Energization of Active Transport by Escherichia coli*

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SUMMARY

An activated membrane state necessary for the active transport of certain amino acids, carbohydrates, and cations by Escherichia coli can be generated by either oxidative energy or phosphate bond energy. Membrane fragment vesicles from E. coli couple oxidative but not phosphate bond energy to the transport of proline. The vesicles show an O₂-dependent transport yet lack measurable capacity for oxidative phosphorylation. Such transport is arsenate-resistant, consistent with the transport being independent of oxidative phosphorylation. Although vesicles possess ATPase activity, added ATP does not stimulate proline transport.

With intact E. coli cells, incubation with high arsenate and low phosphate drastically lowers intracellular ATP and P-enolpyruvate levels in the presence or the absence of O₂. Aerobic proline transport is uninhibited, but in contrast, anaerobic transport is sharply reduced. These results show that intact cells can use energy either from oxidations or from phosphorylations to drive active proline transport. Similarly, active accumulation of leucine, β-methylthiogalactoside, Rb⁺ (as a K⁺ analogue), and α-methylglucoside occurs aerobically in the presence of high arsenate. Uncouplers of oxidative phosphorylation block the use of energy for transport both from oxidations and from ATP, suggesting that a similar or identical energy-conserving membrane state is formed from either energy source.

Transport of α-methyl glucoside by intact E. coli is only partially inhibited by the high arsenate-low phosphate incubation, and further, part of the transported sugar still appears intracellularly in the phosphorylated form. The glucose-6-P level remains high in presence of arsenate and this or other intracellular phosphate compounds may be involved in phosphorylation of the transported sugar by an unknown mechanism. Low concentrations of iodoacetate nearly completely block the α-methylglucoside transport with but little effect on the O₂-driven proline transport.

Until recently, various considerations made the obligatory coupling of active transport by bacteria to phosphate bond energy an attractive hypothesis. Metabolic precedents estabished since Lipmann elaborated the significance of ATP made it likely that ATP could serve as an energy donor for transport. Factors known to affect transport seemed consistent with this view, including the well recognized sensitivity of transport to inhibitors of oxidative phosphorylation. Furthermore, in accord with such possibilities, oxygen uptake required for the transport of a galactoside molecule into E. coli was estimated as equivalent to that required for synthesis of one ATP molecule (1).

Certain observations, however, suggested the possibility that active transport by microorganisms might be driven oxidatively without intervening phosphorylations. Several years ago, Halvorson and Cowie showed that arsenate would inhibit protein synthesis by yeast, but not inhibit the uptake of phenylalanine (2). An arsenate block of ATP production, but not of membrane energization by oxygen uptake, would explain their data. Also, following earlier demonstrations (3, 4), considerable evidence has accumulated showing that O₂ uptake by mitochondria or light absorption by chloroplasts can drive the active transport of ions without intermediary formation of ATP. Mechanistic similarities of the energy-transducing processes in mitochondrial and chloroplast membranes with those of bacterial membranes appear plausible.

In other related studies, Harold et al. more recently demonstrated that uncouplers of oxidative phosphorylation inhibit anaerobically driven transport by Streptococcus faecalis and Escherichia coli (3, 6). These observations point to the formation from ATP of an energized membrane state coupled to transport processes. A unifying hypothesis that emerges is that, in general, either oxidation-reduction reactions or ATP cleavage can serve to energize the membrane, and this high energy membrane state can be coupled to an active transport system (7, 8).

The objective of the experiments reported in this paper was to assess in various ways the possibility that accumulation of amino acids, sugars, and ions by E. coli might be directly coupled either to oxygen uptake or to substrate level phosphorylations. A preliminary report of our results indicating that this is indeed the case has appeared (9). Subsequent reports from our laboratory (10), by Kaback et al. (11, 12), by Hirata et al. (13), and by Konings and Freese (14) have presented additional evidence favoring the concept of direct oxidative energization of active transport.

EXPERIMENTAL PROCEDURE

Reagents—All amino acids used were of L configuration. [¹⁴C]Leucine, α-methyl[¹⁴C]glucoside, and [H]proline were ob-
tained from Amersham-Searle. β-Methyl[14C]thiogalactoside was purchased from New England Nuclear Corp. 86Rb was obtained from Oak Ridge National Laboratory. Luciferin and luciferase were obtained from DuPont, lysozyme from Worthington, and all other enzymes from Sigma. ATP and ADP were purchased from Boehringer and P-enolpyruvate was obtained from Calbiochem.

Preparation of Cells—E. coli ML 308-225 (lac i-α-y+a+) was grown at 30° either in 65 mm potassium phosphate, pH 7, with 7 mm ammonium sulfate and 0.4 mm MgSO4 (Medium A) or in 50 mm Tris-Cl, pH 7.6, supplemented with 0.5 mm potassium phosphate, 7 mm ammonium sulfate, and 0.01 volume of a stock solution containing per liter the following salts: 50 g of potassium chloride, 42.8 g of magnesium chloride, 5 g of sodium citrate, 1 g of ferrous chloride, 1 g of calcium chloride, 1 g of ammonium molybdate, and 0.2 g of cobalt chloride. The carbon source utilized was glucose at 20 mm, unless otherwise specified. Logarithmically growing cells obtained from 10-fold dilutions of overnight cultures were quickly chilled and pelleted. The pellet was washed three times in ice-cold 25 mm Tris-Cl, pH 7.6, and resuspended in the same medium to an A460 of 1.0 (0.23 mg of bacterial protein per ml). Cells were used for the reported experiments immediately after resuspension, although only slight loss in transport capacity was observed during storage for up to 6 hours at 0°.

Transport Assay with Intact Cells—Cells resuspended as described were incubated at 30° for 10 min with 80 µg of chloramphenicol per ml and the desired supplements before adding transport solute. For kinetic determinations of solute uptake, the radioactive solute was added to a small volume of cells, permitting adequate oxygen diffusion, yet of sufficient size for convenient removal of all aliquots from the same suspension. Five hundred-microliter total volumes of cells were used, and uptake of solute was followed by diluting 50-μl aliquots into a-ml volumes of temperature-equilibrated, transport solute-free incubation solution followed by rapid filtration on 0.45-μ Millipore filters. Dilution and filtration took less than 6 s for completion. Radioactivity retained by Millipore filters was counted with a Nuclear Chicago liquid scintillation detector after dissolving the filter in Bray's solution, and the specific uptake was calculated after subtracting background counts retained by filters when sampling was performed in the absence of bacteria. To measure the apparent steady state level of solute accumulation, a single aliquot was assayed as above, 10 min after the addition of radioactive material.

Various points give assurance that the amino acids transported were accumulated in unchanged form. Paper chromatography of cell extracts essentially as described by Piperno and Oxender (15) showed that intracellular proline and leucine accumulated during the transport assays are not chemically modified or incorporated into protein. In studies of carbohydrate transport, the nonmetabolizable glucose and lactose analogues, α-methylglucoside and β-thiogalactoside, respectively, were used routinely. Furthermore, lactose is not metabolized by E. coli MC308225, a strain which lacks β-galactosidase. Hence, lactose rather than the analogue was used routinely to assay transport. In addition, we noted that transport was stimulated markedly by added d-lactate, in harmony with results by Kaback and Milner (11) and was prevented by anaerobic conditions. When glucose was substituted for d-lactate, no stimulation of transport or of O2 uptake.

RESULTS

Experiments with Membrane Vesicles

O2-linked Transport—For meaningful correlations with vesicle phosphorylative activity, the transport capacity of the vesicles needed to be established. The results given in Fig. 1 show the maximum capacity for active transport of proline by the vesicles used. Transport was stimulated markedly by added d-lactate, in harmony with results by Kaback and Milner (11) and was prevented by anaerobic conditions. When glucose was substituted for d-lactate, no stimulation of transport or of O2 uptake...
was observed, indicating the lack of whole cells in the vesicle preparation.

Transport of leucine, glutamate, and alanine by vesicles also was tested. Of these, only transport of alanine was stimulated by \textit{d}-lactate, although glutamate was actively accumulated. Leucine transport was very low and not stimulated by \textit{d}-lactate. Stimulation of all these transport systems by \textit{d}-lactate has been reported \cite{11} in contrast to the present findings.

Maximum capacity for proline accumulation varied with the buffer used. As shown in Fig. 1, vesicles in 50 mM potassium phosphate supplemented with 10 mM MgCl2 and 10 mM \textit{d}-lactate, accumulated a maximum of 2 nmoles of proline per mg of vesicle protein. In other similar experiments, Tris-HCl buffered vesicles showed less than one-tenth, and imidazole-buffered vesicles showed only about one-fourth of the maximum capacity. Vesicles in 50 mM glycylglycine or in 10 mM P1, however, transported at nearly the same maximum capacity. The addition of varying amounts of P1 did not stimulate uptake in any of the nonphosphate buffer systems. Transport capacity decreased when P1 concentration increased to greater than 50 mM.

\textbf{Absence of Oxidative Phosphorylation.—}To detect possible oxidative phosphorylation capacity in vesicles used for transport studies, a conventional hexokinase trap as employed in mitochondrial and bacterial particle systems was used. The disappearance of perchloric acid-soluble P2 in the presence of glucose, hexokinase, and membrane vesicles \cite{Procedure} was followed under various conditions using 50 mM glycylglycine or 10 mM potassium phosphate buffers. Table I summarizes the results of these experiments.

The most important facet shown by the data of Table I is that under conditions permitting active proline transport, but where the P1 present in the medium is very low, no detectable phosphorylation by inorganic phosphate occurred in a sensitive assay. The vesicles did, however, show a weak capacity for phosphorylation by inorganic phosphate in the presence of high P1 levels in the medium that appeared sensitive to 1 mM 2,4-dinitrophenol. This phosphorylation was difficult to measure accurately because it represented only about 0.15\% of the phosphate of the media. Thus, little or no 2,4-dinitrophenol-sensitive uptake was observed.

The nature of the phosphorylated product was not determined. As noted in the table, even if all the observed uptake in the presence of high phosphate were by oxidative phosphorylation, P:O ratio would be low. In addition, with only 1 \textmu M added P1, under conditions similar to those of the transport assay, phosphorylation was only 1\% or less of the capacity for proline transport.

\textbf{ATP and P-Enolpyruvate Levels—}Although \textit{d}-lactate can stimulate proline transport markedly, it has practically no effect on the small amount of ATP and P-enolpyruvate detected in vesicles by the sensitive fluorometric assay. Table II shows that while proline accumulation was increased by 1.9 nmoles...
Arsenate Block of Phosphate Activation—The results of Fig. 3 confirm that arsenate is an effective metabolic inhibitor for an ATP-driven process in intact E. coli cells under conditions of a normal ATP supply, was strikingly sensitive to the addition of exogenous arsenate, even at concentrations as low as 10^-4 M. Arsenate can thus enter the cells used readily, compete with the residual intracellular phosphate, and sufficiently perturb energy metabolism to eliminate protein synthesis.

Since many anabolic and catabolic processes are known to be sensitive to modest changes in energy charge (18), assessing the effectiveness of arsenate as an inhibitor of intracellular P_i uptake required direct measurement of ATP and P-enolpyruvate levels after arsenate addition. Fig. 4 suggests that arsenate quickly and drastically reduced both intracellular ATP and P-enolpyruvate. Repeated measurements with perchloric acid extracts established routine ATP and P-enolpyruvate levels in arsenate-treated cells at 90 to 95% less than the levels found when P_i replaced arsenate.

At a symposium, we reported that incubation with high arsenate and low phosphate apparently did not reduce intracellular P-enolpyruvate levels (10). This report is now known to be in error. In part, assay difficulties arose because extraction of cells with hot aqueous ethanol, 50% (v/v), did not inactivate adenylate kinase.

Arsenate-insensitive Active Transport—Contrary to its inhibitory effect on protein biosynthesis and phosphorylation, arsenate did not interfere with cellular proline transport. As shown in Fig. 5, addition of arsenate did not reduce and, indeed, somewhat increased the capacity of cells to accumulate proline. Although the intracellular ATP decreased 98% to a level of 0.15 n mole per mg of bacterial protein, the maximum steady state accumulation of proline reached a level of 13.8 n moles per mg of bacterial protein. The rate of proline uptake was approximately the same in the presence and absence of arsenate. An additional important point showed by this experiment is that a relatively long exposure to arsenate prior to testing transport did not lower the total accumulation.

Arsenate-insensitive transport is not restricted to proline or other amino acids. Table III shows that various classes of transport ligands are accumulated in the presence of high arsenate. The lactose analogue, β-methylthiogalactoside, and the glucose analogue, α-methylglucoside, as well as proline and leucine were accumulated against a gradient in the relative absence of intracellular phosphate bond energy. Variable sensitivity among the transport system exists, although, with the maximal inhibition of leucine and α-methylglucoside transport frequently exceeding 50%. The continued transport of various solutes did not result because of insufficient concentrations of arsenate; data for α-methylglucoside are shown in Fig. 6.

Effects of Arsenate, NaF, and Iodoacetate on α-Methylglucoside Transport—The observation that α-methylglucoside could be accumulated by cells having almost no P-enolpyruvate is somewhat surprising, as the transport of hexoses in E. coli has been documented extensively as proceeding via a P-enolpyruvate phosphotransferase complex (19). Furthermore, as seen in Fig. 7, cells treated with arsenate appear to establish an intracellular pool of α-methylglucoside phosphate although very little P-enolpyruvate is present. In addition, cells maintained in arsenate medium for extended periods of time contain a near normal level of glucose-6-P, as determined by assay with TPN+ and glucose-6-P dehydrogenase.

Further attempts to inhibit α-methylglucoside transport by reducing intracellular P-enolpyruvate lower than the level obtained by adding arsenate have been unsuccessful. Sodium fluoride, an inhibitor of the enolase reaction, had no effect on α-methylglucoside transport when added at concentrations as high as 100 mM. Differential inhibition of α-methylglucoside and proline transport was obtained with iodoacetate. Low concentrations of iodoacetate are known to inhibit glyceraldehyde 3-phosphate dehydrogenase and thus glucose breakdown. The inhibition of α-methylglucoside but not of proline or lactose transport is shown in Fig. 8. It is of interest that the intracellular P-enolpyruvate level was lowered about 95% by iodoacetate, similar to the reduction obtained when arsenate is added. The differential inactivation effected by iodoacetate could reflect the carboxymethylation of an essential sulfhydryl component of the glucose transport system.
Fig. 4 (left). Reduction of cellular ATP and P-enolpyruvate (PEP) by arsenate. Exponentially growing cells in Tris-Cl medium were harvested and resuspended as described under “Experimental Procedure,” with the addition of 10 mM potassium arsenate to the wash and resuspension buffers. Cells were incubated at 30° for the times indicated. ATP and P-enolpyruvate also were assayed as described under “Experimental Procedure.” Maximum levels represent an ATP concentration of 5.4 nmoles per mg of protein and a P-enolpyruvate concentration of 1.4 nmoles per mg of protein, determined for unwashed exponentially growing cells.

Fig. 5 (center). Retention of proline transport in ATP-depleted cells. Cells resuspended in arsenate as described in the legend of Fig. 4 were incubated at 30°. At the indicated times, transport with arsenate block of phosphorylation

Exponential cells grown at 37° in Medium A were washed twice in cold 50 mM Tris-Cl, pH 7.5, and resuspended in 50 mM Tris-Cl, pH 7.5, with 0.1 mM MgSO4, 7 mM NH4SO4, and either 10 mM potassium arsenate or phosphate. Resuspended cells were incubated at 37° for 5 min except for those used for the β-methylthiogalactoside study which were incubated at 25°. Labeled amino acids or sugars were added (16 μM leucine, 57 μM β-methylthiogalactoside, and 85 μM α-methylglucoside) and steady state uptake capacity was determined for each.

Table III
Transport with arsenate block of phosphorylation

| Conditions of assay | Lecine | β-Methylthiogalactoside | α-Methylglucoside |
|--------------------|--------|------------------------|------------------|
| Phosphate medium... | 29     | 33                     | 105              |
| Arsenate medium.... | 13     | 42                     | 55               |

Rubidium Transport Inhibition by Arsenate—Rubidium ions have been shown to be taken up by bacterial cells, apparently by the K1 uptake system (20). In contrast to the other transport systems tested, Rb+ transport was completely abolished by exposing glucose-grown cells to arsenate as shown in Fig. 9. 2,4-Dinitrophenol at 1 mM also blocked accumulation of Rb+.

Additional interesting aspects of the Rb+ system are its responses to the carbon source of the growth medium. As seen in Fig. 9, cells grown on succinate instead of glucose retain considerable capacity to accumulate Rb+ in the presence of arsenate. Added d-lactate could serve as an energy source for Rb+ transport only in the succinate-grown cells. No similar responses to carbon source in the growth media have been observed with any other transport system so far investigated.

Uncoupler Effects on Arsenate-insensitive Transport—Table IV shows that when a known uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, is added to cells suspended in arsenate, proline is no longer accumulated. In contrast, as noted in Table IV, α-methylglucoside accumulation was not sensitive to 2,4-dinitrophenol under the same conditions. In experiments not detailed here, other uncouplers (azide, m-Cl-carbonylcyanide phenylhydrazone and 5-Cl, 3-t-butyl, 2'-Cl, 4'-NO2-salicylanilide) were similarly effective.

O2 Requirement of Arsenate-insensitive Transport—As illustrated in Fig. 10, another significant characteristic of the arsenate-insensitive proline transport, in addition to being abolished by uncouplers, is that it requires O2. Anaerobiosis eliminates the capacity of cells to couple transport oxidatively. Accordingly, although proline transport is insensitive to the addition of arsenate under aerobic conditions, it is nearly abolished by arsenate under anaerobic conditions. Neither glucose nor d-lactate stimulated transport under these conditions.

A significant point is that the residual ATP measured after anaerobic incubation in presence of arsenate was only 0.25 to 0.5 n mole per mg of bacterial protein. Thus arsenate reduced ATP approximately the same in cells maintained aerobically or anaerobically. This observation gives additional evidence against the possibility that aerobic, arsenate-insensitive proline transport reflects insufficient ATP reduction.

Because anaerobic cells will accumulate proline in a process sensitive to the addition of arsenate, phosphate bond energy must supply the required coupling input. Yet even under con-
Fig. 7 (left). Apparent phosphorylation of \( \alpha \)-methylglucoside in arsenate-treated cells. Cells grown in Medium A were resuspended with the addition of 10 mM arsenate and incubated at 37\( ^\circ \)C for 10 min. \( \alpha \)-Methyl\([\text{\^{14}}\text{C}]\)glucoside was added to 4 ml of cells at a final concentration of 85 \( \mu \)M. At 30 s, 5 min, and 10 min, 1-ml aliquots were withdrawn and 0.5 ml was pipetted into 10 ml of Tris-Cl at 37\( ^\circ \), and 0.5 ml was pipetted into 10 ml of ice-cold 57 mM BaBr\(_2\) in 80\% ethanol. The sample in Tris buffer was filtered rapidly through a Millipore filter and washed with another 10 ml of Tris, while the BaBr\(_2\)-treated sample was filtered after 10 min at 0\( ^\circ \) and the precipitate was washed with 10 ml of ice-cold 80\% ethanol. The \( ^{14}\text{C} \) retained in filtration without prior BaBr\(_2\)-ethanol precipitation was taken as a measure of total intracellular \( \alpha \)-methylglucose phosphate, and the \( ^{14}\text{C} \) in the BaBr\(_2\)-ethanol precipitate was taken as a measure of intracellular \( \alpha \)-methylglucose phosphate.

Fig. 8 (center). Differential inhibition by iodoacetate of \( \alpha \)-methylglucoside, lactose, and proline transport. Resuspended cells grown in Tris-Cl were incubated at 30\( ^\circ \)C for 10 min in the presence of the indicated amounts of iodoacetate. Labeled solutes were added to various suspensions of cells (19 \( \mu \)M \( \alpha \)-methylglucoside, 34 \( \mu \)M lactose, 10 \( \mu \)M proline) and steady state accumulation measured as described under "Experimental Procedure."

Table IV

| Additions | Transport |
|-----------|-----------|
| P\(_i\)  | \( \alpha \)-Methylglucoside |
| m\( \text{M}\) | m\( \text{m}\) | m\( \text{M}\) | m\( \text{m}\) | nmoles/mg | nmoles/mg |
| 25 | 25 | 1 | 25 | 1 | 8.1 | 54 |
| 20 | 25 | 1 | 25 | 1 | 1.3 | 18 |

* DNP, 2,4-dinitrophenol.
Fig. 10 (left). Arsenate inhibition of anaerobic proline transport. Resuspended cells were made anaerobic in the presence of 10 mM potassium arsenate as described in the legend of Fig. 1. Uptake of 10 μM [3H]proline at 25°C was followed.

Fig. 11 (right). Effect of 2,4-dinitrophenol on anaerobic proline and α-methylglucoside transport. Anaerobic uptakes of 10 μM [3H]proline (A) and 10 μM α-methyl-14C]glucoside (B) by whole cells grown in Tris-Cl were measured as described in the legend of Fig. 1. Where indicated, cells were incubated with 1 mM 2,4-dinitrophenol prior to the transport assay.

Fig. 12. Preferential inhibition of anaerobic proline uptake by N,N-dicyclohexylcarbodiimide (DCCD) using intact Escherichia coli cells. MC38225 cells growing exponentially at 37°C in Medium A with glucose were chilled and resuspended in cold medium A. N,N-Dicyclohexylcarbodiimide in an ethanolic solution was added in the concentrations indicated giving a final ethanol concentration of 0.1% in all samples. Ethanol (0.1%) had no effect on transport. The samples were incubated overnight at 4°C and then resuspended in fresh Medium A with 20 mM glucose and 80 μg of chloramphenicol per ml. Aerobic and anaerobic proline transport, 5-min uptakes at 37°C, were assayed by Millipore filtration.

by our experimental findings. First, if energy input from only one source is blocked, then substantial retention, but not necessarily complete retention, of transport capacity should be observed. Second, if both sources of input are blocked, then transport capacity must fall markedly. Third, if the high energy state is dissipated, then transport should be inhibited independent of the energy input source. As shown by our findings, transport systems for proline, leucine, lactose, and under certain conditions, K+, function in harmony with these predictions. The transport of glucose only partially satisfies the predictions, reflecting novel aspects of glucose transport by E. coli.

Inhibition of oxidative input is readily achieved by establishing anaerobic conditions. As noted in Fig. 10 proline uptake by intact cells proceeds at a near-normal level in the absence of oxygen. Vesicles, on the other hand, do not transport well anaerobically, even in the presence of ATP (see Fig. 1). Possibly a coupling factor or factors have been lost during the vigorous disruption and extensive washing required for membrane vesicle preparation. The inability of vesicles to use ATP or other phosphates to stimulate transport of proline, although a significant ATPase activity is present (25), has been noted earlier (9, 11).

Blocking phosphorylative input is more difficult than blocking oxidative input, and requires careful assessment. In our first approach, sensitive measurement of membrane vesicle capacity for oxidative phosphorylation in presence of n-lactate and low Pi concentration revealed little or no uptake of added ADP (Table I). In the low phosphate medium, (1 mM added Pi) as used for transport assay, the molar capacity of the vesicles to produce ATP was at least two orders of magnitude less than the molar capacity to accumulate proline. With much more Pi present (10 mM), a small 2,4-dinitrophenol-sensitive uptake was observed (Table I). Membrane vesicles of E. coli prepared by other methods do have a measurable capacity for oxidative phosphorylation (26, 27) as do many bacterial systems (28), producing sufficient ATP to account stoichiometrically for the proline transport observed in osmotically prepared vesicles. The important result reported here is that n-lactate oxidation in our

Fig. 13. Dual energy input model for active transport.

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system greatly stimulates transport under conditions where capacity for P_i uptake is negligible. Thus in the vesicles, oxidative input for active transport may function near normally, but capacity for phosphorylative input from ATP is negligible.

Another approach to blocking phosphorylative input is to eliminate intracellular energy-rich phosphates, rather than to disrupt the membrane coupling sites. The known uncoupling action of arsenate made this P_i analogue a likely reagent for reducing intracellular energy-rich phosphate pools. However, the relatively poor ability of arsenate to compete with Pi and other factors known to modify arsenate inhibition (29), as well as the inability of arsenate to enter certain strains of E. coli (30), could reduce the effectiveness of arsenate. Adequate assessment of arsenate effects under our conditions thus seemed essential. The results show that incubation with high arsenate concentrations markedly reduces intracellular ATP and P-enolpyruvate levels (Fig. 4). Indeed, metabolically available levels may be reduced even more drastically than indicated by our data because perchloric acid extraction will also remove firmly but noncovalently bound nucleotides that might be present. Loss of phosphate bond energy is also indicated by the complete inhibition of protein synthesis by low concentrations of arsenate. However, even with little or no phosphorylative capacity retained, proline transport proceeds normally, as predicted by the model given in Fig. 13. Under such conditions, leucine, \( \beta \)-methylthiogalactoside, \( \text{Rb}^+ \), and \( \alpha \)-methylglucoside uptake all occur at nearly half or more of the level found in the absence of arsenate. Partial loss of some transport capacities in presence of arsenate does not appear to result from incomplete disruption of phosphorylations; increasing concentration and time of arsenate exposure has limited effect on arsenate-insensitive transport (see Fig. 6).

The differential effects of arsenate on anaerobic and aerobic transport are instructive. Arsenate allows normal proline transport in aerobic cells, but completely eliminates proline accumulation in anaerobic cells, although the same low level of ATP is found under each condition (see Fig. 10). This provides additional strong evidence that the transport dependent on \( \text{O}_2 \) is not occurring via ATP generation. The arsenate-insensitive process requires oxygen and the oxygen-independent process requires generation of phosphate bond energy. In harmony with the model of Fig. 13, either oxidative or phosphorylative energy is minimal requirement for energizing transport. Results with the ATPase inhibitor \( N,N \)-dicyclohexylcarbodiimide are in accord with this conclusion (see Fig. 12).

Transport dependent on phosphorylative input and transport dependent on oxidative input are each sensitive to the action of uncouplers. Although the molecular interpretation of uncoupler action remains an area of considerable speculation, uncouplers functionally appear to prevent the formation of, or cause the dissipation of, high energy membrane states. Thus, the action of uncouplers in eliminating amino acid, \( \beta \)-methylthiogalactoside, and \( \text{Rb}^+ \) transport under a variety of conditions gives strong support for the requirement of a high energy membrane state to couple energy input with many transport systems.

Despite the considerable experimental evidence favoring the exclusive participation of the P-enolpyruvate phosphotransferase system in accumulating certain hexoses in several bacterial species (31, 32), experiments showing the uptake \( \alpha \)-methylglucoside with drastically lowered intracellular P-enolpyruvate and ATP levels (see Figs. 4, 6, 7, and Table III) suggest consideration of the possibility of \( \alpha \)-methylglucoside entry in the free form followed by phosphorylation with an intracellular donor other than P-enolpyruvate. For example, phosphoryl transfer from glucose-6-P to various hexoses can be catalyzed by an enzyme preparation from E. coli (33). Present information leaves unresolved, however, the source of the phosphate for the formation of the phosphorylated \( \alpha \)-methylglucoside. Cells transferred to arsenate media still have considerable amounts of various intracellular phosphate present, and one or more of these by transfer reactions might be involved.

The mechanism by which oxidative uptake is coupled to active transport is unknown. Similar or identical energized compounds or states of membranes appear to be involved in both oxidative phosphorylation and in active transport. The means of coupling oxidative energy to transport thus joins oxidative phosphorylation in presenting the problem of determining the nature of high energy membrane states.

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