BBA 75290

CHARACTERIZATION OF THE PLASMA MEMBRANE OF *MYCOPLASMA LAIDLAWII*

VI. POTASSIUM TRANSPORT

HYONG WON CHO AND HAROLD J. MOROWITZ
Department of Molecular Biophysics, Yale University, New Haven, Conn. (U.S.A.)

(Received February 5th, 1969)

SUMMARY

K^+ has been shown to be an absolute requirement for the growth of *Mycoplasma laidlawii*. A medium has been devised in which K^+ is the growth-limiting constituent. An internal K^+ concentration of about 70 mM is maintained over external concentrations ranging from 0.135 to 5.37 mM. Cells have been adapted to grow at concentrations of 0.034 mM. There is no Na^+ requirement, although there are limitations on the ionic composition of the medium. Rb^+ can partially substitute for K^+. Uptake experiments with $^{42}K^+$ indicate a half-time for K^+ exchange of about 5 min. Studies with inhibitors indicate that the exchange is energy dependent and appears to be mediated by sulfhydryl-containing proteins.

INTRODUCTION

The plasma membrane of procaryotic cells, lacking a cell wall, has been shown to be a very useful system for the elucidation of membrane structure[1]. The ultimate aim of such studies is to relate the biological functions of the membrane to its molecular structure. Membrane-bound enzymes[2] as well as active transport[3,4] have been reported for the Mycoplasma. The purpose of this paper has been to investigate in detail the transport of potassium across the plasma membrane of *Mycoplasma laidlawii*.

MATERIALS AND METHODS

Organism and growth conditions

Cells of *M. laidlawii* were grown statically at 37° in a modified form of the medium developed by Dr. Mark Tourtellotte in which the sole source of K^+ was in KCl which was added in variable amounts. The medium shown in Table I was supplemented with 5.37 mM of KCl for normal growth and for carrying stock cultures. In selected experiments the concentration of KCl was varied or other monovalent cations were substituted in the growth medium.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

TABLE I

THE COMPOSITION OF SEMIDEFINED CULTURE MEDIUM FOR *M. laidlawii*

Component	*Concn. (mg/l)*	*Component*	*Concn. (mg/l)*
L-Tyr	200	Adenosine	5
L-Try	200	Deoxyadenosine	5
L-Glu	200	Guanosine	5
L-Asp	150	Deoxyguanosine	5
L-Asn	150	Cytidine	5
L-Ala	725	Deoxycytosine	5
L-Ser	725	Uridine	5
L-Val	725	Thymidine	5
L-Arg	440	Sodium acetate	50
L-His	310	Na_2HPO_4	120
L-Lys	840	$(NH_4)_2SO_4$	120
L-Thr	460	$MgSO_4 \cdot 7H_2O$	200
L-Leu	1010	Pyridoxine	0.2
L-Ile	480	Niacine	0.2
L-Pro	500	Coenzyme A	0.5
L-Phe	500	Biotine	0.08
Gly	700	Thiamine	0.08
L-Met	325	Riboflavin	0.08
L-Cys Cys	30	NaCl	8 000
L-Gln	1000	Tris	1 000
		Glucose	10 000
		Difco PPLO serum fraction	20 ml
		Distilled water	1 000 ml

Cells were adapted to grow in low K^+ by culturing them in medium containing successively reduced concentrations of KCl. Cells were transferred at a given concentration until good growth was observed by turbidity and then transfers were made to lower K^+ concentrations. The serial transfers in KCl were from 5.37 to 0.537 to 0.269 to 0.135 to 0.068 mM, and at present the cells are being cultured in a medium containing 0.034 mM.

In studies of Na^+ requirement, the medium shown in Table I was modified by replacing Na_2HPO_4 and sodium acetate by their potassium salts. The NaCl concentration in the medium was varied and experiments were carried out replacing NaCl by a series of substituents.

Growth assay

Growth was determined either by absorbance at 420 $m\mu$ measured on a Beckman Model D.U. spectrophotometer or by colony count on a medium containing the following constituents per l: Bacto tryptose 10 g, agar 10 g, NaCl 5 g, Tris 1.5 g, glucose 10 g, Difco serum fraction 20 ml.

K^+ concentration

Direct measurement of K^+ concentrations of media and cell lysates was made with a flame photometer, Model 143 (Instrumentation Laboratory).

$^{42}K^+$ *uptake and concentration studies*

For studying K^+ uptake of normal cells, 50 ml of a log-phase culture growing at 37° in normal medium (Table I) supplemented with 5.37 mM of KCl were mixed at 37° with 50 ml of uninoculated medium containing 2 mC of $^{42}K^+$. At various times 10 ml of the mixture were rapidly filtered through a 0.22-μ pore size filter (Millipore Corp.) which had been previously soaked with wash medium (the wash medium is the same as the culture medium in Table I except that it lacks vitamins and serum fraction). Filtration was carried out under a negative pressure of 70 mm Hg. In previous studies when the filtration of *M. laidlawii* was carried out with a hypodermic syringe and a Swinney adaptor, the fraction of cells passing through a 0.22-μ pore size filter was 0.01 and 0.6 in duplicate experiments[5]. However, in control experiments for the present procedure when the cells were filtered by the application of negative pressure of 70 mm Hg, the values for the fraction of cells passing the filter were $2.1 \cdot 10^{-6}$ and $6.2 \cdot 10^{-6}$. These results indicate that the efficiency of the filtration of *M. laidlawii* depends on the pressure applied during the filtration. For the present experiments, the loss of cells during filtration was negligible. The cells on the filter were rapidly washed with 10 ml of ice-cold wash medium divided into three aliquots. The filters were air dried and placed in 5 ml of 0.25% lubrol solution (Lubrol WX is a nonionic detergent made by Imperial Industries). After 2 h 4 ml of the suspension of cell lysate were transferred to a counter tube and counted for γ-ray activity. As a control for the absorption of $^{42}K^+$ on cells, filters and elsewhere, the uptake experiment was carried out on a suspension of log-phase cells that had been killed by heating to 60° for 30 min.

In one series of experiments $^{42}K^+$ uptake studies were carried out in medium containing 0.537 mM KCl and using both normal cells grown at 5.37 mM and adapted cells grown at 0.068 mM. For the study with unadapted cells, the uptake of $^{42}K^+$ was started by the addition of 12 ml of normal log-phase culture to 188 ml of fresh medium containing 0.25 mC of $^{42}K^+$. For the study with cells adapted to very low K^+ medium, the uptake of $^{42}K^+$ was started by direct addition of 0.25 mC of $^{42}K^+$ to the culture in the logarithmic phase. The final concentration of K^+ in both the test systems was 0.537 mM KCl. Therefore, for the normal cells the concentration of K^+ in the medium was abruptly reduced by 10 times, whereas for the cells adapted to very low K^+ medium, the concentration was abruptly increased by 8 times. $^{42}K^+$ was obtained as the chloride from the Cambridge Nuclear Corp., and counting was carried out on the Automatic gamma counter, Model 4224 (Nuclear Chicago Corp.).

Determination of cell volume

1.5 l of the culture in the logarithmic phase were harvested by centrifugation at 12000 × *g* for 15 min at 4°. The pellet of cells was resuspended in 6 ml of the supernatant, transferred to the calibrated tapered centrifuge tube, centrifuged at 7000 × *g* for 30 min and the volume of the pellet was measured. From the plate-count and pellet volume a rough estimation of the cell volume per colony-forming unit was made.

RESULTS

The growth of unadapted *M. laidlawii* cells as a function of the K^+ concentration of the medium is shown in Table II for two independent experiments. These results indicate that for the medium used K^+ is growth limiting. Additional studies were carried out with K^+ concentration from 5.37 (400 mg/l KCl) to 16.1 mM (1200 mg/l KCl). The net growth as judged by absorbance did not vary over this concentration range.

TABLE II

GROWTH OF *M. laidlawii* AS A FUNCTION OF K^+ CONTENT IN THE MEDIUM

KCl (mM)	*Expt. I (72-h growth)*		*Expt. II (50-h growth)*	
	$A_{420\,m\mu}$	*Viable cells per ml*	$A_{420\,m\mu}$	*Viable cells per ml*
5.37	0.247	$8.6 \cdot 10^8$	0.247	$1.39 \cdot 10^9$
2.69	0.231	$5.5 \cdot 10^8$	—	—
1.35	0.147	$3.3 \cdot 10^8$	0.121	$3.6 \cdot 10^8$
1.01	0.103	$1.5 \cdot 10^8$	0.088	$1.87 \cdot 10^8$
0.67	0.084	$8.5 \cdot 10^8$	0.084	$1.28 \cdot 10^8$
0.40	0.064	$6.0 \cdot 10^7$	0.069	$7.5 \cdot 10^7$
0.27	0.039	$6.9 \cdot 10^6$	0.047	$1.6 \cdot 10^7$
0.20	0.036	$1.1 \cdot 10^6$	0.043	$4.0 \cdot 10^6$
0.134	0.040	$1.2 \cdot 10^5$	0.049	$6.4 \cdot 10^5$
0.067	0.040	0	0.059	$1.05 \cdot 10^5$
0.013	0.039	0	0.047	$9.0 \cdot 10^3$
Initial titre		10^6		10^6

The growth medium for the experiments in Table II had a Na^+ concentration of 137 mM so that it is apparent that Na^+ cannot substitute for K^+. The other monovalent cations were tried by removing K^+ and substituting at the same concentration 5.37 mM. The results are shown in Table III and indicate that Rb^+ can partially substitute for K^+ but Li^+ and Cs^+ had no effect.

TABLE III

SUBSTITUTION OF MONOVALENT CATIONS FOR K^+ IN THE GROWTH OF *M. laidlawii*

Univalent cations (5.37 mM)	$A_{420\,m\mu}$
K^+	0.201
Li^+	0.002
Rb^+	0.068
Cs^+	0.007

A series of experiments was carried out to establish the Na^+ requirement for *M. laidlawii*. The overall results are shown in Table IV. In each case the concentration of Na^+ was decreased from its normal level as indicated in the left-hand column. When no additions were made to the medium the growth was a monotonic function of the Na^+ concentration as indicated in the second column. When K^+ was substituted for Na^+ so as to keep the total concentration at 137 mM in addition to the 5.37 mM of K^+ already in the medium, the growth was independent of Na^+ concentration. Examination of the column headed KCl shows normal growth in the total absence of Na^+.

This point was carefully checked by the use of analytical grade reagents containing insignificant traces of Na^+.

Attempts to substitute xylose, a non-metabolized sugar, for Na^+ were unsuccessful. Thus the role of Na^+ goes beyond the regulation of osmotic pressure and depends on the ionic nature of the salt.

TABLE IV

GROWTH OF *M. laidlawii* IN Na^+- SUBSTITUTED MEDIA

NaCl (*mM*)	$A_{420\ m\mu}$				
	No substitution	*KCl*	*Xylose*	$MgCl_2$	
				Ionic strength	*Osmotic pressure*
136.7	0.151	0.175	0.150	0.149	0.156
68.4	0.113	0.133	0.176	0.183	0.192
34.2	0.061	0.168	0.081	0.165	0.032
17.1	0.054	0.172	0.073*	0.149	0.026
8.6	0.032	0.174	0.077*	0.102	0.013
4.3	0.016	0.175	0.074*	0.058	0.009
2.2	0.012	0.178	0.078*	0.023	0.011
1.1	0.012	0.198	0.077*	0.013	0.013
0.0	0.011	0.182	0.075*	0.011	0.009

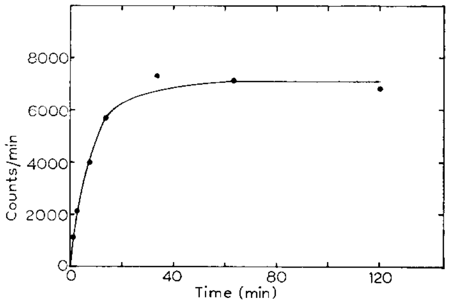
* The medium in the xylose experiments developed a yellow color, so that the absorbance is not a valid measure of cell growth. Below a concentration of 34.2 mM Na^+ there was no visible turbidity in the xylose cultures.

Mg^{2+} was substituted under two different protocols as is indicated in Table IV. In one set of experiments $MgCl_2$ is substituted at one-third the molar concentration of the depleted NaCl in order to maintain constant ionic strength. In the second set $MgCl_2$ is substituted at two-thirds the molar concentration of NaCl in order to maintain constant osmolality. Comparison of Columns 1, 4 and 5 show that Mg^{2+} can in fact partially substitute for Na^+, however, at concentrations of 50 mM and greater Mg^{2+} appears to exert an inhibitory effect. In general the role of NaCl in the medium appears largely in terms of regulating the ionic environment of the cells with no absolute Na^+ requirement for the growth of *M. laidlawii*.

The detailed determination of K^+ content of cells was carried out in two ways. From the steady-state $^{42}K^+$ concentration in uptake curves, the specific activity of the label, and the volume per colony-forming unit as determined by direct volumetric measurement and titre, a K^+ concentration was calculated. For cells grown in medium at 5.37 mM of K^+ the internal concentration was 71 ± 9 mM. A single determination on cells grown at 0.134 mM gave a value of 72 mM.

K^+ levels were also determined by flame photometry and gave values comparable to those obtained by the $^{42}K^+$ measurement. A $^{42}K^+$ uptake curve is shown in Fig. 1. It is corrected for the exponential growth of cells which accompanies the uptake. The mean generation time is 4 h and the times for half-maximal uptake were 5.2 and 4.8 min in two separate experiments.

In a variation of the uptake experiment, cells grown in normal medium and those adapted to 0.067 mM of K^+ were inoculated into medium with a concentration of 0.537 mM. The uptake curves are shown in Fig. 2. The unadapted cells were only able to maintain a K^+ concentration of 19 mM, which is consistent with Table II where unadapted cells experience problems in growing in low K^+ medium. The adapted cells take a longer time to reach steady state but eventually maintain a K^+ concentration of 110 mM.



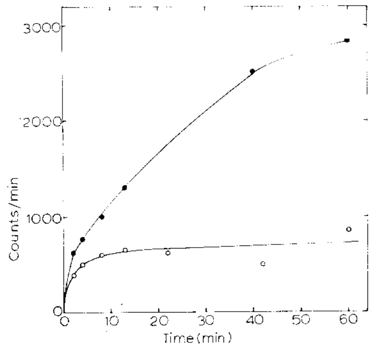


Fig. 1. K^+ influx Expt. I. Uptake of $^{42}K^+$ by exponentially growing cells of *M. laidlawii* in a medium containing 5.37 mM of K^+. The data are corrected for the growth of the cells.

Fig. 2. K^+ influx in adapted and unadapted cells. Uptake of $^{42}K^+$ by *M. laidlawii* in intermediate-level K^+ medium (0.537 mM). Unadapted cells grown in 5.37 mM of K^+ are transferred to the intermediate-level and uptake measured (○—○). Cells adapted to low K^+ medium (0.067 mM) are also transferred to intermediate-level medium and uptake is measured (●—●).

TABLE V

EFFECT OF METABOLIC INHIBITORS ON THE UPTAKE OF $^{42}K^+$ BY *M. laidlawii*

Inhibitor	*Concn. of inhibitor (mM)*	*Inhibition (%)*
Phospholipase A (crude)	1.2 mg/22 ml aliquot	56
Phospholipase C	1.2 mg/22 ml aliquot	9
NaF	25	54
	2.5	5
Iodoacetate	1	94
	0.1	34
	0.01	2
p-Chloromercuriphenylsulfonic acid (sodium salt)	1	99
	0.1	29
	0.05	24
PCMB (sodium salt)	0.05	99
N-Ethylmaleimide	10	98
	1	59
Sodium arsenite	10	5
KCN	10	62
	1	14
Ouabain	1	1
NaN_3	10	11.6
Dinitrophenol	1	0

Effects of metabolic inhibitors on uptake of $^{42}K^+$

Table V shows that the transport of K^+ is strongly inhibited by sulfhydryl blocking agents, *p*-chloromercuriphenylsulfonic acid (sodium salt) 1 mM and *N*-ethylmaleimide 1 mM. NaF strongly inhibited the uptake. Sodium arsenite had little effect at a relatively high concentration of 0.01 M. The uncoupling agents of oxidative phosphorylation, NaN_3 and dinitrophenol, and ouabain, an inhibitor of Na^+-dependent ATPase in the cell membrane had little effect. Phospholipase A inhibited K^+ uptake markedly, whereas phospholipase C had no significant effect. CN^- (0.01 M) exerted a significant inhibition. In order to see that the inhibitory effect by NaF at 25 mM was not due to the increased salt concentration, $^{42}K^+$ uptake in the presence of 25 mM of additional NaCl was carried out and no inhibition of $^{42}K^+$ uptake was observed.

DISCUSSION

For the study of active transport in *M. laidlawii*, K^+ has the advantage of being highly concentrated and not undergoing metabolic breakdown or incorporation. ROTTEM AND RAZIN[4] have reported a K^+ accumulation of 7 mg per g of cell protein of an oral strain of *M. laidlawii*. Assuming that the aqueous portion of the cell is 3 times the protein mass, this corresponds to a concentration of 58 mM. Our values of 70 mM in strain B are in close agreement with their values.

K^+ appears to be an indispensable requirement for microorganisms, plants and animals. The addition of at least one species of mycoplasma to this list makes it likely that K^+ is a ubiquitous substance in cellular biochemistry. An aminoacyl-tRNA transfer enzyme from *Escherichia coli* showed its maximum activity in 0.1 M K^+. The minimum concentration for the activation was 0.04 M (ref. 6).

The K^+ content of cells adapted to 0.068 mM of KCl was 72 mM, essentially identical to that of normal cells. This observation indicates that the adaptation of *M. laidlawii* to grow in the low K^+ medium was made by its development of a greater ability to accumulate K^+ to the level of about 70 mM (rather than being able to develop the ability to grow at the low intracellular K^+), and that for the normal growth and activity of certain enzymes of *M. laidlawii* cellular K^+ concentration of 70 mM is required. K^+ requirement was further shown by the failure of *M. laidlawii* to grow in a medium containing no K^+ after being adapted to grow in only 0.034 mM KCl.

K^+ requirement of *M. laidlawii* could not be replaced with Li^+, Na^+ or Cs^+. Rb^+ was not a good substitute but supported some growth. LESTER[7] reported that Rb^+ supported the growth of gram-positive and gram-negative bacteria when substituted for K^+.

K^+ effectively replaced Na^+ in the growth medium, indicating that Na^+ is not essential for the growth of *M. laidlawii*. Some growth was observed when 75–95% of Na^+ was replaced with Mg^{2+} at equal ionic strength. Upon reduction of Na^+ by 99%, Mg^{2+} was without effect. Xylose did not substitute for Na^+. These observations show that the normal growth of *M. laidlawii* requires a minimum of 50 mM monovalent alkali ion such as K^+, or at least 5 mM K^+ *plus* Na^+; and that the main function of the minimal 50 mM of monovalent ions is not solely to maintain the adequate osmotic pressure of the medium.

Iodoacetate which is particularly active in inhibiting 3-phosphoglyceraldehyde dehydrogenase almost completely inhibits transport at 1 mM. Arsenite had little effect at 0.01 M. Oxidation of ketoacids including pyruvate are particularly sensitive to the action of arsenite[8]. The site of action of arsenite was suggested to be the thiol (–SH) group of lipoic acid[9]. According to TOURTELLOTTE AND JACOBS[10], 90% of carbon of glucose were recovered as lactate *plus* pyruvate and only 2.6% as acetate. This indicates that the role of metabolic pathway leading pyruvate to acetate is relatively minor. The negligible effect of arsenite may be due to the insignificant contribution of pyruvate breakdown to the energy production in *M. laidlawii*. Consistent with the result obtained with oral strain by ROTTEM AND RAZIN[4], ouabain was without effect. Dinitrophenol had no effect on the $^{42}K^+$ transport (Table V) showing that the cytochrome system is not the major source of energy for the active transport in *M. laidlawii*.

VAN STEVENINCK *et al.*[11] have demonstrated that reaction of *p*-chloromercuriphenylsulfonic acid with intact red blood cells was limited to the –SH group on the outer membrane surface area, whereas *p*-chloromercuribenzoate (PCMB) penetrated into the cell. In addition ENGELMAN AND MOROWITZ[12] have reported that cysteine content of membrane of *M. laidlawii* was only 0.19 mole %.

In our study, *p*-chloromercuriphenylsulfonic acid and *N*-ethylmaleimide strongly inhibited K^+ transport at 1 mM. At 50 μM inhibition by *p*-chloromercuriphenylsulfonic acid and PCMB was 24 and 99%, respectively. From these results it is presumed that the effect of PCMB is far stronger than *p*-chloromercuriphenylsulfonic acid, since it easily reacts with both membrane and intracellular sulfhydryl groups. Although cysteine is present in the membrane of *M. laidlawii* at very low ratio it appears to have a significant role in active K^+ transport.

When placed in the $^{42}K^+$ medium containing 0.536 mM K^+, the cells adapted to 0.068 mM K^+ were able to transport $^{42}K^+$ into the cells more rapidly than the unadapted cells; the maximum radioactivity of the adapted cells was reached at 60 min, whereas that of normal cells at 10 min.

The intracellular concentration of K^+ in the adapted and unadapted cells at 60 min were 110 and 19 mM, respectively. Since normal concentration of K^+ in *M. laidlawii* was 71 mM, 19 mM in the unadapted cells indicates that they were unable to take up K^+ fast enough to maintain normal K^+ concentration when suddenly transferred in the medium containing one-tenth the amount of K^+.

The high internal K^+ concentration along with the rapid K^+ exchange suggest that the concentration is maintained by an active transport mechanism working against a leak. Assuming a spherical cell radius r and permeability h, the time constant for the leak, τ, is $r/3h$. For the K^+ transport in *M. laidlawii*, r is $3 \cdot 10^{-5}$ cm and τ is $t/0.693$ or 433 sec for a half-time of 5 min. This gives a permeability value of $2.31 \cdot 10^{-8}$ cm/sec.

Working against this leak each cell must pump $1.83 \cdot 10^{-20}$ moles or $1.10 \cdot 10^4$ K^+ per sec. For an external concentration of 0.068 mM the minimum work per mole transported is $RT \ln(70/0.068)$ or 4280 cal/mole. The minimum energy expenditure of the cell in maintaining its K^+ level is thus $7.83 \cdot 10^{-17}$ cal/sec.

ACKNOWLEDGMENTS

The authors would like to express their thanks to Dr. Joseph F. Hoffman who generously made his laboratory available to us and also provided valuable counsel.

We would also like to thank Raymond L. Drabko and Christina J. Ingram for assistance in setting up the isotope experiments. This work was supported by National Institutes of Health Grant 1-RO1-GM 15600.

REFERENCES

1 D. M. Engelman, T. M. Terry and H. J. Morowitz, *Biochim. Biophys. Acta*, 135 (1967) 381.
2 J. D. Pollack, Ph.D. Dissertation, Univ. of Connecticut, 1967.
3 S. Razin, L. Gottfried and S. Rottem, *J. Bacteriol.*, 95 (1968) 1685.
4 S. Rottem and S. Razin, *J. Bacteriol.*, 92 (1966) 714.
5 H. J. Morowitz, M. E. Tourtellotte and M. E. Pollack, *J. Bacteriol.*, 85 (1963) 134.
6 T. W. Conway, *Proc. Natl. Acad. Sci. U.S.*, 54 (1964) 216.
7 G. Lester, *J. Bacteriol.*, 75 (1958) 426.
8 H. A. Krebs, *Z. Physiol. Chem.*, 217 (1933) 191.
9 O. K. Reiss and L. Hellerman, *J. Biol. Chem.*, 231 (1958) 557.
10 M. E. Tourtellotte and R. E. Jacobs, *Ann. N. Y. Acad. Sci.*, 79 (1960) 521.
11 J. Van Steveninck, R. I. Weed and A. Rothstein, *J. Gen. Physiol.*, 48 (1965) 617.
12 D. M. Engelman and H. J. Morowitz, *Biochim. Biophys. Acta*, 150 (1968) 385.