

## ATP Synthesis by an Uncoupler-resistant Mutant of *Bacillus megaterium*\*

(Received for publication, April 22, 1981)

Arthur A. Guffanti, Hal Blumenfeld, and Terry A. Krulwich‡

From the Department of Biochemistry, Mount Sinai School of Medicine, City University of New York, New York, New York 10029

ATP synthesis has been studied in starved whole cells of *Bacillus megaterium* and its uncoupler-resistant mutant derivative, C8. The latter strain was originally isolated and described by Decker and Lang (Decker, S. J., and Lang, D. R. (1977) *J. Biol. Chem.* 252, 5936-5938; (1978) *J. Biol. Chem.* 253, 6738-6743). In the present studies, ATP synthesis was examined as a function of the transmembrane electrical potential under conditions in which this potential was the sole component of the electrochemical proton gradient ( $\Delta\bar{\mu}_{H^+}$ ). Energization by respiration, with L-malate as substrate, was compared to energization by a valinomycin-induced potassium diffusion potential. Both strains synthesized ATP only at respiration-generated  $\Delta\bar{\mu}_{H^+}$  values above -30 mV. At -120 mV the ratio of the phosphorylation potential to the  $\Delta\bar{\mu}_{H^+}$  was 3-4 for both strains. As the  $\Delta\bar{\mu}_{H^+}$  was dissipated with low, increasing concentrations of uncoupler, ATP synthesis was inhibited in the wild type more profoundly than in C8. By contrast, when energization of ATP synthesis was mediated by a potassium diffusion potential, both strains exhibited little synthesis at  $\Delta\bar{\mu}_{H^+}$  values below -150 mV; C8 showed consistently poorer ATP synthesis than the wild type; there was no resistance of either strain to low concentrations of uncoupler; and the phosphorylation potential/ $\Delta\bar{\mu}_{H^+}$  ratios were consistently lower than observed during respiration.

The mutation in C8 appeared to be a single mutation, with a reversion frequency of  $1$  in  $8 \times 10^5$ . C8 exhibited lower ATPase activity and higher respiratory activity than the wild type. Taken together, the results are consistent with a model in which the energy form that is directly coupled to ATP synthesis is a relatively uncoupler-insensitive microscopic gradient that is produced during natural proton pumping. During establishment of artificial gradients, the uncoupler-sensitive bulk transmembrane gradient might predominate. The mutant strain C8 could possess an alteration in the ATPase so that it is even more selectively coupled than the wild type to the microscopic gradient.

Several years ago, Decker and Lang (1, 2) reported the isolation and characterization of mutant strains of *Bacillus megaterium* which exhibited L-malate-dependent ATP synthesis in the presence of carbonyl cyanide *m*-chlorophenylhy-

drazone. Under their experimental conditions, ATP synthesis by strain C8 at pH 7.4 was resistant to extraordinarily high concentrations of the uncoupler (up to 150  $\mu$ M); the transmembrane electrical potential<sup>1</sup> was reduced to "base-line level" by as little as 5  $\mu$ M CCCP. These findings posed a challenge to the chemiosmotic hypothesis (3, 4) which postulates that ATP synthesis via oxidative phosphorylation is energized by a transmembrane electrochemical gradient of protons ( $\Delta\bar{\mu}_{H^+}$ ) consisting of a  $\Delta$ pH (outside acid) and a  $\Delta\psi$  (outside positive). Uncouplers are posited to abolish these gradients by making the mitochondrial or bacterial cell membrane permeable to protons. Decker and Lang (2) observed that their CCCP-resistant strain had almost no ATPase (hydrolytic) activity. They proposed that the strain had a mutational change in the ATPase such that an uncoupler binding site, as well as hydrolytic activity, had been lost. They further suggested that ATP synthesis could be energized by a chemical intermediate produced during respiration; loss of the uncoupler binding site might facilitate utilization of this putative intermediate in the presence of CCCP.

Because of our interest in oxidative phosphorylation under conditions in which the electrochemical proton gradient is small (5, 6), we wondered whether ATP synthesis by Decker and Lang's mutant (2) might actually depend upon the  $\Delta\bar{\mu}_{H^+}$ , with the mutant able to use a gradient of smaller magnitude than its wild type parent. The results reported here suggest that the mutation in strain C8 indeed affects energy coupling. The mutant synthesizes ATP at slightly lower  $\Delta\bar{\mu}_{H^+}$  values than the wild type when energization is via respiration. Notably, the converse is true, *i.e.* C8 synthesizes less ATP at a given  $\Delta\bar{\mu}_{H^+}$  when energization is mediated by an artificially-induced diffusion potential.

### MATERIALS AND METHODS

**Organisms and Growth Conditions**—Wild type *B. megaterium* (ATCC19213) and its uncoupler-resistant derivative (C8) was used in all studies; the latter strain was generously provided by Dr. Dennis R. Lang, University of Cincinnati Medical School, Cincinnati, Ohio. The organisms were grown on the basal salts medium of Slepecky and Forster (7) as modified by Decker and Lang (8). The medium was supplemented with 10 mM D-fructose, 50 mM DL-malate, and 0.1% (w/v) yeast extract, all of which were added from separate sterile solutions. The organisms were grown with shaking at 200 rpm at 37 °C in a New Brunswick G25 rotary shaker. Growth was followed turbidometrically with a Klett-Summerson colorimeter (No. 42 filter).

**Measurement of ATP Synthesis**—For determinations of L-malate-driven ATP synthesis, cells of both strains were first depleted of ATP by starvation. Mid-logarithmic phase cultures were harvested by

\* This work was supported in part by Research Grant PCM7810213 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ A recipient of Research Career Development Award 5K04GM00020 from the National Institutes of Health.

<sup>1</sup> The abbreviations used are:  $\Delta\psi$ , transmembrane electrical potential; CCCP, carbonyl cyanide *m*-chloro phenylhydrazide; TPMP<sup>+</sup>, triphenylmethylphosphonium bromide; TPP<sup>+</sup>, tetraphenylphosphonium;  $\Delta\bar{\mu}_{H^+}$ , electrochemical proton gradient;  $\Delta$ pH, transmembrane pH gradient;  $\Delta$ Gp, phosphorylation potential.

centrifugation ( $20,000 \times g$ ), washed with and resuspended in 50 mM potassium phosphate buffer, pH 7.4, to 100 Klett units. At intervals, samples were removed from the cell suspensions for determinations of ATP content. Another set of samples from each suspension was incubated with 10 mM L-malate for 1 min at 37 °C with constant oxygenation before the ATP contents were measured. Conditions were sought for each strain such that the level of ATP in the starved cells was approximately 10% of that found after incubation with L-malate. This represented a reduction in cellular ATP from about 0.95 mM to 0.25 mM, which then rose above 2.0 mM upon incubation of either strain with L-malate. The depletion of ATP was accomplished by starving the wild type cells for 30 min, while C8 had to be starved for 60 to 90 min to achieve the same reduction in ATP. The initial and post-starvation ATP levels were monitored in every experiment.

The measurements of ATP were conducted on samples which were pipetted into 30% perchloric acid as described by Cole *et al.* (9) and neutralized with KOH. ATP was assayed by the luciferin-luciferase method using a Beckman LS-230 spectrometer, with the coincidence switch off, as described by Stanley and Williams (10). The assay, in our hands, was linear over at least a 100-fold range of ATP concentrations; a standard curve was conducted with each assay. ADP was determined, as described by Chapman *et al.* (11), by conversion to ATP using pyruvate kinase. The cell volume was determined to be 10  $\mu$ L/mg of cell protein, by the method of Stock *et al.* (12). Protein was determined by the method of Lowry *et al.* (13) using egg white lysozyme as the standard. Phosphate was assayed spectrophotometrically by the method of Fiske and SubbaRow (14). The phosphorylation potential was calculated from the formula  $\Delta G_p = \Delta G^\circ + RT \ln [ATP]/[ADP][P_i]$ . The value for  $\Delta G^\circ$ , assuming 0.2 M ionic strength and 37 °C, was 7.3 Kcal/mol (30.6 kJ/mol), taken from Rosing and Slater (15).

**Assay of ATP Hydrolysis**—ATPase activity was assayed in membrane preparations as described by Decker and Lang (8), except that inorganic phosphate was determined by the method of Fiske and SubbaRow (14).

**Respiratory Rates**—Logarithmically growing cells of the wild type or C8 were washed and resuspended in 50 mM potassium phosphate buffer, pH 7.4, as described above. The rate of oxygen consumption by whole cells was measured in the presence of 3 mM L-malate with a Yellow Springs Instrument Model 53 Clark-type oxygen monitor as described previously (16).

**Determinations of  $\Delta pH$  and  $\Delta \psi$** —The  $\Delta pH$  was measured by the distribution of either the weak acid, [ $^{14}C$ ]5,5-dimethyl-2,4-oxazolidinedione (55  $\mu$ M), or the weak base, [ $^{14}C$ ]methylamine (27  $\mu$ M), in a flow dialysis assay (5, 17). In a variation of the method of Friedberg and Kaback (18), 50 mM potassium phosphate buffer, pH 5.5 or 7.4, was pumped through the dialysis chamber. After the radioactive probe was added and allowed to equilibrate, a cell suspension was added to the upper chamber (to a final concentration of 1.5 mg of cell protein/ml) along with 10 mM L-malate. The suspension was stirred and aerated as described previously (5). Samples were collected and the radioactivity was measured by scintillation spectrometry. Controls and calculations were performed as previously described (5).

The  $\Delta \psi$  was determined from the distribution of 5  $\mu$ M [ $^3H$ ]TPP $^+$  or 6.25  $\mu$ M [ $^3H$ ]TPMP $^+$  using a filtration assay (19). Washed cells were incubated at 37 °C in an appropriate buffer (usually 50 mM potassium phosphate, pH 7.4) with the radioactive probe under constant oxygenation. Samples were removed at intervals and filtered through Millipore cellulose acetate filters (type EH, 0.5  $\mu$ m pore size). The steady state level of accumulation was determined. Respiration-generated  $\Delta \psi$  values were consistently 20 to 25 mV lower when calculated from TPMP $^+$  distribution than when calculated from TPP $^+$  distribution. For experiments in which ATP synthesis and  $\Delta \psi$  measurements were conducted on the same cell preparations, the determinations were always made simultaneously on the same suspensions. Controls were included in which nonradioactive  $\Delta \psi$  probes were present during the incubations to be used for ATP measurements. The probes had no effect under these conditions.

An additional methodological control was conducted, in which the  $\Delta \psi$  values calculated from TPP $^+$  and TPMP $^+$  distribution were compared to the results obtained from a determination of  $^{86}RbCl$  uptake in the presence of valinomycin. For this set of experiments, cells were suspended in 50 mM sodium phosphate buffer, pH 7.4. The distribution of  $^{86}Rb^+$  was assayed using a flow dialysis procedure (20). The  $^{86}Rb^+$  (50  $\mu$ M) was added to the dialysis chamber. The cells were then added, together with 10 mM L-malate and 10  $\mu$ M valinomycin. The values for the  $\Delta \psi$  were calculated from the Nernst relationship as described by Schuldiner and Kaback (19). Average  $\Delta \psi$  values calcu-

lated for washed, unstarved wild type cells, incubated with 10 mM L-malate in sodium phosphate buffer, were: (a)  $-119 \pm 8$  mV using TPMP $^+$ ; (b)  $-147 \pm 14$  mV using TPP $^+$ ; and (c)  $-139 \pm 8$  mV using  $RbCl$ .

**Generation of a Valinomycin-induced Potassium Diffusion Potential**—Logarithmically-growing cells of the wild type or C8 were washed and resuspended in 50 mM potassium phosphate buffer, pH 7.4, to a concentration of 15 to 20 mg of cell protein/ml. The suspension was incubated with 1 mM NaCN for 20 min at 37 °C. The cells were then rapidly diluted at 37 °C either 1000- or 300-fold into 50 mM sodium phosphate buffer, pH 7.4, containing 10  $\mu$ M valinomycin and 1 mM NaCN. The magnitude of the diffusion potential was varied by introducing different amounts of potassium phosphate, pH 7.4, into the dilution buffer. Initially, we attempted to establish the diffusion potential by preincubating cells in high potassium-containing buffer with valinomycin. This approach was unsuccessful with *B. megaterium* because of an extremely rapid efflux of potassium upon dilution into sodium-containing buffer. Similarly, protocols used by others for establishing diffusion potentials in *Escherichia coli* (21) and *Streptococcus lactis* (22) were not applicable here. When cells of *B. megaterium* were incubated in potassium-free buffer, there was too much leakage of intracellular potassium to establish a large diffusion potential upon subsequent addition of valinomycin. The intracellular potassium concentration in unstarved cells of the wild type or C8, as measured with a Beckman KLiNa flame photometer (5), was as high as 600 mM. After a brief starvation in 50 mM potassium phosphate buffer, the internal potassium concentration dropped to between 300 to 350 mM. In view of these fluctuations, we routinely measured the actual intracellular potassium concentrations for purposes of calculating the theoretical magnitude of the diffusion potential for a given experiment. Kashket *et al.* (23) have shown a direct relationship in *S. lactis* cells between the  $\Delta \psi$  calculated from  $[K^+]_{in}/[K^+]_{out}$  and the  $\Delta \psi$  determined by TPP $^+$  distribution; Shioi *et al.* (24) have shown similar correspondence between calculated values and those obtained from TPMP $^+$  distribution up to  $-120$  mV in *Bacillus subtilis*. A similar relationship was found, between  $-75$  and  $-150$  mV, in *B. megaterium* when the  $\Delta \psi$  calculated from  $[K^+]_{in}/[K^+]_{out}$  was compared to the TPP $^+$  or TPMP $^+$  distributions for the same experiments (Fig. 1). Above  $-150$  mV, there was a deviation from linearity, with an apparent failure of the probes to reflect the total  $\Delta \psi$ . There was no significant difference between the  $\Delta \psi$  values measured by TPP $^+$  and TPMP $^+$  in this protocol, in contrast to the results with respiration-induced potentials.

**Chemicals**—Valinomycin, luciferin-firefly tails, ATP (disodium salt), ADP, sodium ascorbate, and CCCP were purchased from Sigma. [ $^{14}C$ ]methylamine hydrochloride (52.2 mCi/mmol),  $^{86}RbCl$  (2 mCi/mmol), [ $^3H$ ]5,5-dimethyl-2,4-oxazolidinedione (8.8 mCi/mmol), and [ $^3H$ ]TPMP $^+$  were obtained from New England Nuclear. [ $^3H$ ]TPP $^+$

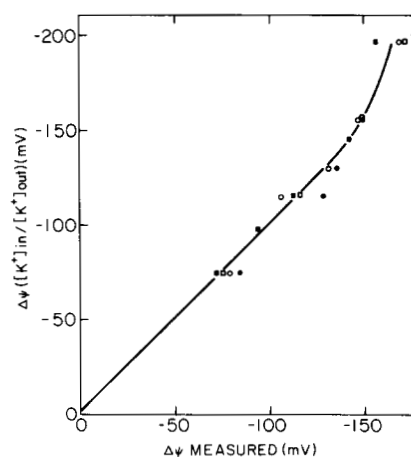


FIG. 1. The relationship between the  $\Delta \psi$  measured using TPP $^+$  and TPMP $^+$  uptake and that calculated for valinomycin-induced potassium diffusion potentials. Cells of the wild type *B. megaterium* ( $\square$ ,  $\blacksquare$ ) or C8 ( $\circ$ ,  $\bullet$ ) were starved in the presence of 1 mM cyanide. The value for  $\Delta \psi$  ( $[K^+]_{in}/[K^+]_{out}$ ) was calculated from the Nernst equation by varying the external  $K^+$  concentration. Intracellular  $K^+$  was determined by flame photometry. The  $\Delta \psi$  was also measured by the steady state distribution of 5  $\mu$ M TPP $^+$  (open symbols) and 6.25  $\mu$ M TPMP $^+$  (closed symbols).

(2.5 Ci/mmol) was the generous gift of Dr. H. R. Kaback (Roche Institute of Molecular Biology, Nutley, NJ).

## RESULTS

**Bioenergetic Characteristics of *B. megaterium* and Its Derivative Strain C8**—As part of an initial characterization of the experimental system, the magnitude of the  $\Delta\bar{\mu}_{H^+}$ , the respiratory rates, and the ATPase activities of the two strains were compared. For determinations of the  $\Delta\bar{\mu}_{H^+}$ , cells of each strain were harvested during logarithmic growth, washed with and suspended in buffer containing 10 mM L-malate. At both pH 5.5 and 7.4, C8 exhibited a  $\Delta\bar{\mu}_{H^+}$  that was slightly higher than that of the wild type strain (Table I). At pH 5.5, the major component of the  $\Delta\bar{\mu}_{H^+}$  was the  $\Delta pH$ , whereas at pH 7.4, the  $\Delta\bar{\mu}_{H^+}$  consisted entirely of a  $\Delta\psi$ . These results differ only a little from the values reported by Decker and Lang (2), who found a small  $\Delta pH$  at pH 7.4 using a different assay procedure. The data shown here are quite similar to those reported by Shioi *et al.* (24) for cells of *B. subtilis*.

Membrane preparations from the two strains differed in ATPase activity as described by Decker and Lang (2) except that the wild type had somewhat less activity under our conditions; the specific activities were 0.16 and 0.04  $\mu\text{mol P}_i$  released/min/mg of protein for the wild type and C8, respectively. The respiratory rates of whole cells, assayed by oxygen consumption, also corresponded to previously reported values (2), 916 natoms 0/min/mg of cell protein for the wild type and a higher value of 1663 natoms 0/min/mg of cell protein for C8.

The frequency of spontaneous reversion was determined by plating large numbers of C8 cells on the standard growth

medium, without yeast extract, and solidified with agar. On this medium, C8 produces reproducibly smaller colonies than the wild type. On this basis, a reversion frequency of one in  $8 \times 10^5$  cells was calculated. Three different revertant strains were isolated. The strains showed ATPase activities (0.16, 0.15, and 0.16  $\mu\text{mol P}_i$ /min/mg of protein) and respiratory rates (922, 984, and 915 natoms 0/min/mg of protein) that were essentially the same as those of the wild type. Moreover, they regained the wild type sensitivity to CCCP (see below).

**L-Malate-dependent ATP Synthesis and the Effect of CCCP**—In preliminary experiments, several *B. megaterium* mutant strains were newly isolated on plates containing 5  $\mu\text{M}$  CCCP. These strains failed to exhibit L-malate-dependent ATP synthesis in the presence of concentrations of CCCP in excess of 5–10  $\mu\text{M}$ . Nor did starved cells of C8 exhibit L-malate-dependent ATP synthesis when more than 10  $\mu\text{M}$  CCCP was present, under our conditions. These conditions differed from those of the original investigators of this strain by: inclusion of a small amount of yeast extract in the medium to achieve comparable growth rates of the two strains; use of constant oxygenation during ATP synthesis and  $\Delta\psi$  measurements; resuspension of the cell pellet during washing procedures; and the use of considerably less dense cell suspensions (0.05 to 0.08 mg of protein/ml). Importantly, while resistance to very high CCCP concentrations was not demonstrable under our conditions, a reproducible CCCP-resistance of C8, relative to the wild type, was documented at concentrations of CCCP from 1 to 5  $\mu\text{M}$ . As shown in Table II, starved whole cells of the wild type and C8 exhibited comparable  $\Delta\psi$  values and levels of steady state ATP synthesis when incubated with L-malate in the absence of CCCP. One-min incubations for the ATP measurements were employed, after initial experiments indicated that a steady state was achieved by this time. With increasing concentrations of CCCP, from 1 to 5  $\mu\text{M}$ , the  $\Delta\psi$  declined in both strains, with the wild type showing a somewhat greater sensitivity to the protonophore. Interestingly, assays using  $\text{TPP}^+$  resulted in consistently higher estimates of  $\Delta\psi$  than assays using  $\text{TPMP}^+$ , although the patterns were the same; there had been no significant difference between the two probes in assays of the magnitude of valinomycin-induced  $\text{K}^+$  diffusion potentials (Fig. 1). With increasing concentrations of CCCP, ATP synthesis by both strains was inhibited, but the inhibition was more pronounced with the wild type than with C8. This relative resistance was even manifest in the  $\Delta G_p$  values, especially at 5  $\mu\text{M}$  CCCP, in which the logarithmic function compresses the magnitude of the

TABLE I

*The  $\Delta\bar{\mu}_{H^+}$  generated by wild type and C8 strains of *B. megaterium**

Cells of the two strains were harvested during logarithmic growth, washed and suspended in 50 mM potassium phosphate buffer, pH 5.5 or 7.4, plus 10 mM L-malate. The  $\Delta\psi$  was determined from  $\text{TPP}^+$  accumulation and the  $\Delta pH$  was assayed by the distribution of 5,5-dimethyl-2,4-oxazolidinedione or methylamine in a flow dialysis assay as described under "Materials and Methods."  $\Delta\bar{\mu}_{H^+}$  is the sum of  $\Delta pH$  and  $\Delta\psi$ .

External pH	Wild Type			C8		
	$\Delta\psi$	$\Delta pH$	$\Delta\bar{\mu}_{H^+}$	$\Delta\psi$	$\Delta pH$	$\Delta\bar{\mu}_{H^+}$
	mV			mV		
5.5	-72	-98	-170	-81	-111	-192
7.4	-146	0	-146	-156	0	-156

TABLE II

*The effect of CCCP on L-malate-dependent ATP synthesis in *B. megaterium* wild type and C8 strains*

Washed cells of the two strains were suspended in 50 mM potassium phosphate buffer, pH 7.4, at 0.05 to 0.08 mg of cell protein/ml. They were starved, as described under "Materials and Methods," until the cellular ATP levels were approximately 0.25 mM; the precise level was determined in each experiment. ATP synthesis by the starved cells was initiated by the addition of 10 mM L-malate. Where indicated, CCCP was added as an ethanolic solution. The suspensions were incubated for 1 min with constant oxygenation. ATP was measured by the luciferin-luciferase assay; the background values of ATP remaining in starved cells (in the absence of added L-malate) were

subtracted. The  $\Delta\psi$  was calculated from the distribution of either  $\text{TPP}^+$  or  $\text{TPMP}^+$  using the same cell preparations as for the ATP synthesis. The  $\Delta G_p$  (in mV) was calculated assuming an  $\text{H}^+$ /ATP stoichiometry of unity. The average intracellular ADP was 5.2 mM and 5.0 mM in starved cells of the wild type and C8, respectively. The decrease in ADP concentration correlated with the ATP formed. The intracellular concentration of inorganic phosphate in both organisms was between 45 and 50 mM. The values are the average of more than ten separate determinations.

CCCP concentration	Wild type					C8				
	ATP synthesized	$\Delta G_p$	$\Delta\psi$			ATP synthesized	$\Delta G_p$	$\Delta\psi$		
			$\text{TPP}^+$	$\text{TPMP}^+$				$\text{TPP}^+$	$\text{TPMP}^+$	
$\mu\text{M}$	$\text{mM}$	$\text{mV}$		$\text{mV}$		$\text{mM}$	$\text{mV}$		$\text{mV}$	
0	2.08	-388	-114	-90		2.61	-398	-120	-98	
1	1.07	-362	-78	-52		2.30	-392	-85	-60	
2	0.50	-340	-60	-38		1.77	-380	-62	-53	
5	0.12	-244	-38	-33		0.85	-356	-51	-45	

difference in the ATP concentrations. The three revertants of C8 all exhibited the wild type pattern of CCCP sensitivity. For example, using the same protocol as in Table II, the steady state concentrations of ATP upon incubation in the presence of L-malate and 2  $\mu\text{M}$  CCCP were: 0.50 mM in the wild type; 1.77 mM in C8; and 0.49, 0.44, and 0.51 mM in the three revertant strains.

The data from Table II can be used to assess the relationship between the  $\Delta G_p$  and the  $\Delta\bar{\mu}_{H^+}$  (which is equal to the  $\Delta\psi$  at pH 7.4). The ratio of these steady state potentials is shown as a function of the magnitude of the  $\Delta\bar{\mu}_{H^+}$  in Fig. 2. In both strains, the highest  $\Delta\bar{\mu}_{H^+}$  values observed upon incubation with L-malate were  $-110$  to  $-120$  mV. At these  $\Delta\bar{\mu}_{H^+}$  values, the  $\Delta G_p/\Delta\bar{\mu}_{H^+}$  ratio was between 3 and 4 for both strains. At lower values of the  $\Delta\bar{\mu}_{H^+}$  (increasing CCCP concentrations), there was a significant rise in the  $\Delta G_p/\Delta\bar{\mu}_{H^+}$  ratio. This rise was steeper in C8 than in the wild type, reflecting more L-malate-dependent ATP synthesis at a given low  $\Delta\bar{\mu}_{H^+}$ .

Although data will not be shown, it was important that qualitatively similar differences between the two strains were demonstrable using 2 mM 2,4-dinitrophenol or 1–2  $\mu\text{M}$  gramicidin instead of CCCP, or using ascorbate (10 mM) plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (1 mM) instead of L-malate. The latter experiments were affected by apparent cytotoxic effects of the electron donor, but the pattern of CCCP resistance was the same as that observed with L-malate.

**ATP Synthesis in Response to a Valinomycin-induced  $K^+$  Diffusion Potential**—If a bulk transmembrane electrochemical gradient is directly coupled to oxidative ATP synthesis, then synthesis should be energized to the same extent by a  $\Delta\psi$  of a given magnitude whether that  $\Delta\psi$  is produced by respiration or by artificially induced ion fluxes. Therefore, we studied ATP synthesis by starved cells of the wild type and C8 upon generation of a valinomycin-induced  $K^+$  diffusion potential. In most of the experiments, the same cell preparations were used for experiments with L-malate and with valinomycin; the ionic composition of the buffer and the inclusion of cyanide in diffusion potential experiments were the only differences in incubation conditions. The results obtained from the two modes of energization, however, were strikingly different. This is first illustrated by time courses of ATP synthesis by the two strains upon generation of diffusion potentials of various magnitudes (Fig. 3). The wild type strain synthesized appreciable amounts of ATP upon energization by a diffusion potential of  $-200$  mV (*top curve*, Fig. 3A) and much lower amounts at  $-150$  and  $-110$  mV (*second and third curves from top*, Fig. 3A). It should be noted that at  $-110$  mV, produced by a diffusion potential, a cellular ATP concen-

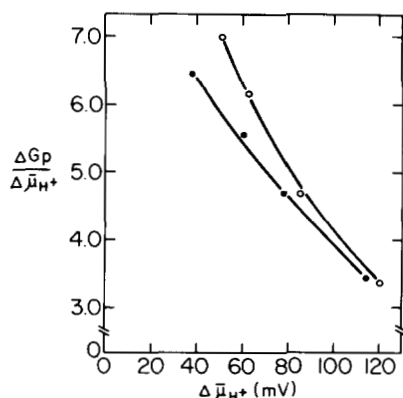


FIG. 2. The  $\Delta G_p/\Delta\bar{\mu}_{H^+}$  ratio as a function of the magnitude of the  $\Delta\bar{\mu}_{H^+}$  generated during respiration. The data are taken from Table II. The  $\Delta\bar{\mu}_{H^+}$  is the value calculated from the  $\text{TPP}^+$  distribution.

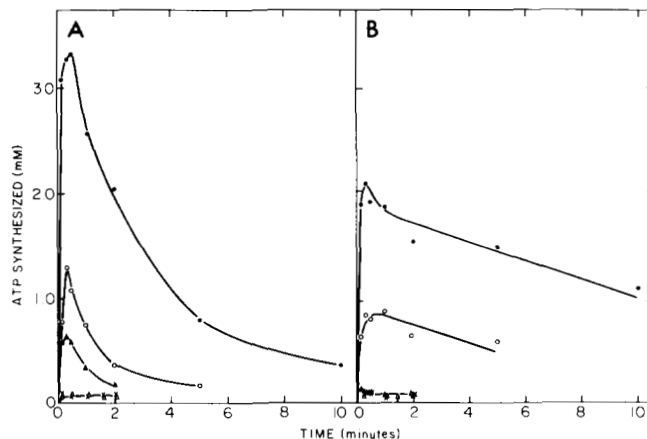


FIG. 3. ATP synthesis energized by valinomycin-induced diffusion potentials of different magnitudes and the effect of CCCP. Cells of the wild type (A) or C8 (B) were starved in the presence of cyanide as described under "Materials and Methods." The size of the potassium diffusion potential was determined by diluting 300-fold into the following buffers at pH 7.4 in the presence of 10  $\mu\text{M}$  valinomycin: 1) 50 mM  $\text{Na}^+$  phosphate ( $\bullet$ ); 2) 49 mM  $\text{Na}^+$  phosphate and 1 mM  $\text{K}^+$  phosphate ( $\circ$ ); 3) 45 mM  $\text{Na}^+$  phosphate and 5 mM  $\text{K}^+$  phosphate ( $\blacktriangle$ ); and 4) 50 mM  $\text{K}^+$  phosphate ( $\times$ ). In addition, cells were diluted in the presence of 10  $\mu\text{M}$  valinomycin into 50 mM  $\text{Na}^+$  phosphate that contained 2  $\mu\text{M}$  CCCP ( $\Delta$ ).

tration of 0.65 mM was observed, as compared to 2.08 mM ATP found during generation of a  $\Delta\psi$  of  $-114$  mV by respiration (Table II). Furthermore, essentially no synthesis of ATP occurred in response to diffusion potentials smaller than  $-50$  mV or to a potential of  $-200$  mV in the presence of 2  $\mu\text{M}$  CCCP (Fig. 3A). The cellular ATP concentration of wild type cells declined rapidly after synthesis, in a manner that correlated with the decline in the  $\Delta\psi$  (data not shown).

In the mutant strain C8, the amount of ATP synthesized in response to comparable diffusion potentials was much lower than in the wild type. Thus at  $-200$  mV, C8 produced ATP concentrations of 2.1 mM, less than that produced by C8 during respiration at a  $\Delta\psi$  of  $-120$  mV (Table II) and less than the 3.3 mM ATP produced by the wild type at a  $-200$  mV diffusion potential (Fig. 3). At a diffusion potential of  $-110$  mV, C8 exhibited virtually no synthesis of ATP (Fig. 3B), even though ATP was synthesized at much lower respiration-generated  $\Delta\psi$  value. Moreover, no resistance of ATP synthesis by C8, at a  $-200$  mV diffusion potential, was observed at 2  $\mu\text{M}$  CCCP. The decline of ATP after synthesis by C8 was considerably slower than in the wild type; this correlates with the lower hydrolytic ATPase activity of the mutant strain.

The synthesis of ATP as a function of the magnitude of the diffusion potential is graphically illustrated in Fig. 4. The wild type synthesized ATP at lower diffusion potentials and also synthesized more ATP at given  $\Delta\psi$  values between  $-110$  and  $-200$  mV. The *inset* to Fig. 4 is a presentation of the  $\Delta G_p/\Delta\psi$  ratio as a function of the  $\Delta\psi$ . Interestingly, these ratios are generally lower than those obtained in experiments using respiration-generated gradients; in fact, the highest ratio in this series is slightly below 3.0, whereas the lowest ratio in the other experimental series is higher than 3.0. At  $-200$  mV diffusion potentials, both strains exhibited  $\Delta G_p/\Delta\bar{\mu}_{H^+}$  ratios quite close to 2 (Fig. 4, *inset*).

In a small number of experiments, ATP synthesis was energized by an artificially-induced pH gradient, produced by a sudden addition of a predetermined aliquot of HCl to the suspending medium. The relative patterns of ATP synthesis by the two strains were the same as observed with the valinomycin-induced  $K^+$  diffusion potential; this included an ab-



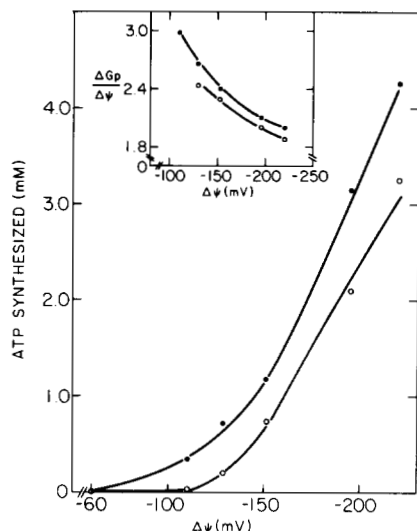


FIG. 4. The relationship between the amount of ATP synthesized and the magnitude of the diffusion potential. Cell suspensions of the wild type (●) or C8 (○) were starved and incubated with cyanide as indicated under "Materials and Methods." Valinomycin-induced potassium diffusion potentials of different magnitudes were established by varying the external  $K^+$  concentration upon dilution of the cell suspensions. The  $\Delta\psi$  value was obtained from the Nernst equation by inserting the values of  $[K^+]_{in}/[K^+]_{out}$ . The amount of ATP synthesized was determined by removing samples at various times after dilution and performing the luciferin-luciferase assay. Care was taken to ascertain peak values of ATP synthesis (see Fig. 3), and background values for cellular ATP (in the absence of a diffusion potential) were subtracted. *Inset*, the relationship between  $\Delta Gp/\Delta\psi$  and  $\Delta\psi$ . The values for ATP synthesis in the wild type (●) and C8 (○) were used to calculate  $\Delta Gp$ , using 5 mM as the starting concentration of ADP in energized cells, and 45 mM as the intracellular concentration of inorganic phosphate. All values represent the average of at least five separate experiments.

sence of ATP synthesis by either strain in the presence of 1–2  $\mu M$  CCCP (data not shown).

#### DISCUSSION

Mitchell proposed that a proton-translocating ATPase couples the  $\Delta\bar{\mu}_{H^+}$  to the synthesis (and hydrolysis) of ATP (3). Assuming that 2 protons are translocated for each ATP formed, he calculated that a  $\Delta\bar{\mu}_{H^+}$  of  $-210$  mV would be required to maintain an  $[ATP]/[ADP]$  ratio of unity when the concentration of  $P_i$  is 10 mM (3, 25). It is inherent in the original formulation of the chemiosmotic hypothesis by Mitchell (3) that the  $\Delta Gp$  bear a direct relationship to the  $\Delta\bar{\mu}_{H^+}$  (26). Thus if ATP synthesis is completely coupled to the  $\Delta\bar{\mu}_{H^+}$ , the  $\Delta Gp$  should approach equilibrium with the  $\Delta\bar{\mu}_{H^+}$ .

Evidence from several different experimental systems, all using artificially-generated gradients, documents a requirement for a  $\Delta\bar{\mu}_{H^+}$  greater than  $-150$  mV for substantial ATP synthesis; results with some of these systems indicate a consistent correlation between the  $\Delta Gp$  and  $\Delta\bar{\mu}_{H^+}$ , and a  $\Delta Gp/\Delta\bar{\mu}_{H^+}$  ratio close to 2. For example, the experiments reported here, using a valinomycin-induced  $K^+$  diffusion potential, indicate a distinct requirement for at least  $-150$  mV for substantial ATP synthesis and a  $\Delta Gp/\Delta\bar{\mu}_{H^+}$  ratio of 2 (at the highest  $\Delta\psi$  values) up to 2.4 (or 3 in C8), over the range of  $\Delta\psi$  values at which synthesis occurred. These data, then, are consistent with a model in which the  $\Delta\bar{\mu}_{H^+}$  is directly coupled to the ATP synthesis, with a threshold of  $-150$  mV, and an  $H^+/ATP$  stoichiometry that varies slightly with  $\Delta\psi$  from 2–2.4 for the wild type and 2–3 for C8. Using submitochondrial

particles, Thayer and Hinkle (27) demonstrated that optimal ATP synthesis occurred with artificially generated  $\Delta\bar{\mu}_{H^+}$  values of about  $-265$  mV. Similarly, Sone *et al.* (28) found a requirement for at least  $-205$  mV for ATP synthesis by reconstituted ATPase ( $TF_0F_1$ ) from thermophilic bacterium PS3. In experiments with whole cells of both *E. coli* (29) and *S. lactis* (30), a gated response of ATP synthesis to artificially-generated  $\Delta\bar{\mu}_{H^+}$  values has been shown at  $-175$  to  $-200$  mV.

On the other hand, a multitude of reports on many different eukaryotic and prokaryotic systems contain disparities between the values of the  $\Delta Gp$  and  $\Delta\bar{\mu}_{H^+}$ , and exhibit  $\Delta Gp/\Delta\bar{\mu}_{H^+}$  ratios that are very high or very variable. Interestingly, these are generally reports of experiments in which the  $\Delta\bar{\mu}_{H^+}$  was generated by natural proton pumps, *e.g.* the respiratory chain, photosystems, or bacteriorhodopsin. Thus, in the current work, the  $\Delta Gp/\Delta\bar{\mu}_{H^+}$  ratios were consistently higher when energization was by L-malate than when a diffusion potential was used. Using L-malate, moreover, the  $\Delta Gp/\Delta\bar{\mu}_{H^+}$  ratios varied over a larger range (3.3 to 7.0), and were higher for C8 than for the wild type, especially at low  $\Delta\bar{\mu}_{H^+}$  values. While no ATP synthesis was observed at  $\Delta\bar{\mu}_{H^+}$  values below  $-30$  mV, there was no apparent threshold in the  $-150$  to  $-200$  mV range. These data are thus markedly different from those observed with the artificially generated gradients. Notably, strain C8 appeared to utilize respiration-generated gradients more efficaciously, and artificial gradients less efficaciously, than the wild type. In mitochondria (31, 32), submitochondrial particles (33, 34), chloroplasts (35), and bacteria such as *Paracoccus denitrificans* (36, 37), *Halobacterium halobium* (38), and *Bacillus alcalophilus* (6),  $\Delta\bar{\mu}_{H^+}$  values derived from natural pumps did not bear a constant relationship to observed  $\Delta Gp$  values or show a high threshold for synthesis. For example, Azzone *et al.* (31) observed very high  $\Delta Gp/\Delta\bar{\mu}_{H^+}$  ratios (up to 18) in mitochondria at  $\Delta\bar{\mu}_{H^+}$  values of about  $-30$  mV, and  $\Delta Gp/\Delta\bar{\mu}_{H^+}$  ratios of 2–3 at much higher  $\Delta\bar{\mu}_{H^+}$  values; these results are similar in pattern to those found here with respiring *B. megaterium* strains. Moreover, Azzone *et al.* (31) found that the decline in the  $\Delta Gp$  upon partial dissipation of the  $\Delta\bar{\mu}_{H^+}$  depended upon the specific agent used for the dissipation rather than solely on the magnitude of the residual  $\Delta\bar{\mu}_{H^+}$ . Similar findings with inhibitors, synthesis of ATP at low  $\Delta\bar{\mu}_{H^+}$  values, and variability in the  $\Delta Gp/\Delta\bar{\mu}_{H^+}$  ratio are reported in the other references cited.

Several modifications of the original chemiosmotic formulation have been suggested to account for disparities of the type found in this and other studies. The  $H^+/ATP$  stoichiometry is, for example, controversial and may even be variable, as proposed for the proton-translocating ATPase of *Neurospora crassa* (39). While a variable  $\Delta\bar{\mu}_{H^+}$ -dependent  $H^+/ATP$  stoichiometry could account for a variable  $\Delta Gp/\Delta\bar{\mu}_{H^+}$  ratio, it could not easily explain the difference found between energization by artificial gradients *versus* natural pumps or between dissipation of a  $\Delta\bar{\mu}_{H^+}$  by different inhibitors (as in Ref. 31). Therefore, several investigators have suggested that the bulk transmembrane gradient, the  $\Delta\bar{\mu}_{H^+}$  as usually measured, may not be the gradient that is directly coupled to ATP synthesis. Williams (40) and Gould and Cramer (41) have proposed that there may be an intramembranal circulation of protons. Rottenberg (42), Gould (43), Van Dam *et al.* (32), Kell (44), and even Mitchell (45) have, in somewhat different ways, posited the existence of functional "microscopic" or "localized" gradients. A microscopic gradient might be the direct product of natural proton pumps and the form of energy that is directly coupled to ATP synthesis. This gradient might not be highly susceptible to dissipation by CCCP (38). The bulk transmembrane gradient, the highly CCCP-sensitive

$\Delta\bar{\mu}_{H^+}$  that is measured experimentally, might be a smaller or different gradient, formed secondarily during natural proton pumping. By contrast, during establishment of artificial transmembrane gradients, the bulk  $\Delta\bar{\mu}_{H^+}$  might be the predominant or only form produced. This  $\Delta\bar{\mu}_{H^+}$  could then energize ATP synthesis only to the extent that a small microscopic component is also formed or that a very large bulk gradient itself can be used.

The results obtained here with wild type and C8 strains of *B. megaterium* can be accommodated by this type of model. The  $\Delta G_p/\Delta\bar{\mu}_{H^+}$  ratios are highest at low  $\Delta\bar{\mu}_{H^+}$  values, i.e. under conditions in which the bulk gradient may have been dissipated much more than an effective microscopic gradient. Both strains exhibit higher  $\Delta G_p$  values at given  $\Delta\bar{\mu}_{H^+}$  values when energization is via respiration than by a diffusion potential. Moreover, CCCP sensitivity is much greater during energization of ATP synthesis by diffusion potentials or pH drops (1) than by respiration. The mutation in C8 could be a change in the ATPase which lowers its hydrolytic activity and which is, importantly, a mutation affecting energy coupling. The mutant strain is even more selective than the wild type in its preference for a respiration-derived (microscopic?) gradient rather than an artificially generated (solely or largely bulk transmembrane?) gradient. The mutation in C8 appears to be a single mutation, and we would speculate that the higher respiratory rates of C8 relative to the wild type could also be a reflection of its tighter coupling to a microscopic gradient, such that a release of some respiratory control occurs.

Future work will include detailed studies of the  $F_0F_1$  ATPases in the two strains, so that the mutation may be characterized biochemically. It will also be of interest to ascertain whether specific reagents can be used to probe putative microscopic gradients. The feasibility of this approach is suggested by results such as those of Azzone *et al.* (31), in which FCCP and nigericin altered the  $\Delta G_p/\Delta\bar{\mu}_{H^+}$  ratio to different extents, and the finding here that TPP<sup>+</sup> and TPMP<sup>+</sup> gave consistently similar measurements of diffusion potentials but not of respiration-induced potentials.

Finally, it is of interest to note that if ATP synthesis is indeed energized directly by some microscopic gradient, then its direct energy source may not be identical with that which functions in the energization of other respiration-dependent membrane processes, e.g. many solute transport systems. Considerable evidence indicates that the bulk transmembrane  $\Delta\bar{\mu}_{H^+}$  directly energizes H<sup>+</sup>/solute symport systems (e.g. 46–49). Possibly, the utilization of somewhat distinct gradients that are only partially in equilibrium results in the insurance of preferred utilization of respiration-derived energy for ATP synthesis.

## REFERENCES

- Decker, S. J., and Lang, D. R. (1977) *J. Biol. Chem.* **252**, 5936–5938
- Decker, S. J., and Lang, D. R. (1978) *J. Biol. Chem.* **253**, 6738–6743
- Mitchell, P. (1966) *Biol. Rev. Camb. Philos. Soc.* **41**, 445–502
- Mitchell, P., and Moyle, J. (1967) *Biochem. J.* **104**, 144–148
- Guffanti, A. A., Susman, P., Blanco, R., and Krulwich, T. A. (1978) *J. Biol. Chem.* **253**, 708–715
- Guffanti, A. A., Bornstein, R. F., and Krulwich, T. A. (1981) *Biochim. Biophys. Acta* **635**, 619–630
- Slepecky, R., and Foster, J. W. (1959) *J. Bacteriol.* **78**, 117–123
- Decker, S. J., and Lang, D. R. (1977) *J. Bacteriol.* **131**, 98–104
- Cole, H., Wimpenny, J. W. T., and Hughes, D. E. (1967) *Biochim. Biophys. Acta* **143**, 445–453
- Stanley, P. E., and Williams, S. G. (1969) *Anal. Biochem.* **29**, 381–392
- Chapman, A. G., Fall, L., and Atkinson, D. E. (1971) *J. Bacteriol.* **108**, 1072–1086
- Stock, J. B., Rauch, B., and Roseman, S. (1977) *J. Biol. Chem.* **252**, 7850–7861
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Fiske, C. H., and SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- Rosing, J., and Slater, E. C. (1972) *Biochim. Biophys. Acta* **267**, 275–290
- Lewis, R. J., Belkina, S., and Krulwich, T. A. (1980) *Biochem. Biophys. Res. Commun.* **95**, 857–863
- Ramos, S., Schuldiner, S., and Kaback, H. R. (1979) *Methods Enzymol.* **55**, 680–688
- Friedberg, I., and Kaback, H. R. (1980) *J. Bacteriol.* **142**, 651–658
- Schuldiner, S., and Kaback, H. R. (1975) *Biochemistry* **14**, 5451–5461
- Ramos, S., Schuldiner, S., and Kaback, H. R. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 1892–1896
- Wilson, D. M., Alderete, J. F., Maloney, P. C., and Wilson, T. H. (1976) *J. Bacteriol.* **126**, 327–337
- Kashket, E. R., and Wilson, T. H. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70**, 2866–2869
- Kashket, E. R., Blanchard, A. G., and Metzger, W. C. (1980) *J. Bacteriol.* **143**, 128–134
- Shioi, J.-I., Matsuura, S., and Imae, Y. (1980) *J. Bacteriol.* **144**, 891–897
- Greville, G. D. (1969) *Curr. Top. Bioenerg.* **3**, 1–77
- Slater, E. C. (1979) *Methods Enzymol.* **55**, 235–245
- Thayer, W. S., and Hinkle, P. C. (1975) *J. Biol. Chem.* **250**, 5330–5335
- Sone, N., Yoshida, M., Hirata, H., and Kagawa, Y. (1977) *J. Biol. Chem.* **252**, 2956–2960
- Maloney, P. C. (1978) *Biochem. Biophys. Res. Commun.* **83**, 1496–1501
- Maloney, P. C. (1977) *J. Bacteriol.* **132**, 564–575
- Azzone, G. F., Pozzan, T., and Massari, S. (1978) *Biochim. Biophys. Acta* **501**, 307–316
- Van Dam, K., Wiechmann, A. H. C. A., Hellingwerf, K. J., Arents, J. C., and Westerhoff, H. V. (1978) in *Membrane Proteins* (Nicholls, P., Moller, J. V., Jorgensen, P. C., and Moody, A. J., eds) Vol. 45, pp. 121–132, Pergamon Press, Elmsford, N. Y.
- Azzone, G. F., Pozzan, T., Viola, E., and Arslan, P. (1978) *Biochim. Biophys. Acta* **501**, 317–329
- Sorgato, M. C., Ferguson, S. J., Kell, D. B., and John, P. (1978) *Biochem. J.* **174**, 237–256
- Pick, U., Rottenberg, H., and Avron, M. (1974) *FEBS Lett.* **48**, 32–36
- Deutsch, C. J., and Kula, T. (1978) *FEBS Lett.* **87**, 145–151
- Kell, D. B., John, P., and Ferguson, S. J. (1978) *Biochem. J.* **174**, 257–266
- Michel, H., and Oesterheld, D. (1980) *Biochemistry* **19**, 4615–4619
- Warncke, J., and Slayman, C. L. (1980) *Biochim. Biophys. Acta* **591**, 224–233
- Williams, R. J. P. (1978) *Biochim. Biophys. Acta* **505**, 1–44
- Gould, J. M., and Cramer, W. A. (1977) *J. Biol. Chem.* **252**, 5875–5882
- Rottenberg, H. (1979) *Biochim. Biophys. Acta* **549**, 225–253
- Gould, J. M. (1979) *J. Bacteriol.* **138**, 176–184
- Kell, D. B. (1979) *Biochim. Biophys. Acta* **549**, 55–99
- Mitchell, P. (1977) *FEBS Lett.* **75**, 1–20
- Kashket, E. R., and Wilson, T. H. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70**, 2866–2869
- Harold, F. M. (1977) *Curr. Top. Bioenerg.* **6**, 83–149
- Ramos, S., and Kaback, H. R. (1977) *Biochemistry* **16**, 848–854
- Ramos, S., and Kaback, H. R. (1977) *Biochemistry* **16**, 854–859