Biochimica et Biophysica Acta, 1019 (1990) 81–90
Elsevier

BBABIO 43250

^{31}P and ^{39}K nuclear magnetic resonance spectroscopy studies of halobacterial bioenergetics

Philip G. Quirk * and Iain D. Campbell

Department of Biochemistry, University of Oxford, Oxford (U.K.)

(Received 3 March 1990)

Key words: NMR; pH gradient, transmembrane; Uncoupling agent; Cation transport; (*H. halobium*); (*H. marismortui*)

The bioenergetics of two extreme halophiles, *Halobacterium halobium* and *Halobacterium marismortui*, have been investigated by respiratory studies and in vivo nuclear magnetic resonance (NMR) spectroscopy. The phosphorus resonances of both species were very broad under anaerobic conditions, but sharpened somewhat on initiation of respiration and considerably upon cooling; changes attributable in part to slow chemical exchange processes. Transmembrane pH gradients (ΔpH) of *H. marismortui* were somewhat lower than those of *H. halobium*. Uncoupling agents caused ATP to disappear and inhibited respiration in both species, but dissipated ΔpH at best only partially. Uptake of Mn^{2+} by *H. halobium* was unaffected by the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), whereas *H. marismortui* appeared not to transport the ion, even in the absence of uncoupler. FCCP caused a slow efflux of K^+ from *H. halobium*, but no K^+ was lost from *H. marismortui*. These findings are discussed with particular reference to the intracellular environments of the two organisms.

Introduction

The obligate halophiles of the genus *Halobacterium* provide an impressive illustration of cellular transport capabilities. Although grown in media containing 3–4 M NaCl and as little as 20 mM K^+, cells of *Halobacterium halobium* and related species contain 3–4 M K^+ and less than 1.5 M Na^+ (reviewed in Refs. 1, 2). Metabolic energy is derived from heterotrophic metabolism, normally aerobic, supplemented in some species under certain circumstances by bacteriorhodopsin-driven photophosphorylation [3].

A particularly interesting species is *Halobacterium marismortui*, whose membrane is reported to have a very high electrical conductance [4] and to be unusually permeable to ions and sugars [5,6]. *H. marismortui* nevertheless maintains high concentrations of intracellular K^+ and low concentrations of Na^+, even after starvation or uncoupler treatment [5], both of which dissipate the transmembrane ion gradients of *H. halobium* [5,7]. To reconcile these apparently contradictory findings, it has been suggested that the cell water of *H. marismortui* is divided into two phases: one, designated W, exchangeable with the extracellular medium and containing most cell Na^+, and a 'structured' phase, designated SW, which retains the bulk of the cell K^+ close to the acidic groups of cell proteins [8].

Here, we report on in vivo nuclear magnetic resonance (NMR) spectroscopic studies of *H. halobium* and *H. marismortui*. This non-invasive technique can provide considerable information on the surroundings of sensitive nuclei (pH, complexation with ions, association/dissociation rates, molecular motion) [9,10]. Surprisingly, no phosphorus spectra of halobacterial cells appear to have been published, although previous NMR studies have investigated the mobility of intracellular water and cations [11–15]. We have also employed ^{39}K-NMR spectroscopy to monitor directly the changes in cell K^+ resulting from starvation and uncoupler treatment, allowing an independent reassessment of the earlier work.

Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; MDP, methylenediphosphonic acid; MeP, methylphosphonic acid; TTFB, 4,5,6,7-tetrachloro-2′-(trifluoromethoxy)benzimidazole; ΔpH, transmembrane pH gradient.

* Current address: Department of Biochemistry, Mount Sinai School of Medicine, New York, NY, U.S.A.
Correspondence: I.D. Campbell, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, U.K.

0005-2728/90/$03.50 © 1990 Elsevier Science Publishers B.V. (Biomedical Division)

An abstract of some of this work has been published: 5th European Bioenergetics Conference (1988), Short Report No. 159.

Materials and Methods

Cell culture. Halobacterium halobium R1 [16] and the Ginzburg strain of *Halobacterium marismortui* [17] were grown aerobically without illumination on a New Brunswick orbital shaker (37 °C, 130 rpm). *H. halobium* medium (pH 7) comprised (mmolal) 4270 NaCl, 81 $MgSO_4$, 27 KCl, 1.5 $CaCl_2$ and 9.1 g/l tryptone (Difco). *H. marismortui* medium (pH 7) comprised (mmolal) 3900 NaCl, 150 $MgSO_4$, 1.5 $CaCl_2$, 1.5 Na_2HPO_4 and 10% fresh yeast autolysate/3.5 molal NaCl. The trace element supplements used previously [18] were omitted in order to minimize intracellular levels of paramagnetic ions. Bacteriorhodopsin synthesis in *H. halobium* was not induced.

Both species reached optical absorbances of around 0.75 (600 nm) after 40–48 h growth (late log phase), and were then harvested by centrifugation at 11 300 × *g* for 10 min at 4 °C. The cell pellets were removed with a spatula and resuspended with gentle stirring in a minimal volume of basal salt medium (*H. halobium* growth medium lacking tryptone, but with 50 mM Tris-maleate buffer (pH 7) added). A trace of DNAse was added, as a small proportion of the cells often lysed.

Assays. Protein was estimated by the Lowry assay [19], with bovine serum albumin as standard.

Inorganic phosphate was assayed essentially by the method of Baginski et al. [20], except that the final absorbance was monitored at 750 nm. Using a similar assay, Zoratti and Lanyi [21] reported results in good agreement with ^{31}P-NMR analyses of cell extracts. Cell volumes of 2.5 μl/mg protein (*H. halobium* [22,23]) and 3 μl/mg protein (*H. marismortui* [18]) were assumed.

Oxygen electrode. Respiratory studies employed a Clark-type oxygen electrode (Yellow Springs YSI 5331) inserted into a water-jacketed, 3.3 ml reaction chamber, maintained at 37 °C. Cells were inoculated to a final concentration of 0.3 mg protein/ml.

NMR spectroscopy. The spectrometer was constructed around an Oxford Instruments 8.4 T wide-bore superconducting magnet, interfaced to Nicolet/General Electric software. Resonance frequencies were 145.8 MHz for ^{31}P and 16.8 MHz for ^{39}K.

Most spectra were obtained with a single pulse sequence (90° for ^{39}K; 40° for ^{31}P) and comprised, typically, 1024 individual acquisitions, collected in 4k of memory. Recycling times of 900 ms for ^{31}P and 300 ms for ^{39}K caused negligible saturation of intracellular resonances. Prior to Fourier transformation, data were multiplied by a decaying exponential equivalent to a line-broadening of 20 Hz. Phosphorus chemical shifts are quoted relative to an external standard at 0.00 ppm of methylene diphosphonic acid (MDP), comprising (mM) 190 MDP, 150 KCl, 50 Hepes, 5 EDTA (pH 7). MDP resonates 17.13 ppm downfield of 85% H_3PO_4.

Spin-lattice (T_1) relaxation constants were measured by the inversion-recovery method (180–t_D–90). Spectra comprised 128 or 256 transients accumulated with a relaxation delay of 4 s, while t_D was varied between 1 ms and 4 s. Resonance intensity data were fitted by computer to a three-parameter exponential function [24].

Most cell samples were of 2.95 ml initial volume in 10 mm diameter NMR tubes, comprising (ml): cells plus basal salt medium, 2.5; ^2H_2O/3.5 M NaCl (frequency standard/lock), 0.3; silicone antifoam, 0.1; 200 mM methylphosphonate (MeP: pH indicator), 0.05. In a few cases, 14.9 ml samples were examined in 20 mm diameter NMR tubes, and the amount of each component was scaled up accordingly. Respiration was initiated by the addition of glycerol and bubbling with 95% O_2 : 5% CO_2 at 10 ml/min. Optimal cell protein concentration was found to be in the range of 25–40 mg/ml.

Spectral analysis. Phosphorus resonances were assigned in perchloric acid cell extracts by consideration of chemical shift, multiplicity and pH sensitivity. Assignments were confirmed by the addition of authentic compounds.

Calibration curves of chemical shift against pH were prepared for inorganic phosphate and MeP in basal salt medium. The pK_{a2} values of both compounds in this medium (phosphate, 5.6; MeP, 6.4) were much lower than in more dilute media (phosphate, 6.8–7.2; MeP, 7.65 [25]). The dilute media values were independent of ionic composition, but in media of higher ionic strength, Na^+ lowered pK_a values more than K^+ (Fig. 1). The phosphate pK_{a2} in 3.5 M KCl was measured as 6.2, and the calibration curve from this medium was used to estimate intracellular pH. As halobacteria contain some Na^+ as well as K^+, these pH values may be slight overestimates. Values of extracellular pH estimated from MeP and phosphate chemical shifts agreed well with each other and with pH-electrode (Wilmad type 6030-02) measurements.

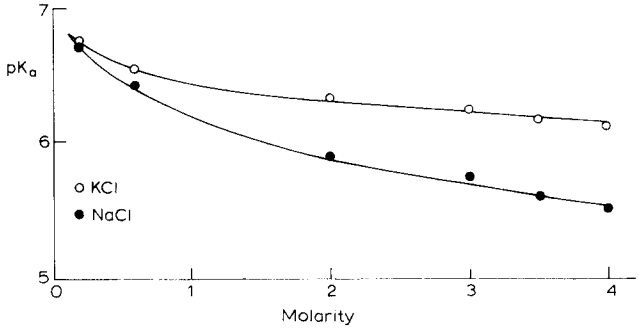

Fig. 1. Variation of phosphate pK_{a2} with ionic strength. At high concentrations, NaCl lowers the pK_{a2} more effectively than KCl.

Intracellular resonances were often broad and overlapping, particularly in the phosphomonoester/phosphate/phosphodiester region of the spectrum. To overcome these problems, many spectra were simulated using the Nicolet/General Electric computer programs NTCCAP and GEMCAP. Each simulated Lorentzian resonance was adjusted manually to give the best match with the spectrum (lowest root-mean-square area deviation). The line-width, area and chemical shift of each spectral component could therefore be estimated. The components of simulated spectra were generally consistent with the observed resonances of perchloric acid cell extracts (data not shown); one exception concerned the β-nucleoside triphosphate resonance, which in cells at low temperatures was sometimes resolved into two overlapping components (see below).

Atomic absorption spectroscopy. Manganese and iron were assayed in an extract from each species, using a Perkin-Elmer model 306 atomic absorption spectrophotometer. Results were: *H. halobium,* 0.5 mM Mn, 2.5 mM Fe; *H. marismortui,* 0.2 mM Mn, 0.9 mM Fe.

Results

^{31}P-NMR spectra of halobacteria

Fig. 2A and B show typical spectra, acquired at 25°C, of *H. halobium* (Fig. 2A) and *H. marismortui* (Fig. 2B) respiring on glycerol. Resonances of both species are broad; in these examples, intracellular phosphate line-widths were 200 Hz for *H. halobium* and 560 Hz for *H. marismortui,* cf. 50–70 Hz for *Escherichia coli* [26]. Nucleoside di-/triphosphates (NDP/NTP), readily discernible in *H. halobium,* were barely visible in the *H. marismortui* spectrum. The ratio of NTP:(NDP + NTP), measured from the β-NTP and γ-NTP + β-NDP resonances, averaged 0.63 in respiring *H. halobium,* suggesting adequate oxygenation (Table I). Spectra of anaerobic cells of either species showed virtually no β-NTP resonance. The intracellular phosphate: β-NTP ratio in *H. halobium* spectra averaged 10:1; cell extracts assayed chemically and by NMR gave a ratio averaging 25:1 (Table I), in agreement with the literature [23,27,28]. Hydrolysis of NTP during the extraction process was probably insignificant, since the NTP:(NDP + NTP) ratio was little changed. Thus, a large proportion of *H. halobium* intracellular phosphate appears invisible to NMR spectroscopy, NTP visibility appears higher, the resonance intensity being consistent with the expected concentration of 2–5 mM [23,27,28] (Table I). Similar problems with phosphate and nucleo-

TABLE I

Concentrations and concentration ratios of inorganic phosphate (P_i), nucleoside diphosphate, and nucleoside triphosphate in H. halobium and H. marismortui

Values from ^{31}P-NMR spectra, with number of determinations in brackets. NTP was measured from the β-phosphate resonance, while NTP + NDP was estimated from the combined γ-NTP and β-NDP resonance. Phosphate in extracts was also measured chemically; results were in good agreement with NMR spectra.

	H. halobium	*H. marismortui*
P_i in extracts (mmol/kg cell H_2O)	88 ±6 (8)	76 ±9 (9)
P_i/NTP		
in vivo	10.2 ±2.1 (14)	4.2 ±0.8 (15)
in extracts	25 (3)	22 (5)
NTP/(NDP + NTP)		
in vivo	0.63 ± 0.2 (14)	0.83 ± 0.2 (15)
in extracts	0.67–0.77 (3)	0.56–0.91 (5)

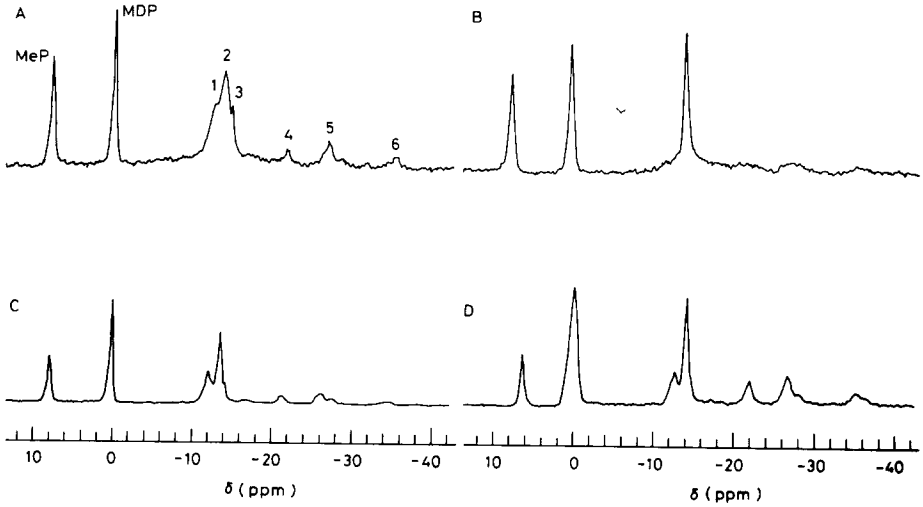

Fig. 2. Effect of temperature on the ^{31}P-NMR spectra of aerated halobacteria. All samples were of 3.05 ml volume, including 0.1 ml glycerol. (A) *H. halobium*, 25°C; (B) *H. marismortui,* 25°C; (C) *H. halobium,* −12°C; (D) *H. marismortui,* −12°C. Assignments: (1) Phosphomonoesters (two major components, partially resolved in (D); (2) intracellular phosphate; (3) extracellular phosphate; (4) γ-NTP, β-NDP; (5) α-NTP, α-NDP, NAD(P)(H), NDP-glucose (the two major components are α-NTP (downfield) and NDP-glucose (upfield)); (6) β-NTP.

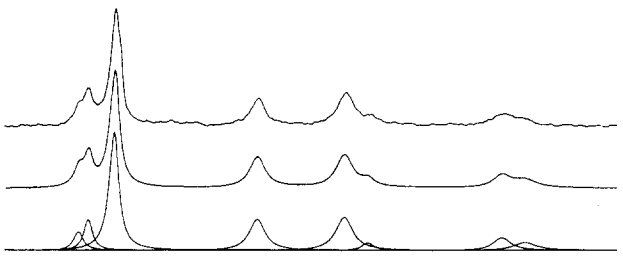

Fig. 3. A simulation of the *H. marismortui* spectrum, Fig. 2D, constructed using the computer program NTCCAP. Top trace: original spectrum; middle trace: simulated spectrum; lower trace: the individual components of the simulation. In this case, resolution of intra- and extracellular phosphate was not possible, but the two components of the β-NTP resonance were separable.

tide NMR-visibility have been reported for various eukaryotic systems [9] and in the cyanobacterium *Anacystis nidulans* [46]. In the latter case, paramagnetic ions were blamed.

Spectral line-widths were affected by the cellular metabolic state. The intracellular phosphate resonance narrowed by 100–200 Hz when anaerobic cells of either species were energised at 25 °C, and broadened again when oxygenation was stopped or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added (e.g., Fig. 9).

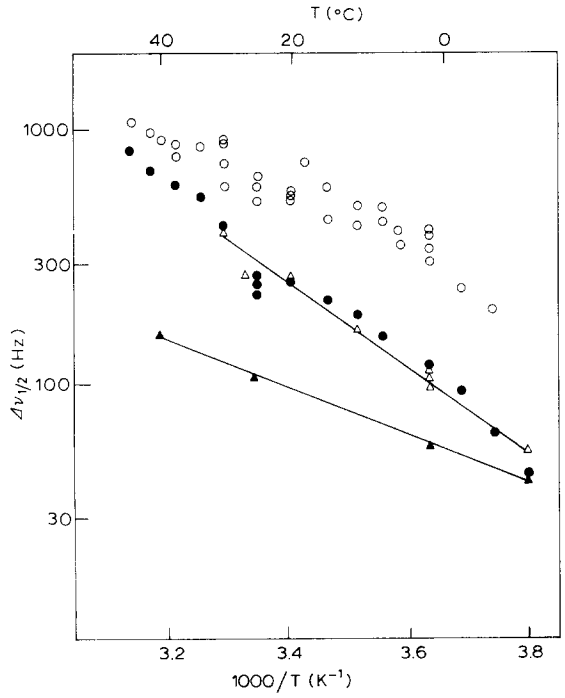

Fig. 4. Temperature-dependence of phosphate linewidths. Key: (●) anaerobic *H. halobium* intracellular phosphate; (○) anaerobic *H. marismortui* intracellular phosphate; (△) phosphate in basal salt solution + 65 μM Mn^{2+}; (▲) phosphate in 3.9 M NaCl + 65 μM Mn^{2+} (no Mg^{2+} present). Straight lines have been fitted to the solution data. Substitution of KCl for NaCl had little effect on linewidths. See text and Table II for further details.

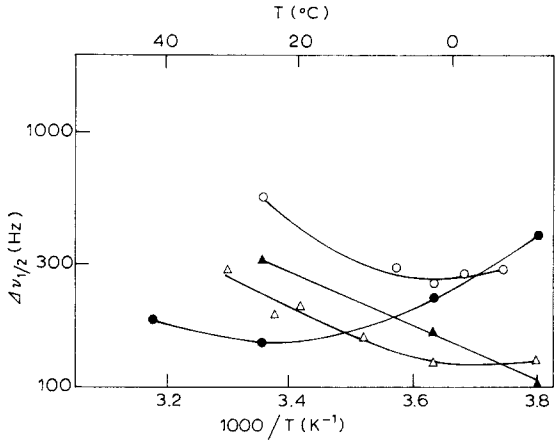

Fig. 5. Temperature-dependence of β-NTP linewidths. Key: (●) aerobic *H. halobium;* (○) aerobic *H. marismortui;* (△) ATP in basal salt solution + 65 μM Mn^{2+}; (▲) ATP in 3.9 M NaCl + 65 μM Mn^{2+} (no Mg^{2+} present). KCl salt solutions behaved similarly.

Low-temperature

The intracellular phosphate resonances of both species sharpened considerably upon cooling: at −12 °C, linewidths were 70 Hz for *H. halobium* (Fig. 2C) and 90 Hz for *H. marismortui* (Fig. 2D). The nucleotide resonances of *H. marismortui* became much clearer, the substantial β-NTP resonance confirming the initial viability of the sample, and implying adequate oxygenation during the prior incubation at 25 °C. The NTP : (NDP + NTP) ratio was slightly higher than in *H. halobium.* One feature of *H. marismortui* spectra was that the phosphate : NTP ratio was often as low as 3 : 1. As this ratio was 22 : 1 in extracts (Table I), an even higher percentage of intracellular phosphate than in *H. halobium* appeared to be NMR-invisible. Below −10 °C, the β-NTP resonance of *H. marismortui* was resolved into two components, believed to represent Mg-bound (downfield) and non-bound nucleotide (upfield) in slow exchange (Fig. 3). Such separation was not achieved with *H. halobium.*

These observations were surprising because resonances are generally broadened at low temperatures by the more efficient spin-spin relaxation resulting from reduced thermal motion. The narrowing of phosphate resonances in the cooled halobacterial cells almost certainly results from the slowing of chemical exchange processes between two or more intracellular pools. One pool may be associated with paramagnetic ions, as both species contained substantial amounts of iron and manganese (see Materials and Methods), and the spectra of cell extracts could be sharpened considerably by addition of the chelating agent EDTA. A slow exchange process between Mn^{2+} and ATP has been documented in media of relatively low ionic strength [29,30], in which the Mn-bound species is so broad as to be invisible. An interaction of this type may explain the smaller than expected halobacterial phosphate signals,

TABLE II

Temperature-dependence of phosphate linewidth

Data from Fig. 4 and other spectra (not shown). Salt solutions were at pH 7. *H. marismortui*: data from six experiments (four anaerobic, two oxygenated), external pH 6–6.5. *H. halobium*: data from four experiments (two anaerobic, two oxygenated), external pH 6–6.5.

Conditions	Apparent activation energy: $-\text{slope}/2.3R$ (kJ/mol)
Basal salt (3.9 M Na^+ + 70 mM Mg^{2+})	0
Basal salt + 65 μM Mn^{2+}	33
3.9 M K^+ + 65 μM Mn^{2+}	21
3.9 M K^+ + 65 μM Mn^{2+} + 70 mM Mg^{2+}	30
H. halobium P_i, anaerobic cells	29
H. halobium P_i, oxygenated cells	29
H. marismortui P_i, anaerobic cells	20
H. marismortui P_i, oxygenated cells	30

but other aspects of the intracellular environment might also be important.

Modelling the intracellular environments

Line-widths of intracellular phosphate and β-NTP resonances were determined over the range −12°C to 40°C, using the simulation programmes to analyse each spectrum. To correct for line-broadening arising from magnetic field inhomogeneities and susceptibility effects, a factor of 50 Hz was subtracted from all intracellular line-width data prior to graphical analysis. Solutions of NaCl and KCl containing Mg^{2+}, Mn^{2+}, phosphate and ATP were also studied in attempts to model the behaviour of the cells. Results for phosphate (Fig. 4) and β-NTP/ATP (Fig. 5) are presented as graphs of $\log_{10}$ (line-width) against $1/T$. For two-site exchange, the slope of this Arrhenius-type plot gies the activation energy of the process. In halobacteria, however, there are probably multi-component exchanges, with several ions competing to associate with phosphate and NTP. Comparison of the slopes was still useful for phosphate, which gave reasonably linear plots, and data are presented as 'apparent' activation energies in Table II. Interestingly, the omission of Mg^{2+} from a KCl solution containing Mn^{2+} reduced the apparent activation energy of phosphate by 30%. As the slope obtained from a KCl solution containing Mg^{2+} and Mn^{2+} was very similar to those from *H. halobium* and from aerobic *H. marismortui*, it appears that the salt solution represents a reasonable approximation to the intracellular environments, and that both halobacteria contain significant amounts of free Mg^{2+}. In anaerobic *H. marismortui*, the phosphate line-width was broadened to a greater extent at lower temperatures, resulting in a reduced slope, possibly due to increased binding of Mg^{2+}.

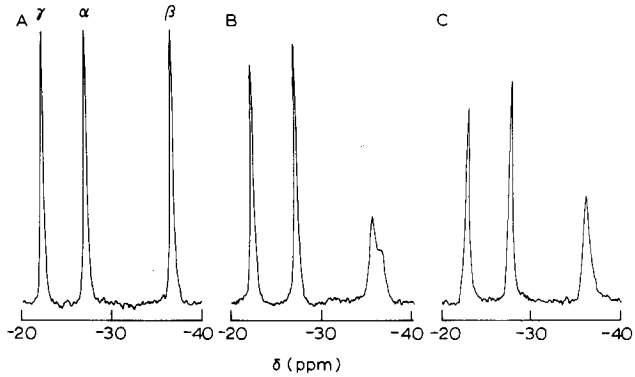

Fig. 6. Broad-band ^1H-decoupled ^{31}P-NMR spectra of ATP at Mg^{2+} : ATP ratios of: (A) 0; (B) 1.5; (C) 4. The sample comprised initially (mM): 2850 KCl, 2.5 K_2HPO_4, 5 ATP, 2.5 AMP (resonance not shown); $MgCl_2$ was added as required. Temperature: −12°C; pH 6.87. All spectra are plotted to the same scale.

Data for the intracellular β-NTP resonance were less reproducible than those for phosphate, for reasons which remain unclear. Consideration of individual cell samples, however, reveals a tendency for the resonance to broaden at low temperatures in *H. halobium* (Fig. 5), and to resolve into two components in *H. marismortui*, as discussed above (Fig. 3). A likely explanation for these phenomena is the slowing of a previously rapid exchange process between Mg^{2+} and NTP. This exchange remains fast in media of low ionic strength [31]. Modelling of the intracellular α- and γ-NTP resonances was not pursued, as their signals could not be resolved from those of α- and β-NDP.

There are some indications that *H. marismortui* may contain less free Mg^{2+} than *H. halobium*, at least at low temperatures. The broad appearance of the β-NTP resonance of *H. halobium* at −12°C (Fig. 2C) was best duplicated in solutions with an Mg : ATP ratio exceeding 4 : 1, whereas the best modelling of the *H. marismortui* resonance, clearly resolved into two components at this temperature (Figs. 2D, 3), was in solutions with a Mg : ATP ratio of around 1.5 : 1 (Fig. 6). Assuming that both species, when energised, contain similar amounts of NTP (around 3 mM [22,23], Table II), free Mg^{2+} may be estimated at 10–15 mM in *H. halobium* and around 5 mM in *H. marismortui*. Lower limits for free Mg^{2+} may be set at 3 mM in *H. halobium*, where all the visible NTP is complexed with the ion, and around 2 mM in *H. marismortui*. In theory, free Mg^{2+} may also be estimated from the difference in chemical shift between the α- and β-phosphate resonances of intracellular NTP [9,29], but this parameter may be affected by several factors in vivo, such as overlap of the α-NTP and α-NDP resonances and variations in intracellular ion concentrations, making accurate measurements impossible in our halobacteria.

A comparison of spin-lattice (T_1) relaxation rates of phosphate and ATP in vivo and in vitro is presented in

TABLE III

Spin-lattice relaxation times of phosphorus compounds

Salt solutions (pH 6.9) comprised (mM): 2700 KCl, 10 MeP, 10 AMP, 10 K_2HPO_4, 10 ATP, 10 $MgCl_2$, and included 10% ^2H_2O/3.5 M NaCl. $MnCl_2$ (17 μM) was added when required. External pH of cell samples: *H. halobium*, 6.2; *H. marismortui*, 7.0. Abbreviations: *H.h.*, *H. halobrium;* *H.m.*, *H. marismortui;* NTP, nucleoside triphosphate; MeP, methylphosphonate. Cells were oxygenated throughout, with glycerol added periodically (0.3 mmol glycerol sufficed for 4 h). Errors for intracellular compounds estimated at $\pm$0.005 s; extracellular $\pm$0.05 s.

Resonance	Environment	T_1 (s)		
		−12 °C	0 °C	15 °C
MDP	capillary	1.5	3.0	4.0
Phosphate	2.7 M KCl		4.5	3.9
	2.7 M KCl, 17 μM Mn^{2+}		0.6	0.6
Extracellular phosphate	*H.h.*	1.2	1.0	0.8
	H.m.	1.4	1.1	0.7
Intracellular phosphate	*H.h.*	0.04	0.09	0.06
	H.m.	0.03	0.05	0.03
γ-NTP + β-NDP	*H.h.*	0.02	0.02	0.02
	H.m.	0.02	0.01	0.01
γ-ATP	2.7 M KCl, 17 μM Mn^{2+}		0.05	0.03
α-NTP + α-NDP	*H.h.*	0.10	0.11	0.09
	H.m.	0.04	0.03	0.03
α-ATP	2.7 M KCl, 17 μM Mn^{2+}		0.06	0.04
β-NTP	*H.h.*	0.01	0.01	0.01
	H.m.	0.02	0.01	0.01
β-ATP	2.7 M KCl, 17 μM Mn^{2+}		< 0.01	< 0.01

Table III. Relaxation was rapid in both halobacterial species (cf., *E. coli* T_1 values of 0.4 s for phosphate and 0.2 s for β-NTP: [32]), with rates generally 2–3-times faster in *H. marismortui* than in *H. halobium.* Relaxation rates of both phosphate and ATP were increased in salt solutions containing 17 μM Mn^{2+}. Under these conditions, the T_1 of ATP was similar to that of intracellular NTP, but the phosphate T_1 remained an order of magnitude larger than that of intracellular phosphate.

Certain aspects of the behaviour of intracellular phosphate and NTP can thus be explained at least qualitatively in terms of free paramagnetic ions and Mg^{2+}. However, discrepancies remain, and relaxation may be affected by factors not yet incorporated into our models. These include viscosity and the effects of soluble proteins, which in halobacteria contain a large excess of acidic residues [33]. Proteins such as serum albumin sequester Mn^{2+} in vitro, making it unable to associate with ATP, conversely, Mn^{2+} bound to the outer surface of a protein could produce enhanced spin–spin relaxation. A phase of structured water might also be expected to enhance the relaxation of any associated metabolites, and could alter chemical shifts. A further problem is to explain the narrowing of resonances, particularly phosphate, when cells are energised. One possibility is that the production of ATP from ADP and phosphate causes increased chelation of paramagnetic ions, leaving fewer available to associate with the remaining phosphate.

Manganese transport

Respiring cells were treated with small aliquots of Mn^{2+} from a stock solution of 5 mM $MnCl_2$. On addition of 33 μM Mn^{2+} to *H. halobium,* intracellular resonances broadened rapidly, while extracellular phosphate broadened initially (not shown) but then sharpened again, indicating uptake of the ion (Figs. 7A, B). As 20 μM Mn^{2+} was sufficient to broaden MeP and phosphate resonances in free solution, at least 13 μM Mn^{2+} must have been taken up over the first 2 min; a transport rate exceeding 0.26 nmol/min per mg protein. The cells could accumulate Mn^{2+} to at least 3.8 mM (a transmembrane gradient exceeding 190 : 1) and retain it for at least 90 min in the presence of 48 μM FCCP (Fig. 7C). Preincubation for 30 min with 48 μM FCCP also failed to affect Mn^{2+} uptake (data not shown). With *H. marismortui,* in contrast, Mn^{2+} caused only extracellular resonances to broaden, and was apparently not taken up (Figs. 7D, E).

Transmembrane pH gradient (ΔpH)

Many determinations of ΔpH were made as described in the Materials and Methods section, using spectral simulation procedures when the phosphate resonances overlapped. The most reliable data were obtained when intracellular pH was reasonably close to the estimated phosphate pK_{a2} of 6.2, i.e., with the extracellular pH in the range 5–6.5. Homeostasis of pH appeared poor in this region, energised cells instead maintaining fairly constant transmembrane gradients of 1.2 pH units (*H. halobium*) or 0.7 pH units (*H. marismortui*) (Fig. 8A). At higher extracellular pH, these gradients became smaller, consistent with the literature [22,23]. Values of ΔpH obtained with anaerobic cells were more variable (due partly to the increased phosphate line-widths, which made simulation more difficult), but appeared reduced to around 0.8 pH units in *H. halobium* and little changed in *H. marismortui* (Fig. 8B).

Effects of uncoupling agents

Addition of 8 μM FCCP to respiring *H. halobium* caused the intra- and extracellular phosphate resonances to merge, along with a gradual disappearance of NTP and NDP (Fig. 9). While some reduction in ΔpH had obviously occurred, quantitation of this required a

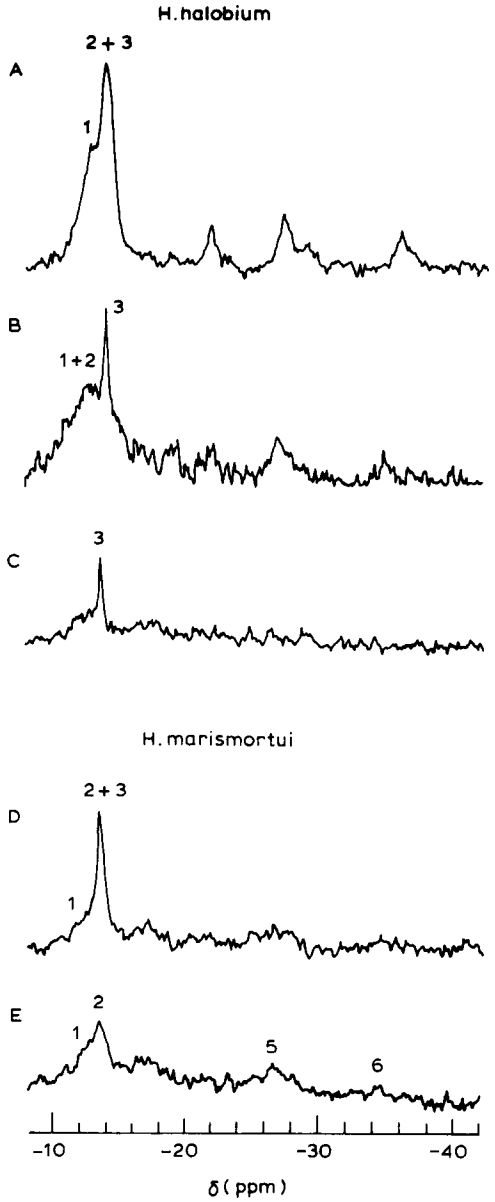

Fig. 7. Effects of Mn^{2+} on ^{31}P-NMR spectra of respiring halobacteria. Assignments as in Fig. 2. Sample for traces (A)–(C): 3.05 ml oxygenated, glycerol-fed *H. halobium*, external pH 6.15, temperature 23°C. (A) Initial spectrum (1024 transients); (B) spectrum (256 transients) acquired 1–3 min after addition of 33 μM Mn^{2+}. Note the broadening of intracellular phosphate and loss of β-NTP intensity. (C) Manganese was added to a final concentration of 240 μM. FCCP (48 μM) was added 10 min later, and the spectrum (1024 transients) was acquired after a further 90 min. Only extracellular phosphate remained visible. Sample for traces (D) and (E): As above but *H. marismortui*. (D) Initial spectrum (1024 transients). (E) Spectrum (1024 transients) acquired 8–15 min after addition of 33 μM Mn^{2+}. Extracellular phosphate remained broad, while intracellular resonances (although broad to start with), appeared unaffected.

knowledge of the intracellular K^+ concentration; uncouplers would be expected to dissipate the transmembrane gradients of Na^+ and K^+ by abolishing protonmotive force-dependent secondary transport. However, potassium NMR studies indicated that even 48 μM FCCP caused only a slow efflux of cell K^+ (see below), and we therefore continued to estimate intracellular pH from the phosphate titration curve in 3.5 M KCl. FCCP appeared to cause only a partial dissipation of ΔpH in *H. halobium*; for example, at an external pH of 6, a ΔpH averaging 1.2 units was reduced by FCCP to 0.8 units (Fig. 8B). A possible explanation of this incomplete dissipation of ΔpH is the build-up of a reversed membrane potential, positive in, opposing further influx of protons (see, for example, Ref. 34). FCCP had little or no effect on ΔpH in *H. marismortui* (Fig. 8B).

Respiratory studies revealed that as little as 3 μM FCCP or 3 μM TTFB caused 50% inhibition of endogenous respiration in both species (*H. halobium* from 40 to 20 ngatom O/min per mg protein; *H. marismortui* 30 to 15). A respiratory response to glycerol was still observed at these concentrations, but this was abolished by 10 μM of either uncoupler and by 6 μM nigericin, which also inhibited respiration. Valinomycin (3 μM) had no detectable effect on respiration. The respiratory inhibition caused by FCCP and TTFB could be substantially relieved by adding bovine serum albumin to sequester the uncouplers. Growth of both species was abolished by 1 μM FCCP or by 2 μM TTFB.

Interestingly, similar values of ΔpH were measured in cells deprived of oxygen or treated with uncoupler (Fig. 8B); the two regimens also caused similar NTP losses in both species. Many of our findings could thus be interpreted in terms of FCCP and TTFB acting primarily as respiratory inhibitors rather than as protonophores.

Potassium NMR spectroscopy

The ^{39}K nucleus (spin 3/2) possesses an electric quadrupole moment, which affords a mechanism of fast spin-spin relaxation not available for spin 1/2 nuclei such as ^{31}P. Quadrupolar relaxation has little effect on the signal intensity from K^+ in free solution (essentially 100% visible), but causes around 60% of the signal from intracellular K^+ to broaden and become invisible [15,35]. Thus, any K^+ leaving cells immediately produces 2.5-times more observable signal. This enabled us to compare the retention of K^+ by *H. halobium* and *H. marismortui*. The high concentration of extracellular Na^+ made it impracticable to employ shift reagents [36] to resolve intra- and extracellular K^+ directly.

Respiring cells were treated with either FCCP or an equal volume of ethanol (controls) and maintained at 21°C. Spectra were acquired periodically and peak areas were determined by cutting and weighing. Extracellular K^+ was determined following rapid centrifugation of the cells (Eppendorf microfuge), while total sample K^+ was measured following 2.5 min sonication. Line-widths of intracellular K^+ (80–100 Hz, cf. 30 Hz in supernatant samples) were similar in both species.

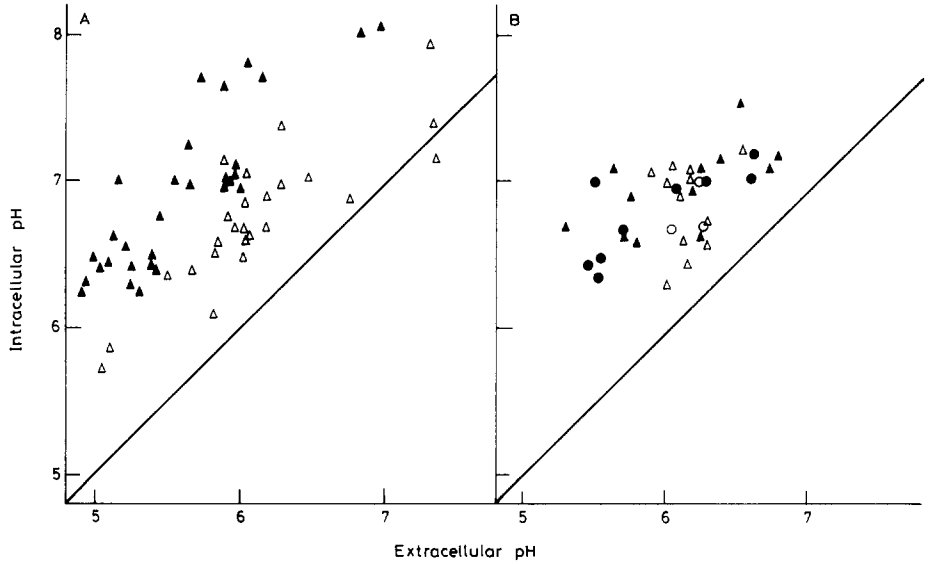

Fig. 8. Transmembrane pH-gradients of halobacteria estimated from phosphate chemical shifts, using calibration curves in basal salt (extracellular pH) and 3.5 M KCl (intracellular pH). (A) ΔpH in respiring samples, 21–25 °C. Key: (▲) *H. halobium;* (△) *H. marismortui.* (B) ΔpH in samples either anaerobic or treated with FCCP (8 μM). Key: (▲) anaerobic *H. halobium;* (△) anaerobic *H. marismortui;* (●) FCCP-treated *H. halobium;* (○) FCCP-treated *H. marismortui.* The diagonal lines represent a ΔpH of zero. See text for discussion.

Treatment with 48 μM FCCP caused the loss of virtually all the intracellular K^+ from *H. halobium* (Table IV), but the process was much slower ($T_{1/2} \approx 10$ h) than the disappearance of the β-NTP resonance ($T_{1/2} \approx 15$ min). In contrast, there was no increase in the K^+ signal from FCCP-treated *H. marismortui,* indicating that no efflux had occurred. Similar results were obtained when as much as 1.6 mM TTFB was added; in three experiments, *H. marismortui* lost no K^+ within 10 h, while *H. halobium* lost all intracellular K^+ within 2 h. Control samples took up some K^+. The visibility of intracellular K^+ (calculated from cytocrit data to be initially 3.5–4 M in both species) was 30–40%, consistent with literature data on *H. halobium* [15].

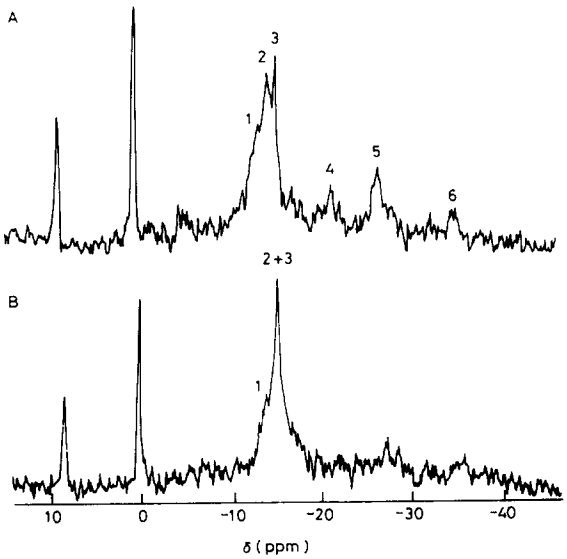

Fig. 9. ^{31}P-NMR spectra showing the effect of FCCP on *H. halobium.* (A) Respiring cells (1024 transients), external pH, 5.03; internal pH, 5.72. (B) Spectrum (1024 transients) acquired 30–37 min after addition of 8 μM FCCP. Oxygenation was continued throughout. External pH, 5.39. Assignments as in Fig. 2. Intracellular phosphate linewidths were: (A) 420 Hz; (B) 490 Hz.

Discussion

The halobacteria have proven rather challenging organisms with which to work. Not only are the cells extremely fragile, making sample preparation difficult, but their spectra at room temperature are characterised by broad, poorly resolved resonances. Nevertheless, extensive modelling studies and the use of computer-aided spectrum simulation routines have allowed us to make some progress towards understanding the internal environment of *H. halobium* and *H. marismortui,* a pre-

TABLE IV

Effects of FCCP on cell K^+

Cells were oxygenated throughout, with glycerol added periodically. Similar results were obtained in duplicate experiments, but the initial concentration of extracellular K^+ varied somewhat between samples, reflecting differing periods of storage and varying degrees of cell lysis during harvesting. Quantitation errors estimated as ±5%.

Conditions	Observed K^+ signal (% of total sample K^+)			
	0 h	4 h	8 h	20 h
H. halobium control	65	61	55	53
H. halobium + 48 μM FCCP	64	64	76	99
H. marismortui control	77	76	72	65
H. marismortui + 48 μM FCCP	79	74	69	68

requisite for extracting further useful information from the NMR spectra.

The phosphorus spectra of both halobacteria are greatly influenced by complex processes of chemical exchange, with the possible involvement of several cations (H^+, Na^+, K^+, Mg^{2+}, and one or more paramagnetic species such as Mn^{2+}). Comparable analyses of other systems are few, but the relaxation behaviour of HeLa cell metabolites has been accounted for by a combination of paramagnetic ions and the presence of chemical shift envelopes resulting from heterogeneity in intracellular environment [37]. The situation is more complicated with the halobacteria, where the data, in particular the short T_1 of phosphate relative to that of β-NTP, may not be explicable solely in these terms. Environmental heterogeneity probably contributes relatively little to halobacterial line-widths; at least at low temperatures, where the intracellular phosphate resonance is narrow. The invisibility of a large proportion of intracellular phosphate in both species is also puzzling; were this caused solely by paramagnetic ions, then one would expect an even larger proportion of NTP to be invisible, since these have a greater affinity for ions such as Mn^{2+}, at least in media of low ionic strength [38]. Below $-10\,^\circ C$, a signal from NTP unassociated with Mg^{2+} was noted in *H. marismortui,* but not in *H. halobium*. Under these conditions, *H. marismortui* may contain less free Mg^{2+} than *H. halobium,* or might sequester some of its NTP in a pool inaccessible to Mg^{2+}. The possibility of slowing Mg^{2+}/NTP exchange by cooling as low as $-20\,^\circ C$ makes halobacteria particularly suitable for studies of divalent cation exchange by NMR spectroscopy.

During the course of this work, it was hoped that *H. halobium* would serve as a 'control' organism, against which to assess the less well characterised *H. marismortui*. Our observations concerning *H. halobium* bioenergetics were generally in accordance with previous reports. Using benzoic acid, Stoeckenius' group [23] measured a ΔpH of 1.2 at external pH 6.2, in good agreement with our observations. Literature data at external pH values below 6 suggest the maintenance of intracellular pH well above 7 [22,29], but may be less reliable than NMR measurement, the accuracy of which is aided by the low pK_{a2} of intracellular phosphate. Our NMR studies also confirm the only partial dissipation of ΔpH in *H. halobium* by CCCP or FCCP [22,23]. These reagents have been thought to act primarily as protonophores in halobacteria, but in our hands, FCCP and TTFB inhibited respiration severely at concentrations little higher than those required to prevent growth (where the uncoupler : cell ratio is at least 10-fold higher). Despite some uncertainties in the NMR measurements owing to broad resonances, *H. marismortui* appeared not to maintain a constant intracellular pH below external pH 6.5, in this respect behaving similarly to *H. halobium*. The ΔpH of *H. marismortui,* however, seemed insensitive to metabolic state, being virtually unaltered by anaerobiosis or uncoupler treatment.

Further differences between the species emerged when manganese transport was studied. Our experiments appear to represent the first investigation of Mn^{2+} metabolism in halobacteria. Manganese is regarded as essential for growth of *H. halobium* [40], but the failure of up to 48 μM FCCP to inhibit its uptake was surprising. Eubacterial transport of Mn^{2+} is energy-dependent and inhibited by uncouplers [41,42]. In view of the concentration gradients attained, Mn^{2+} seems unlikely to be transported passively into *H. halobium,* but its mode of entry remains unclear. *H. marismortui*, in contrast, appeared not to take up Mn^{2+} under these conditions, although the fact that intracellular Mn^{2+} is detected by atomic absorption spectrometry suggests that an uptake system also exists in this species. Halobacterial Mn^{2+} metabolism deserves further study, and NMR spectroscopy may offer a useful approach to this problem.

The metabolic state of halobacteria might reasonably be expected to influence their internal ionic composition. Starvation of *H. halobium* for 24 h at room temperature causes a substantial loss of K^+ and an influx of Na^+ [5,27], although cultures kept at $4\,^\circ C$ retain K^+ for much longer, remaining viable for many months. Our results are consistent with these studies; FCCP causes K^+ efflux from *H. halobium,* albeit rather slowly. It has been suggested that K^+ functions as an energy store, its gradual electrogenic escape allowing starving cells to maintain their membrane potential [7,27,43]. In contrast, *H. marismortui* maintained large Na^+.K^+ gradients when starved for up to 3 weeks (room temperature or $0\,^\circ C$) [5,44], and in the absence of any detectable metabolism [45]. Our ^{39}K-NMR studies have provided independent confirmation of this phenomenon, with even 1.6 mM TTFB (or 1 mM FCCP: data not shown) failing to cause any measurable K^+ efflux. Intracellular binding of K^+ may thus be important [5].

Our studies have highlighted certain similarities and differences between *H. halobium* and *H. marismortui.* In general, the ^{31}P-NMR spectra of the two species were similar, although the lower ΔpH of *H. marismortui* meant that the resonances from intra- and extracellular phosphate always overlapped. The respiratory properties of the two species; stimulation by glycerol, inhibition by uncoupling agents, appeared virtually identical. The Mn^{2+} transport results, in contrast, were very different, suggesting that there might be some significant differences between the membrane properties and/or internal environments of the two species (cf. Ref. 5). Our data allow some bounds to be placed on the membrane permeability of *H. marismortui*: cells appear not to be freely permeable to Mn^{2+}. Furthermore, the narrow line-width of extracellular phosphate indicated

that transmembrane phosphate exchange was not fast enough to be detected by NMR (i.e., a rate constant of efflux $< 10\ s^{-1}$). Further study is needed to reconcile these results with previous data suggesting a generally high permeability [4,5,8]. Although the identical appearance of the ^{39}K-NMR resonances suggests that the internal environment of K^+ may be similar in both species, some form of water structuring [5,8] in one or both cannot be ruled out, and could account for the tenacity of K^+ retention in *H. marismortui*.

Finally, it is interesting that in both species, there appeared to be little relationship between ΔpH and ATP synthesis, and it would be of value to measure their membrane potentials, particularly in *H. marismortui*, where ΔpH appeared unchanged by uncoupler-treatment. Even in *H. halobium*, there is a large and unexplained difference between the time-scales of ATP disappearance and K^+ efflux following FCCP treatment. NMR spectroscopy may be useful in the further studies required to resolve these problems.

Acknowledgments

We thank SERC and the Wellcome Trust for financial support, N. Soffe and Dr. J. Boyd for constructing the NMR probes and for expert assistance with the spectrometer, and S. Wyatt for performing the atomic absorption analyses.

References

1 Bayley, S.T. and Morton, R.A. (1978) CRC Crit. Rev. Microbiol. 6, 151–205.
2 Kushner, D.J. (1985) in The Bacteria, Vol. 8 (Woese, C.R. and Wolfe, R.S., eds.), pp. 171–214, Academic Press, London.
3 Oesterhelt, D. and Stoeckenius, W. (1971) Nature New Biol. 233, 149–152.
4 Morgan, H., Ginzburg, M. and Ginzburg, B.-Z. (1987) Biochim. Biophys. Acta 924, 54–66.
5 Ginzburg, M. (1978) in Energetics and Structure of Halophilic Microorganisms (Caplan, S.R. and Ginzburg, M., eds.), pp. 561–577, Elsevier, Amsterdam.
6 Ginzburg, M. and Ginzburg, B.-Z. (1979) Biochim. Biophys. Acta 584, 398–406.
7 Wagner, G., Hartmann, R. and Oesterhelt, D. (1978) Eur. J. Biochem. 89, 169–179.
8 Ginzburg, M. and Ginzburg, B.-Z. (1975) Biomembraneas 9, 218–251.
9 Avison, M.J., Hetherington, H.P. and Shulman, R.G. (1986) Annu. Rev. Biophys. Biophys. Chem. 15, 377–402.
10 Brindle, K.M. and Campbell, I.D. (1987) Q. Rev. Biophys. 19, 159–182.
11 Cope, F.W. and Damadian, R. (1970) Nature 228, 76–77.
12 Edzes, H.T. (1976) Doctoral Thesis, University of Groningen.
13 Edzes, H.T., Ginzburg, M., Ginzburg, B.-Z. and Berendsen, H.J.C. (1977) Experientia 33, 732–734.
14 Magnuson, J.A. and Magnuson, N.S. (1973) Ann. NY Acad. Sci. 204, 297–310.
15 Shporer, M. and Civan, M.M. (1977) J. Membr. Biol. 33, 385–400.
16 Stoeckenius, W. and Kunau, W.H. (1968) J. Cell. Biol. 38, 337–357.
17 Ginzburg, M. (1969) Biochim. Biophys. Acta 173, 370–376.
18 Ginzburg, M., Sachs, L. and Ginzburg, B.-Z. (1970) J. Gen. Physiol. 55, 187–207.
19 Ohnishi, S.T. and Barr, J.K. (1978) Anal. Biochem. 86, 193–200.
20 Baginski, E.S., Foa, P.P. and Zak, B. (1967) Clin. Chem. 13, 326–332.
21 Zoratti, M. and Lanyi, J.K. (1987) J. Bacteriol. 169, 5755–5760.
22 Bakker, E.P., Rottenberg, H. and Caplan, S.R. (1976) Biochim. Biophys. Acta 440, 557–572.
23 Helgerson, S.L., Requadt, C. and Stoeckenius, W. (1983) Biochemistry 22, 5746–5753.
24 Levy, G. and Peat, I. (1975) J. Magn. Reson. 18, 500–521.
25 Gillies, R.J., Alger, J.R., Den Hollander, J.A. and Shulman, R.G. (1982) in Intracellular pH: Its Measurement, Regulation, and Utilisation in Cellular Functions (Nuccitelli, R. and Deamer, D.W., eds.), pp. 79–104, Alan R. Liss, New York.
26 Quirk, P.G., Jones, M.R., Haworth, R.S., Beechey, R.B. and Campbell, I.D. (1989) J. Gen. Microbiol. 135, 2577–2587.
27 Michel, H. and Oesterhelt, D. (1980) Biochemistry 19, 4607–4614.
28 Helgerson, S.L. and Stoeckenius, W. (1985) Arch. Biochem. Biophys. 241, 616–627.
29 Cohn, M. and Hughes, T.J., Jr. (1962) J. Biol. Chem. 237, 176–181.
30 Brown, F.F., Campbell, I.D., Henson, R., Hirst, C.W.J. and Richards, R.E. (1973) Eur. J. Biochem. 38, 54–58.
31 Misawa, K., Lee, T.-M. and Ogawa, S. (1982) Biochim. Biophys. Acta 718, 227–229.
32 Brown, T.R., Ugurbil, K. and Shulman, R.G. (1977) Proc. Natl. Acad. Sci. (USA) 74, 5551–5553.
33 Eisenberg, H. and Wachtel, E.J. (1987) Annu. Rev. Biophys. Biophys. Chem. 16, 69–92.
34 Goulbourne, E., Jr., Matin, M., Zychlinsky, E. and Matin, A. (1986) J. Bacteriol. 166, 59–65.
35 Edzes, H.T. and Berendsen, H.J.C. (1975) Annu. Rev. Biophys. Bioeng. 4, 265–285.
36 Gupta, R.K. and Gupta, P. (1982) J. Magn. Reson. 47, 344–350.
37 Evans, F.E. (1979) Arch. Biochem. Biophys. 193, 63–75.
38 Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1986) Data for Biochemical Reseach (3rd Edn.), pp. 408 and 414, Clarendon Press, Oxford.
39 Michel, H. and Oesterhelt, D. (1976) FEBS Lett. 65, 175–178.
40 Grey, V.L. and Fitt, P.S. (1976) Can. J. Microbiol. 22, 440–442.
41 Silver, S., Johnseine, P. and King, K. (1970) J. Bacteriol. 104, 1299–1306.
42 Jasper, P. and Silver, S. (1978) J. Bacteriol. 133, 1323–1328.
43 Arshavsky, V.Y., Baryshev, V.A., Brown, I.I., Glagolev, A.N. and Skulachev, V.P. (1981) FEBS Lett. 133, 22–26.
44 Ginzburg, M., Sachs, L. and Ginzburg, B.-Z. (1971) J. Membr. Biol. 5, 78–101.
45 Kirk, R.G. and Ginzburg, M. (1972) J. Ultrastruct. Res. 41, 80–94.
46 Nicolay, K. (1983) Ph.D. thesis, University of Groningen.