J. Membrane Biol. 33, 385–400 (1977)

Pulsed Nuclear Magnetic Resonance Study of ^{39}K within Halobacteria

Mordechai Shporer* and Mortimer M. Civan

Departments of Physiology and Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pa. 19174

Received 27 September 1976

Summary. The ^{39}K contents of isolated pellets and supernatant solutions from suspensions of *Halobacterium halobium* were studied at 21–22 °C by pulsed NMR spectroscopy. The rates of transverse relaxation were measured directly from the free induction decay (FID). The rate of longitudinal relaxation was measured by studying the FID after pairs of pulses of approximately 90°. Care was exercised to minimize the effect of magnetic field inhomogeneity; its contribution to the FID was approximately 25–30 sec^{-1}. The transverse relaxation process was found to consist of at least two components, whose rates were 321–449 sec^{-1} and 1,122–2,067 sec^{-1}. In one preparation where the longitudinal relaxation process was studied, the data could be well fit to a single exponential relaxing at 253 ± 33 (mean $\pm 95\%$ confidence limits) sec^{-1}. Comparison of the relative intensities of the NMR signals with the results of atomic absorption photometric analyses indicated that the great bulk of the intracellular ^{39}K was detected by the NMR techniques used. The data obtained from the current NMR of *H. halobium* are consistent with: (1) fractional binding of $<3\%$ of the total intracellular K^+, (2) a small ordering factor characterizing all of the intracellular K^+, or (3) some combination of the two.

The halobacteria have stimulated considerable recent interest because of the striking bioenergetic properties of the purple membrane of certain species (Oesterhelt & Stoekenius, 1973). However, the ionic compositions of the intracellular and extracellular milieux of these organisms in nature is perhaps even more remarkable. The extremely halophilic bacteria can grow in media nearly saturated in NaCl. The intracellular K^+ concentration can be maintained at similarly high levels, even when the external K^+ is less than 1 mM (Christian & Waltho, 1962; Ginzburg, Sachs & Ginzburg, 1970; Gochnauer & Kushner, 1971; Lanyi &

* On leave from the Department of Isotope Research, The Weizmann Institute of Science, Rehovot, Israel.

Silverman, 1972). Another unusual characteristic of these bacteria is that they can undergo cell swelling without necessarily rupturing their elastic cell-envelope; their cell volume is highly dependent upon the ionic composition and pH of the external bathing medium (Ginzburg & Ginzburg, 1976).

From their studies of lysed freeze-thawed cells, using ion-specific electrodes and atomic absorption, Lanyi and Silverman (1972) concluded that the intracellular K^+ of halobacteria is free. On the other hand, two previous studies of halobacteria based on the techniques of nuclear magnetic resonance (NMR) have suggested that binding or immobilization of intracellular K^+ is largely responsible for the differences in intracellular and extracellular ionic composition. Using continuous wave (cw) NMR, Magnuson and Magnuson (1973) observed only a single narrow spectral line for ^{39}K within preparations of *Halobacterium salinarium*. It should be emphasized that, when a sample is characterized by a superposition of spectral lines, the wider lines may be easily overlooked in the cw mode of NMR detection. Thus, the small narrow line observed by Magnuson and Magnuson may have reflected ^{39}K in the extracellular space. The interstitial space in pellets of halobacteria is large; even after centrifugation at $13{,}000 \times g$ for 20 min, the interstitial volume has been measured to be $28 \pm 3\%$ of the total pellet weight (Ginzburg & Ginzburg, 1976). Magnuson and Magnuson (1973) also lysed samples of bacteria, and then added sufficient KCl to produce a 0.9 M ^{39}K solution; however, they were only able to detect approximately half of the added ^{39}K with their NMR techniques. This effect may well have reflected a first-order nuclear quadrupole interaction of the ^{39}K, analogous to that characterizing intracellular ^{23}Na (Shporer & Civan, 1972; Berendsen & Edzes, 1973; Shporer & Civan, 1974).

Cope and Damadian (1970) have applied pulsed NMR techniques to the ^{39}K of *Halobacterium halobium*. They have reported simple exponential behavior of the transverse relaxation process proceeding at a rate $(1/T_2)$ of 2,857 sec^{-1} (presumably at room temperature); this reported value is two orders of magnitude greater than that characterizing ^{39}K in aqueous solution. As discussed in detail elsewhere (Civan, McDonald, Pring & Shporer, 1976), the equipment used by these investigators seems to have been characterized by a large inhomogeneity of magnetic field, so that the true rate of transverse relaxation may be considerably lower than their measured value.

In order to examine this problem in greater detail, the following studies of *Halobacterium halobium* were performed. Considerable care

was taken to minimize magnetic field inhomogeneity. Sample volumes were reduced to a value 4–5 times smaller than that used by Cope and Damadian (1970). In addition, the superconducting magnet used was part of a high resolution NMR system designed specifically to provide highly homogeneous fields.

Materials and Methods

Biological Specimens

Halobacterium halobium, generously provided by Joan J. Englander and Dr. S.W. Englander, was grown for 4–7 days at 37 °C using the technique described by Oesterhelt and Stoekenius (1974). A slightly modified incubation medium was used, consisting of (in mM): Na^+, 4,288.1; Mg^{++}, 81.2; K^+, 26.8; Ca^{++}, 1.8; Cl^-, 4,308.3; SO_4^{--}, 81.2; citrate, 10.2; supplemented with 10 g/liter of bacteriological peptone (code 49-111-03, Inolex Corp., Glenwood, Ill.).

Pellets of bacteria were isolated as described by Ginzburg *et al.* (1970) and Ginzburg and Ginzburg (1976), spinning the bacterial suspension at $13{,}000 \times g$ for approximately 20 min in a refrigerated centrifuge. Some samples were subsequently resuspended by scraping the cells off the walls of the centrifuge bottle with a spatula into an added volume of supernatant, mixing with a Vortex-Genie stirrer (Scientific Industries, Inc., Bohemia, N.Y.) and finally stirring with a magnetic stirrer.

Chemical Analysis

The K^+ contents of several bacterial pellets and supernatant solutions were analyzed by techniques similar to those commonly used in this (Ginzburg *et al.*, 1970) and other tissues (Macknight, Civan & Leaf, 1975). The wet weight of each pellet was initially determined by weighing the sample in a previously tared flask; the samples were chosen to be approximately 0.5 g in weight. The dry weight was determined after drying the pellet to constant weight at 105 °C. Each sample was extracted in 150 cc of deionized water for 7 days, following which aliquots were taken for analysis with a flame photometer (Model 343, Instrumentation Laboratories, Inc., Lexington, Mass.). The presence of Na^+ in the fluid for analysis alters the flame photometric signal observed for K^+. For this reason, sufficient NaCl was added to each aliquot to provide a large stable background level of Na^+ (of 219–241 mM), which was 1–2 orders of magnitude larger than the unknown K^+ concentrations. Supernatant samples and standards were prepared in a similar way. Determinations were performed in duplicate, and proved to be in uniformly good agreement with each other within a range of $\pm 1\%$.

For certain of the calculations, it was necessary to know the specific gravity of the bacterial pellets. This was measured by weighing the same NMR sample tubes first with deionized water, and later with the same volume of pellet. From an average of two determinations, the specific gravity was found to be 1.21; this value is in agreement with a value of 1.18 obtained by Ginzburg *et al.* (1970) under similar conditions for a different species of halobacterium.

NMR Techniques

All NMR measurements were performed with a pulse spectrometer (Model CPS-2, Spin-Lock Instruments, Ltd., Port Credit, Ontario, Canada) operating at 10.2 MHz. A magnetic field of 51.7 kgauss was generated by the superconducting magnet of a high resolution spectrometer (Varian HR 220). The sample chamber was an rf coil (10 mm in inner diameter and 15 mm in length), which in turn was introduced into the clear bore of the magnet, with its principal axis oriented perpendicular to the superconductor solenoid. The chamber consisted of a hollow cylinder constructed from Lucite, whose inner diameter was 8 mm, inner length 8.5 mm, and volume therefore 0.43 cm^3.

The apparent transverse relaxation time T_2^* was measured directly from the free induction decay (FID) following a pulse 50–60 μsec in duration; this duration was adjusted to produce a 90° pulse in an aqueous standard containing 4 M KCl. From measurements of the apparent rates of transverse $(1/T_2^*)$ and longitudinal relaxation $(1/T_1)$ of ^{39}K in such aqueous standards under the current operating conditions, we have found that the magnetic field inhomogeneity contributes only 25–30 sec^{-1} to $(1/T_2^*)$ (Civan *et al.*, 1976). As will be apparent in the results section, this contribution constituted a negligible fraction of the observed rates of transverse relaxation of the biological samples.

T_1 was measured by studying the free induction decay following pairs of pulses of approximately 90°.

The output from the detector was filtered with an RC network whose time constant was 10 μsec.

Data Acquisition

As previously described (Civan *et al.*, 1976), a Varian 620*i* computer, equipped with a 13 bit 50 kHz conversion rate analog-to-digital converter (ADC) and with 9 and 14 bit digital-to-analog converters (DAC), was used for noise reduction and data display. A command pulse from the computer was transmitted to the spectrometer, initiating a single pulse train which was then under the control of the pulse programmer. The programmer signalled the computer which would then begin data acquisition after an optional acquisition delay period. Following the data acquisition, a pulse delay ensued: the magnitude of that delay was chosen, so that the total duration of each transient study was 4–5 times longer than the value of T_1 for the sample.

Noise was reduced by means of a double-precision (30 bit) summation type program. The program provided a baseline correction by sampling the receiver output in the absence of pulses. Bias in the ADC and the DC offset in the receiver output were subtracted from the observed signals. As many as 5,000 free induction decays were sampled in a single measurement of T_2. Therefore, a secondary baseline correction was included after every hundredth FID, and a tertiary baseline correction after every thousandth FID to deal with the small round-off error in the original correction factor.

The maximum sampling rate was limited by the software used to 180 μsec per point.

Immediately following an applied pulse, a signal artifact was recorded even in the absence of a sample. After averaging over tens of thousands of pulse sequences, the pulse artifact was still clearly detectable 400 μsec after the end of the 90° pulse. For this reason, data were analyzed only after 600 μsec had elapsed.

Data Reduction

The NMR data obtained were fitted with a standard multiexponential optimization program available at the Medical School Computer Facility to the following functional

forms:

$$M_x = M_z e^{-(1/T_2)t} + A \tag{1}$$

and

$$M_x = M_z e^{-(1/T_2)t} + M_z' e^{-(1/T_2')t} + A. \tag{2}$$

In studies of T_1, a series of free induction decays was studied after the second of paired pulses of approximately 90°, separated by a variable interval τ. The values for M_z were fitted by the multiexponential optimization program to the forms:

$$M_z = M_0(1 - e^{-\tau/T_1}) + A \tag{3}$$

and

$$M_z = M_0(1 - e^{-\tau/T_1} - e^{-\tau/T_1'}) + A. \tag{4}$$

As described previously (Civan *et al.*, 1976), the program used Fletcher and Powell's (1963) modification of Davidon's function minimization method in the form of a locally improved version of that available in the IBM Scientific Subroutine Package. In addition to estimating the parameters, this procedure estimates the variance-covariance matrix of the optimization; the latter was used to calculate confidence limits for the parameter estimates.

When a series of measurements at various values of τ was analyzed for the muscle samples, the values obtained for M_x 0.6 msec after the end of the second pulse were taken as a measure of M_0, and fitted to Eqs. (3) and (4) as functions of τ.

Each value calculated with the computer program is presented in the results section in the form of a mean $\pm$the 95% confidence limits.

Results

Fig. 1 illustrates a representative free induction decay for ^{39}K in a pellet of halobacteria (Exp. C_1 of Table 1). The data could be fit reasonably well with a single exponential, whose apparent rate of relaxation ($1/T_2^*$) was $553 \pm 17\,sec^{-1}$. However, the fit was appreciably improved when two exponentials were used. In Figs. 1*c* and 1*d*, the solid lines have been calculated on the basis of two approximately equal fractions relaxing at $1{,}374 \pm 130$ sec^{-1} and at 409 ± 17 sec^{-1}. The degree of the improvement of fit was estimated from the ratio (d^2) of the sum of the squares of the deviations calculated for the data fit with two exponentials to that fit with one; thus, a value of $d^2 < 1$ implies an improved fit with two exponentials. For the data of Fig. 1, $d^2 = 0.090$.

In each of the preparations studied (Table 1) d^2 was $\ll 1$, indicating that the transverse relaxation process consists of at least two exponential components. In all, three batches of bacteria (A, B and C) were studied. The data of experiments A, B and C_0 were obtained under comparable conditions; the measurements were carried out immediately after isolating the cell pellets. Part of the bacterial suspension studied in experiment

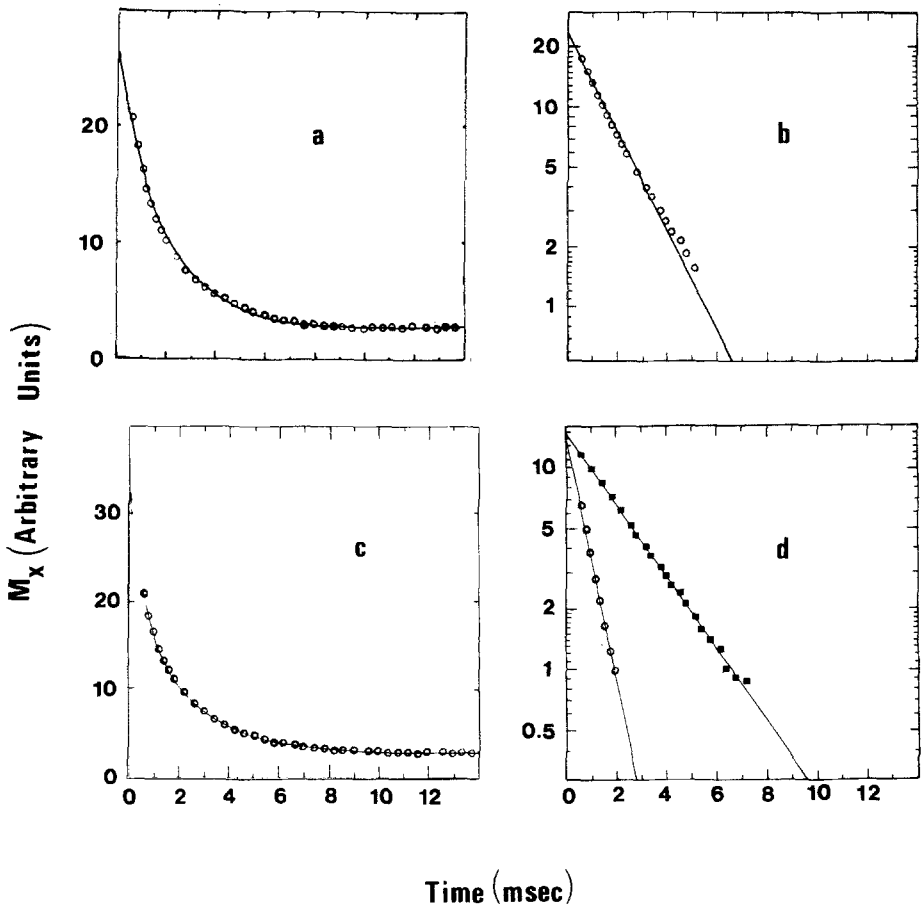

Fig. 1. Transverse relaxation of ^{39}K in a pellet of *Halobacterium halobium*. The data were obtained from Exp. C_1 of Table 1. The magnetization (M) is presented as a function of time (t) following a single pulse of approximately 90°. All panels constitute fits of the same data points. The ordinate scales of Figs. 1*a* and 1*c* are arithmetic, while those of Figs. 1*b* and 1*d* are logarithmic. In panels *a* and *b* the results have been fit to a single exponential:

$$M_x = (23.7 \pm 0.57)\, e^{-(0.55 \pm 0.017)t} + (2.85 \pm 0.059).$$

In panels *c* and *d*, the data have been fit to two exponentials:

$$M_x = (14.9 \pm 0.82)\, e^{-(1.4 \pm 0.13)t} + (14.9 \pm 1.1)\, e^{-(0.41 \pm 0.017)t} + (2.71 \pm 0.024).$$

In each equation, the uncertainties have been expressed as ±95% confidence limits

C_0 was stored at 4 °C for four days before isolating the bacterial pellets. This material was used in determining the rate of longitudinal relaxation ($1/T_1$) of the intracellular ^{39}K. Both C_1 and C_2 were studies of free induction decays following a single 90° pulse applied to the same pellet.

Table 1. Transverse relaxation of ^{39}K in pellets of *Halobacterium halobium*[a]

Exp.	Single exponential	Two exponentials					d^2
	$(1/T_2^*)$ (sec^{-1})	M_0 (Arbitrary	M_0' units)	$\frac{M_0'}{M_0+M_0'}$	$(1/T_2^*)$ (sec^{-1})	$(1/T_2^{*\prime})$ (sec^{-1})	
A	708±37	8.3±0.50	4.8±0.30	0.37	2,095±181	477±20	0.042
B	614±33	13.4±1.7	8.1±0.68	0.38	1,933±299	421±23	0.13
C_0	486±17	16.6±0.69	13.4±0.80	0.45	1,258±79	348±12	0.058
C_1	553±17	14.9±0.82	14.9±1.1	0.50	1,374±130	409±17	0.090
C_2	523±15	13.4±0.90	12.2±1.2	0.48	1,150±130	369±20	0.096

[a] Three batches of bacteria (A, B, and C) were studied. Experiments A, B and C_0 were performed directly after preparing the cell pellets. Part of the bacterial suspension studied in Exp. C_0 was stored at 4 °C for four days before isolating and analyzing the bacterial pellets for Exps. C_1 and C_2. The results tabulated for the latter two experiments were obtained from free induction decays conducted approximately 4 hr apart. All entries appear as means ± the 95% confidence limits.

The data from each experiment were fit both to a single exponential relaxing at a rate $(1/T_2^*)$ and to two exponentials relaxing at rates $(1/T_2^*)$ and $(1/T_2^{*\prime})$. The apparent rates of transverse relaxation, $(1/T_2^*)$ and $(1/T_2^{*\prime})$ reflected both the true rates of transverse relaxation, $(1/T_2)$ and $(1/T_2')$, respectively, and the effect of magnetic field inhomogeneity. The latter amounted to only 25–30 sec^{-1}, thus contributing $<10\%$ to the total measured rates. The absorption coefficients for the two rates of relaxation, $(1/T_2^*)$ and $(1/T_2^{*\prime})$, are entered under M_0 and M_0', respectively.

In each experiment, the free induction decays were better fit by two exponentials than by one. The degree of improvement of fit is quantified by (d^2), the ratio of the sum of the squares of the deviations calculated for the results fit with two exponentials to that fit with one.

However, an interval of approximately 4 hr separated the two determinations.

With time, over the course of the day, there seem to have been changes in the transverse relaxation rates $(1/T_2^*)$ and $(1/T_2^*)'$ of the faster and slower relaxing fractions, respectively. However, the maximal variation in the values of these rates over a period of 4–5 days was only 17–18% of the initial values.

For the three preparations studied, $(1/T_2^*)$ ranged from 1,150 to 2,095 sec^{-1}, and $(1/T_2^*)'$ ranged from 348 to 477 sec^{-1} (Table 1). With the current apparatus, the contribution of the field inhomogeneity to the free induction decay is 25–30 sec^{-1} (Civan *et al.*, 1976). Subtracting 28 sec^{-1} from the observed apparent rates of relaxation, the true rates of transverse relaxation are calculated to be 1,122–2,067 sec^{-1} and 320–449 sec^{-1} for the faster and slower relaxing fractions, respectively. The latter constituted 37–50% of the total observed intensity (M_0+M_0') (Table 1).

Table 2. Comparison of photometric and NMR determinations of the ^{39}K content of supernatants, pellets and suspension of *Halobacterium halobium*[a]

Sample	Photometric analysis (mM)	NMR analysis (mM)
Supernatant I	38.0	38.0 (standard)
Pellet I	1.8×10^3	2.1×10^3
Suspension	280	244
Supernatant II	100	97.9
Pellet II	1.4×10^3	–

[a] The intensity of the NMR signal obtained with Supernatant I was used as the reference standard for NMR analysis of the other samples.

From these data, it appeared that a large fraction of the observed signal was undergoing transverse relaxation at a rate an order of magnitude slower than that reported by Cope and Damadian (1970). However, it was possible that the total NMR signal observed represented only a fraction of the total spectrum of the intracellular ^{39}K. In order to examine this possibility, aliquots from the same specimens were separately analyzed by NMR spectroscopy and flame photometry (Table 2). After an initial centrifugation at $13{,}000 \times g$, aliquots of the pellet (Pellet I) and supernatant (Supernatant I) were studied. Some of the bacterial pellet was resuspended and analyzed (Suspension sample). Subsequently, the suspension was centrifuged a second time and samples of this supernatant (Supernatant II) were subjected to photometric and NMR analysis, as well; the second pellet (Pellet II) was analyzed only by flame photometry.

The NMR results are presented in Fig. 2. Panels 2*a*, 2*b*, 2*c* and 2*d* present the free induction decay obtained with Pellet I, Suspension, Supernatant II, and Supernatant I, respectively, using the same scale for each ordinate. The free induction decays of Pellet I and Suspension were fitted to two exponentials; those of the two supernatants were fitted to a single exponential. The relative intensities of the different samples were obtained from the intercepts of the empirical curves with the y-axis. From these NMR data alone, it is clear that a large fraction of the intracellular ^{39}K was NMR-visible.

The relative intensities calculated from the NMR data were normalized to the volume (0.43 cm^3) of the sample chamber. On the other hand, the high viscosity of the pellets and suspension precluded taking precise

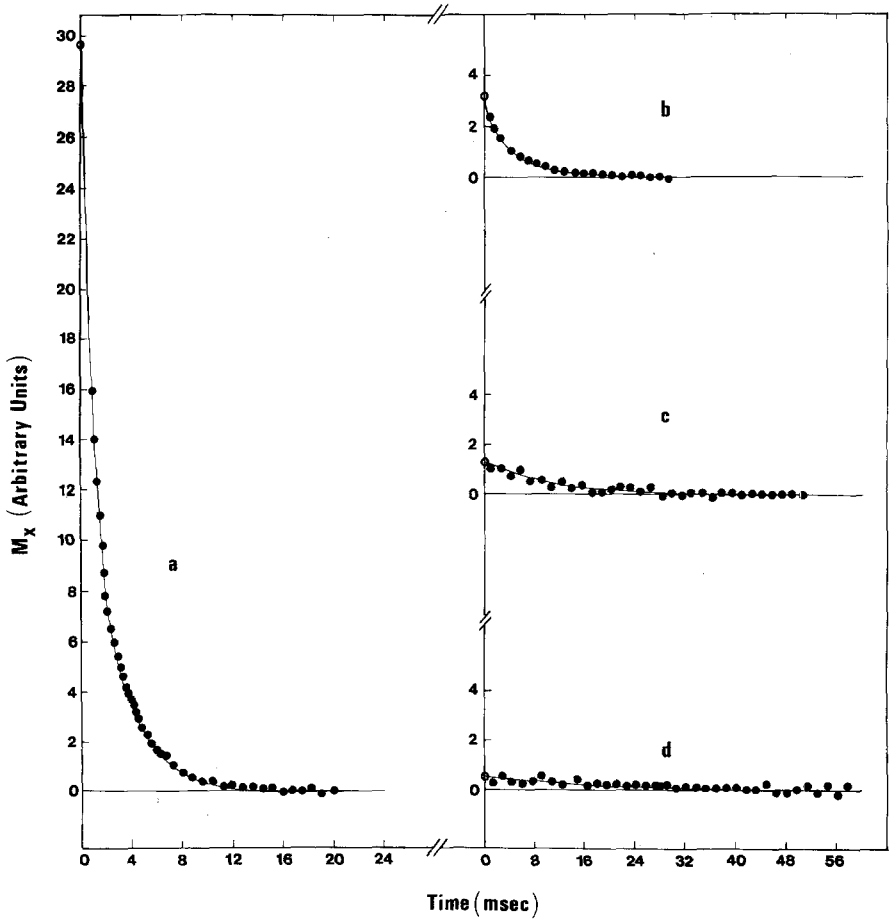

Fig. 2. Transverse relaxation of ^{39}K in pellet, supernatants and suspension from same preparation of *Halobacterium halobium*. The aliquots studied were taken from preparation C of Table 1. The magnetization (M_x) is presented as a function of time (t) following a pulse of 90°. The same ordinate scale is used in all four panels in order to present clearly the relative magnitudes of the NMR signals from the different samples. The time scale for panels *b*, *c* and *d* is twice as compressed as that for *a*. The data of panels *a*, *b*, *c* and *d* were taken from Pellet I, Suspension, Supernatant II and Supernatant I, respectively, of Table 2. The results were fit to the following equations:

(*a*) $M_x=(16.3\pm0.67)\,e^{-(1.3\pm0.10)t}+(13.1\pm0.78)\,e^{-(0.35\pm0.012)t}$;

(*b*) $M_x=(1.6\pm0.19)\,e^{-(0.7\pm0.21)t}+(1.7\pm0.24)\,e^{-(0.13\pm0.020)t}$;

(*c*) $M_x=(1.40\pm0.093)\,e^{-(0.09\pm0.010)t}$;

(*d*) $M_x=(0.50\pm0.066)\,e^{-(0.04\pm0.015)t}$. The noise levels for the four curves are different, reflecting the different numbers of transients averaged in accumulating the data

aliquot volumes for the flame photometric analyses; instead, the aliquots were normalized on the basis of their weight. In order to relate the two sets of analyses to each other, it was necessary to measure the specific gravities of the pellet and supernatant. The specific gravity of the

bacterial cell pellet δ_c was measured by weighing the same sample tube when filled with cell pellet and when filled with deionized water; δ_c was found to be 1.207. The specific gravity of the supernatants (δ_s) was measured by weighing 10 cc aliquots, and found to be 1.165. The total volume (V_T), total weight (w_T), weight of cell pellet (w_c) and weight of supernatant (w_s) within a given sample of suspension were related by the following simple expression:

$$w_T = \frac{V_T}{\frac{f_c}{\delta_c} + \frac{1-f_c}{\delta_s}} \tag{5}$$

$$w_c = f_c w_T \tag{6}$$

$$w_s = (1-f_c) w_T \tag{7}$$

where f_c is the fractional weight of the pellet within the suspension. f_c was determined to be 0.137 by weighing the suspension in a tared centrifuge tube, centrifuging at $13{,}000 \times g$ for 20 min, removing the supernatant, and reweighing the tube.

The results of the NMR and photometric analyses are presented in Table 2. The relative intensity of the NMR signal of ^{39}K within the various samples has been related to that obtained with the initial supernatant (Supernatant I); thus, this intensity was set equivalent to 38.0 mM (the value obtained from photometric analysis of the same material). As will be appreciated from Table 2, results of the two analyses are in satisfactory agreement. If anything, the K^+ concentration of the cell pellet was calculated to be slightly higher by NMR measurement than by flame photometric analysis. We conclude, therefore, that the great bulk of the intracellular ^{39}K was detected by the present NMR techniques.

It should be added that the various measurements obtained by flame photometry appeared to be internally consistent. Specifically, we would expect the measurements to conform to the following expression of material balance for K^+:

$$(1/V_T)[(f_c w_T/\delta_c)(K^+)_{cII} + (1-f_c)(w_T/\delta_s)(K^+)_{sII}] = (K^+)_{susp} \tag{8}$$

where $(K^+)_{cII}$, $(K^+)_{sII}$ and $(K^+)_{susp}$ are the K^+ concentrations in millimoles/liter for the Pellet II, Supernatant II, and Suspension samples, respectively. From the data presented above and in Table 2, the left-hand side of Eq. (8) may be calculated to be 263 mM, only 6% less than the

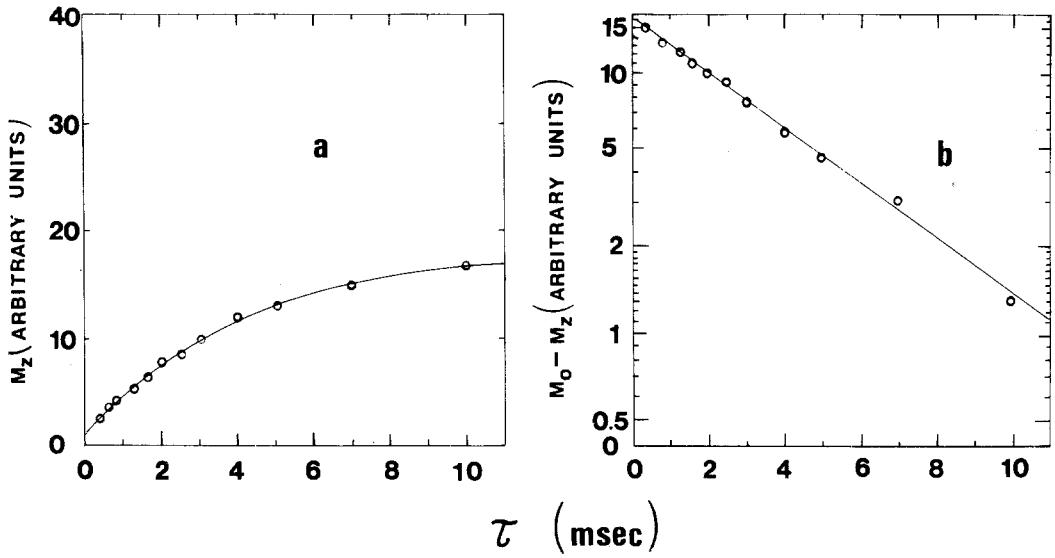

Fig. 3. Longitudinal relaxation of ^{39}K in a pellet of *Halobacterium halobium*. The z-component of the magnetization (M_z) following pairs of pulses is plotted as a function of the time interval (τ) separating those pulses. The data were obtained from preparation C, during the period between Exps. C_1 and C_2 (Table 1). The data have been fit to a single exponential:

$$M_z = (16.5 \pm 0.73)(1 - e^{-(0.25 \pm 0.033)\tau}) + (1.2 \pm 0.47).$$

The fit was not improved by using two exponentials

value actually measured for $(K^+)_{susp}$ (Table 2). This agreement suggests that the degree of ionic extraction from the tissues and actual analysis by flame photometry were technically satisfactory.

In order to measure $(1/T_1)$, a series of experiments with various values of τ were fitted to Eqs. (3) and (4). Fractional distortion of the values for M_z resulting from deletion of the first 0.6 msec should have been constant, so that the estimates of $(1/T_1)$ obtained with functions 3 and 4 should not have been distorted. Fig. 3 presents these data for M_z as a function of τ obtained from the preparation of Exps. C_1 and C_2. The fit to a single exponential decaying with a rate of longitudinal relaxation of 253 ± 33 sec^{-1} was excellent. No improvement in fit resulted from using two exponentials (function 4) instead of a single exponential (function 3).

Discussion

The present study demonstrates that the great bulk of the intracellular ^{39}K of *Halobacterium halobium* is NMR-visible. The trans-

verse relaxation process consists of at least two components, whose rates are $(1/T_2)_a = 320$–449 sec^{-1} and $(1/T_2)_b = 1{,}122$–$2{,}067$ sec^{-1} at 21–22 °C. Between 37 and 50% of the total signal relaxed at rate $(1/T_2)_a$, which is an order of magnitude slower than that previously reported (Cope & Damadian, 1970). In the single preparation where the longitudinal relaxation process was studied, the data could be well fit to a single exponential relaxing at a rate $(1/T_1) = 253 \pm 33$ sec^{-1}. The rates of nuclear relaxation of ^{39}K at this temperature in free solution are 15 ± 2 sec^{-1} (Civan *et al.*, 1976).

These data may be further analyzed by means of the equations presented below. In general, for nuclides such as ^{39}K and ^{23}Na with spin numbers (I) of 3/2, both $(1/T_1)$ and $(1/T_2)$ consist of two components (Hubbard, 1970; Rubinstein, Baram & Luz, 1971):

$$(1/T_1)_{\mathrm{I}} = \left[\frac{(e^2 qQ)^2}{10}\right]\left[1+\frac{\eta^2}{3}\right]\tau_c\left[\frac{1}{1+(\omega_0\tau_c)^2}\right] \tag{9}$$

$$(1/T_1)_{\mathrm{II}} = \left[\frac{(e^2 qQ)^2}{10}\right]\left[1+\frac{\eta^2}{3}\right]\tau_c\left[\frac{1}{1+4(\omega_0\tau_c)^2}\right] \tag{10}$$

where (e^2qQ) is the nuclear quadrupole coupling constant, η is an asymmetry factor characterizing the electric field gradient imposed on the ^{39}K nucleus (Shporer & Civan, 1976), and τ_c is the correlation time. Only 20% of the signal intensity decays with the longitudinal relaxation rate $(1/T_1)_{\mathrm{I}}$, while the remaining 80% decays at the rate $(1/T_1)_{\mathrm{II}}$. Since the two rates differ at most by a factor of 4, and since $(1/T_1)_{\mathrm{I}}$ reflects only 20% of the total signal, $(1/T_1)_{\mathrm{I}}$ is not readily detectable.

The two rates of transverse relaxation for nuclides with I = 3/2 are:

$$(1/T_2)_{\mathrm{I}} = \left[\frac{(e^2 qQ)^2}{20}\right]\left[1+\frac{\eta^2}{3}\right]\tau_c\left[\frac{1}{1+(\omega_0\tau_c)^2}+\frac{1}{1+4(\omega_0\tau_c)^2}\right] \tag{11}$$

$$(1/T_2)_{\mathrm{II}} = \left[\frac{(e^2 qQ)^2}{20}\right]\left[1+\frac{\eta^2}{3}\right]\tau_c\left[1+\frac{1}{1+(\omega_0\tau_c)^2}\right]. \tag{12}$$

$(1/T_2)_{\mathrm{I}}$ characterizes the decay of 40% of the signal and can be attributed to the transition between the $+(1/2)$ and $-(1/2)$ Zeeman energy levels. The remaining 60% of the signal decays at the rate $(1/T_2)_{\mathrm{II}}$, and can be attributed to the transitions between the $+(3/2)$ and $+(1/2)$, and between the $-(1/2)$ and $-(3/2)$ energy levels.

If all the intracellular ^{39}K consisted of a single population of immobilized K^+, the measured rate $(1/T_2)_a$ could then be identified as $(1/T_2)_{\mathrm{I}}$

and $(1/T_2)_b$ as $(1/T_2)_{II}$ in Eqs. (11) and (12). The measured rate $(1/T_1)$ would be identical with $(1/T_1)_{II}$ of Eq. (10). Within this framework, any one of the three possible pairs of equations from Eqs. (10)–(12) could be used to solve for τ_c and $\left[(e^2qQ)\sqrt{1+\frac{\eta^2}{3}}\right]$. As can be appreciated from Table 1, the relaxation parameters characterizing ^{39}K within the halobacteria varied somewhat from sample to sample. To reduce this source of variation, the analysis was carried out on data obtained from a single sample (Exps. C); in this case, $(1/T_2)_a$ was 381 sec^{-1}, $(1/T_2)_b$ was 1,346 sec^{-1}, and $1/T_1$ was 253 sec^{-1}. However, even with this precaution, the calculated results were not internally consistent. Application of the three pairs of equations led to estimates of $\left[(e^2qQ)\sqrt{1+\frac{\eta^2}{3}}\right]$ ranging from 0.15 to 0.22 MHz, depending upon the pair chosen. This value is 3.6–5.3 times smaller than the lower bound estimated for intracellular ^{23}Na (Shporer & Civan, 1977). The estimate for the $\left[(e^2qQ)\sqrt{1+\frac{\eta^2}{3}}\right]$ of ^{39}K must be even higher since the nuclear magnetic relaxation rates of ^{23}Na and ^{39}K are similar in aqueous solution, but the τ_c characterizing ^{39}K is expected to be shorter than that characterizing ^{23}Na (Samoilov, 1972). Thus, the results of the present study cannot be accommodated within the framework of a single immobilized intracellular phase of K^+.

The data may be interpreted within a variety of alternative models. The second simplest concept would be that each of the measured rates of relaxation [$(1/T_1)$, $(1/T_2)_a$ and $(1/T_2)_b$] consists of two components, one of which (R) is characterized by a very short correlation time of the same order of magnitude as that characterizing ^{39}K in aqueous solution, and is therefore common to all three. The second component is considered characterized by a longer correlation time, rendering the three measured rates of nuclear relaxation different. Within this framework, the measured parameters would be related to the relaxation rates of Eqs. (10)–(12) by the expressions:

$$(1/T_1)=(1/T_1)_{II}+R \tag{13}$$

$$(1/T_2)_a=(1/T_2)_I+R \tag{14}$$

$$(1/T_2)_b=(1/T_2)_{II}+R. \tag{15}$$

From Eqs. (10)–(15) and the measured values of $(1/T_1)$, $(1/T_2)_a$ and $(1/T_2)_b$ cited above, τ_c may be calculated to be 3.32×10^{-8} sec, the apparent quadrupolar coupling constant is 0.125 MHz, and R is 215 sec^{-1}.

There are at least two simple physical models which could serve as the basis for the above formalism. First, a rapid exchange might exist between two populations of intracellular ^{39}K. Distinct populations of free and bound ^{39}K would be characterized by very short and by relatively longer correlation times, respectively. In this case,

$$R=\frac{P_f}{T_{1f}}=\frac{P_f}{(T_2)_{If}}=\frac{P_f}{(T_2)_{IIf}}=215\,\text{sec}^{-1} \tag{16}$$

where P_f is the relative mole fraction of free ^{39}K, and the subscript f refers to the free state. Here, the apparent quadrupolar interaction would correspond to $\left[\sqrt{P_b}(e^2qQ)\sqrt{1+\frac{\eta^2}{3}}\right]$ (Shporer & Civan, 1974), where P_b is the relative mole fraction of bound ^{39}K, and $\left[(e^2qQ)\sqrt{1+\frac{\eta^2}{3}}\right]$ is the true quadrupolar interaction in the bound state. Using our conservatively low estimate of $\left[(e^2qQ)\sqrt{1+\frac{\eta^2}{3}}\right]$, we find the upper limit for P_b to be 2.4%. It should be noted that the relaxation rate R of 215 sec^{-1}, ascribed in this model to ^{39}K in the free phase, is much higher than the nuclear relaxation rates of ^{39}K in aqueous solution (Civan *et al.*, 1976); this enhancement might reflect an electrostatic interaction between K^+ and intracellular polyelectrolytes.

A second simple model could also provide the physical basis for the formalism of Eqs. (13)–(15). Cations, including ^{39}K, could diffuse between domains of ordered macromolecules (Berendsen & Edzes, 1973). Within this framework, R would represent the nuclear relaxation rates in the domains. These rates are larger than in aqueous solution because of ion condensation effects in the vicinity of macromolecular polyelectrolytes. Such enhancements have been noted for ^{23}Na in simple polyelectrolyte solutions (Shporer & Civan, 1977). Here, the apparent quadrupolar interaction would reflect incomplete averaging of the field gradient in each of the domains. Thus, the ordering factor (Shporer & Civan, 1976) ascribed to the domains would be small. The relatively long correlation time reflects diffusion of ^{39}K between the domains.

It will be appreciated that the above analysis constitutes only the simplest possible approach to the data. Eqs. (10)–(12) were developed on the presumption that reorientation of the electric field gradient applied to the ^{39}K nucleus, rather than fluctuations in its magnitude, constitutes the major mechanism for the rates of nuclear relaxation (Sutter &

Harmon, 1975). Actually, both τ_c and the ordering factor characterizing ^{39}K within the presumed domains may not constitute single values, but may consist of distributions of values. In addition, models can be formulated incorporating intermediate rates of exchange between free and immobilized fractions of K^+.

Because of these considerations, a unique mechanism cannot be defined by the data presented. However, the results clearly indicate that the immobilized fraction of intracellular K^+ within halobacteria cannot be more than a few per cent of the total. Thus, the current NMR data are consistent with the analyses of Lanyi and Silverman (1972), using ion-selective electrodes and atomic absorption spectroscopy, suggesting that the great bulk of the intracellular K^+ is in free form.

We are grateful to Joan J. Englander and Dr. S.W. Englander for their advice, for providing us with samples of *H. halobium*, and for making their laboratory facilities available to us. We also thank Miss Kim Peterson for her skillful technical assistance, and Mr. Joel DeLong for carrying out the atomic absorption photometric measurements.

Supported, in part, by a research grant from the National Institutes of Health (1 RO1 AM 1658603 A1). M.S. received partial salary support from the University of Pennsylvania-Israel Exchange Program.

References

Berendsen, H.J.C., Edzes, H.T. 1973. The observation and general interpretation of sodium magnetic resonance in biological material. *Ann. N.Y. Acad. Sci.* **204**:459

Christian, J.H.B., Waltho, J. 1962. Solute concentrations within cells of halophilic and non-halophilic bacteria. *Biochim. Biophys. Acta* **65**:506

Civan, M.M., McDonald, G.G., Pring, M., Shporer, M. 1976. Pulsed nuclear magnetic resonance study of ^{39}K in frog striated muscle. *Biophys. J.* **16**:1385

Cope, F.W., Damadian, R. 1970. Cell potassium by ^{39}K spin echo nuclear magnetic resonance. *Nature (London)* **228**:76

Fletcher, R., Powell, M.J.D. 1963. A rapidly convergent descent method for minimization. *The Computer J.* **6**:163

Ginzburg, M., Ginzburg, B.Z. 1976. Regulation of cell volume and ion concentrations in a *Halobacterium*. *J. Membrane Biol.* **26**:153

Ginzburg, M., Sachs, L., Ginzburg, B.Z. 1970. Ion metabolism in a *Halobacterium*. I. Influence of age of culture on intracellular concentrations. *J. Gen. Physiol.* **55**:187

Gochnauer, M.B., Kushner, D.J. 1971. Potassium binding, growth and survival of an extremely halophilic bacterium. *Can. J. Microbiol.* **17**:17

Hubbard, P.S. 1970. Non-exponential nuclear magnetic relaxation by quadrupole interactions. *J. Chem. Phys.* **53**:985

Lanyi, J.K., Silverman, M.P. 1972. The state of binding of intracellular K^+ in *Halobacterium cutirubrum*. *Can. J. Microbiol.* **18**:993

Macknight, A.D.C., Civan, M.M., Leaf, A. 1975. The sodium transport pool in toad urinary bladder epithelial cells. *J. Membrane Biol.* **20**:365

Magnuson, J.A., Magnuson, N.S. 1973. NMR studies of sodium and potassium in various biological tissues. *Ann. N.Y. Acad. Sci.* **204**:297

Oesterhelt, D., Stoeckenius, W. 1973. Functions of a new photoreceptor membrane. *Proc. Nat. Acad. Sci. USA* **70**:2853

Oesterhelt, D., Stoeckenius, W. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods in Enzymology.* **21**:667

Rubinstein, M., Baram, A., Luz, Z. 1971. Electronic and nuclear relaxation in solutions of transition metal ions with spin $S=3/2$ and 5/2. *Molec. Phys.* **20**:67

Samoilov, O.Ya. 1972. Residence times of ionic hydration. *In:* Water and Aqueous Solutions: Structure, Thermodynamics, and Transport Processes. R.A. Horne, editor, p. 597. Wiley-Interscience, New York

Shporer, M., Civan, M.M. 1972. NMR of ^{23}Na linoleate-water: Basis for an alternative interpretation of ^{23}Na spectra within cells. *Biophys. J.* **12**:114

Shporer, M., Civan, M.M. 1974. Effects of temperature and field strength on the NMR relaxation times of ^{23}Na in frog striated muscle. *Biochim. Biophys. Acta* **354**:291

Shporer, M., Civan, M.M. 1977. The state of water and alkali cations within the intracellular fluids: The contribution of NMR spectroscopy. *Curr. Top. Membr. Trans.* **9**:1

Sutter, E.J., Harmon, J.F. 1975. Molecular motion in supercooled liquids. I. Pulsed nuclear magnetic resonance of lithium 7 in 11 M aqueous lithium chloride. *J. Phys. Chem.* **79**:1958