Energy Coupling in the Transport of β-Galactosides by *Escherichia Coli*: Effect of Proton Conductors

EMILIE PAVLASOVA AND F. M. HAROLD

Division of Research, National Jewish Hospital, and Department of Microbiology, University of Colorado School of Medicine, Denver, Colorado 80206

Received for publication 18 November 1968

*Escherichia coli* accumulates thiomethyl-β-D-galactoside against a concentration gradient under anaerobic conditions. The accumulation was abolished by carbonylcyanide m-chlorophenylhydrazone, tetrachlorosalicylanilide, 2,4-dinitrophenol, and other uncouplers of oxidative phosphorylation even though oxidative phosphorylation would not be expected to occur anaerobically. In the presence of the uncouplers, the β-galactoside carrier remained functional and catalyzed equilibration of thiomethylgalactoside across the membrane. The uncouplers did not inhibit the generation of adenosine triphosphate or protein turnover, or the accumulation of α-methylglucoside and glycerol by phosphorylation. We conclude that, at least anaerobically, uncouplers of oxidative phosphorylation do not interfere with energy metabolism in general, but prevent the utilization of metabolic energy for the active transport of galactosides. The uncouplers also facilitate passage of protons across the membrane. Various hypotheses are considered to explain why a proton-impermeable membrane may be required for active transport of galactosides and other substrates.

It has been known for many years that 2,4-dinitrophenol (DNP) and other uncouplers of oxidative phosphorylation block the accumulation of β-galactosides (15, 25) and of other metabolites by *Escherichia coli*. In the presence of inhibitors of energy metabolism, the active transport system is converted into one which mediates facilitated diffusion of β-galactosides across the membrane (16, 32). There is disagreement as to whether metabolic energy, presumably in the form of adenosine triphosphate (ATP; reference 26), reduces the affinity of the β-galactoside carrier for exit (16, 32) or increases its affinity for entry (26, 27). Be this as it may, uncouplers of oxidative phosphorylation are commonly assumed to abolish the formation of ATP and thus indirectly prevent coupling of transport to energy metabolism.

An alternative explanation for the inhibition of active transport by uncouplers of oxidative phosphorylation was developed by Mitchell (19–21). According to this view, the respiratory carriers are so arranged as to generate $H^+$ and $OH^-$ on opposite sides of the membrane. By virtue of associated membrane carriers, the resulting proton gradient may be utilized directly to drive active transport. It was proposed that uncouplers of oxidative phosphorylation facilitate the passage of protons across the membrane and thus collapse the gradient.

There is a considerable body of evidence that uncouplers of oxidative phosphorylation do in fact accelerate proton translocation across a variety of membranes (1, 8, 13, 19–21). In a previous report from this laboratory, we demonstrated that a number of such compounds, including tetrachlorosalicylanilide (TCS), carbonylcyanide-m-chlorophenylhydrazone (CCCP) and tetramethyldipircylamine (TMPA) inhibit energy-dependent transport processes in the fermentative organism, *Streptococcus faecalis* (8). These findings prompted us to examine the effects of uncouplers on β-galactoside transport in *E. coli* under anaerobic conditions. We shall demonstrate that under these conditions, uncouplers of oxidative phosphorylation do not interfere with ATP metabolism but still abolish energy coupling and conduct protons across the membrane.

**MATERIALS AND METHODS**

**Organisms.** Unless otherwise stated, we employed a strain of *E. coli* W1895 constitutive for both β-galactosidase and permease (lac $T^y^+$; met$^-^+$). The permease-negative strain ML35 was used in a few experiments.
An aerobic experiments. All experiments were conducted, as far as possible, under anaerobic conditions. The organisms were grown overnight at 37°C in a tightly stoppered bottle filled to the top; dissolved oxygen was driven out by passing nitrogen through the medium before inoculation. Overnight cultures were diluted with fresh medium and allowed to grow in the same way until the late exponential phase. Medium M56 (23) was used throughout, supplemented with methionine and with 0.01 mM glucose as energy source.

The bacteria were harvested by centrifugation, washed, and resuspended in fresh M56. To measure accumulation of 14C-thiogalactalactoside (TMG) or hydrolysis of o-nitrophenyl-galactoside (ONPG), glucose and methionine were omitted and chloramphenicol (50 μg/ml) was added. Suspensions were incubated at 22°C in stoppered tubes under continuous nitrogen perfusion; substrates were added after 10 min. Samples were withdrawn with a syringe through a narrow port in the stopper, filtered through membrane filters (Millipore Corp., Bedford, Mass.), and washed on the filter three times with chilled basal M56. Internal concentrations of 14C-TMG are expressed as micromoles per ml of cell water, assuming a water space of 2.7 ml/g, dry weight (32). Accumulations of 14C-α-methylglucoside and the capture of 14C-glycerol were followed by the same procedure.

It was not feasible to exclude air during centrifugation and sampling. We therefore stress that our conclusions in no way depend upon rigorous anaerobiosis at any stage of the experiments.

Production of acid from internal reserves or from glucose was followed by automatic addition of alkali through a pH-stat. The cells were suspended in 0.15 M KCl, with nitrogen perfusion, at pH 7.5. ATP was extracted with sulfuric acid and determined by a modification of the firefly-luminescence method (3). Protein synthesis was followed by incorporation of [14H]-leucine into the fraction insoluble in hot trichloroacetic acid.

Proton conductance. Permeability of the cells to protons was measured by following equilibration of the pH after addition of a pulse of HCl (8, 22). For reasons to be discussed below, cells grown on medium M56 were washed with tris(hydroxymethyl)aminomethane (Tris) and treated with ethylenediaminetetraacetate (EDTA) as described by Leive (18). The suspension was diluted with 0.05 M KCl and centrifuged; the cells were resuspended in KCl at 2 mg/ml (dry weight). Changes in pH were followed under continuous nitrogen perfusion. Sometimes valinomycin was added to permit rapid efflux of K⁺ (8, 22); E. coli is normally resistant to this antibiotic but was rendered sensitive by treatment with Tris-EDTA.

Chemicals. 14C-TMG and 14C-α-methylglucoside were gifts from H. V. Rickenberg; 14C-l-glycerol was from M. L. Morse. Inhibitors were donated by the following investigators: J. C. MacDonald (valinomycin), T. S. Meyer (TMAP), and R. C. S. Woodruffe (TCS). CCCP and ONPG were commercial products.

O-methyl TCS was prepared by M. B. Goren by methylation of TCS with a 20-fold excess of diazomethane in methanol-ether for 18 hr; it crystallized as needles from methanol (melting point, 150.5 to 152°C). The infrared spectrum (KBr pellet) showed no —OH absorption at 3,500 cm⁻¹, but did have the —NH band at 3,360 cm⁻¹.

RESULTS

Effect of uncouplers of oxidative phosphorylation on anaerobic accumulation of TMG. Our basic finding is illustrated in Fig. 1. Cells of the constitutive strain accumulated 14C-TMG anaerobically. Uncouplers of oxidative phosphorylation prevented accumulation if added prior to the substrate (Fig. 1A). Addition of uncouplers to cells which had first been allowed to accumulate 14C-TMG brought about rapid loss of radioactivity (Fig. 1B). In both cases, the final level of 14C-TMG within the cells was approximately equal to that in the medium. O-methyl TCS did not inhibit accumulation of TMG.

Effect of uncouplers on energy generation. It was conceivable that uncouplers of oxidative phosphorylation interfere in some unknown way with anaerobic generation of ATP. Several lines of evidence suggest that this is not the case.

(i) TCS, CCCP, DNP, and TMPA did not significantly inhibit anaerobic production of acid from endogenous energy reserves or from external glucose. Concentrations of uncouplers which completely abolished accumulation of 14C-TMG had no effect on the ATP content of the cells (Table 1).

(ii) Uncouplers inhibited not only the accumulation of TMG but that of amino acids as well.

![Fig. 1. Inhibition of anaerobic accumulation of 14C-TMG by uncouplers. Cells of E. coli W1895 lac (1 were suspended in medium M56 (no glucose) at 0.5 mg/ml (dry weight) and incubated under nitrogen. (A) The cells were incubated with the uncouplers for 10 min. At this time, designated 0 min, 14C-TMG was added to 5 × 10⁻⁴ M. (B) The cells were incubated with 5 × 10⁻⁴ M 14C-TMG for 10 min. At this time (0 min) the uncouplers were added. Symbols: ○, control; •, CCCP (2 × 10⁻⁴ M); Δ, TCS (10⁻⁴ M); ▲, DNP (2 × 10⁻⁴ M).]
TABLE 1. Effect of uncouplers on ATP levels and TMG accumulation under anaerobic conditions

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>ATP(^b)</th>
<th>(^{14})C-TMG(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.1</td>
<td>3.3</td>
</tr>
<tr>
<td>DNP (10(^{-3}) M)</td>
<td>3.1</td>
<td>0.42</td>
</tr>
<tr>
<td>CCCP (2 (\times) 10(^{-4}) M)</td>
<td>2.9</td>
<td>0.18</td>
</tr>
<tr>
<td>TMPA (10(^{-4}) M)</td>
<td>3.2</td>
<td>0.17</td>
</tr>
<tr>
<td>TCS (10(^{-4}) M)</td>
<td>Not done</td>
<td>0.18</td>
</tr>
</tbody>
</table>

\(^{a}\) Cells of E. coli W1895 lac \(\text{I}^{-}\) were incubated anaerobically in medium M56 (no glucose), with or without inhibitors, at a density of 1 mg/ml (dry weight). \(^{14}\)C-TMG (10\(^{-4}\) M) was added to all; samples were taken for analysis 10 min later.

\(^{b}\) Expressed as micromoles per gram of cells.

\(^{c}\) Expressed as micromoles per milliliter of cell water.

(unsupervised data). However, even at concentrations twice as high as those used to block TMG uptake, TCS and CCCP did not interfere with protein synthesis. Cells were allowed to accumulate \(^{3}\)H-leucine in the presence of chloramphenicol and were then transferred to fresh anaerobic medium supplemented with TCS, CCCP, or chloramphenicol. Transfer of \(^{3}\)H-leucine from the acid-soluble pool to the protein fraction was blocked by chloramphenicol but was unaffected by CCCP and TCS (Fig. 2). Protein turnover, rather than net synthesis, was followed in this experiment.

(iii) Accumulation of \(\alpha\)-methylglucoside is apparently mediated by a phosphotransferase system in which phosphoenolpyruvate serves as the phosphoryl donor (14, 28, 29). Similarly, the capture of glycerol depends upon phosphorylation of glycerol by ATP (11). Neither of these processes was inhibited by TCS or CCCP (Fig. 3).

Effect of uncouplers on the carrier protein. Some uncouplers of oxidative phosphorylation, including TCS and CCCP, bind strongly to proteins. They inhibit certain enzymes (6, 33) and are thought to induce conformational changes in membrane proteins (30). The M protein specified by the lac \(\gamma\) gene (4, 5) is thought to be the carrier for \(\beta\)-galactosides. It was therefore necessary to consider the possibility that the inhibition of TMG accumulation results from inhibition or denaturation of the carrier protein itself. A series of experiments modeled after the elegant studies of Winkler and Wilson (32) and of Koch (16) led us to conclude that TCS and CCCP do not inhibit the carrier but strictly abolish energy coupling.

(i) It is well known that the rate of ONPG hydrolysis by intact cells is limited by the transport of the substrate (16). In accordance with Koch's work (16), ONPG hydrolysis was almost abolished by the inhibitors of transport, \(p\)-chloromercuribenzoate (PCMB) and formaldehyde; hydrolysis by the permease-deficient strain, ML-35, was very slow. TCS and CCCP did inhibit anaerobic hydrolysis of ONPG by about 50% (Table 2). A comparable degree of inhibition was reported by Koch (16) for aerobic cells with DNP and sodium azide. The carrier thus con-

![FIG. 2. TCS and CCCP do not inhibit protein turnover. Cells were incubated anaerobically at 22 °C in M56 (plus glucose and 30 μg of chloramphenicol per ml; no methionine). After 10 min, \(^{3}\)H-leucine (10\(^{-4}\) M) was added; 5 min later, the cells were quickly filtered, washed with chilled M56, and transferred to fresh anaerobic M56 (plus glucose and methionine). Samples were incubated at 22 °C. Symbols: ○, control; ●, plus 50 μg of chloramphenicol per ml; △, plus 2 \(\times\) 10\(^{-4}\) M TCS; ▲, plus 4 \(\times\) 10\(^{-4}\) M CCCP. Samples were taken at intervals for determination of the \(^{3}\)H incorporated into the protein fraction.](http://jb.asm.org/)

![FIG. 3. Effect of uncouplers on anaerobic accumulation of \(^{14}\)C-\(\alpha\)-methylglucoside and of \(^{14}\)C-glycerol. Cells and procedure as in Fig. 1A. (A) \(^{14}\)C-\(\alpha\)-methylglucoside (2.5 \(\times\) 10\(^{-4}\) M) added at 0 min. (B) \(^{14}\)C-glycerol (3 \(\times\) 10\(^{-4}\) M) added at 0 min. Symbols: ○, control; ●, CCCP 4 \(\times\) 10\(^{-4}\) M; △, TCS (2 \(\times\) 10\(^{-4}\) M).](http://jb.asm.org/)

![FIG. 4. Effect of uncouplers on anaerobic accumulation of \(^{14}\)C-\(\alpha\)-methylglucoside and of \(^{14}\)C-glycerol. Cells and procedure as in Fig. 1A. (A) \(^{14}\)C-\(\alpha\)-methylglucoside (2.5 \(\times\) 10\(^{-4}\) M) added at 0 min. (B) \(^{14}\)C-glycerol (3 \(\times\) 10\(^{-4}\) M) added at 0 min. Symbols: ○, control; ●, CCCP 4 \(\times\) 10\(^{-4}\) M; △, TCS (2 \(\times\) 10\(^{-4}\) M).](http://jb.asm.org/)
continues to function in the presence of the uncouplers, though possibly at a reduced rate. (An alternative explanation for the partial inhibition of ONPG hydrolysis would be as follows. Although ONPG hydrolysis is thermodynamically passive, energy coupling may be expected to stimulate it, simply because efflux of substrate via the carrier would be minimized.) The fact that the uncouplers somewhat inhibit the hydrolysis of ONPG indicates that they do not simply render the membrane grossly leaky to small molecules.

(ii) The conclusion that the carrier itself remained functional in the presence of TCS and CCCP was confirmed by means of counterflow experiments. Cells were loaded anaerobically, in the presence of TCS, with a high concentration of unlabeled TMG; the cells were then filtered and resuspended in fresh anaerobic medium containing TCS and 14C-TMG. Transient uptake of 14C-TMG was observed under these conditions; no such transient uptake occurred in cells not preloaded with TMG, nor in the transport-deficient strain ML35 (Fig. 4). Similar results were obtained with CCCP. Counterflow is a criterion for carrier-mediated transport (31) and is thus strong evidence for the persistence of the carrier (32).

(iii) The final level of TMG in inhibited cells was approximately equal to, or slightly higher than, that in the medium. This equilibration and residual uptake of 14C-TMG were dependent upon the carrier (Fig. 5); in the presence of both TCS and CCCP, equilibration was inhibited by thiogalactoside (TGD), a competitive inhibitor of transport, by PCMB, and also by formaldehyde. In the transport-negative strain, ML35, equilibration was very slow and was not affected by CCCP in either the presence or absence of TGD and PCMB.

Proton conduction. Upon addition of a pulse of acid to a suspension of E. coli, the pH fell initially and then rose slowly as H+ passed into the cells. TCS and CCCP had very little effect on this, presumably because the cells are largely impermeable.

---

**Table 2. Effect of uncouplers on hydrolysis of ONPG**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>ONPG hydrolyseda (i/z y)</th>
<th>W1895 (i/z y)</th>
<th>ML-35 (i/z y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>425</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>CCCP (2 × 10⁻⁶ M)</td>
<td>224</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>TCS (10⁻⁵ M)</td>
<td>206</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate (10⁻⁴ M)</td>
<td>17</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Formaldehyde (10⁻³ M)</td>
<td>50</td>
<td>19</td>
<td>19</td>
</tr>
</tbody>
</table>

* Cells were incubated anaerobically at 22°C with uncouplers or inhibitors as shown. ONPG (2 × 10⁻⁴ M) was added after 10 min.

b Expressed as micromoles of ONPG hydrolyzed per gram of cells per 5 min.
to both cations and anions, and proton movements are thus restricted by the development of a membrane potential (22). The measurements were therefore repeated with organisms pretreated with Tris-EDTA by the procedure of Leive (18). Such cells were more permeable to ions and, in addition, were sensitive to valinomycin which renders membranes freely permeable to K\(^+\). Valinomycin, by itself, caused some extrusion of H\(^+\); the reason for this has not been explored. When valinomycin and an uncoupler were both present, the pH rose rapidly. Valinomycin facilitates K\(^+\) movements and would thus permit more rapid exchange of H\(^+\) for K\(^+\). Such observations strongly suggest that TCS and CCCP increase the permeability of the membrane to protons, but that net movement of H\(^+\) is possible only if K\(^+\) can move out simultaneously to preserve electroneutrality. Significantly, O-methyl TCS was not a proton-conductor and did not inhibit accumulation of \(^{14}\)C-TMG.

Strictly speaking, proton conduction by TCS and CCCP was demonstrated only for EDTA-treated cells, and we must extrapolate this conclusion to the normal cells used in the earlier experiments. This is warranted by the finding (18), confirmed here, that EDTA-treated cells were still capable of accumulating TMG; accumulation was abolished by TCS and CCCP.

![Fig. 6. Proton conduction by uncouplers. Cells were harvested during exponential growth, washed twice with Tris buffer (0.12 M, pH 8), and treated for 2 min with EDTA (0.2 mM EDTA in Tris buffer, 97 C). They were then diluted 10-fold into 50 mM KCl, washed twice, and resuspended in 50 mM KCl at 2 mg/ml. The suspension was made anaerobic and adjusted to an initial pH of 6.9. A pulse of HCl was then added, followed by uncouplers at arrows. The pH was recorded continuously from the expanded scale. (a) TCS (10\(^{-4}\) M); (b) valinomycin (VAL) (1 \(\mu\)g/ml) followed by TCS (10\(^{-4}\) M); (c) CCCP (2 \(\times\) 10\(^{-4}\) M); (d) valinomycin (VAL) (1 \(\mu\)g/ml) followed by CCCP (2 \(\times\) 10\(^{-4}\) M).](https://jb.asm.org/)
$O\text{-methyl TCS}$, in which the acidic OH group is blocked, was at least 100-fold less active than TCS. Our results thus confirm one of the predictions of the Chemiosmotic Hypothesis (19–21), that a membrane relatively impermeable to protons is required for active transport of TMG. The ultimate reason for this is uncertain. It is possible that a proton gradient across the membrane is directly involved in energy coupling (19) or in the maintenance of an energized membrane conformation (10). We should also recall that energy-rich intermediates which can arise either by electron transport or via ATP have been implicated in both transport and energy conservation by mitochondria (17); uncouplers may catalyze the hydrolysis or discharge of such intermediates by facilitating access of protons to sensitive sites. Perhaps the most attractive hypothesis is derived from the work of Harris, Höfer, and Pressman (9). Proton conductors permit passive entry of $H^+$ into the cells; extrusion of protons, and active transport in general, involve energy-rich intermediates or states of the membrane. The uncouplers would thus set up a proton cycle which continuously consumes and dissipates the energized state.

Finally, we call attention to the ambiguity of the concept of "energy requirement" for transport. In general, it is clear that the response of any given transport process to a particular metabolic inhibitor will depend on the precise chemical mechanism of the energy coupling. Accumulation of glycerol involves its phosphorylation by ATP (11); this process obviously requires metabolic energy but apparently does not involve a specific transport catalyst. Accumulation of $\alpha$-methylglucoside and of other substrates of the phosphotransferase system (28, 30) requires metabolic energy in the form of phosphoenolpyruvate; the work of Kaback (14) strongly suggests that phosphorylation is a vectorial process which facilitates both the entry and the retention of the substrate. However, it has long been known that accumulation of $\alpha$-methylglucoside is relatively resistant to certain metabolic inhibitors, including azide and DNP (12); this is confirmed here. Finally, the transport of many amino acids, sugars and inorganic ions by $E. coli$ is inhibited by uncouplers of oxidative phosphorylation (15). These experiments were usually conducted under aerobic conditions, and inhibition may thus simply be due to the lack of ATP. However, it may well turn out that even aerobically the transport block is a direct consequence of the conduction of protons across the membrane.

ACKNOWLEDGMENTS
We thank H. V. Rickenberg and J. Janeček for $^{14}$C-TMG, $^1$H-leucine, and for many helpful suggestions, and M. B. Goren for the synthesis and characterization of $O\text{-methyl TCS}$. J. C. MacDonald, T. S. Meyer, and R. C. S. Woodruff generously donated antimicrobial agents used in this and previous investigations.

This work was supported by Public Health Service grant AI-03568 from the Institute of Allergy and Infectious Diseases.

LITERATURE CITED
20. Mitchell, P. 1966. Chemiosmotic coupling in oxidative and