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SITE OF ENERGY COUPLING IN THE CARRIER MECHANISM FOR β-GALACTOSIDE TRANSPORT

J. TZE-FEI WONG, ALEXANDRA PINCOCK AND PATRICIA M. BRONSKILL
Department of Biochemistry, University of Toronto, Toronto (Canada)
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SUMMARY

A kinetic analysis of the effects of energy poisons on β-galactoside influxes into *Escherichia coli* cells has been directed toward locating the site of energy coupling in the carrier mechanism for β-galactoside transport. Cyanide inhibited the influx of thiomethylgalactoside but not nitrophenylgalactoside. The inhibition of nitrophenylgalactoside influx by thiomethylgalactoside was not affected by cyanide, and nitrophenylgalactoside also failed to relieve the cyanide inhibition of thiomethylgalactoside influx. On the basis of the steady-state kinetic predictions for a monovalent carrier mechanism, these observations have been interpreted as pointing toward the intracellular dissocation of the carrier–galactoside complex as an important site of energy coupling.

INTRODUCTION

The active transport of β-galactosides into *Escherichia coli* cells is dependent on the y-gene, or permease, function of the lactose operon[1–3]. In recent years, striking progress has been achieved in the discovery of possible components of this system, including a protein product of the y-gene[4], a sugar phosphotransferase complex[5] as well as unsaturated fatty acids[6]. The exact functions of such components in bringing about the catalysis of transport and accumulation are as yet incompletely understood, and different models have been advanced for the role of energy coupling in converting the transport system from one of facilitated diffusion into one of active accumulation.

To define the role of energy coupling, the kinetic influences of energy metabolism on galactoside influxes and effluxes have been extensively analysed. A stimulation of efflux by energy poisons has been established by KOCH[3] and WINKLER AND WILSON[7], but their finding that influx was little affected has been questioned. KOCH[3] followed the influx of *o*-nitrophenylgalactoside on the basis of its hydrolysis by intact cells, and WINKLER AND WILSON[7] compared the influx into active unloaded cells with poisoned cells preloaded with galactosides; both types of influx measurements are open to complications[8]. Thus SCARBOROUGH *et al.*[9] and SCHACHTER and co-workers[8,10] have demonstrated a variation of the influx rate with the energy status of the cells. Aside from this question of dependence of influx on energy metabolism, in most of these studies there

has been little attempt to utilize unsimplified steady-state carrier equations to define the energy-linked steps in the transport mechanism for galactosides. Accordingly, in the present study the usefulness of carrier kinetics for this purpose has been exploited, and the relative effects of energy poisons on the influxes of *o*-nitrophenylgalactoside and thiomethylgalactoside are found to be particularly informative.

MATERIALS AND METHODS

Chemicals

Thiomethylgalactoside and *o*-nitrophenylgalactoside were purchased from Mann Laboratories, [^{14}C]thiomethylgalactoside from New England Nuclear, and [1-^{14}C]-lactose from Nuclear Chicago Corporation. Metricel GA-6 filters, 2 inches in diameter with 0.45-μm pores, were obtained from Gelman Co., and Millipore AA filters, 25 mm in diameter with 0.8-μm pores, from Millipore Co.

Cultures

E. coli strain K12 Leu$^-$, a leucine auxotroph kindly supplied by Dr. A. GOLDSTEIN[11] was grown at 37° in Medium 63 (ref. 12) containing 20 μg/ml leucine and 0.5 % glycerol. After induction by 0.4 mM thiomethylgalactoside for 40 min, except where otherwise specified, 50 ml portions of cells containing about $2 \cdot 10^8$ cells/ml were placed on a Metricel GA-6 filter and washed with warm Medium 63 (37°) for 4 min. After resuspension in Medium 63 *plus* 0.5 % glycerol to $0.8 \cdot 10^8$ cells/ml they were stored at 0° until use.

Assay of thiomethylgalactoside transport

Cells suspended in Medium 63 *plus* 0.5 % glycerol were incubated at 20° for 10 min before [^{14}C]thiomethylgalactoside (4.05 mC/mmole) was added to a final concentration of 0.1 mM. At different times, a 1-ml aliquot was removed, mixed with 10 ml of ice-cold Medium 63, placed on a Millipore filter, and washed 3 times with ice-cold Medium 63. The washed filter was transferred to a scintillation vial and 10 ml of a counting mixture of 5 g 2,5-diphenyloxazole and 0.5 g 1,4-bis-2-(5-phenyloxazolyl)-benzene in 1 l of toluene–ethanol (3:1, v/v) was added. Scintillation counting of radioactivity at optimized settings in a Nuclear Chicago Mark II counter gave 70 % efficiency. To obtain progress curves of influx, the cellular radioactivities at different times were in all cases corrected for the radioactivity in the zero-time sample, obtained by adding [^{14}C]thiomethylgalactoside to cells already mixed into ice-cold Medium 63.

Assay of nitrophenylgalactoside hydrolysis

Cell suspensions were assayed either intact, or toluenized by mixing 1-ml samples with 0.05 ml toluene and continually inverting on a rotating rack for 1 h. Hydrolysis by toluenized cells was carried out in accordance with the method of PARDEE *et al.*[13] and gave a measure of the β-galactosidase in the cells; 1 unit of activity equals 1 nmole nitrophenylgalactoside hydrolysed per min. Hydrolysis by intact cells was carried out at 20° according to the method of KOCH[3], using a Gilford Recording Spectrophotometer to monitor absorbance changes at 420 mμ. The initial slope of the absorbance change gave the hydrolytic rate.

Assay of lactose hydrolysis

In this assay 10 nmoles of [^{14}C]lactose (10.2 mC/mmole) were incubated with $7 \cdot 10^9$ cells in 0.5 ml. At different times, the incubation mixture was brought to 100° for 2 min in order to stop the enzymic hydrolysis. After chilling and centrifugation to remove precipitated cells, 20 μl of the supernatant were spotted on a paper chromatogram (Whatman No. 1) along with added galactose, glucose and lactose as markers. Development with the upper phase of ethyl acetate–pyridine–water (2:1:2, by vol.) as solvent[14] for 24 h completely resolved lactose from glucose and galactose (R_{glc} = 0.94 for galactose and 0.31 for lactose). After drying, the markers were located by spraying with aniline–diphenylamine[15], and the lactose spot was cut out and counted by scintillation counting. Such measurements provided a time course for the total lactose hydrolysed and metabolized by the cells.

RESULTS

Dependence of thiomethylgalactoside influx on permease concentration

Before examining the effects of energy poisons on the initial rate of thiomethylgalactoside influx, it was necessary to establish that adequate measurements of the initial rate were obtainable under the experimental conditions employed. Accordingly, the extent of permease induction was varied by changing either the induction time or the inducer concentration. In both instances, the progress curves of influx were well

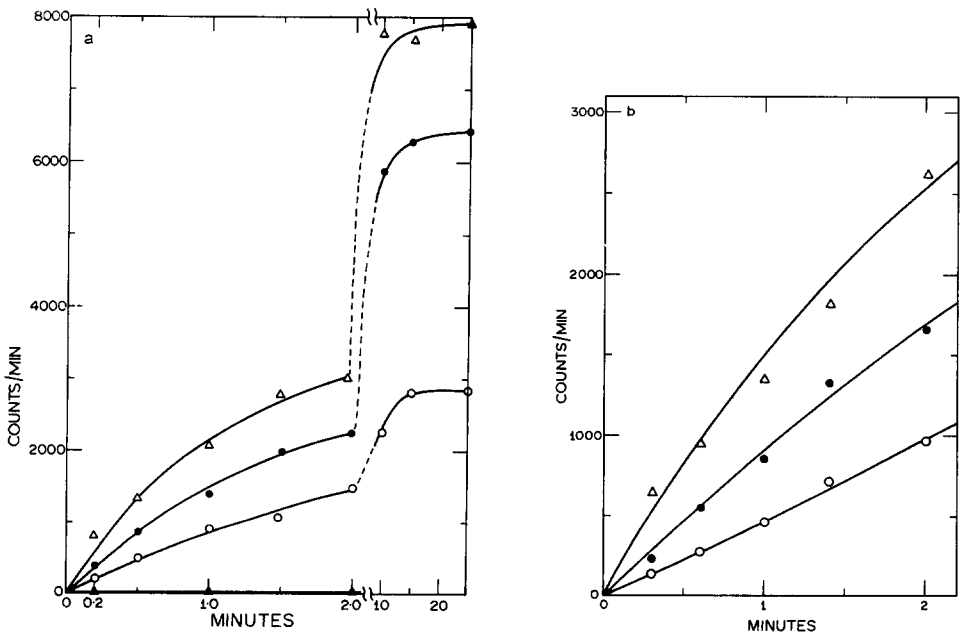

Fig. 1. Influx of [^{14}C]thiomethylgalactoside into differently induced cells. (a) Cells were induced by 0.4 mM thiomethylgalactoside for different intervals before harvested and assayed for uptake of [^{14}C]thiomethylgalactoside as described in MATERIALS AND METHODS: △—△, 60 min; ●—●, 30 min; ○—○, 15 min; ▲—▲, 0 min. (b) Cells were induced for 20 min by different concentrations of thiomethylgalactoside: △—△, 0.8 mM; ●—●, 0.4 mM; ○—○, 0.2 mM. In all cases, 100 counts/min in the cell sample would correspond to 0.10 mM [^{14}C]thiomethylgalactoside in cellular water and therefore equilibration with extracellular medium.

behaved, and the initial slopes determined from the curves in Figs. 1a and 1b were linearly related to the cellular concentration of β-galactosidase (Fig. 2). This linear dependence strongly indicates a proportionality both between permease and β-galactosidase inductions, as well as between permease concentration and the initial influx rate.

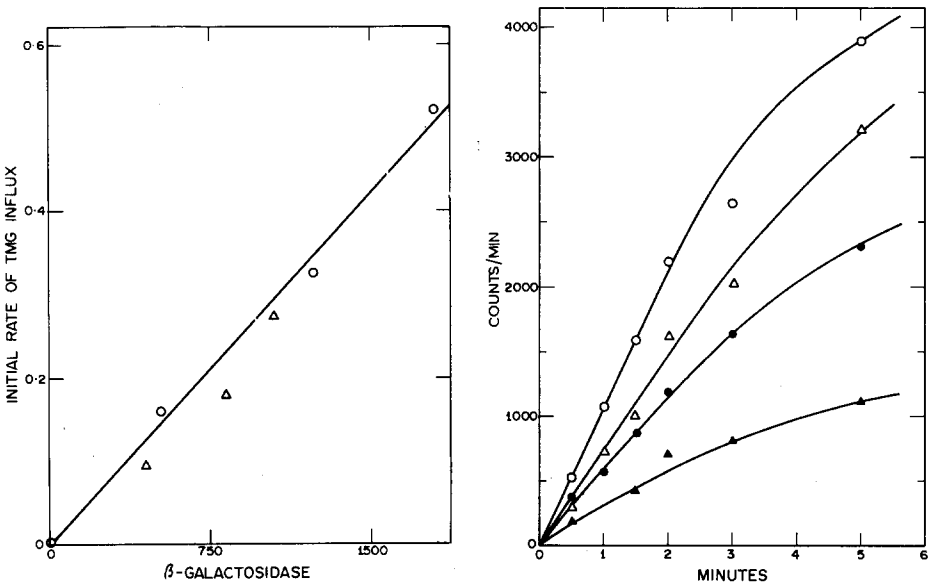

Fig. 2. Relationship between initial rates of thiomethylgalactoside (TMG) uptake and induced concentration of β-galactosidase. For each of the cell preparations used in Figs. 1a and 1b, the concentration of induced β-galactosidase was determined using toluenized cells and plotted against the initial slope of the [^{14}C]thiomethylgalactoside uptake curve for the same cell preparation. The β-galactosidase concentration is expressed as Pardee units per 10^8 cells, and the initial rate of uptake expressed as nmoles thiomethylgalactoside per min per 10^8 cells. ○, cells from Fig. 1a; △, cells from Fig. 1b.

Fig. 3. Effects of cyanide on influx of [^{14}C]thiomethylgalactoside. Different concentrations of cyanide were added to cell preparations 10 min before transport assays were carried out: ○—○, 0 mM; △—△, 0.03 mM; ●—●, 0.1 mM; ▲—▲, 0.2 mM.

Influence of cyanide on influx rates

In the presence of increasing cyanide concentrations, the progress curves of thiomethylgalactoside uptake remained well behaved. As can be seen from Fig. 3, the initial slope was extensively inhibited by 0.2 mM cyanide. In contrast, lactose influx (Fig. 4) and nitrophenylgalactoside influx, measured on the indirect basis of their hydrolysis and disappearance, were little affected at the same cyanide concentration. The thiomethylgalactoside rates obtained from Fig. 3 were plotted in Fig. 5 along with the nitrophenylgalactoside rates for the sake of comparison. It might be noted that nitrophenylgalactoside hydrolysis by intact cells was only 20% as rapid as by toluenized cells; consequently its uptake was rate-limiting relative to hydrolysis, and the rate of hydrolysis by intact cells could be used as an indirect monitor for influx[3].

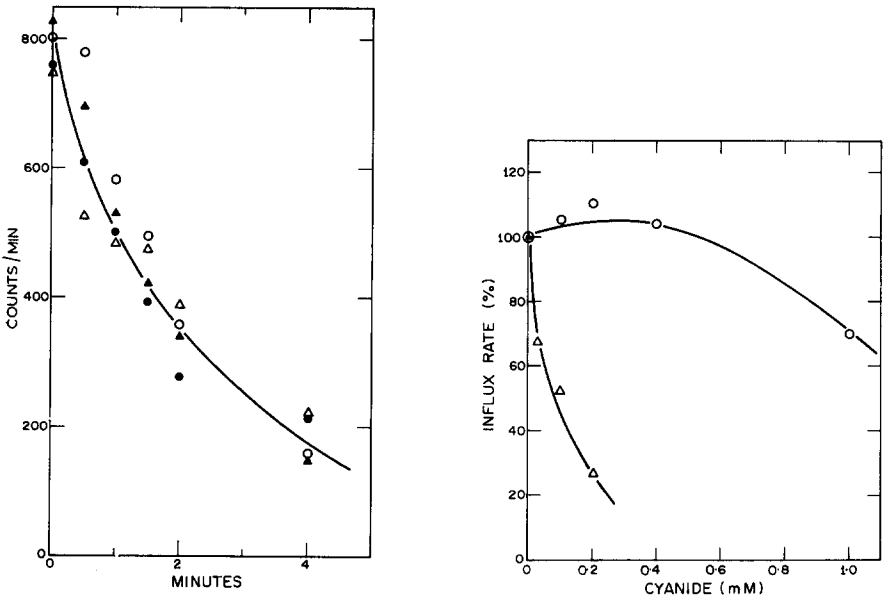

Fig. 4. Effects of cyanide on lactose uptake. Disappearance of lactose was assayed as described in MATERIALS AND METHODS, and the radioactivity remaining in the lactose spot on the chromatogram is plotted against incubation time. The incubation mixtures contained various concentrations of cyanide: ○—○, 0 mM; ▲—▲, 0.1 mM; △—△, 0.15 mM; ●—●, 0.2 mM.

Fig. 5. Relative effects of cyanide on thiomethylgalactoside and *o*-nitrophenylgalactoside influxes. The thiomethylgalactoside influx rates (△—△) are given by the initial slopes in Fig. 3, and the *o*-nitrophenylgalactoside influx rates (○—○) were measured as described in MATERIALS AND METHODS. In each case the control rate in the absence of cyanide is taken as 100%.

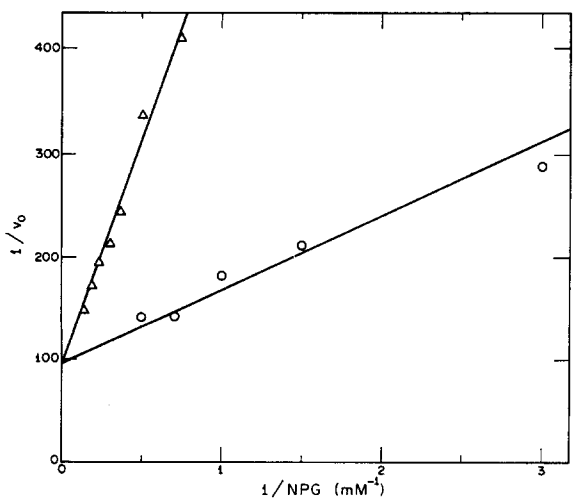

Fig. 6. Double reciprocal plot of thiomethylgalactoside inhibition of *o*-nitrophenylgalactoside(NPG) influx. v_0, the initial *o*-nitrophenylgalactoside influx rate is expressed in terms of change in absorbance units at 420 $m\mu \cdot min^{-1}/10^8$ cells$\cdot ml^{-1}$. (○—○, v_0 measured in the absence of thiomethylgalactoside; △—△, v_0 measured in the presence of 20 mM thiomethylgalactoside.)

Transport interactions between galactosides

Since cyanide affected differently the influxes of thiomethyl- and nitrophenylgalactosides, it became useful to examine the influence of cyanide on the transport

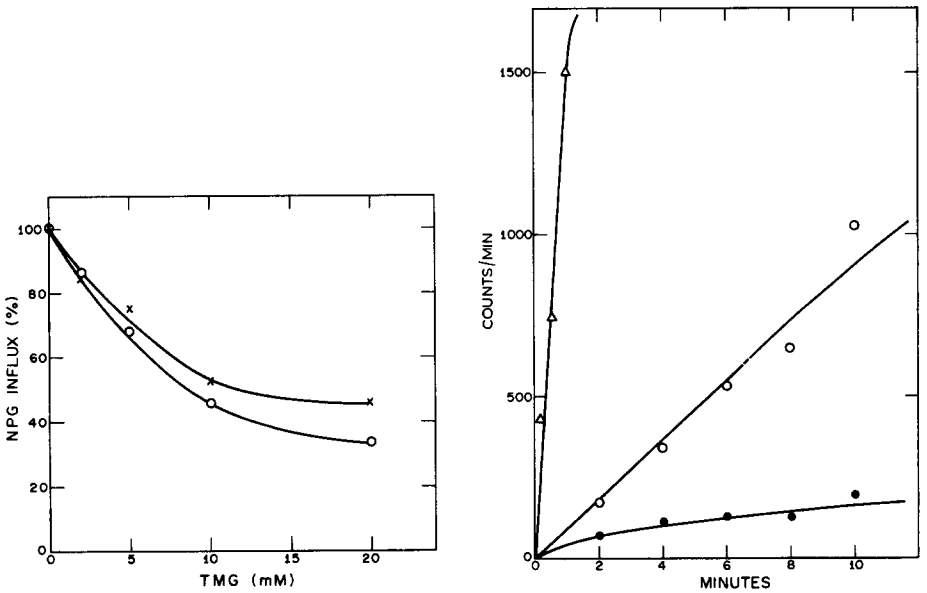

Fig. 7. Effects of cyanide on thiomethylgalactoside (TMG) inhibition of *o*-nitrophenylgalactoside (NPG) influx. *o*-nitrophenylgalactoside influx was measured at different thiomethylgalactoside concentrations both in the absence (×—×) and presence (○—○) of 1 mM cyanide. In either case, *o*-nitrophenylgalactoside influx in the absence of thiomethylgalactoside is taken as 100%.

Fig. 8. Effects of *o*-nitrophenylgalactoside on cyanide inhibition of [^{14}C]thiomethylgalactoside influx: △—△, no *o*-nitrophenylgalactoside or cyanide added; ○—○, 2 mM *o*-nitrophenylgalactoside added; ●—●, 2 mM *o*-nitrophenylgalactoside and 0.2 mM cyanide added.

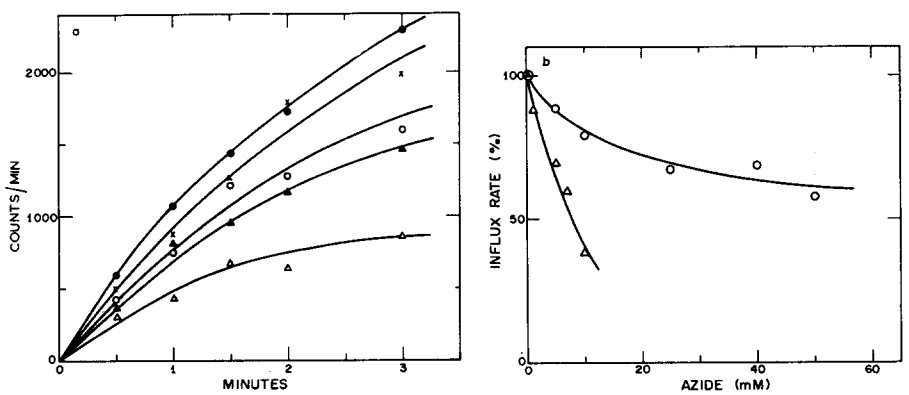

Fig. 9. Effects of azide on thiomethylgalactoside and *o*-nitrophenylgalactoside influxes. (a) Different concentrations of azide were added to cell preparations 10 min before assays of thiomethylgalactoside transport: ●—●, 0 mM; ×—×, 1 mM; ○—○, 5 mM; ▲—▲, 7 mM; △—△, 10 mM. (b) The initial thiomethylgalactoside influx rates (△—△) from part (a) as well as initial *o*-nitrophenylgalactoside influx rates (○—○) measured separately, are plotted against azide concentration.

interactions between these substrates. In Fig. 6 the uptake of nitrophenylgalactoside is seen to be competitively inhibited by thiomethylgalactoside. The extent of inhibition was not diminished even by 1 mM cyanide (Fig. 7). Since 1 mM cyanide would severely interfere with the intracellular accumulation of thiomethylgalactoside, it is evident that this inhibiton was exerted extracellularly rather than intracellularly.

Just as thiomethylgalactoside inhibited nitrophenylgalactoside uptake, a comparison of the top and middle curves in Fig. 8 shows that the reverse was also observed. A comparison of the middle and bottom curves further indicates that the presence of nitrophenylgalactoside failed to relieve the cyanide inhibition of thiomethylgalactoside influx to any considerable extent.

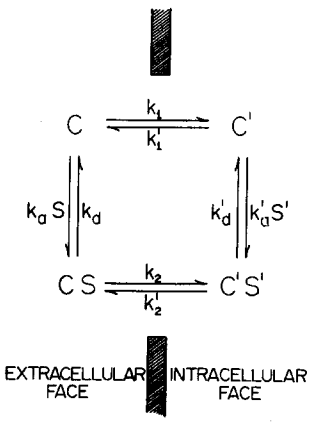

Fig. 10. Monovalent carrier model.

Influence of azide on influx rates

As in the case with cyanide, azide inhibited thiomethylgalactoside influx more severely than nitrophenylgalactoside influx (Fig. 9). However, the latter influx was relatively more affected by low concentrations of azide than by low concentrations of cyanide.

DISCUSSION

Analysis of carrier kinetics

The formulation of transport carrier mechanisms has permitted the application of steady-state kinetic analysis to a wide range of transport problems[16–19], and its basic assumption that the substrate traverses the cell membrane in combination with a specific carrier has been supported by the isolation of a series of protein molecules capable of binding transport substrates[20]. Consequently, although details of the galactoside transport mechanism in *E. coli* cells remain to be established, carrier kinetics would serve at least for an initial description of the system. The reaction mechanism for a monovalent carrier with a single substrate site is shown in Fig. 10. This mechanism together with related modifications have been applied to the galactoside system by a number of workers. KEPES[2] and KOCH[3] had considered the possibility of the carrier being distinct from the y-gene product of the lactose operon, whereas FOX AND KENNEDY[4] had considered the possibility of the two being identical. WINKLER AND

WILSON[7] employed simplified rate equations for the mechanism, and SCHACHTER AND MINDLIN[8] allowed the transport of the carrier–substrate complex but not the free carrier across the cell membrane. In one important aspect, however, the mechanism in Fig. 10 might under-represent the galactoside carrier. The presence of two distinct substrate binding sites on the M-protein of FOX AND KENNEDY[4] is suggestive of a divalent galactoside carrier, and we have shown previously that divalent carriers are far more versatile than monovalent ones in predicting interactions between transport substrates either on the cis- or trans-side of the cell membrane[19].

In the carrier mechanism in Fig. 10, carrier C binds substrate S either on the extracellular (unprimed) or intracellular (primed) face of the membrane, and both C and CS can commute between the two faces. The steady-state initial rate v_0 for the influx of S under the zero-time condition of $S' = 0$ has been given by JACQUEZ[18]:

$$v_0 = \frac{k_a k_2 k'_d k'_1 S}{k_a S(k_2 k'_d + k_2 k'_1 + k'_d k'_1 + k'_2 k'_1) + (k_1 + k'_1)(k_2 k'_d + k_d k'_d + k_d k'_2)} \quad (1)$$

As JACQUEZ[18] has pointed out, all the primed rate constants in the mechanism equal the unprimed counterparts in the absence of energy input, and the carrier catalyses facilitated diffusion but not active transport against a concentration gradient. To achieve active transport, it is necessary through coupling to energy metabolism to increase one or more of the influx constants k_a, k_2, k'_d and k'_1, or to decrease one or more of the efflux constants k'_a, k'_2, k_d, and k_1. Accordingly, the addition of energy poisons is expected to decrease the influx constants or increase the efflux constants. A kinetic approach toward defining the site of energy coupling, therefore, would proceed by identifying the rate constant altered by the presence of energy poisons.

Exclusion of k'_a

Of the eight elementary rate constants in the monovalent carrier mechanism, k'_a is uniquely absent from Eqn. 1. S' being zero in unloaded cells, the intracellular binding step $k'_a S'$ would be negligible regardless of any alteration in k'_a; the initial influx rate v_0 is, therefore, independent of k'_a. Accordingly, if v_0 is completely unaffected by energy poisons, energy coupling through k'_a would be strongly indicated. Conversely, if v_0 is affected by energy piosons, k'_a is strongly excluded as the only site of energy coupling. Early studies on galactoside transport had suggested an independence of v_0 of energy metabolism. KOCH[3] followed nitrophenylgalactoside uptake on the basis of its hydrolysis. WINKLER AND WILSON[7] compared influxes into metabolically active cells and poisoned cells, but the latter were preloaded with nonradioactive galactoside. Both works indicated that the influx parameters were largely unaffected by energy poisons, but the validity of the methodologies has been questioned by SCHACHTER AND MINDLIN[8]. In contrast, a dependence of v_0 on the energy status of the cells has now been demonstrated in three types of cell preparations. SCARBOROUGH *et al.*[9] observed a stimulation of nitrophenylgalactoside uptake by ATP in cells treated with Tris. SCHACHTER and co-workers[8,10] observed an influence on thiomethylgalactoside uptake by energy source and showed an inhibition of v_0 in heavily poisoned cells which catalysed mainly facilitated diffusion. Finally, in the present study a progressive inhibition of v_0 by increasing concentrations of cyanide and azide is seen even in cells still actively accumulating thiomethylgalactoside. In view of these developments,

evidence favours a dependence of v_0 on energy metabolism, and therefore an exclusion of k'_a as the only site of energy coupling.

In this study K12-Leu$^-$ cells were employed. Protein synthesis could be arrested without the addition of an inhibitor such as chloramphenicol, simple by omitting leucine from the suspension medium (the kinetics of thiomethylgalactoside transport were uninfluenced by the presence or absence of leucine). After induction, the cells also were washed with warm medium in order to remove any intracellular inducer which could exchange with the transport substrate. Under such conditions, two lines of evidence support the validity of the initial rates of thiomethylgalactoside influx measured. First, the progress curves exhibited a recognizably linear portion, and an estimate of v_0 can be obtained without serious extrapolations. Secondly, a linear relationship between the measured v_0 and the induced concentration of permease, as indicated by that of β-galactosidase, could be obtained (Fig. 2). Since interpretations of influx kinetics critically rely on the validity of v_0, these results are reassuring concerning the v_0 measurements.

In Fig. 2, v_0 varied with permease concentration, but so did the final level of substrate accumulation. If permease should represent the galactoside carrier, its function in galactoside transport is expected to be a catalytic one, and the number of catalytic units on the cell membrane is not expected to influence the equilibrium level of accumulation. Thus the variation of the final level with permease remains unexplained. One possibility is that the permease does not represent the carrier, but governs some step in the carrier mechanism[2,3]. Another is that an alternate exit mechanism operates in the cell, so the final level of accumulation is only a steady-state level and not an equilibrium level[21]. In any event, because of additional complications due to exit, kinetic interpretations based on levels of accumulation would be even more difficult than those based on v_0.

Exclusion of k_a and k_d

In the inhibition of thiomethylgalactoside influx by energy poisons, k'_a cannot be the target rate constant because of its absence from Eqn. 1. A further definition of the target site is furnished by the interactions between transport substrates in the cyanide system, where 0.2 mM vastly inhibited thiomethylgalactoside but not nitrophenylgalactoside influx. From Fig. 6, thiomethylgalactoside is seen to be a competitive inhibitor of nitrophenylgalactoside transport, and this inhibition was not diminished by cyanide (Fig. 7). Since cyanide affected thiomethylgalactoside accumulation, the inhibition of nitrophenylgalactoside uptake must be exterted by extracellular thiomethylgalactoside in a competition for binding to the carrier. If cyanide specifically inhibited thiomethylgalactoside influx through an increase in k_d or a decrease in k_a, or both, inhibition of nitrophenylgalactoside transport by extracellular thiomethylgalactoside should be reduced. The results of Fig. 7 showing the inhibition to be unaffected even by 1 mM cyanide therefore excludes any alteration of k_a and k_d by cyanide.

Possibility of k'_1 or k'_d as the coupling site

With k'_a, k_a and k_d excluded as being the target sites, a further location of the target site depends on several lines of evidence.

WINKLER AND WILSON[7] reported that energy poisons drastically decreased the

Michaelis constant K'_m for the efflux process. In terms of the monovalent carrier mechanism of Fig. 10, the expression for the efflux K'_m is:

$$K'_m = \frac{(k_1 + k'_1)(k'_2k_d + k_dk'_d + k_2k'_d)}{k'_a(k'_2k_d + k'_2k_1 + k_dk_1 + k_2k_1)} \quad (2)$$

From Eqn. 2 it is clear that a decrease in K'_m can be brought about most simply by a decrease in k'_1 or k'_d, which appear exclusively in the numerator, or an increase in k'_a, which appears exclusively in the denominator. Since the constants k'_2, k_d, k_1 and k_2 enter into both numerator and denominator terms, alterations in these also can bring about a decrease in K'_m. Experimentally, the lowering of K'_m by poisons was not confirmed by KEPES[21].

SCHACHTER AND MINDLIN[8] found that energy poisons altered the temperature profile of galactoside influx and increased the substrate protection of carrier against inactivation by *N*-ethylmaleimide. These results were interpreted as favouring the existence of a dual pathway at some influx step in the mechanism. In terms of the monovalent mechanism of Fig. 10, one of the influx constants k_a, k_2, k'_d and k'_1 would have an energy-dependent component as well as an energy-independent one. The authors pointed out that their experiments did not allow an identification of the influx constant so affected, but arbitrarily chose k'_d to illustrate the point. At present, the interpretation of temperature profiles in terms of elementary steps in enzymic or carrier mechanisms requires extensive kinetic analysis[22], and the observations on galactoside transport can be related to the individual steps of the carrier mechanism only if the rate-limiting steps in effluxes and influxes become known. Substrate protection of transport carrier entails even greater difficulties. For the monovalent carrier mechanism, the following equation describes the ratio between ecomplexed and uncomplexed carriers in the presence of both S and S':

$$\frac{CS + C'S'}{C + C'} = \frac{k_ak'_aSS'(k_2 + k'_2) + k'_1k_aS(k'_2 + k'_d + k_2) + k_1k'_aS'(k_2 + k_d + k'_2)}{k_dk'_d(k_1 + k'_1) + k_dk'_2(k'_aS' + k'_1 + k_1) + k'_dk_2(k_aS + k_1 + k'_1)} \quad (3)$$

An increase in the above ratio corresponds to an increased substrate protection of the carrier. Since all eight rate constants appear in both the numerator and the denominator of the expression, an increased protection brought about by energy poisons is not easily traced to alteration in any particular constant. Any reduction of S' by poisons also influences the ratio.

A lowering of K'_m in poisoned cells favours k'_1, k'_d and k'_a as the coupling site, and the experiments of SCHACHTER AND MINDLIN[8] favour k_a, k_2, k'_d and k'_1, but neither line of evidence rules out the remainder constants. A more decisive argument in this regard is provided by the occurence of counterflow[7,21,23]. As observed by WINKLER AND WILSON[7], preloading of poisoned cells with one galactoside can drive the entry of another galactoside. In terms of the mechanism of Fig. 10, the presence of an intracellular substrate T' can relieve the blocked entry of another substrate S. There are two possible mechanisms for such relief actions[24]. First, if energy poisoning should lower k'_1, the empty carrier C' would not be transferred to the extracellular phase efficiently. By combining with C', T' can facilitate the exit of the carrier in the form

of $C'T'$ and hence prepare for the entrance of S. Secondly, if energy poisons should lower k'_d the dissociation step of $C'S'$, the inward flow of S would be obstructed. By directly displacing S' from the $C'S'$ complex to form $C'T'$, T' can effectively overcome the blockade of S entrance. Since both modes of T' action involves the formation of $C'T'$, these two mechanisms are not readily distinguished on the basis of available data. Other types of counterflow mechanisms also are not totally excluded. If the carrier for galactoside should be dimeric and carry two substrate binding sites, the possible modes of interaction between S and T' will further multiply[19,25]. In any event, the phenomenon of counterflow is compatible with k'_1 or k'_d as the coupling site.

Evidence for k'_d

In the experiments of Figs. 4, 5 and 9, the hydrolysable nitrophenylgalactoside and lactose are seen to be more resistant than the non-hydrolysable thiomethylgalactoside to influx inhibition by energy poisons. It is necessary to distinguish between two types of explanations for such resistance[10]. First, the resistance might reflect the maintenance of influx through counterflow driven by the exit of the products of hydrolysis *i.e.* galactose and nitrophenol in the case of nitrophenylgalactoside. Secondly, the resistance might stem from the act of hydrolysis freeing S' from $C'S'$ and therefore activating the k'_d step; in this regard β-galactosidases are known to attack protein-bound galactoside linkages in the case of glycoproteins[26]. In the experiment of Fig. 9, if counterflow by galactose or nitrophenol is driving the entrance of nitrophenylgalactoside in the presence of cyanide, it should drive likewise the entrance of thiomethylgalactoside. Since there was no increased resistance of thiomethylgalactoside influx to cyanide poisoning in the presence of nitrophenylgalactoside, counterflow does not appear adequate to maintain nitrophenylgalactoside influx. Consequently, the freeing of S' from $C'S'$ by the act of hydrolysis may make an important contribution to the resistance of hydrolysable substrates to influx poisoning.

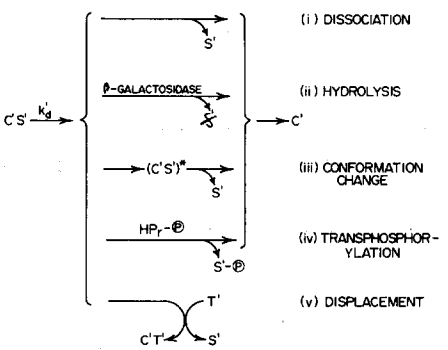

Fig. 11. Possible pathways for the energized extraction of S' from $C'S'$.

To summarize, reaction steps k'_a, k_a and k_d appear highly unlikely to be coupling sites. Of the remainder five steps, the occurence of counterflow suggests the consideration of k'_1 and k'_d, and the experiment of Fig. 9, by questioning the adequacy of counterflow to explain fully nitrophenylgalactoside influx into poisoned cells, points to the involvement of k'_d. However, it should be noted that the process of galactoside exit

appears a complex one[21], and counterflow experiments necessarily involve both entrance and exit processes.

Energized extractions as a coupling mechanism

The suggestion that energy coupling at the k'_d step may be effectively substituted for by the act of hydrolysis focuses attention on the many modes of reaction possibly open to the $C'S'$ complex. As shown in Fig. 11, these possibilities include (i) simple dissocation, (ii) hydrolysis by β-galactosidase, and (iii) an energised conformation change in $C'S'$ promoting the dissociation of S' from the complex[4,8]. From the work of KUNDIG *et al.*[5] and KABACK[27] the phosphoenolpyruvate phosphotransferase enzymic system has been demonstrated to participate in the active transport of various sugars into bacterial cells. Enzyme II of this system is responsible for substrate specificity, and fulfills the carrier function in the transport process. Where this system should participate in sugar uptake, a transphosphorylation between the enzyme II-bound substrate and the phosphorylated heat-stable protein of the system will contribute reaction (iv) for the complex $C'S'$. Finally, in the counterflow exchange between an intracellular substrate T' and an extracellular substrate S, T' may bind to the empty carrier C' and facilitate its exit, or actually react with $C'S'$ to displace S' and furnish reaction (v) for the $C'S'$ complex.

The multiple pathways (ii)–(v) in Fig. 11 can all energize the extraction of S' from $C'S'$, respectively by the free energy of S' hydrolysis, by an energy-linked conformation change, by the transphosphorylation reaction, or by the concentration gradient of T'. The problem of β-galactoside transport into *E. coli* cells therefore requires a continued evaluation of k'_d as coupling site and the likelihood of additional sites, as well as an assessment of the quantitative contributions made to k'_d by each of the multiple pathways such as (ii)–(v). Since both lines of enquiry involve examining carrier rate constants as functions of physiological and experimental variables, kinetic analysis of the system will have to become an increasingly important tool toward understanding the relationship between transport and energetic processes.

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