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EFFECT OF UNCOUPLERS ON THE BIOENERGETIC PROPERTIES OF A CARBONYL CYANIDE *m*-CHLOROPHENYLHYDRAZONE-RESISTANT MUTANT *ESCHERICHIA COLI* UV6

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The effects of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and tri-*n*-butyltin chloride (Bu_3SnCl) on proline transport, proton uptake and the transmembrane pH gradient in intact cells have been compared in a CCCP-resistant mutant strain *Escherichia coli* UV6, and its parent strain, AN180. CCCP and Bu_3SnCl inhibited proline uptake in AN180 but the pH gradient was affected only by CCCP. Neither uncoupler affected the pH gradient in UV6 although inhibition of proline uptake occurred at high concentrations. CCCP caused efflux of accumulated proline in both strains but Bu_3SnCl was ineffective. Bu_3SnCl did not prevent the efflux of proline induced by CCCP, indicating that Bu_3SnCl had not inactivated the transport carrier. In contrast with the parent strain, CCCP failed to reverse the oxidation-dependent inhibition of the phosphotransferase system in UV6 even at concentrations causing inhibition of proline uptake. The phosphorylation potential of UV6 with succinate as substrate was lower than in AN180. This was associated with a 10-fold higher concentration of phosphate in succinate-grown UV6 than in AN180. These results suggest that CCCP and Bu_3SnCl have different sites of action on the membrane energization system of intact cells of *E. coli*. A possible explanation of the differences between AN180 and UV6 is that the energization system is altered in the CCCP-resistant mutant. Both uncouplers stimulated the uptake of protons by intact cells to the same extent in UV6 as in AN180. In UV6, and in AN180 with Bu_3SnCl, this was not accompanied by effects on the transmembrane pH gradient. The extent of proton uptake appeared to be related to the level of the highly anionic membrane-associated oligosaccharides in the periplasmic space. It is proposed the outer membrane acts as a partial barrier to protons and that the uncouplers can equilibrate protons between the extracellular medium and the periplasmic space in intact cells.

Introduction

According to the chemiosmotic hypothesis of Mitchell [1], membrane energization for ATP synthesis or solute transport involves the translocation of protons across the energy-transducing membrane. ATP synthesis requires the use of the electrochemical potential gradient of protons by the ATP-synthase complex. Uncouplers dissipate the energized membrane state and so prevent ATP formation and inhibit the transport of those solutes which are energized by it [1]. It has been proposed that uncouplers dissolve in the membrane lipid and act as mobile proton conductors to equilibrate the proton gradient [1–3]. However, there is some evidence from the use of photoaffin-

Abbreviations: Bu_3SnCl, tri-*n*-butyltin chloride; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TCS, $3,3^1,4^1,5$-tetrachlorosalicylanilide; Mops, 4-morpholinepropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ity labelling uncouplers that uncouplers react with membrane proteins [4–8].

One possible approach to determining the mechanism of action of uncouplers, and so increasing our knowledge of the process of energy transduction, is to isolate bacterial mutants which are resistant to uncouplers. Decker and Lang [9,10] described a mutant of *Bacillus megaterium* which was resistant to the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). ATP synthesis in this mutant was resistant to concentrations of CCCP sufficient to diminish the ATP pool of the parent strain. Furthermore, the mutant could maintain its ATP pool in the absence of a significant electrochemical proton gradient [11,12]. The biochemical mechanism of resistance was not elucidated. Date et al. [13] have isolated a CCCP-resistant mutant of *Escherichia coli* which they used to study the energy-dependent processing of precursors of M13 coliphage coat protein. CCCP-resistant and tributyltin chloride (Bu_3SnCl)-resistant mutants have also been isolated by Ito et al. [14,15]. One of the CCCP-resistant mutants was investigated in more detail. This strain showed cross-resistance of growth to a number of uncouplers. It was suggested that this mutant had a defect in a subunit of ATP-synthase complex but direct evidence for this was not presented.

In the present paper, we describe some bioenergetic properties of a CCCP-resistant mutant of *E. coli*. Measurements of the phosphorylation potential of intact cells, and of the effects of the uncouplers CCCP and Bu_3SnCl on proline transport, proton uptake and the transmembrane pH gradient, suggests that the mutant has an alteration in its membrane energization systems. Moreover, CCCP and Bu_3SnCl do not have identical sites of action on these systems. Besides acting at the level of the cytoplasmic membrane, uncouplers were found also to equilibrate protons across the outer membrane of intact cells between the extracellular medium and the periplasmic space. The extent of proton uptake appeared to be related to the level of the highly anionic membrane-associated oligosaccharides in the periplasmic space.

Materials and Methods

Bacterial strains. E. coli AN180 (*thi argE mtl xyl rpsL*) is the parent of the CCCP-resistant strain UV6 used in this study. The isolation and characteristics of UV6 will be described elsewhere. *E. coli* BB 26-36 (*plsB*) was a generous gift of Dr. R.M. Bell (Duke University). *E. coli* JF568 (CGSC 6041) (*proC aroA his purE ilv metB lacY xyl rpsL cycA tsx*), JF701 (CGSC 6045) (as JF568 but *ompC*), JF699 (CGSC 6043) (*proC his purE ilv metB lacY xyl rpsL cycA tsx ompA*) and JF703 (CGSC 6046) (*proC his purE ilv metB lacY xyl rpsL cycA tsx ompF*) were kindly supplied by Dr. B. Bachmann.

Growth of cells. AN180 and UV6 were grown at 37°C with aeration from a 1% (v/v) inoculum either on Penassay Broth (Difco) or on a minimal medium [16] supplemented with thiamine (1 μg/ml) and arginine (50 μg/ml) and using 0.4% glucose as growth substrate. UV6 was grown initially in the presence of 50 μM CCCP. This culture was used to give a 1% inoculum for larger scale growth.

BB 26-36 (100 ml) was grown at 37°C with aeration on the low osmolarity medium of Kennedy [17] supplemented where indicated with 0.05, 0.1, 0.2 or 0.4 M NaCl. 0.5 mM glycerol containing 20 μCi [2-^{3}H]glycerol was also present.

The cells were harvested by centrifugation and washed with an appropriate buffer as indicated in the legends to the figures and tables.

Measurements of uptake of proline (leucine or succinate). For the experiments shown in Fig. 1, cells were grown to the late exponential phase with shaking in Penassay Broth at 37°C.

(a) The cells sedimented from 80 ml broth culture by centrifugation at 10000 × *g* for 15 min were washed once by resedimentation from 50 mM Tris-HCl (pH 7.4) containing 50 mM KCl, and resuspended in 2 ml 2 mM Tris-HCl (pH 6.6) containing 50 mM KCl. Uptake was measured at 37°C in a 2 ml system containing 2 mM Tris/50 mM KCl buffer (pH 6.6), 20 μM L-proline containing 0.5 μCi [U-^{14}C]proline and 0.2 ml cell suspension. Samples (0.1-ml) were removed at intervals and filtered through a 0.45 μm Millipore filter (Type HA). The filters were washed with 3 ml 0.1 M LiCl, dried, and the radioactivity was measured by scintillation counting in ACS fluid (Amersham).

(b) The cells sedimented from 40 ml broth culture were washed by resedimentation from 50

mM Tris-HCl (pH 8.0) and then from 50 mM potassium phosphate (pH 6.6). The cells were suspended in 8 ml of phosphate buffer. Uptake of proline was measured as above with 10 mM glucose, 44 μM L-proline containing 0.5 μCi [U-^{14}C]proline and 0.25 ml cell suspension in a total volume of 2 ml 50 mM potassium phosphate (pH 6.6). 0.1-ml samples were removed.

Uptake experiments with [U-^{14}C]leucine and (2,3-^{14}C]succinate were carried out at 22°C as described in (b) using a concentration of 20 μM L-leucine or succinate.

Uptake of α-methylglucoside (and proline). The cells sedimented from 50 ml broth culture by centrifugation at $10000 \times g$ for 15 min were washed twice by resedimentation from 0.9% NaCl, and finally suspended in 20 mM Mops-KOH buffer (pH 6.6) containing 50 mM KCl and 80 μg/ml chloramphenicol. Uptake was measured at 22°C in the Mops buffer above with 20 μM α-methyl D-glucoside containing 0.4 μCi α-methyl [U-^{14}C]glucoside. The total volume ws 1.0 ml. In some experiments, sodium formate (30 mM) and CCCP were present where indicated in the legends. The reaction was started by the addition of 0.05 ml cell suspension. At intervals, 0.2 ml samples wre removed and filtered through a 0.45 μm Millipore filter (Type HA). The filters were washed with two 2 ml vol. 0.1 M LiCl and the radioactivity on the filter was determined after drying by scintillation counting.

At the same time as the above experiment, the uptake of L-proline was measured using the same system but with α-methylglucoside replaced by 20 μM L-proline (containing 0.5 μCi [U-^{14}C]proline) and in the presence of 30 mM sodium formate. Samples were treated as before.

Proton uptake by intact cells ('proton pulse' experiments). The cells from 50 ml of an exponential phase Penassay Broth culture were washed twice by resedimentation from the appropriate 'assay' buffer (see below). The cells were suspended in assay buffer at 2 mg protein/ml. In some cases, the cells were washed once with 50 mM Tris-HCl (pH 6.8) and suspended in 10 ml 2 mM Tris-HCl (pH 6.8) containing 50 mM KCl and 2.5 mM EDTA. After incubation at 22°C for 10 min, the treated cells were sedimented and resuspended in the 2 mM Tris/50 mM KCl buffer.

Proton translocation in response to a proton pulse was measured with a combination pH electrode as described before [18]. A full scale deflection on the chart recorder was equivalent to 0.2 pH units. The assay system contained 0.2 ml cell suspension in 2.0 ml 2 mM Hepes-KOH buffer (pH 6.8) (containing 50 mM KCl). 10 μl 10 mM HCl was added as the 'proton pulse' followed by 4 μl 5 mM CCCP or Bu_3SnCl in ethanol. The changes in the pH of the medium were recorded.

Parallel measurements of proline uptake, Δ pH and proton uptake by intact cells. The cells from 100 ml Penassay Broth culture grown aerobically at 37°C were washed twice by sedimentation from 0.9% NaCl and resuspended in 2.5 ml 20 mM Tris-HCl buffer (pH 6.6) containing 50 mM KCl and chloramphenicol (50 μg/ml) to give a protein concentration in the range of 22–28 mg/ml. The basic system for each of the above measurements at 22°C consisted of 1.9 ml Tris-KCl/chloramphenicol buffer to which 0.1 ml of the cell suspension was added at zero time.

In the proton uptake experiments, metabolism of the glucose (0.2 μmol) acidified the medium. When a stable pH had been achieved, CCCP or Bu_3 SnCl was added and the uptake of protons was observed as described above. The initial rate of proton uptake was measured using small additions of HCl to calibrate the system.

To measure ΔpH, [7-^{14}C]benzoic acid (0.25 μCi) was added to the buffer prior to the addition of glucose (0.2 μmol) and cells at zero time. Three 0.2-ml samples were removed at 5 min. (Control experiments had shown that an equilibrium distribution of benzoic acid had been achieved by this time.) The samples were filtered through Bio-Rad Uni-Pore polycarbonate membranes (0.4 μm). The filters were not washed [19]. The filters were dried and the radioactivity was determined by scintillation counting in ACS fluid (Amersham). ΔpH ($pH_{in} - pH_{out}$) was calculated as described by Rottenberg [20] using a value of 5 μl/mg protein for the internal volume of the cells [21].

Proline uptake was measured following addition of glucose (0.2 μmol) and cells to the buffer system with 20 μM L-proline containing 1 μCi [U-^{14}C]proline. 0.2-ml samples were removed at intervals and filtered through a 0.45 μm Millipore filter (Type HA). The filters were washed with 3

ml 0.1 M LiCl, dried, and the radioactivity was measured by scintillation counting in ACS fluid (Amersham).

Measurement of uptake and efflux of proline. The cells from 50 ml minimal salts/glucose medium were washed by sedimentation once from 0.9% NaCl and once from 0.9% NaCl containing 80 μg/ml chloramphenicol. The cells were suspended in 1.5 ml 50 mM potassium phosphate buffer (pH 6.6) (for experiments with CCCP and TCS) or 1.25 ml 20 mM Tris-HCl buffer (pH 6.6) containing 50 mM KCl (for experiment with Bu_3SnCl). Both suspension buffers contained chloramphenicol. Samples (0.2-ml) were removed at intervals from the uptake system incubated at 22°C and containing 20 mM glucose, 20 μM L-proline containing 1 μCi [U-^{14}C]proline and 0.1 ml cells in a total volume of 2 ml of cell-suspension buffer. The samples were filtered and their radioactivity was determined as above.

For the proline efflux experiments, cells were allowed to accumulate proline for 10 min in 4 ml of the uptake system. The cells were then sedimented by centrifugation at $10\,000 \times g$ for 10 min. The inside of the tube was carefully wiped to remove excess fluid and the cell pellet then suspended in 5 ml of the cell-suspension buffer (phosphate for experiments with CCCP and TCS; Tris-KCl for the experiments with Bu_3SnCl). Efflux was followed by removing 0.2-ml samples at intervals from 1-ml portions of the cell suspension to which various concentrations of uncoupler had been added. The samples were filtered and their radioactivity was determined as above.

Phosphorylation potential. 0.04 g (wet weight) of cells, which had been washed three times with 0.9% NaCl, in 0.36 ml 20 mM Tris-HCl (pH 6.6) containing 50 mM KCl, was shaken in air at 22°C for 10 min before the addition of 0.04 ml 40% perchloric acid. The suspension was kept for 10 min at 0°C and then the precipitated material was removed by centrifugation at $10\,000 \times g$ for 10 min. The supernatant was analyzed for ATP, ADP and inorganic phosphate as described previously [22,23]. The internal volume of the cells was determined from the protein content of the cell suspension [24] using a value of 5 μl interval vol./mg protein [21]. The phosphorylation potential ΔG_p^1 was calculated from the equation $\Delta G_p^1 = \Delta G_0^1 + RT\ln([ATP]/[ADP][P_i])$. ΔG_0^1 was taken as 30.6 kJ/mol.

Measurement of membrane-derived oligosaccharides and proton uptake. 100-ml cultures of *E. coli* BB 26-36 were grown overnight with vigorous aeration at 37°C in low osmolarity buffer containing different levels of NaCl in the presence of [2-^{3}H]glycerol as described above. Before harvesting, 5-ml samples of the cultures were removed and the concentration of the membrane-derived oligosaccharides in the cells was determined as described by Kennedy [17]. The remainder of each culture was harvested. The cells were washed twice by sedimentation from 0.9% NaCl and suspended in 1.5 ml 20 mM Tris-KCl buffer (pH 6.6) containing 50 mM KCl and 80 μg/ml chloramphenicol. Proton uptake was measured with a pH electrode in a system containing 1.9 ml Tris-KCl/chloramphenicol buffer to which 0.1 ml cell suspension had been added. When the pH was stable, 2 or 3 μl 0.1 M HCl was added to give a change of about 0.1 pH unit. Proton uptake was initiated by the addition of 40 μM CCCP or 0.5 μM Bu_3SnCl. The change in pH was followed as described above.

Materials. The suppliers of the following materials are indicated in brackets: [2-^{3}H]glycerol, L-[^{14}C]leucine, [2,3-^{14}C]succinic acid (Amersham); L-[U-^{14}C]proline (ICN); [7-^{14}C]benzoic acid (New England Nuclear); [^{3}H]tetraphenylphosphonium bromide (New England Nuclear); CCCP (Sigma); Bu_3SnCl (Alfa Products); TCS (Eastman).

Results

Properties of uncoupler-resistant mutant UV6

E. coli UV6 was obtained from strain AN180 as a spontaneous CCCP-resistant isolate able to grow on Penassay Broth in the presence of 250 μM CCCP. Since the isolation and characterization of this mutant will be described in more detail elsewhere, only a brief account of its properties will be given. UV6 showed cross-resistance to FCCP, a more potent structural analog of CCCP, and to Bu_3SnCl, but was not more resistant to growth inhibition by 2,4-dinitrophenol, 2,4-dibromophenol, azide, *N*-ethylmaleimide, phenethyl alcohol, polymyxin B and chloramphenicol. The resistance to CCCP, FCCP and Bu_3SnCl did not involve a nonspecific increase in resistance to per-

meation of inhibitors in general.

As will be discussed later, the outer membrane presents a partial barrier to uncouplers. Therefore, its composition was of interest. No significant differences were seen in the ratios of protein, lipopolysaccharide and phospholipid between the parent and mutant strain. The fatty acid composition and relative amounts of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin were similar. No differences could be detected in the outer membrane proteins present in parent and mutant by one-dimensional or two-dimensional isoelectric focusing-SDS gel electrophoresis. The monosaccharide compositions of the lipopolysaccharide were identical. Furthermore, both strains were equally sensitive to phages T2, T3, T4, T5, T6 and T7. These results apply to mutant cells grown in the absence of CCCP, the conditions for most of the experiments to be described in this paper. As will be described elsewhere, growth in the presence of CCCP alters the composition of the outer membrane.

Inhibition of proline uptake by CCCP and Bu_3SnCl

The effect of CCCP on proline uptake in An180 and UV6 is shown in Fig. 1. In the parent strain, uptake of proline was abolished at 6.25 μM CCCP when 2 mM Tris/50 mM KCl buffer (pH 6.6) was used. By contrast, this level of uncoupler had no effect on the mutant strain. Similar results were obtained if the experiments were carried out in 50 mM potassium phosphate (pH 6.6) but cells of AN180 were noticeably less sensitive. Similar results were observed for the uptake of leucine and succinate. Fig. 1 demonstrates a phenomenon also shown by our other CCCP-resistant mutants. Uptake of proline occurred more rapidly and to a greater level in the mutant than in the parent cells. This resembles the results of Guffanti et al. [11] with an uncoupler-resistant mutant of *Bacillus megaterium* in which respiration-driven membrane energization was used more effectively for ATP synthesis than in the wild-type strain.

Proline uptake in AN180 was not inhibited if the experiments were carried out at pH 7.5. In a similar manner, the uptake of proline by AN180 was inhibited by Bu_3SnCl to a greater extent at pH 6.6 than at pH 7.5 (Table I). Bu_3SnCl is believed to act as an uncoupler by catalyzing the

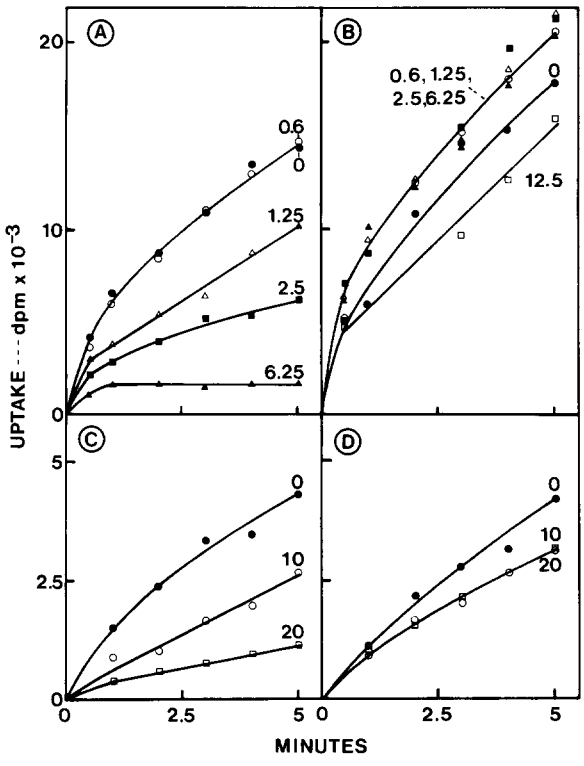

Fig. 1. Effect of CCCP on the uptake of proline by intact cells of AN180 (A, C) and UV6 (B, D). The concentration of CCCP as indicated on the lines is expressed in μM. Uptake was measured at pH 6.6 in 2 mM Tris/50 mM KCl (A, B) and in 50 mM potassium phosphate buffers (C, D), respectively, as described in Materials and Methods.

transmembranous exchange of chloride and hydroxyl ions [25]. The chloride-ion-dependence of the inhibition of proline uptake by Bu_3SnCl is shown in Table II. Inhibition occurs both with AN180 and UV6. This table also demonstrates the stimulatory effect of KCl on proline uptake. NaCl also was stimulatory.

Effect of CCCP on inhibition of the phosphotransferase system

Oxidation of substrates by the respiratory chain of *E. coli* inhibits the uptake of α-methyl-D-glucoside through the phosphotransferase system. Uncouplers reverse the inhibition. This result has been interpreted as indicating that the energized state of the membrane can affect the activity of the phosphotransferase system [26]. As shown in Ta-

TABLE I

EFFECT OF pH ON THE INHIBITION OF PROLINE UPTAKE BY CCCP AND Bu_3SnCl

For the experiments with CCCP, the cells were grown on Penassay broth, washed with 50 mM potassium phosphate buffer (pH 6.6 or 7.5) and suspended in the same buffer. Proline uptake was measured in phosphate buffer at pH 6.6 or 7.5. With Bu_3SnCl, the cells were grown in the minimal medium with glucose, washed twice with 0.9% NaCl, and suspended in 20 mM Tris-HCl buffer, containing 50 mM KCl and chloramphenicol (80 μg/ml), at pH 6.6 or 7.8. Proline uptake was measured in the same buffer at pH 6.6 or 7.8. Values for proline uptake are expressed as percentages of those observed in the absence of CCCP or Bu_3SnCl. Values from proline uptake curves were taken at 5 min. The 100% values (dpm/0.1 ml sample removed) are: AN180 (pH6.6, CCCP), 23300; AN180 (pH 7.5, CCCP), 15100; UV6 (pH 6.6, CCCP), 19200; UV6 (pH 7.5, CCCP), 20400; AN180 (pH 6.6, Bu_3SnCl), 33000; AN180 (pH 7.8, Bu_3SnCl), 53000.

Strain	pH	Uncoupler	Concentration (μM)	Proline uptake (%)
AN180	6.6	CCCP	0	100
			10	29
			20	8
	7.5	CCCP	0	100
			10	69
			20	41
UV6	6.6	CCCP	0	100
			10	91
			20	76
	7.5	CCCP	0	100
			10	87
			20	90
AN180	6.6	Bu_3SnCl	0	100
			0.5	68
			2.5	35
			10	23
	7.8	Bu_3SnCl	0	100
			0.5	100
			2.5	104
			10	95

TABLE II

REQUIREMENT FOR CHLORIDE IONS FOR INHIBITION OF PROLINE UPTAKE BY Bu_3SnCl

The cells were grown on 50 ml minimal salts/glucose medium, washed twice in 20 mM Tris-H_2SO_4 buffer (pH 6.6) containing 80 μg/ml chloramphenicol, and suspended in 1.25 ml of the same buffer. Uptake was measured as described in Materials and Methods except that the buffer was 20 mM Tris-H_2SO_4 (pH 6.6) containing 40 mM KCl where indicated. The concentration of Bu_3SnCl was 2 μM. Proline uptake is expressed as dpm accumulated in 5 min per 0.2 ml sample of reaction mixture.

Strain	KCl	Bu_3SnCl	Proline uptake
AN180	−	−	39600
	−	+	40000
	+	−	88300
	+	+	61300
UV6	−	−	22400
	−	+	23300
	+	−	118400
	+	+	68000

ble III, formate oxidation inhibited the uptake of α-methylglucoside in AN180 and UV6. CCCP reversed the inhibition in AN180. However, a reversal of inhibition was not observed in the uncoupler-resistant mutant even at the high concentrations of CCCP which were required for inhibition of proline uptake. Evidently, the absence of reversal of the formate oxidation-dependent inhibition of the uptake of α-methylglucoside in UV6 was not due to the inability of CCCP to reach the cytoplasmic membrane.

Effect of CCCP and Bu_3SnCl on the transmembrane proton gradient

Experiments with membrane vesicles suggest that the transmembrane pH gradient and the membrane potential both contribute to the driving force for proline uptake at pH 6.6 [27]. The membrane potential is the sole component at pH 7.5. Thus, the greater sensitivity of proline uptake to inhibition by CCCP at pH 5.5 compared with pH 7.5 would be consistent with CCCP causing dissipation of the pH gradient. The effect of CCCP on the pH gradient in AN180 and UV6 is shown in Fig. 2.

Uncouplers can equilibrate a pH gradient across a phospholipid bilayer and across many naturally-occurring membranes [2,3]. Measurement of the ability of CCCP to stimulate uptake of protons from the medium was measured in 'proton pulse' experiments. Since these necessitated the use of a weakly buffered system, this system was used in all of the experiments shown in Fig. 2 in order to facilitate comparison of the relative

TABLE III

COMPARISON OF EFFECT OF CCCP ON REVERSAL OF FORMATE-OXIDATION DEPENDENT INHIBITION OF α-METHYLGLUCOSIDE UPTAKE AND ON PROLINE UPTAKE

The experiment was carried out as described in Material and Methods. Uptake is expressed as percentage of that observed in the control sample after 2 min. +, 30 mM sodium formate present.

Strain	Additions		Uptake (% control value)	
	formate	CCCP (μM)	proline	α-methylglucoside
AN180	−	−	100	
	−	2	37	
	−	5	13	
	−	15	4	
	−	50	3	
AN180	−	−		100
	+	−		44
	+	2		49
	+	5		70
	+	15		97
	+	50		93
UV6	−	−	100	
	−	2	94	
	−	5	84	
	−	15	48	
	−	50	24	
UV6	−	−		100
	+	−		39
	+	2		38
	+	5		37
	+	15		36
	+	50		42

uncoupler sensitivities of the processes measured. The experiments were carried out in parallel with the same batch of cells. The transmembrane pH gradient was measured from the equilibration of [^{14}C]benzoic acid [19,20]. The membrane potential could not be measured satisfactorily, since intact cells do not readily take up the tetraphenylphosphonium cation. (We obtained values for the membrane potential of about 50 mV in untreated cells of both AN180 and UV6.) EDTA treatment is usually employed to permeabilize the outer membrane of cells in such experiments [19,20]. As will be discussed later, this treatment was considered to be undesirable in our experiments with uncouplers. The equilibration of benzoic acid in

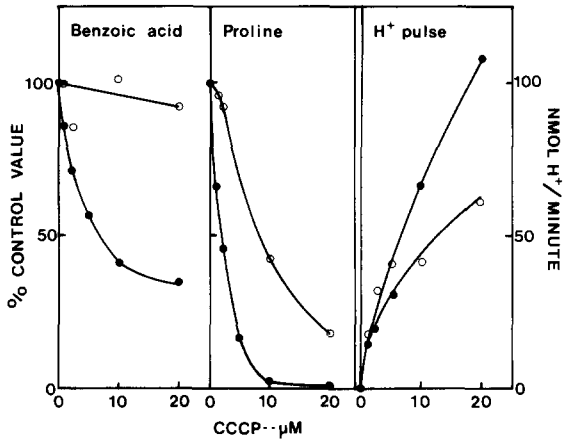

Fig. 2. Effect of CCCP on the uptake of benzoic acid, proline and protons in intact cells of *E. coli* AN180 and UV6. The experiments were carried out with the same batch of cells and using the same buffer system as described in Materials and Methods. Values for uptake of benzoic acid and proline are expressed as percentages of those observed in the absence of CCCP. Values from uptake curves were taken at 5 min. Proton uptake ('H^+ pulse') is given as the initial rate of uptake observed following addition of the uncoupler. Open symbols, UV6; closed symbols, AN180.

response to the pH gradient has been expressed in terms of uptake in order to facilitate direct comparison with effects on proline uptake. The 100% value shown in Fig. 2 is equivalent to a pH gradient of 0.70 pH units. The results of proton pulse experiments are expressed as the initial rate of proton uptake by the cell suspension induced by the addition of uncouplers. Acidification of the medium was achieved by metabolism of added glucose.

As shown in Fig. 2, uptake of proline in the CCCP-resistant mutant UV6 was less sensitive to inhibition by CCCP than in its parent strain AN180. Uptake of protons was stimulated by CCCP in both strains in the proton pulse experiments. The difference between the curves for AN180 and UV6 in the proton pulse experiment shown in Fig. 2 is much greater than what has been observed in other experiments (see later). CCCP had little effect in UV6 on the pH gradient measured by benzoic acid uptake. By contrast, the pH gradient of AN180 was diminished by CCCP.

A similar experiment to the above using

Bu_3SnCl is shown in Fig. 3. Proline uptake was somewhat less sensitive to Bu_3SnCl in UV6 than in AN180, whereas Bu_3SnCl was equally effective in causing the uptake of protons in pulse experiments with both strains. The pH gradient was unaffected by Bu_3SnCl in AN180 and UV6. Although not shown in Fig. 3, the effects of Bu_3SnCl were dependent on the presence of chloride ions.

Effect of uncouplers on the efflux of proline from intact cells

Efflux of proline accumulated in intact cells of AN180 in the presence of chloramphenicol could be induced by the addition of the uncouplers CCCP and TCS (Fig. 4). As shown by experiments carried out at the same time, and in the same buffer system, uptake of and efflux of proline had a similar degree of sensitivity to these two uncouplers in this strain. Uptake and efflux of proline were more resistant in UV6. By contrast, although uptake of proline was inhibited in both strains by Bu_3SnCl, addition of this uncoupler did not induce efflux of accumulated proline under the same conditions.

The inhibitory effect of Bu_3SnCl on proline uptake required chloride ions and thus was likely due to chloride-hydroxyl ion exchange catalyzed by the uncoupler. The possibility that reaction with Bu_3SnCl was immobilizing the proline-transport carrier was tested as follows. Intact cells of AN180 were allowed to accumulate proline in the presence of chloramphenicol. They were washed and incubated with a concentration of Bu_3SnCl sufficient to inhibit uptake of proline but not to cause efflux. CCCP was then added after 5 min of incubation. Prior treatment with Bu_3SnCl did not block the ability of CCCP to cause efflux of accumulated proline. It is unlikely that Bu_3SnCl fails to cause efflux because it immobilizes the proline-transport carrier.

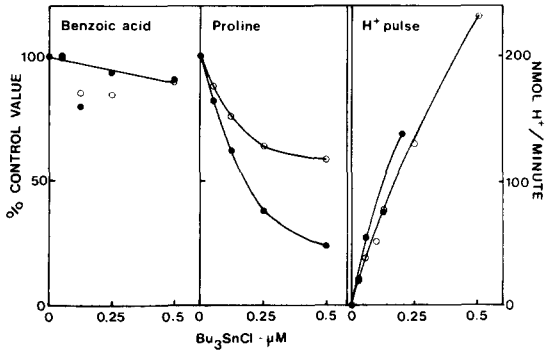

Fig. 3. Effect of Bu_3SnCl on the uptake of benzoic acid, proline and protons in intact cells of *E. coli* AN180 and UV6. The experiments were carried out as described in the legend to Fig. 2. Open symbols, UV6; closed symbols, AN180.

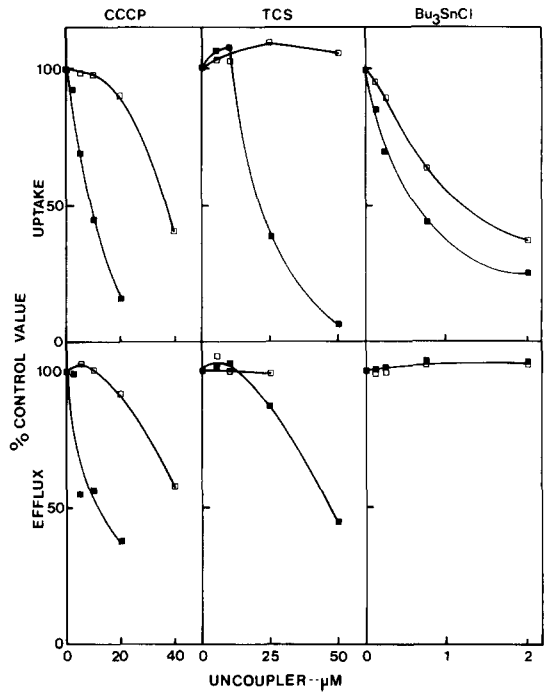

Fig. 4. Effect of CCCP, TCS and Bu_3SnCl on the uptake and efflux of proline in intact cells of *E. coli* AN180 and UV6. The experiment was carried out as described in Materials and Methods. Values for proline uptake and efflux are expressed as percentages of those observed in the samples without uncoupler. Values from uptake and efflux curves were taken at 5 min. Open symbols, UV6; closed symbols, AN180.

Phosphorylation potential in normal and mutant cells

The results discussed above suggested that UV6 had an alteration in the membrane-energization system. This should be most evident when the cells were grown under conditions (e.g., on succinate) where ATP was supplied by oxidative phosphorylation. Further support for this hypothesis was obtained from mesurements of the phosphoryla-

tion potential in normal and mutant cells. The phosphorylation potential ΔG_0^1 is the Gibbs free energy of ATP synthesis and equals $\Delta G_0^1 + RT\ln([ATP]/[ADP][P_i])$ where ΔG_0^1 is the standard free energy of ATP synthesis [19]. The phosphorylation potential maintained by the metabolism of endogenous substrates was calculated from the concentrations of ATP, ADP and inorganic phosphate in cells which had been incubated at 22°C with shaking in air. The phosphorylation potential (38.9 kJ/mol) of UV6 grown on succinate was lower than the value (45.2 kJ/mol) obtained with AN180 (Table IV). Little difference between the two strains was seen when the growth substrate was glucose. The lower phosphorylation potential in cells of succinate-grown UV6 was associated with the high concentration of inorganic phosphate present in these cells. We have consistently observed that succinate-grown UV6 maintains a 10-fold higher concentration of phosphate than AN180. This difference was shown at all stages of growth and was unaffected by incubation of the cells with glucose, succinate and formate prior to assay.

ATPase activity, the oxidase activities with NADH, formate and succinate as substrates, and the cytochrome content of AN180 and UV6 were similar (results not shown).

Proton equilibration across the outer membrane

As shown above, CCCP and Bu_3SnCl stimulated the uptake of protons from the medium by UV6 cells in proton pulse experiments but had no effect on the transmembrane pH gradient measured by the equilibration of benzoic acid. This is understandable if proton pulse experiments measure the equilibration of protons across the outer membrane only. As shown in Fig. 5, the addition of CCCP or Bu_3SnCl markedly increased the rate of passive influx of protons into the cells from a weakly buffered medium to which a small pulse of HCl had been added to set up a pH gradient. Similar effects were observed both with AN180 and its CCCP-resistant mutant grown in the absence of CCCP. The ability of both of these uncouplers to equilibrate the pH gradient was much reduced in mutants grown with CCCP. Since EDTA-treatment of cells in the latter experiment greatly increased the effectiveness of the uncoupler (result not shown) it was considered likely that the outer membrane was acting as a barrier to the uncouplers in these cells. As will be described elsewhere, growth of UV6 with CCCP results in alterations in the composition of the outer membrane.

This role of the outer membrane was examined using mutants which lacked major outer membrane proteins. As shown in Fig. 6, treatment of the cells of the parent strain with EDTA increased the rate at which the pH gradient was equilibrated following addition of CCCP. The rate of equilibration was low in untreated *ompF* and *ompC* strains lacking outer membrane proteins 1a and 1b, respectively (nomenclature of Lee et al. [28]). By contrast, the pH gradient was rapidly equilibrated in untreated cells of an *ompA* strain which lacks the outer membrane protein 3a. (In agreement with this finding, the growth of this strain was

TABLE IV

ENDOGENOUSLY MAINTAINED LEVELS OF ADENINE NUCLEOTIDES AND INORGANIC PHOSPHATE AND THE PHOSPHORYLATION POTENTIAL (ΔG_p^1) IN CELLS OF AN180 and UV6

The cells were grown at 37°C on the substrate indicated. They were harvested by centrifugation and washed three times with 0.9% NaCl. Values are expressed as mean of determinations with three different batches of cells. The assays were carried out as described in Materials and Methods.

Strain	Growth substrate	Intracellular concentration (mM)			ΔG_p^1 (kJ/mol)
		ATP	ADP	phosphate	
AN180	glucose	0.47	0.15	14	43.9
UV6	glucose	0.51	0.13	15	44.4
AN180	succinate	0.29	0.13	5.8	45.2
UV6	succinate	0.42	0.20	75	38.9

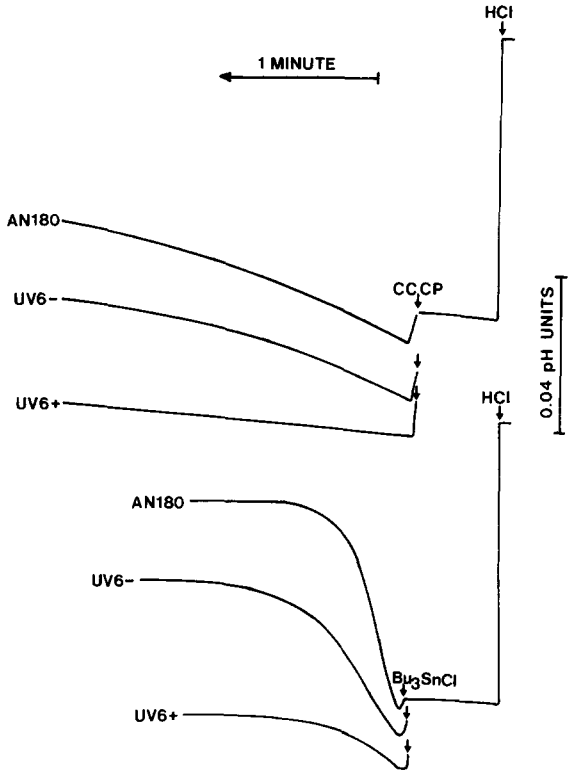

Fig. 5. Uptake of protons induced by addition of the uncouplers CCCP and Bu_3SnCl to intact cell suspensions of *E. coli* AN180, UV6 (UV6−) and UV6 grown in the presence of 50 μMCCCP (UV6+). 10 μl 10 mM HCl and 4 μl 5 mM CCCP or Bu_3SnCl were added where indicated. The time axis runs from right to left. The experiment was carried out as described in Materials and Methods using 2 mM Hepes/50 mM KCl buffer (pH 6.8).

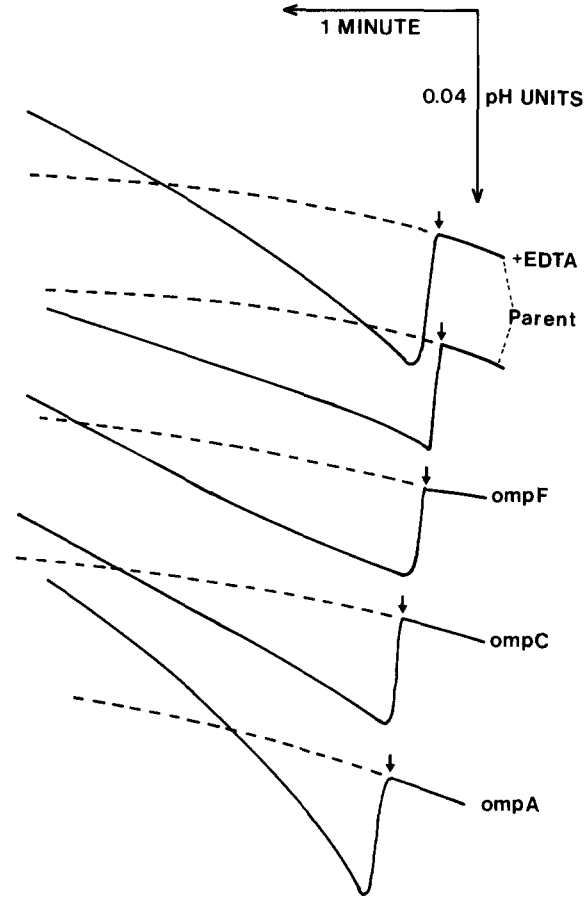

Fig. 6. Uptake of protons induced by addition of CCCP to suspensions of intact or EDTA-treated cells of *E. coli* outer membrane protein mutants. The experiment was carried out as described in Materials and Methods and in the legend to Fig. 5 but using 2 mM Tris/50 mM KCl (pH 6.8) as buffer. The pH change due to the prior addition of HCl is not shown. CCCP was added at the arrow in the experiments shown by the solid lines. The broken lines show uptake of protons in the absence of added CCCP.

much more sensitive to inhibition by CCCP compared with the other strains.) These results are consistent with the hypothesis that the outer membrane does not permit free movement of protons from the medium into the periplasmic space, and that uncouplers facilitate proton movement across this membrane.

Kennedy [17] has described a class of oligosaccharides – membrane-derived oligosaccharides – which are located in the periplasmic space. They are multiply substituted with *sn*-1-phosphoglycerol and with *O*-succinyl ester residues and so are highly anionic. Cells grown in a medium of low osmolarity contain more membrane-derived oligosaccharide than those grown at higher osmolarity. Kennedy has suggested that membrane-derived oligosaccharide is the principle fixed anion in the periplasmic space and acts to maintain the high osmotic pressure and Donnan membrane potential of the periplasmic compartment [17]. The amount of membrane-derived oligosaccharide present can be readily determined in the *pls B* strain *E. coli* BB 26-36 which requires exogenous glycerol for growth. The *sn*-1-phosphoglycerol residues of

membrane-derived oligosaccharide are measured from the extent of incorporation of [2-^{3}H]glycerol into the membrane-derived oligosaccharide fraction. (Only glycerol residues are labelled from [2-^{3}H]glycerol since tritium is lost during metabolism of glycerophosphate to dihydroxyacetone phosphate [17].)

The relationship between the uncoupler-induced uptake of protons by intact cells in proton pulse experiments and the level of membrane-derived oligosaccharide was examined using strain BB 26-36 grown in Kennedy's low osmolarity medium and following the addition to it of 0.05, 0.1, 0.2 and 0.4 M NaCl. The relative amounts of membrane-derived oligosaccharide formed in the presence of NaCl, expressed as a percentage of the level of membrane-derived oligosaccharide present in the absence of added salt, were 71, 57, 35 and 14%, respectively. As shown by the curves in Fig. 7, the extent and/or rate of proton uptake following addition of Bu_3SnCl or CCCP diminished progressively as the membrane-derived oligosaccharide content of the cells was decreased by growth at higher osmolarities.

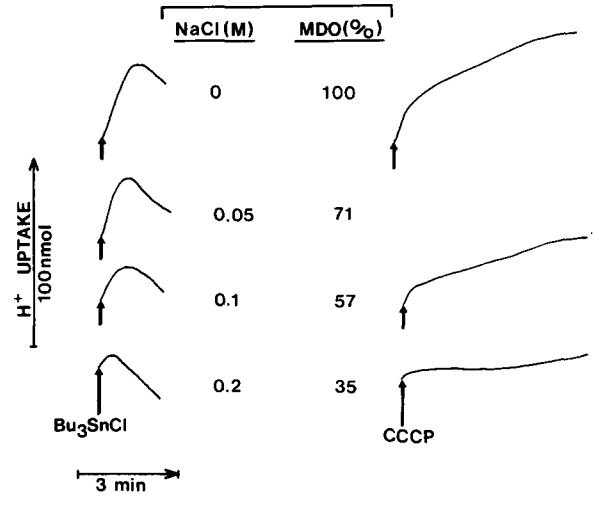

Fig. 7. Uptake of protons induced by the addition of Bu_3SnCl or CCCP to intact cell suspensions of *E. coli* BB 26-36 grown at different concentrations of NaCl. The amounts of membrane-derived oligosaccharides (MDO) present in these cells are expressed as percentages of those observed in the cell suspensions grown without added NaCl. The experiment was carried out as described in Materials and Methods. The pH change induced by the addition of HCl is not shown.

Discussion

In addition to growth, the uptake of proline, leucine and succinate, and the reversal of oxidation-dependent inhibition of the phosphotransferase system, were all more resistant to the action of CCCP in the uncoupler-resistant mutant UV6. Two possible explanations for the observed resistance to CCCP are (a) an increased resistance of the outer membrane to permeation of uncoupler, and (b) a resistance to the uncoupler at the level of the energization system. We have excluded a mechanism for resistance which involves alterations in the phospholipid composition of the inner (plasma) membrane since we could find no distinctive differences between the lipids of the inner membranes of UV6 and its parent strain (unpublished data).

The outer membrane undoubtedly does form a partial barrier to uncouplers since removal of lipopolysaccharide by treatment with EDTA increased the effectiveness of CCCP. Moreover, a strain lacking major outer membrane protein 3a was usually sensitive to CCCP. Deletion of major outer membrane proteins 1a or 1b did not have a similar effect so that the significance of the increased sensitivity in the strain lacking protein 3a is not clear. The increase in sensitivity was not associated with changes in the ratio of the outer membrane proteins, phospholipid and lipopolysaccharide (unpublished data).

Although not documented in detail in this paper, it is unlikely that the increased resistance of CCCP-resistant strain UV6 was due to an outer membrane barrier for the following reasons. (a) The lack of cross-resistance of growth to some other uncouplers indicates that there was some specificity in the nature of the resistance. Nonpolar substances are likely to permeate through the lipid and so the barrier should be relatively nonspecific. (b) No marked differences in the amount and composition of the outer membrane proteins, lipopolysaccharide and phospholipids were detected between AN180 and UV6. (c) In proton pulse experiments, CCCP and Bu_3SnCl were effective in causing the uptake of protons equally well with AN180 and with UV6. As discussed below, it is likely that the equilibration of protons is occurring across the outer membrane.

A site(s) of resistance to uncoupler at the level of the energization system was suggested by the following evidence: (a) The uptake of proline, leucine and succinate, and the uncoupler-dependent reversal of inhibition of the phosphotransferase system, were resistant to CCCP in uncoupler-resistant strains. A common feature of these systems is the energization of the cell membrane. (b) The phosphorylation potential ΔG_p^1 was lower in succinate-grown UV6 than in AN180. This was associated with a 10-fold higher concentration of phosphate in the cells of UV6 compaed with AN180.

The effect of uncouplers on the energization system was analyzed in more detail. CCCP and Bu_3SnCl represent two types of uncoupling agents. According to the chemiosmotic hypothesis, it is likely that CCCP would act as a mobile lipid-soluble proton carrier to equilibrate the electrochemical potential gradient of protons across the cytoplasmic membrane of *E. coli* [1–3]. Bu_3SnCl brings about the transmembrane exchange of hydroxyl and chloride ions, and so could also dissipate the proton gradient across the cytoplasmic membrane [25]. It was surprising therefore to find that the two uncouplers have different effects on the uptake and efflux of proline, and on the pH gradient in AN180 and UV6. (In mitochondria, Bu_3SnCl has a further site of action. It inhibits the ATP synthase [36]. Bu_3SnCl at the concentration used in our experiments has no effect on the ATPase activity of inner membranes of AN180 and UV6 (unpublished data).)

The uptake of proline was inhibited by both CCCP and Bu_3SnCl to a greater extent at pH 6.5 compared with pH 7.5. This would be consistent with a primary effect of both uncouplers on the ΔpH component of the electrochemical gradient of protons since ΔpH is close to zero at pH 7.5 [29]. However, measurement of ΔpH from the accumulation of [^{14}C]benzoic acid did not support this interpretation. Although the extents of inhibition of proline and benzoic acid uptake by CCCP in AN180 were similar, inhibition of the uptake of proline occurred in UV6 without an effect on ΔpH. Similarly, ΔpH was unaffected in both strains by Bu_3SnCl while proline uptake was inhibited. A further distinction between CCCP (or TCS) and Bu_3SnCl was seen when the effects of these uncouplers on the efflux of proline from strains AN180 and UV6 were compared. CCCP (or TCS) induced efflux of proline in both strains with a concentration dependency similar to that for the inhibition of proline uptake, whereas Bu_3SnCl was ineffective.

These results are more consistent with the existence of more than one site of action of uncouplers than with a common site of action involving the translocation of protons through the phospholipid bilayer. The existence of individual sites of action would permit the selection of uncoupler-resistant mutants with a specificity for a limited group of uncouplers. For example, the CCCP-resistant strain UV6 was more resistant to CCCP than to 2,4-dinitrophenol (unpublished data).

Further support for the presence of different sites of action of uncouplers comes from the uncoupler-dependent reversal of inhition of the phosphotransferase system. CCCP reversed the inhibition of methyl-α-D-glucoside uptake caused by respiratory chain-mediated oxidation of substrate in wild-type cells. This reversal was not observed in UV6 even at a concentration of CCCP sufficient to cause inhibition of proline uptake. This suggests that the target sites for CCCP-dependent inhibition of proline uptake and for reversal of inhibition of the phosphotransferase are different. The mechanism of inhibition of the phosphotransferase system is unclear. It is probably due to the generation of an energized state, which is inhibitory and which can be dissipated by uncouplers [26]. There is some evidence that specific dithiol-disulfide interconversions may be involved in this process [30]. Perhaps the target sites of the uncouplers are at this level since there is some evidence that both CCCP and Bu_3SnCl can react with sulfhydryl groups of proteins [31,32]. It seems less likely in the present case that the uncouplers were reacting with the transport carrier [33]. Reaction with concentrations of CCCP able to block uptake completely induced a rapid efflux of accumulated proline. Covalent modification of the transport carrier might also be expected to diminish the rate of exit via the carrier. Reaction with concentrations of Bu_3SnCl able to block uptake of proline, although not inducing efflux of proline, also did not prevent CCCP-induced efflux of this amino acid. CCCP-induced efflux would not have been expected if

Bu_3SnCl had reacted with the carrier to block proline uptake and efflux. Furthermore, the inhibitory effects of Bu_3SnCl were chloride-dependent and consistent with the proposed mechanism of hydroxyl-chloride ion exchange.

Our results showed a discrepancy between the effect of uncouplers on ΔpH and on proton uptake in proton pulse experiments with intact cells. For example, the uptake of protons was stimulated by the addition of Bu_3SnCl without ΔpH being affected. This result is understandable if the periplasmic space serves as a distinct compartment for protons and there is a rate-limiting movement of protons across the outer (and cytoplasmic) membrane. Under these conditions, the proton pulse experiments would be measuring the equilibration by the uncouplers of protons from the medium into the periplasmic space. This would not necessarily affect the observed ΔpH immediately, particularly if the protons were reacting with fixed anionic groups within the periplasm. A possible pool of protonatable anionic groups could be the membrane-derived oligosaccharides characterized by Kennedy [17]. Our finding that there was a decrease in the rate and extent of equilibration of protons by uncouplers as the level of membrane-derived oligosaccharide in the periplasmic space was diminished is consistent with this interpretation. Gould [34] pulsed anaerobic suspensions of intact *E. coli* with oxygen and observed that H^+/O ratios were much below the expected values. The addition of very low levels of uncoupler FCCP increased the magnitude of the H^+/O ratio whereas high concentrations diminished it. Since the protons are discharged across the cytoplasmic membrane in response to respiration induced by the oxygen pulse and yet are measured by the pH electrode in the medium outside of the cells, they have to traverse the periplasmic space and the outer membrane. Uncouplers in Gould's experiments could be facilitating the equilibration of protons between the periplasmic space and the outer medium.

It is perhaps surprising that the outer membrane appears to act as a barrier to protons since it contains pore-forming proteins. However, it is likely that these pores are 'gated' and so may not be open at all times [35]. An alternative explanation is that the membrane-derived oligosaccharide is so organized within the periplasmic space as not to readily exchange protons with the medium. Perhaps uncouplers could facilitate this exchange.

An implication of these experiments with respect to the chemiosmotic hypothesis is that the proton gradient of interest is between the cytoplasm and the components of the periplasmic space. Failure of these protons to enter the external medium would make the size of the gradient difficult to assess.

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