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Coupling of Energy to Active Transport of Amino Acids in *Escherichia coli*

(mutants/membrane vesicles/Ca,Mg-ATPase/electron transport/D-lactate dehydrogenase)

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ABSTRACT **Active transport of amino acids in isolated membrane vesicles of *E. coli* ML 308-225 is stimulated by oxidation of D-lactate, and this stimulation is dependent on electron transport [Kaback, H. R. & Milner, L. S. (1970) *Proc. Nat. Acad. Sci. USA* 66, 1008]. In attempting to relate these results to amino-acid transport in intact cells, we isolated mutants of *E. coli* ML 308-225 that contain defects in D-lactate dehydrogenase (EC 1.1.2.4) and electron transport. Intact cells of these mutants are normal for transport of proline and alanine. We also isolated mutants defective in Ca,Mg-stimulated ATPase (EC 3.6.1.3), which is responsible for coupling electron transport to the synthesis of ATP. These mutants are defective in their ability to transport proline and alanine, as measured both in cells and isolated membrane vesicles. A possible role for the ATPase in coupling energy to active transport is discussed.**

A great deal of attention has been given to the general problem of energy transductions in biological systems. Mitochondrial synthesis of ATP has received much of this attention. In the past few years, however, attention has turned to the problem of the mechanism of coupling metabolic energy to achieve the movement of solute across biological membranes against a concentration gradient.

In eukaryotic cells, it appears that ATP can serve as the direct source of energy for active transport of alkali metal ions. The work of Hokin and collaborators with sodium transport in erythrocytes has provided evidence suggesting that the mechanism of ATP-mediated active transport involves the direct phosphorylation-dephosphorylation of the carrier molecule (1).

The mechanism of energy coupling to active transport in bacteria is less well understood. The work with isolated membrane vesicles of several bacteria provides evidence that ATP is not required for coupling of energy to the transport of various amino acids and sugars (2–4). It appears from these studies with isolated vesicles that oxidation of various substrates by the electron-transport chain can provide energy for active transport without concomitant oxidative phosphorylation (3, 4). Although attempts to test whether exogenous ATP can substitute for electron transport in vesicles have been negative (5), one must be cautious in interpreting these negative experiments in the light of permeability problems with ATP.

A far more basic problem becomes apparent, however, when attempts are made to relate the work with vesicles to the behavior of intact cells. There are some cases where electron transport is clearly not required for active transport in intact cells. The active transport of lactose analogues in intact cells of *Escherichia coli* is not affected by anaerobiosis (6), even though uptake by the same system in vesicles is very sensitive to the lack of oxygen (3). *Streptococcus faecalis*, an anaerobe that contains no electron-transport system, can carry out active membrane transport, presumably using the energy-yielding reactions of glycolysis (7). It has become essential to relate the observations obtained with isolated vesicles to physiological realities of intact cells. We have isolated cells that contain mutations in various components involved in aerobic metabolism and have tested the effects of these mutations on the ability to perform active transport of amino acids.

Abbreviation: DCI, dichlorophenolindophenol.

MATERIALS

[*U*-^{14}C]Proline (233 Ci/mol) and [*U*-^{14}C]alanine (156 Ci/mol) were obtained from New England Nuclear Corp. All other chemicals were of the highest purity available from commercial sources.

METHODS

Growth of cells

All strains of *E. coli* used in these experiments were grown on minimal salts medium [50 mM KPO_4 buffer (pH 7.5)–0.2% $(NH_4)_2SO_4$–0.01% $MgSO_4$] containing glucose as carbon source at 37° on a gyrotory shaker at 150 rpm. Inocula were grown overnight in 2-liter flasks containing 600 ml of salts solution with limiting amounts of glucose so that growth stopped at a Klett reading (540 filter) of about 100. The amount of glucose added varied with the growth yield for each strain (see Fig. 1). In the morning, additional glucose was added to a final concentration of 0.2% and growth resumed immediately. The cultures were allowed to grow exponentially for about one generation. Cells were chilled in ice, harvested, washed once, and suspended with the above salts solution. At this stage aliquots were removed for transport studies with intact cells, and the remainder was processed for preparation of vesicles.

Isolation of mutants

An overnight culture of *E. coli* ML 308–225, grown on salts solution plus 0.2% D-lactate, was incubated with one drop of ethyl methanesulfonate for 2 hr at 37°. Aliquots of the mutagenized culture (0.2 ml) were then transferred to each of 20 tubes containing 5 ml of salts plus 0.5% glucose and allowed to grow overnight. After washing twice with salts solution, each of the 20 cultures was suspended to 5 ml and incubated for 1 hr at 37°. 0.2 ml was then transferred to tubes containing 200 units/ml of penicillin G and 0.2% D-lactate. After incubation for 12 hr at 37°, appropriate dilutions of the penicillin-treated cultures were spread onto agar plates containing the minimal salts solution and 0.5% glucose. The resultant colonies were allowed to grow for 48 hr, after which they were replica-plated to plates containing 0.2% D-lactate and incu-

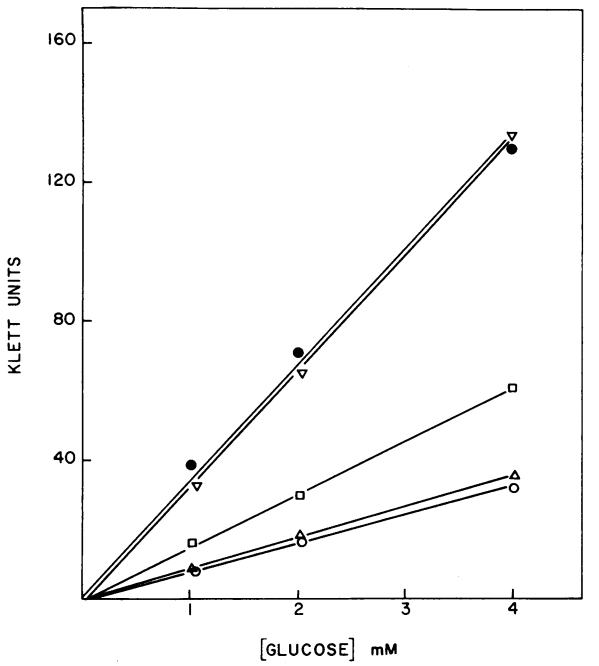

FIG. 1. Growth yields of various strains of *E. coli* on limiting concentrations of glucose. The experiment was conducted as described in *Methods* for the parental strain ML 308-225 (●——●); DL-13, D-lactate dehydrogenase mutant (▽——▽); DL-54, Ca,Mg-ATPase mutant (□——□); S2-21, electron-transport mutant (△——△); and the parental strain ML 308-225 (anaerobic) (○——○).

bated for 12 hr. Those colonies that failed to grow on the D-lactate plates were purified by two single-colony isolations. All strains were stored on nutrient agar slants containing 0.5% glucose. Permanent cultures were kept as stabs in small vials of the same medium that were sealed with Parowax (product of American Oil Co.). The electron-transport-negative strains and, to a lesser degree, the Ca,Mg-ATPase-negative strains showed a relatively high rate of reversion, so all cultures were tested for revertants by plating them on a carbon source on which they would not grow. After the isolation of each strain, it was characterized according to which carbon sources could support growth. This was done by making a master plate of the strains to be tested and replica plating to minimal plates containing the following carbon sources: 0.5% galactose, 0.5% glycerol, 0.2% pyruvate, 0.2% D-lactate, 0.5% succinate, and 0.5% malate. In addition, each strain was tested for sensitivity to chlorate (in the presence of air) on 1% nutrient agar plates containing 0.2% $KClO_3$ and 0.2% glucose (9). Plates were scored after 24 hr incubation at 37°.

Growth yields

We determined growth yields for each strain on glucose by growing the cells overnight in 10-ml salts solution plus 1, 2, or 4 mM glucose in 125-ml sidearm flasks at 37° with shaking at 150 rpm. The optical density was read in a Klett colorimeter until three successive hourly readings showed no further change. Anaerobic growth yields were determined in a similar way, except that the flasks were filled with media, deaerated by boiling, and tightly stoppered during sterilization and growth.

Transport experiments with intact cells

Cells were suspended in the minimal salts solution to a density equivalent to a Klett reading (540 filter) of 800. Each transport incubation contained 0.5-ml cells, 5-ml salts solution, and glucose to a final concentration of 0.1%. The addition of chloramphenicol at 50 μg/ml had essentially no effect on the transport measurements and was not routinely used. After a 15-min incubation at 25°, [*U*-^{14}C]proline or [*U*-^{14}C]alanine was added to a final concentration of 50 μM, and 1-ml aliquots (about 0.2 mg dry weight) were removed at each time point, filtered on Millipore filters (0.45 μm), and washed three times with 1 ml of salts solution, all at 25°. The filters were dried and counted in toluene liquid-scintillation fluid.

Membrane isolation for transport and enzyme assays

After an aliquot of cells was removed for transport measurement, the remainder was used for isolation of membrane vesicles by the procedure of Kaback (8). One culture flask yielded sufficient membranes for several transport experiments and enzyme assays. Transport experiments were conducted as described by Kaback (8). D-Lactate was added to a final concentration of 20 mM.

Oxygen consumption experiments

The rate of oxygen consumption in intact cells was determined with 1 ml of cell culture at a Klett reading (540 filter) of 100–200, 4-ml salts solution, and glucose to a final concentration of 0.2%. The incubation was allowed to equilibrate at 25° for 3–5 min, after which the rate of oxygen consumption was followed, with a YSI oxygen monitor, until a constant rate was reached.

Oxygen consumption by isolated membranes was determined in essentially the same way except that 50 mM potassium phosphate buffer (pH 7.3) with 10 mM $MgSO_4$ was used as a diluent. The amount of membrane protein used varied between 0.25 and 1.5 mg, and electron donors were used at a concentration of 20 mM (D-lactate, succinate) or

TABLE 1. *Use of carbon sources for growth by various strains of E. coli*

Strain	Glucose	Galactose	Maltose	Glycerol	Pyruvate	Succinate	D-Lactate	Malate	Chlorate
ML 308-225 (parent)	+	+	+	+	+	+	+	+	*R*
DL-54 (Ca,Mg-ATPase-negative)	+	+	+	+	+	−	−	−	*R*
S2-21 (electron-transport deficient)	+	+	+	−	−	−	−	−	*S*
DL-13 (D-lactate dehydrogenase-negative)	+	+	+	+	+	+	−	+	*R*

The experiment was conducted as described in *Methods*. Carbon sources were scored + for growth, or − for no growth. The sensitivity of each strain to chlorate was scored as resistant (*R*) or sensitive (*S*).

5 mM (NADH). The oxygen tension used for calculations was 0.47 μg-atom/ml of reaction mixture (13).

Enzyme assays

D-Lactate Dehydrogenase (EC 1.1.2.4). Isolated membranes were assayed for the presence of this enzyme with dichlorophenolindophenol (DCI) as electron acceptor. Assays contained membranes (50–100 μg of protein), 50 mM KPO_4 buffer with 10 mM $MgSO_4$, and 0.002% DCI in a final volume of 1 ml. The decrease in optical density was read at 620 nm in a Gilford spectrophotometer. Assays were conducted at 25°, and the rates of reaction were constant with time of incubation and proportional to protein concentrations. A mM extinction coefficient of 16.1 for DCI was used.

Ca,Mg-ATPase (EC 3.6.1.3). Membranes were washed free of phosphate buffer and suspended in 10 mM Tris·HCl (pH 8.0). Ca,Mg-ATPase was assayed by the procedure of Butlin *et al.* (10) with about 0.5 mg of membrane protein.

RESULTS

The mutant-selection procedure described is nonspecific and yields various mutations in aerobic metabolism. We have isolated and partially characterized three classes of mutants and studied the effects of these mutations on the ability of the cells and isolated membrane vesicles to perform active transport. The phenotypes of the three classes of mutants have been examined and are described in Table 1. A mutant missing the NAD-independent D-lactate dehydrogenase cannot use D-lactate as a carbon source. It can, however, grow normally on all other carbon sources tested. The mutants deficient in either Ca,Mg-ATPase or electron transport are unable to grow on those carbon sources that require electron transport and/or oxidative phosphorylation in order to obtain energy. The response to chlorate is interesting and is discussed later.

The biochemical defect of each class of mutants is given in Table 2. It can be seen that DL-13 is missing >90% of D-lactate dehydrogenase activity, measured by the reduction of DCI. In addition, membranes from these strains do not exhibit D-lactate-dependent oxygen uptake. The remaining strains have normal D-lactate dehydrogenase activity as measured by both assays.

Mutant strain DL-54 is missing the Ca,Mg-stimulated ATPase originally described by Evans (10). A similar mutant has been described by Butlin *et al.* (11). These workers have demonstrated that mutants missing this activity cannot carry out oxidative phosphorylation and are thus uncoupled.

Strain S2-21 shows a general defect in electron transport that is not yet biochemically defined. Intact cells of this mutant show a low rate of oxygen consumption. In addition, isolated membranes are deficient in their ability to oxidize NADH, D-lactate, and succinate. This result suggests that the mutation in this strain has affected a component in the common part of the electron-transport chain, between cytochrome *b* and O_2. It may be similar to the ubiquinone mutants described by Cox *et al.* (12). An interesting property of these electron-transport mutants is that they appear to synthesize the nitrate reductase complex in the presence of oxygen. Wild-type cells of *E. coli* are not sensitive to chlorate in the presence of O_2 (see Table 1). The reason for this insensitivity appears to be that chlorate must be converted to chlorite (or some subsequent product) by the nitrate reductase system, the synthesis of which is repressed in the presence of oxygen. The fact that the electron-transport mutants are sensitive to chlorate in the presence of oxygen suggests that the repression of nitrate reductase synthesis is not due to oxygen *per se*, but rather to its role as terminal electron acceptor in an intact electron-transport chain. In addition, the chlorate sensitivity of these strains provides evidence that these cells are indeed "anaerobic." The additional feature of chlorate sensitivity is that it provides a convenient means of

TABLE 2. *Biochemical characterization of various strains of E. coli*

Strain	D-Lactate dehydrogenase (μmol DCI reduced/mg of protein per 15 min)	Ca,Mg-ATPase		O_2 consumption by isolated membrane		O_2 consumption by intact cells (ng-atom/mg of dry weight per min)
		Additions	μmol Pi released/mg of protein per 30 min	Substrate	ng-atoms/mg weight per min	
ML 308-225 (parent)	0.125	—	0.24	D-Lactate	35	410
		Mg	10.1	NADH	160	
		Ca	8.5	Succinate	50	
DL-54 (Ca,Mg-ATPase-negative)	0.10	—	<0.1	D-Lactate	38	600
		Mg	0.48	NADH	115	
		Ca	0.36	Succinate	76	
S2-21 (Electron-transport-negative)	0.145	—	0.21	D-Lactate	7	100
		Mg	9.4	NADH	8	
		Ca	8.9	Succinate	9	
DL-13 (D-Lactate dehydrogenase-negative)	<0.01	—	0.18	D-Lactate	<5	420
		Mg	8.6	NADH	170	
		Ca	7.8	Succinate	70	

All assays were conducted as described in *Methods*.

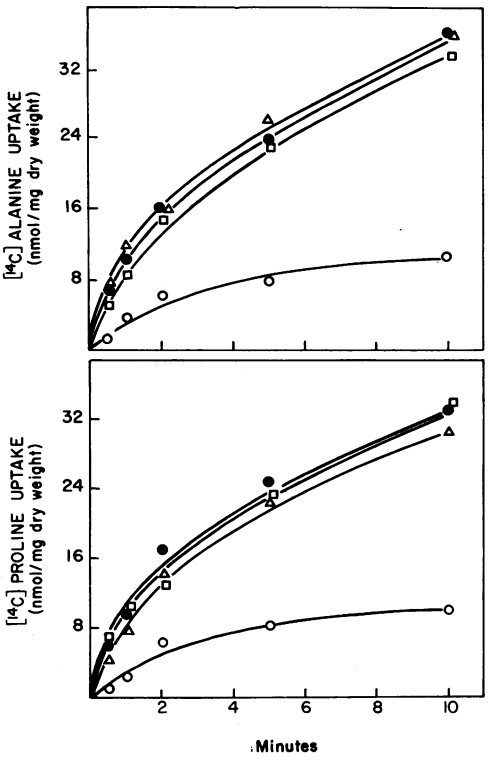

FIG. 2. Uptake of [^{14}C]proline and [^{14}C]alanine by intact cells of various strains of *E. coli*. The experiment was conducted as described in *Methods* for the parental strain, ML 308-225 (●——●); the D-lactate dehydrogenase mutant, DL-13 (△——△); the electron-transport mutant S2-21 (□——□); and the Ca,Mg-ATPase mutant, DL-54 (○——○). Amino acids were tested at 50 μM.

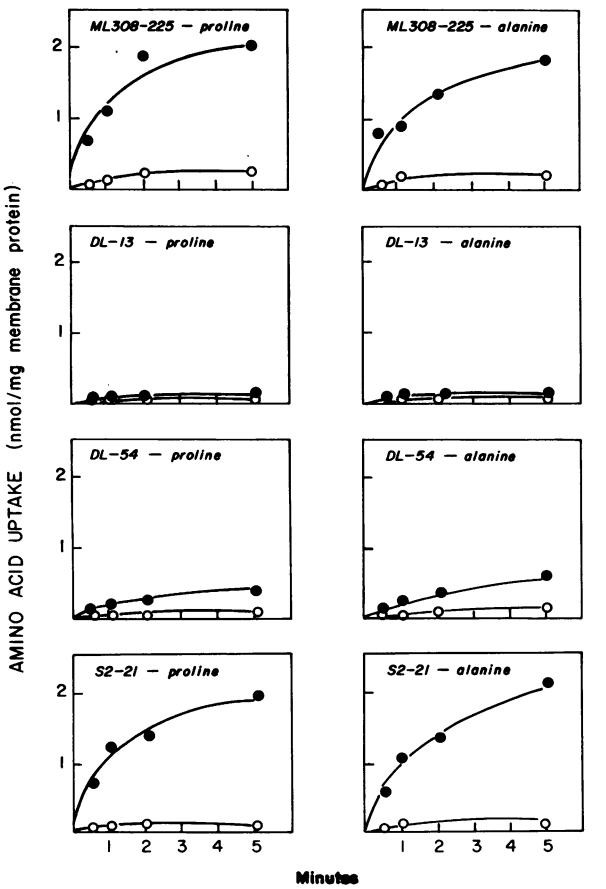

FIG. 3. Uptake of [^{14}C]alanine and [^{14}C]proline by preparations of membrane vesicles of various strains of *E. coli*. The experiment was conducted as described in *Methods* both in the presence (●——●) or absence (○——○) of D-lactate. The final concentrations of amino acids were 5 μM [^{14}C]proline and 10 μM [^{14}C]alanine.

distinguishing electron-transport mutants from those missing Ca,Mg-ATPase.

The effects of these various mutations on the overall energetics of the cell were estimated by determination of the growth yield of each strain on limiting concentrations of glucose (Fig. 1). The D-lactate dehydrogenase mutants have normal growth yield. The growth yields in the Ca,Mg-ATPase mutants are about half the parental values, reflecting the contribution of oxidative phosphorylation to the efficiency of glucose use. Electron-transport mutants are more seriously affected and show poorer growth yields than ATPase mutants. It appears that, in addition to oxidative phosphorylation, electron transport may provide energy to the cell in other ways. Butlin *et al.* have suggested that reactions such as the NAD(P) transhydrogenase may function to provide energy to the cell (11). Fig. 1 shows that the growth yield of the electron-transport mutant is essentially the same as that for an anaerobic culture of the parent, providing more evidence for the deficiency of oxidative energy in this strain.

It is important to establish that the phenotype of each mutant described is the result of a single mutation. Although data are only presented for one mutant of each class, we have isolated at least three independent strains of each type, all of which exhibit the phenotypes as described. In addition, we have analyzed spontaneous revertants for each class and obtained full recovery of the parental phenotype. Each strain had reversion frequencies between 1 in 10^6 to 1 in 10^8. We have not yet established that these mutations affect structural genes.

The isolation of these mutants provides a convenient way to evaluate the source of energy for active transport in intact cells without resorting to the use of metabolic poisons. We have used two amino acids, alanine and proline, as substrates for these experiments. In addition, several other amino acids were tested in some of the experiments and all results are consistent with those reported. We have conducted transport experiments in both cells and isolated membrane vesicles in order to relate the vesicle work to cellular physiology.

Kaback and his collaborators have interpreted their results, with isolated membrane vesicles from *E. coli* ML 308-225, to indicate that the oxidation of D-lactate by the electron-transport chain is directly coupled to the transport process (5). These workers have further suggested that the transport carrier itself is a component of the electron-transport chain, and functions between D-lactate dehydrogenase and cytochrome *b*. We have repeated their experiments, using vesicle preparations from the same strain, and obtained essentially the same results. The transport of alanine and proline in vesicles of *E. coli* ML 308-225 is markedly stimulated by the addition of D-lactate (Fig. 3). As expected, vesicles isolated from the D-lactate dehydrogenase mutant do not exhibit the D-lactate stimulation of transport (Fig. 3). However, when

one examines intact cells of this D-lactate dehydrogenase mutant, no transport defect can be detected (Fig. 2). It thus appears that the *physiological* significance of the D-lactate effect observed in membrane vesicles is uncertain.

It was then of interest to examine the mutant with impaired electron transport, S2-21. As can be seen in Fig. 2, intact cells of this mutant also do not exhibit a transport defect. It appears that in intact cells active transport is not obligatorily coupled to electron transport. Caution must be exercised in this interpretation since the mutants so far isolated are not completely deficient in electron transport. Physiologically, however, the mutation is equivalent to anaerobiosis (see growth yields). One might predict from the results obtained with the D-lactate dehydrogenase mutant that, although no effect on active transport could be seen with intact cells of the electron-transport mutant, there should be an effect when isolated membrane vesicles are tested. This was not observed, however, and vesicle preparations of S2-21 show normal transport (Fig. 3). Although the explanation for this observation is not clear, one important factor must be considered. The approximate rates of transport in membrane vesicles, although comparable to those reported (5), are less than 2% of that observed with intact cells when calculated on the basis of membrane protein (compare Figs. 2 and 3). It is possible that the small amount of remaining electron-transport activity in these membranes may be sufficient to support this low rate of transport.

An interesting effect is observed with the mutants that are uncoupled for oxidative phosphorylation due to a defect in Ca,Mg-ATPase. Intact cells show a substantial decrease in their ability to perform active transport (Fig. 2). It is unlikely that the transport deficiency is the result of a loss of oxidative ATP, since the electron-transport mutant (S2-21) had normal transport activity. When membrane vesicles were examined, the effect was even more striking. The level of transport in the absence of D-lactate was consistently well below that for the parent strain. The addition of D-lactate showed some stimulation of uptake, but only to about one-fourth the level of the parent.

In order to determine that the transport defects observed in these strains were not the result of some nonspecific effect, we have tested all strains, both cells and isolated vesicles, for the ability to transport methyl α-D-glucopyranoside. This substrate serves as a glucose analogue for the PEP-glucose phosphotransferase system, which is responsible for the transport of many carbohydrates by a group-translocation mechanism and, therefore, should be unaffected by a loss of the energy coupling reactions of active transport. These strains were normal for this transport process.

DISCUSSION

The data presented in this paper permit the following conclusions.

(*i*) D-Lactate oxidation is not required for active transport of several amino acids in intact cells of *E. coli* ML 308-225.

(*ii*) A mutation resulting in defective electron transport, while rendering the cells energetically equivalent to cells under anaerobiosis, has no effect on active transport in intact cells. It may thus be suggested that electron transport *per se* is not required for active transport in many bacteria, including *E. coli*—a conclusion reached by others (6, 7, 14).

(*iii*) Mutations affecting Ca,Mg-ATPase, an enzyme that is the coupling factor for oxidative phosphorylation, markedly alters active transport in both intact cells and isolated vesicles. It appears that, in isolated membrane vesicles where the only source of energy is from electron transport, this coupling factor can couple electron-transport energy directly to active transport without going through ATP. Whether the role of the Ca,Mg-ATPase in cells is the same as in vesicles is not clear.

These results appear to differ from those reported by Hirata *et al.* for the uptake of proline by isolated membrane vesicles of *Mycobacterium phlei* (4). These workers have been able to dissociate the coupling factor from the membrane vesicles without obtaining a decrease in active-transport ability. The resolution of this difference must await a more detailed analysis of the transport role of the ATPase in *E. coli*.

Since mutations affecting the electron-transport system do not affect active transport while the ATPase mutation does, it is conceivable that the ATPase is capable of coupling energy from both electron transport and glycolysis (?ATP) to the transport system. The conclusion that a high energy state required for active transport can be generated by either electron flow or glycolysis was reached by Kashket and Wilson (14) as the result of their studies with *Streptococcus lactis*.

Our results also indicate that intact cells of *E. coli* may obtain energy for active transport from both sources. In addition, it appears that Ca,Mg-ATPase may be capable of coupling the energy of electron flow to both ATP synthesis and active transport.

While these studies, as yet, shed little light on the mechanism of the energy transduction process, whether it be an electrochemical gradient or high energy intermediate, they promise to yield a clearer understanding of the components involved.

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