flux through *i* and *h* represent a sizable part of total flux of P and S, respectively. The accurate evaluation of the partition of outflux of P and of S is very difficult; knowing the transport capacity of channels *i* and *h* from exit experiments with PCMB-inactivated cells, this partition of efflux would be between a quarter to one-third of the total flux. From oxygen uptake experiments, one has the feeling that the flux through *d, e, f* is at least more than half of the total flux. From measurement of K_m of the steady state pool (Fig. 10), one would conclude that efflux by the nonspecific leak channels is predominant, and flux through c' b' a' is

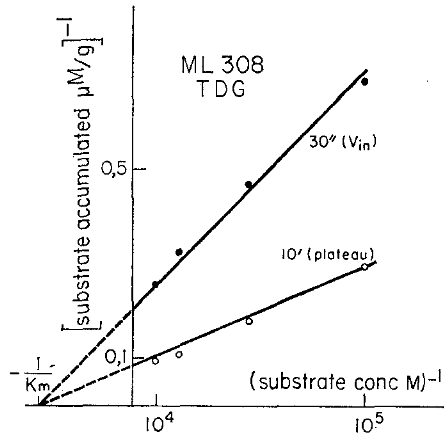


Fig. 10. Concentration dependence of velocity of uptake and steady state accumulatio Shown is a double reciprocal plot of intracellular TDG accumulated in 30 sec and 10 min by *E. coli* ML 308. The two straight lines give the same K_m

rather negligible. The three types of evaluations have a limit set to the accuracy. Rates of exit vary from one experiment to another within a factc of two; oxygen uptake experiments have about the same variability, wit the additional variability of the basal oxygen uptake and the uncertaint about a possible contribution of permease at rest to the basal respiratioı K_m values of the steady state pool are more accurate, but some uncertaint remains when the plateau is not completely horizontal. This is a commo observation in kinetic experiments at 37 °C that the plateau is decreasin slowly, and at low temperatures it has a tendency to increase slowly. Th commonly used temperature of permease experiments is 25 °C where th plateau is usually more stable, but since the reasons for the instability ar obscure, the arbitrary choice of the temperature is not a guarantee that th best parameters are measured. In view of these uncertainties, it is not certai that the three kinds of estimation of the part of steady efflux carried b channels *i* and *h* are really conflicting.

One way of reconciling these different estimations would be to admi that the energized carrier P ~ A can also combine with intracellular substrat and contribute to the efflux as supposed in the model of Winkler and Wilsoı [43, 44].

In view of the insensitivity of net efflux to PCMB (*see* above), this contri bution must be negligible.

Overshoot experiments were done with cells preloaded at high concen tration of nonradioactive substrate. Net exit is initiated by dilution in

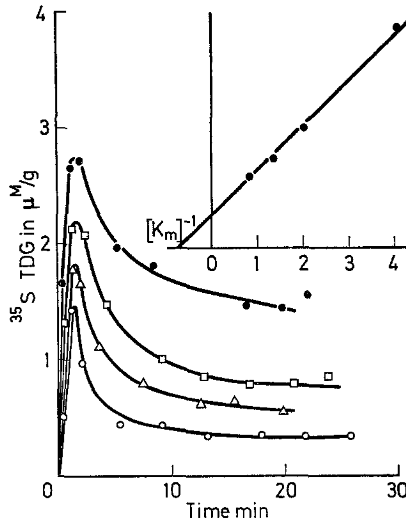


Fig. 11. Overshoot experiments. A concentrated suspension of *E. coli* 3,300 in medium 63 containing 40 mM sodium azide was loaded with 100 mM nonradioactive TMG during 40 min. Portions were diluted in 400 volumes of medium 63 containing 40 mM azide, radioactive TMG, and nonradioactive TMG to make up final TMG concentrations of 1.25, 0.75, 0.50 and 0.25 mM, respectively (from top to bottom). In each case, the final specific activity of TMG (including the preload) was 0.5 μc per μmole. The efflux of cold TMG causes a temporary active uptake of radioactive TMG in spite of the energy inhibitor. After 20 min, the intracellular concentration of radioactive TMG drops to equilibrium level. *Insert:* double reciprocal plot of initial velocities

medium containing uncoupling agents plus trace amounts of a radioactive substrate. During intense net exit of the preloaded sugar, the tracer is taken up against its concentration gradient. Once net exit becomes small enough, the radioactive tracer leaves the cell until equilibrium with external concentration is reached. The situation is the same as in net exit with energy-poisoned cells, except that free P can be recycled by reaction g and by reactions a, b, c in association with the external tracer substrate. The total efflux cannot be harnessed to activate influx since part of it goes through channel i. The part which follows pathway c' b' a' is itself not entirely used for counter transport since part of the free carrier recycles through reaction g', unless external radioactive substrate is saturating. This can be used and should give somewhat higher overshoot in absolute value, but becomes smaller and less demonstrative relative to the final equilibrium values overshoot.

In vivo hydrolysis of ONPG (Fig. 12) in an initial period before a pool of galactose is built up follows the same mechanism as net uptake of a non-hydrolyzable thiogalactoside, via pathway a, b, c, d, e, f, and, in the presence of

an uncoupling agent, it is inhibited and follows the same pathway as net u take in the presence of an uncoupling agent via *a*, *b*, *c*, *g*. After the buildu of a pool of galactose, ONPG uptake follows the same pathways but r cycling of free permease to the outer face also occurs via *c'*, *b'*, *a'* in associ tion with galactose. This overcomes the rate limiting reaction *g* in the energ poisoned state, and the reaction sequence is similar to exchange diffusio The finding that, with severe conditions of energy inhibition, ONP hydrolysis can be decreased to nearly zero seems to indicate that the transpo capacity of reaction *g* is even lower than with other estimations.

Some Open Problems

This model describes in a fairly consistent way all the situations whic have been experimentally scrutinized. It accounts for the main feature namely the Michaelis-type concentration dependence of initial uptake an steady-state pool, equality between rate of uptake and rate of turnove first-order concentration dependence of efflux, insensitivity of efflux to S reagents, exchange diffusion, counterflux with overshoot and the ambiguit of energy dependence of ONPG hydrolysis. It is also consistent with th observations on increased oxygen uptake. In quantitative adjustments c the various transmembrane fluxes, some minor discrepancies have to awa

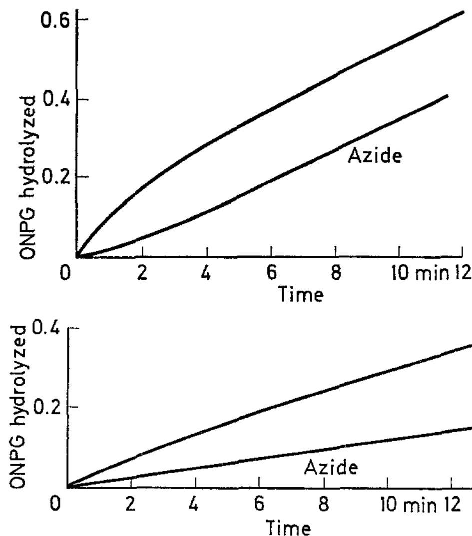


Fig. 12. Recording spectrophotometer traces of the time course of ONPG hydrolysi by whole cells with or without 60 mM azide. *Upper figure: E. coli* 3,300 grown on mediur 63 fructose. *Lower figure: E. coli* 3,300 grown on medium 63, fructose and fucose Fucose is inducing the enzymes of galactose metabolism

more accurate evaluations. The difficulty with these evaluations increases because of the fact that energy poisons have at least two sites of action: they inhibit the coupling reaction *d* and one of the nonspecific leak channels *h*.

The question can be asked if it is worthwhile building such sophisticated models at the present stage of our knowledge. Nothing sure is known in particular about the energy-coupling and the energy-dissipating reactions.

In this field, a too well specified model runs the risk of being contradicted by new findings. But the model can guide new experiments designed to demonstrate or to disprove its assertions.

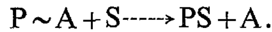
The most unwarranted part of the model is the sequence of reactions *d*, *e*, *f*. In a previous model, these steps were not distinguished, and there is no experimental fact at present to distinguish them. Also, in the flow diagrams represented above, the sequence *d*, *e*, *f* is always undissociated and functional as a whole or inhibited as a whole. In contrast, the distinction of the energized form from the free permease is rather amply documented inasmuch as the shuttling of the latter appears as rate limiting at a lower flux than the former in net exit and net uptake in the energy-poisoned state.

The kinetics of the energy-coupling reaction as a function of free intracellular P and concentration of energy donor A$\sim$B are unknown. The competition for free intracellular P of the coupling reaction *d* and the substrate binding reaction *c′* is unknown *a fortiori*. The important question in this connection would be to know whether the energy coupling reaction requires an auxiliary enzyme (a kinase?) or is permease the enzyme and the acceptor at the same time?

This question might be answered in the near future by the mapping of mutations such as the one reported recently by Wilson, which results in a deficiency of active transport, without an impairment of downhill transport. The simple finding of the mutant does not answer the question, since it is conceivable that the two functions of permease protein – namely substrate binding and energy coupling – are dissociated by a limited change in configuration owing to a mutation. If the mutation maps outside the *y* cistron, then the hypothesis of an auxiliary enzyme will be reinforced. One must not forget, however, that the low K mutation in sheep was shown not to be a mutation of the Na, K-dependent ATPase nor an alteration of another normal component of the cation pump.

The same question can be raised in connection with the energy-dissipating reaction *f*. An auxiliary enzyme built in the membrane in an unsymmetric way would be helpful in polarizing the dissipation reaction and save waste of energy when permease is at rest which would result if coupling and dissipation

can both occur on the inner side. Nevertheless, the saving would still not b complete since free permease could revert back inside through reactio g' and be energized again. One full safety against energy waste would b that the energy-dissipating reaction be substrate dependent, namely



$$P \sim A + S \dashrightarrow PS + A.$$

This would introduce a similarity with the early model presented in 196 [21], where the energy-dissipation step of the unspecific carrier T wa catalyzed by the permease, an "auxiliary enzyme" in the presence of sub strate, to give TS the carrier substrate complex.

It is difficult to test this possibility unless the energized form of th permease has a distinctively different rate of reaction with SH reagent than does the deenergized free permease or has a distinctively differen affinity for substrate. In view of the problem in quantitating accuratel inactivation kinetics, we tried to define the apparent K_m of deenergize permease. In overshoot experiments, the K_m for TMG was found abou two times higher than in active uptake, but again, overshoot experiment are difficult to quantitate. The K_m of net uptake in the energy-poisone state has been also impossible to measure until now. When low concen trations of substrate are used, equilibrium is reached too fast; with hig concentrations, the K_m cannot be properly established. Moreover, durin these attempts it was noticed that 2 mM DNP or 40 mM sodium azid did not completely abolish active transport.

There remain a few facts unexplained by the present model some o which well established. They concern the outflux caused by glucose and th inhibition of ONPG hydrolysis by α-methyl glucoside. These do not seen to throw light on the mechanism of transport as visualized presently an they might reflect superimposed regulatory phenomena. They will gai primary interest, once the basic mechanism of transport is complete elucidated.

The present work was supported by the Delegation Générale à la Recherche Scientifique et Technique and by the Commissariat à l'Energie Atomique.

The author is greatly indebted to Miss Michelle Nardon for her expert technical assistence.

References

1. Bangham, A. D., Standish, M. M., Watkins, J. C. 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **13**:238.
2. Barnes, E. M., Kaback, H. R. 1970. β-Galactoside transport in bacterial membrane preparations: Energy coupling via membrane-bound-D-lactic dehydrogenase. *Proc. Nat. Acad. Sci.* **66**:1190.

3. Britten, R. J., Roberts, R. B., French, E. F. 1955. Amino acid adsorption and protein synthesis in *Escherichia coli*. *Proc. Nat. Acad. Sci.* **41**:363.
4. Chance, B., Williams, G. R. 1956. The respiratory chain and oxidative phosphorylation. *In:* Advances in Enzymology, F. F. Nord, editor. p. 65. Interscience publishers Inc., New York.
5. Cohen, G. N., Monod, J. 1957. Bacterial permeases. *Bacteriol. Rev.* **21**:169.
6. — Rickenberg, H. V. 1955. Etude directe de la fixation de l'inducteur de la β-galactosidase par les cellules d'*Escherichia coli*. *Compt. Rend. Acad. Sci.* **240**:466.
7. Danielli, J. F., Davson, H. 1934. A contribution to the theory of permeability of thin films. *J. Cell. Comp. Physiol.* **5**:495.
8. Deere, C. J., Dulaney, A. D., Michelson, I. D. 1939. The lactase activity of *Escherichia coli* mutabile. *J. Bacteriol.* **37**:355.
9. Englesberg, E., Novotny, C. P. 1966. The L-arabinose permease system in *Escherichia coli* B/r. *Biochim. Biophys. Acta* **117**:217.
10. Fox, C. F., Kennedy, E. P. 1965. Specific labeling and partial purification of the M protein. A component of the β-galactoside transport system of *Escherichia coli*. *Proc. Nat. Acad. Sci.* **54**:891.
11. Gorter, E., Grendel, F. 1925. On biomolecular layers of lipoids on the chromocytes of the blood. *J. Exp. Med.* **41**:439.
12. Herzenberg, L. A. 1959. Study on the induction of β-galactosidase in a cryptic strain. *Biochim. Biophys. Acta* **31**:525.
13. Kaback, H. R. 1968. The role of the phosphoenolpyruvate-phosphotransferase system in the transport of sugars by isolated membranes prepartions of *Escherichia coli*. *J. Biol. Chem.* **243**:3711.
14. — 1969. Studies on sugar transport by isolated bacterial membrane. *In:* Molecular Basis of Membrane Function, D. C. Tosteson, editor. p. 421. Prentice Hall, Englewood Cliffs, N. J.
15. — Kostellow, A. B. 1968. Glycine uptake in *Escherichia coli*. I—Glycine uptake by whole cells of *Escherichia coli* W^+ and a D-serine resistant mutant. *J. Biol. Chem.* **243**:1384.
16. Kauzman, W. 1959. Some factors in the interpretation of protein denaturation. *Advanc. Prot. Chem.* **14**:1.
17. Kennedy, E. P. 1966. Biochemical aspects of membrane function. *In:* Current Aspects of Biochemical Energetics. p. 433. Academic Press, New York.
18. — 1969. Studies on the lactose transport system in *Escherichia coli*. *J. Gen. Physiol.* **54**:91_s–93_s.
19. — 1970. The lactose permease system of *Escherichia coli* in the lactose operon. J. R. Beckwith and D. Z. Zipser, editors. Cold Spring Harbor Monograph p. 49–92.
20. Kepes, A. 1957. Metabolisme oxydatif lié au fonctionnement de la galactoside perméase d'*Escherichia coli*. *Compt. Rend. Acad. Sci.* **244**:1550.
21. — 1960. Etudes cinétiques sur la galactoside perméase d'*Escherichia coli*. *Biochim. Biophys. Acta* **40**:70.
22. — 1961. Bacterial permeases. *In:* Biochemie des Aktiventransport. p. 100. Springer Verlag, Berlin-Heidelberg.
23. — 1964. The place of permeases in cellular organization. *In:* The Cellular Functions of Membrane Transport. J. F. Hoffman editor. p. 155. Prentice Hall, Englewood Cliffs, N. J.
24. — 1969. Carrier properties of β-galactoside permease. The role of permease in the leak of β-galactosides from *Escherichia coli*. *In:* Molecular Basis of Membrane Function. D. C. Tosteson, editor. p. 353. Prentice Hall, Englewood Cliffs, N. J.

25. Kepes, A. Galactoside permease of *Escherichia coli*. *In:* Current Topics in Membranes and Transport. p. 101 (*in press*). F. Bonner and A. Kleinzeller, editors. Academic Press, New York.
26. — Monod, J. 1957. Etude du fonctionnement de la galactoside permease d'*Escherichia coli*. *Compt. Rend. Acad. Sci.* **244**:809.
27. Koch, A. L. 1964. The role of permease in transport. *Biochim. Biophys. Acta.* **79**:177.
28. — 1969. Energy is needed for downhill galactoside transport in *Escherichia coli*. *Fed. Proc.* p. 868.
29. Milner, L. S., Kaback, H. R. 1970. The role of phosphatidylglycerol in the vectorial phosphorylation of sugar by isolated bacterial membrane preparations. *Proc. Nat. Acad. Sci.* **65**:683.
30. Mueller, P., Rudin, D. O., Tien, H. T., Wescott, W. C. 1962. Reconstitution of cell membrane structure *in vitro* and its transformation into an excitable system. *Nature* **194**:979.
31. Overton, E. 1899. Ueber die allgemeinen osmotischen Eigenschaften der Zelle, ihre vermutlichen Ursachen und ihre Bedeutung. für die Physiologie. *Vierteljahr. Naturforsch. Gessel.* (*Zürich*) **44**:88.
32. Pavlasova, E., Harold, F. M. 1969. Energy coupling in the transport of β-galactosides by *Escherichia coli:* effect of proton conductors. *J. Bacteriol.* **98**:198.
33. Post, R. L., Sen, A. K., Bader, H. 1967. Insight into a pump mechanism through the reactivity of a phosphorylated intermediate in (sodium-potassium)-dependent adenosine triphosphate. *In:* Protides of the Biological Fluids. **15**:237–250.
34. Rickenberg, H. V., Cohen, G. N., Buttin, G., Monod, J. 1956. La galactoside permease d'*Escherichia coli*. *Ann. Inst. Pasteur.* **91**:829.
35. Robertson, J. D. 1959. The ultrastructure of cell membranes and their derivatives. *Bioch. Symp.* (*London*) **16**:3.
36. Rotman, B., Guzman, R. 1961. Transport of galactoside from the inside to the outside of *Escherichia coli*. *Pathol. Biol.* **9**:806.
37. Schachter, D., Johnson, N., Kirkpatrick, M. A. 1966. Energy coupling for galactoside accumulation in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **25**:603.
38. — Mindlin, A. J. 1969. Dual influx model of thiogalactoside accumulation in *Escherichia coli*. *J. Biol. Chem.* **244**:1808.
39. Sistrom, W. R. 1958. On the physical state of the intracellularly accumulated substrates of β-galactoside permease in *Escherichia coli*. *Biochem. Biophys. Acta* **29**:579.
40. Sjöstrand, F. S. 1967. Molecular Structure and Function of cellular membranes. Protides of the Biological Fluids, 15th colloquium Bruges 15–36.
41. Skou, J. C. 1960. Phase investigation as a magnesium activated adenosine triphosphatase, possibly related to the active linked transport of Na^+ and K^+ across the nerve membrane. *Biochim. Biophys. Acta* **42**:6.
42. Wallach, D. F., Zahler, P. A. 1966. Protein conformations in cellular membranes. *Proc. Nat. Acad. Sci.* **56**:1552.
43. Winkler, H. H., Wilson, T. H. 1966. The role of energy coupling in the transport of β-galactoside by *Escherichia coli*. *J. Biol. Chem.* **241**:2200.
44. — — 1967. Inhibition of β-galactoside transport by substrates of the glucose transport system in *Escherichia coli*. *Biochim. Biophys. Acta* **135**:1030.
45. — Wong, P. T. S., Kashket, E. R., and Wilson, T. H. 1970. Energy coupling in the lactose transport system of *Escherichia coli*. *Proc. Nat. Acad. Sci.* **65**:63.