Biochimica et Biophysica Acta, 600 (1980) 998—1006

BBA 78888

A NOVEL METHOD FOR THE DETERMINATION OF ELECTRICAL POTENTIALS ACROSS CELLULAR MEMBRANES

II. MEMBRANE POTENTIALS OF ACHOLEPLASMAS, MYCOPLASMAS, STREPTOCOCCI AND ERYTHROCYTES *

ULRICH SCHUMMER, HANS-GERD SCHIEFER and URSULA GERHARDT

Institut für Medizinische Mikrobiologie, Justus Liebig Universität, Schubertstrasse 1, D-6300 Giessen (F.R.G.)

(Received January 24th, 1980)

Key words: Membrane potential; Potassium distribution; Atomic absorption spectroscopy; Valinomycin; Donnan equilibrium; (Acholeplasma, Mycoplasma, Streptococcus, Erythrocyte)

Summary

The membrane potentials of *Acholeplasma laidlawii*, *Mycoplasma mycoides* subsp. capri, *Mycoplasma gallisepticum*, *Streptococcus faecalis* and human erythrocytes have been determined by applying a novel technique. The membrane potentials were calculated simply from potassium concentrations determined by atomic absorption spectroscopy, and gravimetry. The versatility of the new technique is demonstrated by comparing our results with data obtained by different techniques.

Introduction

In the preceding paper [1] the theoretical basis of a novel technique for the determination of electrical potentials across cellular membranes had been developed. The intracellular potassium concentration ($K_{int,o}$) and the critical extracellular potassium concentration ($K_{out} = K_{crit}$), at which no change in the intracellular potassium concentration (and hence no change in membrane potential) occurs upon addition of valinomycin [2], are determined from measurements of potassium concentrations, and gravimetry. From these data the membrane potential is easily calculated according to the Nernst equation, as described by Hoffman and Laris [3]. The potassium concentration, K_{crit}, has essentially the

* Our paper is dedicated to Professor Hansjürgen Staudinger on his 65th birthday.

same meaning as the 'null point concentration' [4] as determined by fluorescence techniques. Our new technique needs the naturally occurring K^+ as 'label'. Besides the measurement of membrane potentials, it allows the determination of whether at all, and to what extent, cells lose their intracellular potassium when they are removed from nutrient solutions with physiological saline concentrations, and brought to media containing no or low $[K^+]$. In this paper we demonstrate the versatility of our method by determining the membrane potentials of *Acholeplasma laidlawii*, *Mycoplasma mycoides* subsp. capri, *Mycoplasma gallisepticum*, *Streptococcus faecalis* and erythrocytes, and compare these results with data obtained by different techniques.

Mycoplasmas are the smallest procaryotic micro-organisms capable of autonomous growth and reproduction [5]. They lack a rigid cell wall but are endowed with a single plasma membrane more stable than that of eucaryotic cells. Streptococci are Gram-positive bacteria and were studied as representatives of micro-organisms with a more complex cell wall structure.

Materials and Methods

I. Cultivation and harvest of organisms

M. mycoides subsp. capri, *M. gallisepticum* and *A. laidlawii* were originally obtained from Drs. Freundt and Chanock [6]. *M. mycoides* subsp. capri was grown in a medium consisting of 350 ml of PPLO broth (Difco), 10 ml of normal horse serum (Flow), 50 ml of 25% yeast extract (Flow), 10 ml of 50% glucose, 10 ml of 0.1% phenol red, and 12.5 ml of 2% thallium acetate; the pH was adjusted to 7.8. The growth medium of *M. gallisepticum* contained 20 ml of normal horse serum. The medium for *A. laidlawii* contained 5 ml of bovine PPLO serum fraction (Difco) instead of horse serum, and only 2 ml of 25% yeast extract. Cells were grown in suspension at 37°C until the growth medium started to change colour.

S. faecalis was grown in liquid tryptic soy broth (TSB, Difco) medium and harvested in the late exponential phase.

The organisms were harvested by centrifugation, washed twice, and finally suspended in fresh growth medium. The final concentration was about 200 mg wet wt./ml. For some experiments, *M. mycoides* subsp. capri was washed and suspended in isotonic $CaCl_2$ solution (116 mM).

Freshly drawn human erythrocytes were washed twice and finally suspended in 145 mM NaCl, 2 mM Tris, pH 7.0.

II. Preparation of specimens

Valinomycin (Sigma) was dissolved in ethanol.

(a) Acholeplasma laidlawii. Micro test-tubes (Eppendorf) denoted as p0; p1; ...; pn; ...; p6, and p0v; p1v; ...; pnv; ...; p6v, contained 1 ml of buffer solution composed as follows: $15 \cdot n$ mM KCl plus $14.5 \cdot (10 - n)$ mM NaCl plus 2 mM Tris, pH 7.0, with n = number of tubes = 0, 1, ..., 6. 50 μl of the *A. laidlawii* suspension were added to each tube. Samples denoted by a 'v' (e.g., p4v) in addition contained 1 μl valinomycin, final concentration 7.5 μM. After a 10 min incubation period at 20°C the specimens were processed as described under 'f'.

(b) Mycoplasma mycoides subsp. capri. The micro test-tubes denoted as p0; p1; ...; pn; ...; p10 and p0v; p1v; ...; pnv; ...; p10v, contained buffer solution of the following composition: $5 \cdot n$ mM KCl plus $(145 - 5 \cdot n)$ mM NaCl plus 2 mM Tris, pH 7.0, with n = number of tubes = 0, 1, 2, ..., 10. Each tube contained 50 μl of the *M. mycoides* subsp. capri suspension. Tubes denoted by a 'v' contained in addition 5 μl of a valinomycin solution, final concentration 37.5 μM. After an incubation period of 15 min at 20°C the specimens were processed as described under 'f'.

(c) Mycoplasma gallisepticum. The number of specimens and the composition of buffer solutions were exactly the same as described for *A. laidlawii* (a). Each micro test-tube contained 50 μl of the *M. gallisepticum* suspension. 5 μl valinomycin, final concentration 37.5 μM, were added to each tube denoted by a 'v'. After an incubation period of 30 min at 37°C the specimens were processed as described under 'f'.

(d) Streptococcus faecalis. The specimens were prepared as described for *M. mycoides* subsp. capri (b), except that the buffer solutions contained $10 \cdot n$ mM KCl plus $(145 - 10 \cdot n)$ mM NaCl plus 2 mM Tris, pH 7.0, with n = number of tubes = 0, 1, 2, ..., 10.

(e) Erythrocytes. The specimens were prepared as described for *S. faecalis* (d), except that the incubation period was 30 min.

(f) Further processing of the specimens. At the end of the incubation period the specimens were centrifuged at $8000 \times g$ for 2.5 min in an Eppendorf 3200 centrifuge, and the supernatant was carefully removed. This procedure was repeated twice. After determination of the wet mass (m_{ww}) the specimens were lyophilized, and the dry mass (m_{dw}) was measured. 1 ml of 0.1 N HCl was added to the dry residue which was subsequently disintegrated by ultrasonic irradiation (Branson Sonifier B-12, equipped with a micro tip). The insoluble remainders were sedimented by centrifugation at $8000 \times g$ for 2.5 min. The supernatants were diluted, usually 1 : 10, with twice-distilled water to give a final potassium concentration ranging from 0.03 to 0.15 mM.

The potassium concentrations of the specimens were determined by atomic absorption spectroscopy using a Unicam SP 90 A Series 2 Atomic Absorption Spectrophotometer, analyzer settings: slit width 0.2; wavelength 766 nm, equipped with an interference filter (λ_{max} = 766 nm).

III. Mathematical calculations

In Ref. 1 it had been shown that the potassium concentration of cells after incubation in a medium with the potassium concentration, K_{out}, is given by:

$$K(K_{out}) = (V_{ex}/V_L)K_{out} + (V_{int}/V_L)K_{int,o} \quad (1)$$

where $K(K_{out})$ is the potassium concentration of the specimens; V_L, the volume of the disintegrating solution; V_{ex}, the extracellular, and V_{int}, the intracellular water volume, respectively; $K_{int,o}$, the intracellular potassium concentration of native cells; and K_{out}, the potassium concentration of the incubation medium. As described above, the potassium concentration of the incubation medium was changed according to $K_{out} = k \cdot n$ (where k is the step width, and n = 0; 1; ...). The specimens differed in wet mass, therefore all parameters were normalized with respect to the wet mass, whenever necessary. The normalized param-

eters were given by the following set of equations:

$K_N(K_{out}) = K(K_{out})/m_{ww}$ $V_{ex,N} = V_{ex}/m_{ww}$ $V_{int,N} = V_{int}/m_{ww}$

$m_{dw,N} = m_{dw}/m_{ww}$ $m_{ww,N} = m_{ww}/m_{ww} = 1$

By introducing the normalized values into Eqn. 1, one obtains:

$$K_N(k \cdot n) = (V_{ex,N}/V_L) \cdot k \cdot n + (V_{int,N}/V_L) \cdot K_{int,o} \quad (2)$$

$V_{ex,N}$ is determined by the slope s of the curve:

$$s = (V_{ex,N}/V_L) \cdot k; \text{ and } V_{ex,N} = s \cdot V_L/k \quad (3)$$

The normalized intracellular volume $V_{int,N}$ is calculated as follows:

From $m_{ww} = (V_{ex} + V_{int}) \cdot \rho + m_{dw}$ with ρ = density of intra- and extracellular fluid, here assumed to be 1 g/ml, it follows that:

$$1 = (V_{ex,N} + V_{int,N}) \cdot \rho + m_{dw,N} \text{ and } V_{int,N} = (1 - m_{dw,N})/\rho - V_{ex,N} \quad (4)$$

The intracellular potassium concentration, $K_{int,o}$, can be calculated from the intersection point of the curve and the ordinate:

$$K_N(0) = (V_{int,N}/V_L) \cdot K_{int,o} \quad (5)$$

$$K_{int,o} = (K_N(0) \cdot V_L)/V_{int,N} \quad (6)$$

The critical potassium concentration, K_{crit}, has been proved to equal the abscissa of the intersection point of the two curves, recorded from specimens incubated in media which contain or are free of valinomycin, respectively. From the measured and calculated data the membrane potential can be calculated according to the modified Nernst equation:

$$\psi = (RT/F) \cdot \ln (K_{crit}/K_{int,o}) = -59 \text{ mV} \cdot \log(K_{int,o}/K_{crit}) \quad (7)$$

where $T = 293$ K, R is the gas constant and F is Faraday's constant.

Results and Discussion

(a) Acholeplasma laidlawii

The results of a typical experiment are presented in Fig. 1. The normalized potassium concentration was plotted vs. the potassium concentration of the incubation media which contain no (●———●, curve b) or 7.6 μM valinomycin (○———○, curve a), respectively. The curves were calculated according to the method of linear regression. When calculating the regression curve b, the first value ($K_N(0)$) was omitted for reasons discussed later. The normalized extracellular water volume was calculated from the slope of curve b according to Eqn. 3. One obtains $V_{ex,N} = 0.58$ ml/g. From gravimetric measurements the normalized dry mass was determined to be $m_{dw,N} = 0.18$. According to Eqn. 4, the normalized intracellular water volume was calculated as $V_{int,N} = (1 - 0.18)/(1 \text{ g/ml}) - 0.58 \text{ ml/g} = 0.24$ ml/g. From the intersection point of curve b and the ordinate, the normalized potassium content of *A. laidlawii* was computed to be $K_N(0) = 35.5$ mM/g. By introducing these values into Eqn. 6, one has for the potassium concentration in native cells: $K_{int,o} = (35.5 \text{ mM/g}) \cdot 1 \text{ ml}/(0.24 \text{ ml/g}) = 148$ mM.

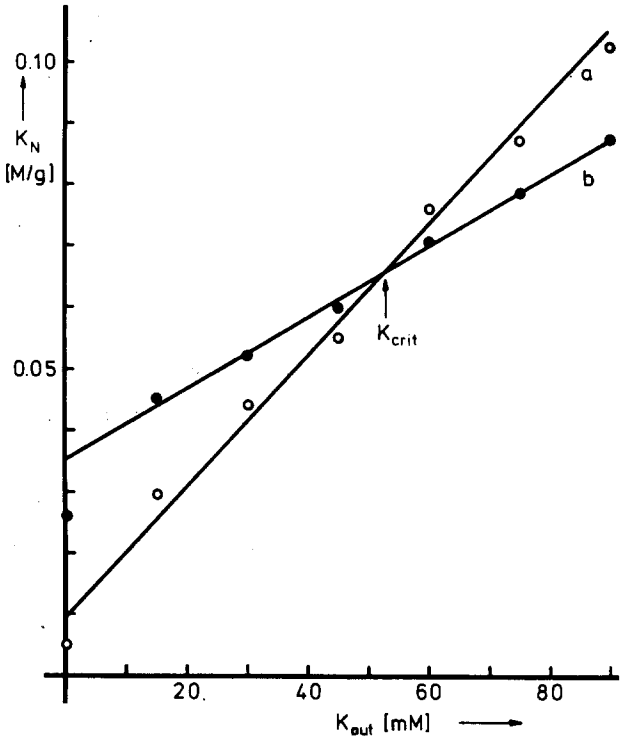

Fig. 1. Plot of the normalized potassium concentration of *A. laidlawii* vs. the potassium concentration of the incubation medium. The organisms were incubated for 10 min at 20°C. Curve a, organisms incubated in media containing valinomycin, final concentration 7.5 μM; curve b, organisms incubated in media without valinomycin. From the abscissa of the intersection point of both curves (arrow), K_{crit} was determined to be 52.5 mM.

As mentioned above, the results obtained for *A. laidlawii* cells incubated in potassium-free medium did not fit the regression curve. $K_N(0)|_{measured}$ was only 26 mM/g, thus indicating that the organisms had lost part of their potassium upon incubation. As demonstrated by the excellent fit of the other points with the regression curve, a potassium leakage is completely prevented when potassium is present in the incubation medium, at least at a concentration of equal or greater than 15 mM. From the abscissa of the intersection point of curves a and b, the critical potassium concentration was determined to be K_{crit} = 52.5 mM. By using the modified Nernst equation (Eqn. 7), the membrane potential was calculated as $\psi = -59\ mV \cdot \log(148/52.5) = -27\ mV$. This is in good agreement with $\psi = -28$ mV obtained by fluorescence measurements [7]. All data are compiled in Table I.

(b) Mycoplasma mycoides subsp. capri

M. mycoides subsp. capri, when removed from nutrient medium, loses part of its internal K^+. By washing in isotonic $CaCl_2$ solution, the organisms are 'stabilized', and potassium leakage is prevented [8,9]. Therefore, parallel experiments to determine the intracellular potassium have been performed with (i) organisms washed in $CaCl_2$ solution (stabilized organisms) and (ii) non-stabilized organisms ('native' organisms). The results are given in Fig. 2, curve a,

TABLE I

PHYSICAL AND CHEMICAL DATA COMPILED FOR THE CALCULATION OF MEMBRANE POTENTIALS

$m_{dw,N}$ denotes the normalized dry mass, m_{dw}/m_{ww}; m_{ww}, the wet mass; $V_{int,N}$, the normalized intracellular water volume; ρ, the density of intra- and extracellular fluid; $K_{int,o}$, the intracellular potassium concentration; K_{crit}, the critical potassium concentration, i.e., the potassium concentration of the incubation medium at which no net flow of potassium occurs upon addition of valinomycin; ψ, the membrane potential; and ψ_{ref}, the respective membrane potential, as taken from the literature. All data are mean values ± S.D. of n experiments ($n \geq 8$).

Organism	$m_{dw,N}$	$V_{ex,N} \cdot \rho$	$V_{int,N} \cdot \rho$	$K_{int,o}$ (mM)	K_{crit} (mM)	ψ (mV)	ψ_{ref} (mV)	Reference
A. laidlawii	0.17 ± 3%	0.63 ± 9%	0.20 ± 9%	216 ± 10%	83 ± 8%	−25 ± 8%	−28	7
M. mycoides subsp. capri	0.19 ± 3%	0.40 ± 8%	0.41 ± 7%	208 ± 6%	33 ± 7%	−48 ± 6%	−48	7
M. gallisepticum	0.22 ± 8%	0.47 ± 9%	0.30 ± 10%	202 ± 11%	47 ± 4%	−37 ± 10%	−48	7
S. faecalis	0.20 ± 4%	0.56 ± 8%	0.24 ± 8%	483 ± 7%	112 ± 8%	−37 ± 9%	−38	10
Human erythrocytes	0.33 ± 1%	0.09 ± 8%	0.58 ± 5%	133 ± 5%	89 ± 7%	−11 ± 6%	−9	3

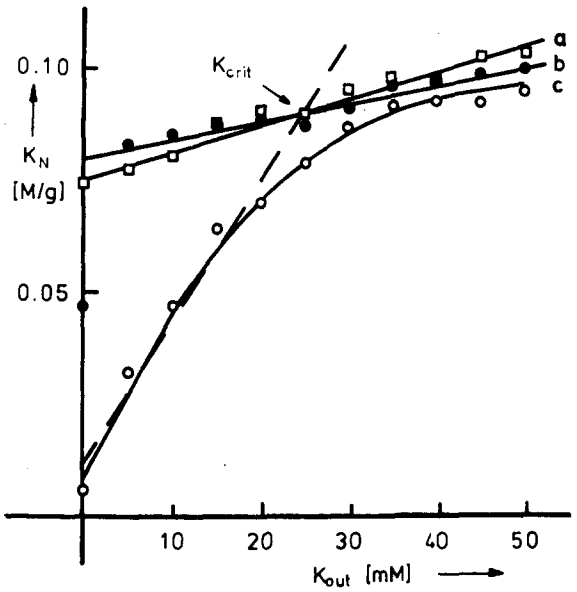

Fig. 2. Plot of the normalized potassium concentration of *M. mycoides* subsp. capri vs. the potassium concentration of the incubation medium. The organisms were incubated for 15 min at 20°C. Curve a, stabilized organisms, i.e., organisms washed in isotonic $CaCl_2$ solution before incubation in media without valinomycin; curve b, native organisms, i.e., untreated organisms incubated in media without valinomycin; curve c, native organisms incubated in media containing valinomycin, final concentration 37.5 μM. The dashed line was extrapolated from the first five points of curve c. From the abscissa of the intersection point (arrow) of this line and curve b, K_{crit} was determined to be 25 mM.

□———□ (stabilized organisms); and b, ●———● (native organisms). With only one exception, all points fit the respective regression curves. The extrapolated normalized potassium concentrations, $K_N(0)|_{extrapol.}$, were 81 and 74 mM/g for native and stabilized organisms, respectively. The normalized potassium concentrations, $K_N(0)|_{measured}$, obtained for native and stabilized organisms were 52 and 74 mM/g, respectively. This shows that native *M. mycoides* subsp. capri loses about 40% of the internal potassium upon incubation in potassium-free medium, while no loss of potassium occurs for stabilized organisms. Furthermore, as indicated by curve a, potassium leakage is completely prevented when potassium is present in the incubation medium at a concentration as low as 5 mM.

The curve resulting from our measurements of *M. mycoides* subsp. capri incubated in media containing valinomycin (○———○, curve b), considerably deviates from the straight line predicted by theory. At potassium concentrations greater than or equal to 30 mM the internal potassium concentration of the organisms did not change. This points to a decrease in the membrane potential which, in turn, may be due to the low stability of the membrane. However, the first five points (specimens p(0), ..., p(4)) could be approximated by a regression curve, and for these specimens obviously the theoretical premises are sufficiently fulfilled, so that K_{crit} could be determined by the intersection point of this curve and curve b. One obtains K_{crit} = 25 mM, and hence ψ = −53 mV. This is in good agreement with ψ = −48 mV as obtained by fluorescence measurements [7].

(c) Mycoplasma gallisepticum

Experiments performed with valinomycin-free media to determine $K_{int,o}$ were in good agreement with theory. All points fit the regression curve, thus indicating that *M. gallisepticum* did not lose potassium. Our experiments with valinomycin, however, met with difficulties. When the organisms were incubated for a short time the potassium distribution did not reach Donnan equilibrium and the points deviated considerably from a straight line. Prolonged incubation shifted the intersection point of the curves towards higher K_{out} values, thus indicating a partial breakdown of the membrane potential. The mean value of five identical experiments was K_{crit} = 53 mM, and the resulting membrane potential was ψ = −35 mV. This result deviates from data obtained by fluorescence techniques (ψ = −48 mV); however, both results are of the same order of magnitude.

(d) Streptococcus faecalis

In Fig. 3 the results obtained with *S. faecalis* are presented. Curve b was calculated from data of organisms incubated in valinomycin-free media, curve a from those incubated in media containing valinomycin. The internal potassium concentration was about 480 mM, the critical potassium concentration about

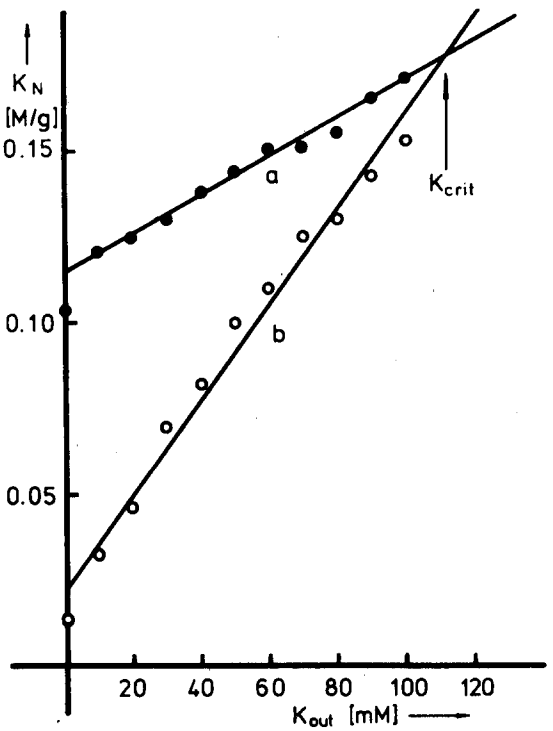

Fig. 3. Plot of the normalized potassium concentration of *S. faecalis* vs. the potassium concentration of the incubation media. The organisms were incubated for 15 min at 20° C. Curve a, organisms incubated in media without valinomycin; curve b, organisms incubated in media containing valinomycin, final concentration 37.5 μM. From the abscissa of the intersection point of both curves (arrow), K_{crit} was determined to be 112 mM.

110 mM, and hence the electrical membrane potential about −37 mV. This is in good agreement with data given in the literature [10].

(e) Erythrocytes

The results of our measurements on erythrocytes are compiled in Table I. Again the results (ψ = −10.6 mV) fit well with the data obtained by different techniques [3].

Conclusion

In a preceding paper the theoretical basis of a new method for the simultaneous determination of the extra- and the intracellular water volumes, the intracellular potassium concentration and the critical potassium concentration had been presented. In this paper the method has been applied to four different micro-organisms and human erythrocytes. In each case, the extra- and intracellular water volumes and the intracellular potassium concentration were easily determined with high accuracy. In addition, potassium leakage of organisms could be detected and its influence eliminated. The critical potassium concentrations could readily be obtained, at least for three of the organisms studied. With one organism (*M. mycoides* subsp. capri) we met with difficulties which could be overcome, and only with *M. gallisepticum* were the results unsatisfactory. Our technique obviously provides a new and simple tool for the determination of transmembrane electrical potentials.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft.

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