BBA 75678

EXCHANGEABILITY OF CHLORIDE IN EHRLICH ASCITES TUMOR CELLS

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(Received April 7th, 1971)

SUMMARY

In Ehrlich cells 30–50 % of cell Cl^- has been reported to be non-exchangeable. In the present study the possibility of protein interference with the Cl^- titration was eliminated by deproteinization with $HClO_4$, or with $ZnSO_4$–NaOH followed by perborate oxidation, or by alkaline dry ashing. Cell Cl^- is demonstrated to be completely exchangeable with $^{36}Cl^-$ and with NO_3^-, and there is no evidence of compartmentation. However, protein interference with the argentimetric titration may introduce substantial error, mimicking a fraction of non-exchangeable cell Cl^-.

For cells equilibrated at 38° in sodium Ringer solution with a Cl^- concentration of 151 mM, the Cl^- concentration was 58 μmole/ml cell water, and this value is consistent with a passive distribution of Cl^-.

INTRODUCTION

Conflicting results have been reported on the exchangeability of Cl^- in Ehrlich cells. Complete exchange with $^{36}Cl^-$ was reported by GROBECKER *et al.*[1]. Later, a value of 30 % non-exchangeable cell Cl^- was reported[2], the inaccessibility for exchange being suggested to be temperature dependent[3]. KROMPHARDT[4] confirmed the non-exchangeability of 30–55 % of cell Cl^-. The cell content of non-exchangeable Cl^- was reported to stay fairly constant at 100 μequiv per g dry wt. independent of variations in the content of exchangeable Cl^-. 30–40 % of cell Cl^- remained in the cells in Cl^--free nitrate Ringer solution[3–5]. This residual cell Cl^- was non-exchangeable with $^{36}Cl^-$ (refs. 2 and 4).

Calculating the equilibrium potential for Cl^- (E_{Cl^-}), AULL[2] considers the concentration of exchangeable Cl^- in total cell water, leading to $E_{Cl^-} = -33.5$ mV. This value is incompatible with reported membrane potentials[2,6] of —11 to —12 mV, assuming passive distribution of Cl^-. KROMPHARDT[4], however, calculates E_{Cl^-} to be —11.6 mV, assuming two compartments of cell Cl^- with equal Cl^- concentration. In relation to microelectrode measurements on Ehrlich cells[7,8] with potentials of about —24 mV, the possible existence of non-exchangeable cell Cl^- was reexamined.

MATERIALS AND METHODS

Ehrlich mouse ascites tumor cells (hyperdiploid strain) were maintained on NMRI mice, and harvested in ice-cold Ringer solution containing heparin. The cells

were washed once, and incubated at a cytocrit of 5–10 % at 38°, with pre-incubation for 15–30 min before the experimental period. The standard Ringer solution (sodium or chloride Ringer solution) contained: 148 mM Na^+; 5.2 mM K^+; 151 mM Cl^-; 1.7 mM Ca^{2+}; 1.2 mM Mg^{2+}; 1.2 mM SO_4^{2-}; and 3.0 mM orthophosphate (pH 7.40). K^+ was substituted for Na^+ in potassium Ringer solution, and NO_3^- for Cl^- in nitrate Ringer solution. $^{36}Cl^-$ (Risö, Roskilde, Denmark) was added as neutral isotonic NaCl solution. Tritiated methoxyinulin (NEN, Boston, Mass., U.S.A.) was used as a marker of trapped volume in cell pellets. 500-μl samples of incubate were centrifuged for 30 sec at 18000 × g. The pellet of cells was lyzed in 10–20 vol. of deionized water.

Deproteinization was carried out using $HClO_4$ (final concn., 7 %) unless otherwise stated, or with $ZnSO_4$–NaOH followed by alkaline perborate oxidation[9]. The recovery of $^{36}Cl^-$ was virtually complete with both procedures. Alkaline dry ashing was carried out by heating the samples with added alkali in platinum crucibles at 550° for 24–36 h. The recovery was higher than 59 % for cell lysate and 93 % for medium (concerning incomplete recovery of tissue Cl^- in particular, *cf.* ref. 9). Isotope dilution analysis with alkaline dry ashing was performed following the procedure of COTLOVE[9]. Cl^- was titrated in duplicate with 2.5 mM $AgNO_3$ in 0.75 M H_2SO_4, with potentiometric end-point detection. The equipment used was from Radiometer, Copenhagen, Denmark: pH-meter PHM 25 SE, Titrator TTT 11, Autoburette ABU 1 (total vol. 0.25 ml), Ag electrode (P 4312), and Hg/Hg_2SO_4 reference electrode (K 6112). ^{36}Cl and ^3H were counted in duplicate in a liquid scintillation spectrometer (Packard Tri-Carb Model 3314) using a standard toluene–ethanol-based counting solution. A slow decay of measured ^3H activity was observed (*cf.* ref. 10).

From the ^3H activity of medium and cell lysate the trapped medium in the cell pellet was calculated, and the appropriate correction was made in calculations of cell Cl^- and $^{36}Cl^-$. The influx of $^{36}Cl^-$ into the cells was treated as steady-state exchange in a closed two-compartment system. The relative specific activity (the specific activity of cells expressed as a fraction of the specific activity of medium at isotopic equilibrium) was followed with time, and fitted to a single exponential function by computer least squares analysis[11] (*cf.* Fig. 1).

The results are given as mean ± S.E., or as the total range, with the number of experiments in parentheses.

RESULTS AND DISCUSSION

The steady-state exchange of $^{36}Cl^-$ in Ehrlich cells was followed under various conditions, using deproteinized samples for $^{36}Cl^-$ and Cl^- analyses. At isotopic equilibrium the relative specific activity of cells was close to unity (Table I), which is incompatible with the existence[2,4] of a non-exchangeable fraction of cell Cl^-.

In the study of GROBECKER *et al.*[1] the specific activity was determined in deproteinized samples, whereas in the studies of AULL[2] and KROMPHARDT[4] water extracts of wet or freeze-dried cells were employed without any statement concerning precipitation of protein being given. On this basis, the influence of deproteinization was investigated (Fig. 1). The influx of $^{36}Cl^-$ was followed in the same samples with (A), and without (B), deproteinization. Both influx curves fit single exponential functions with similar time constants. However, the extrapolated relative specific activity

TABLE I

RELATIVE SPECIFIC ACTIVITY OF CELLS AT ISOTOPIC EQUILIBRIUM AS DETERMINED IN DEPROTEINIZED SAMPLES

The cells were equilibrated with $^{36}Cl^-$ for 60 min or more. Number of experiments is given by *n*.

Experimental conditions	*Temp.* (°)	*Relative specific activity*	*n*
Sodium Ringer solution	38	0.982 ± 0.007	14
Sodium Ringer solution	20	0.986 ± 0.004	6
Ouabain, 0.5 mM*	38	0.99	2
2,4-Dinitrophenol, 0.1 mM*	38	1.02	1
Potassium Ringer solution	38	0.98	1
Mean		0.985 ± 0.004	24

* Experiments in sodium Ringer solution.

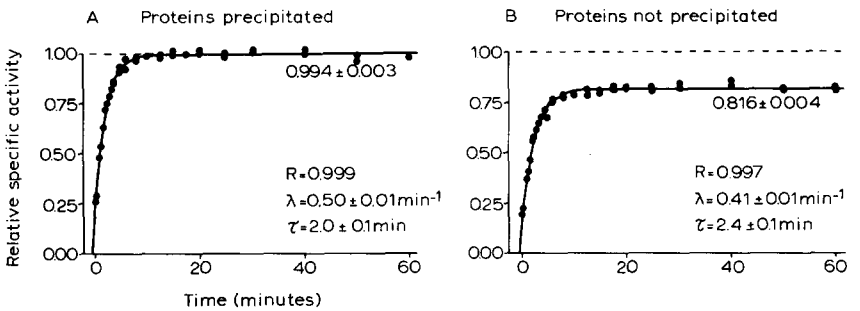

Fig. 1. $^{36}Cl^-$ influx curve in sodium Ringer solution at 38°. The relative specific activity (the specific activity of cells expressed as a fraction of the specific activity of medium at isotopic equilibrium) was followed with time in the same samples with (A) and without (B) deproteinization. The single exponential function with the best fit was calculated by iterative, unweighted, non-linear regression analysis[11], the variables being the crude rate constant (λ), the "true" zero time, and the extrapolated value of the relative specific activity (shown on the graph). τ ($= 1/\lambda$) is the time constant, R is the multiple correlation coefficient.

is 0.994 ± 0.003 (A) and 0.816 ± 0.004 (B), respectively. Comparing samples of cell lysate and medium with and without precipitation of protein, virtually no quenching of the protein-containing samples of cell lysate was demonstrated. Thus, the discrepancy must be caused by a difference in real or apparent Cl^- content, *i.e.* either protein interference with the Cl^- titration, as discussed by COTLOVE[9], or inadvertent removal of protein-bound, non-exchangeable Cl^- by the deproteinization. Therefore, the true specific activity of cells equilibrated with $^{36}Cl^-$ was determined with alkaline dry ashing to eliminate possible protein interference (Table II). The specific activity in deproteinized samples (B) was identical with the true value (C), whereas protein-containing samples (A) gave a value 7–8 % too low. The counting efficiency showed no difference. The deproteinized samples thus represent the true $^{36}Cl^-$ specific activity and Cl^- content, the values from protein-containing samples being distorted by protein interference with the argentimetric titration. The values in Table II are uncorrected for Cl^- in trapped medium. Correction with representative values gives an apparent

TABLE II

SPECIFIC ACTIVITY AT ISOTOPIC EQUILIBRIUM

The cells were equilibrated with $^{36}Cl^-$ in sodium Ringer solution at 20° for 100 min. Aliquots of medium and lysate of packed cells were analyzed in quadruplicate. The specific activity was determined with and without deproteinization with $HClO_4$, and with alkaline dry ashing. The values are uncorrected for trapped medium. The counting efficiency was determined with internal ^{36}Cl standard.

	Specific activity (counts/min per μmole)	*Relative specific activity*	*Counting efficiency (%)*
(A) Proteins not precipitated			
Cells	12 271 ± 83	0.924 ± 0.006	83.4 ± 0.2
Medium	13 277 ± 46		83.8 ± 0.1
(B) Proteins precipitated			
Cells	13 252 ± 30	0.997 ± 0.002	83.1 ± 0.2
Medium	13 295 ± 4		83.4 ± 0.2
(C) Alkaline dry ashing			
Cells	13 298 ± 41	1.002 ± 0.003	83.8 ± 0.1
Medium	13 266 ± 56		83.8 ± 0.1

TABLE III

THE Cl^- CONCENTRATION IN CELL LYSATE AFTER DIALYSIS

The cells were equilibrated with sodium Ringer solution at 38°. A lysate of packed cells was dialyzed in duplicate for 24 h in a cellophane bag against deionized water. The Cl^- concentration was determined with and without deproteinization, with alkaline dry ashing, and by $^{36}Cl^-$ isotope dilution analysis with alkaline dry ashing. Number of experiments is given by *n*.

Cell lysate	*Procedure*	*Cl^- concn.*		*n*
		μmoles/ml	*Relative concn.*	
Before dialysis	Proteins not precipitated	7.82–7.98	1.06–1.09	4
	Proteins precipitated	7.34–7.37	1.00	2
After dialysis	Proteins not precipitated	0.52–1.01	0.07–0.14	4
	Proteins precipitated	<0.15	<0.02	4
	Alkaline dry ashing*	<0.15	<0.02	7
	Isotope dilution analysis*	<0.15	<0.02	7

* Recovery > 95%.

specific activity of protein-containing samples (A), referring to intracellular Cl^-, of 0.82–0.84, consistent with the values found in Fig. 1.

Virtually complete removal of Cl^- from the cell lysate was obtained by dialysis (Table III). The true Cl^- concentration determined by isotope dilution analysis with alkaline dry ashing[9] was below 2% of the Cl^- concentration in deproteinized samples before dialysis. This value is consistent with the results obtained with deproteinization and with alkaline dry ashing. However, the protein-containing samples of the dialysate showed an apparent Cl^- concentration of 7–14%, to be compared with a

TABLE IV

EXCHANGEABILITY OF CELL Cl^- WITH EXTRACELLULAR NO_3^-

Two portions of a cell suspension, pre-incubated in chloride Ringer solution at 38°, were centrifuged, and the cells washed and resuspended in nitrate Ringer solution. After 15 min the cells were again washed and resuspended. The third portion of the cell suspension (control) was taken through an equal number of washes, but with chloride Ringer solution. After equilibration for 30 min in the final medium, Cl^- was determined in duplicated aliquots of cell lysate with and without deproteinization as stated. The values are uncorrected for trapped medium. Values in parentheses indicate number of experiments.

	Cl^- concn. (μmoles/g wet wt.)		*Relative Cl^- concn.*	
	Chloride Ringer solution	*Nitrate Ringer solution**	*Chloride Ringer solution*	*Nitrate Ringer solution**
Proteins not precipitated	82.9 ± 0.8 (2)	19.1 ± 0.4 (4)	1.25 ± 0.01 (2)	0.29 ± 0.01 (4)
Deproteinization with $HClO_4$	68.9 ± 0.3 (2)	2.1 ± 0.1 (4)	1.036 ± 0.004 (2)	0.031 ± 0.003 (4)
Deproteinization with $ZnSO_4$–NaOH, perborate oxidation	66.5 ± 0.7 (2)	<0.5 (4)	1.00 ± 0.01 (2)	<0.01 (4)

* Cl^- concn. of the medium at the end of incubation less than 1 % of the control.

protein interference of 6–9 % before dialysis. Again, this result excludes the hypothetical removal of protein-bound Cl^- during the protein precipitation procedure.

Cells equilibrated in nitrate Ringer solution were essentially Cl^- free (Table IV), in conflict with earlier reports[2–5]. The true Cl^- concentration was evaluated from samples deproteinized with $ZnSO_4$–NaOH and further oxidized with alkaline perborate to eliminate the possibility of sulfhydryl-group interference[9]. Protein-containing samples, however, indicated 25–30 % residual apparent cell Cl^-. Incomplete deproteinization and interference from sulfhydryl-groups in the supernatant may account for the slightly higher (3–4 %) Cl^- concentration in samples deproteinized with $HClO_4$. (*cf.* Table I, where the relative specific activity was slightly below unity.)

In Ehrlich cells Cl^- is generally considered to be in electrochemical equilibrium[2,4]. In the present study the Cl^- concentration in cell water was about 58 mM for cells equilibrated at 38° in sodium Ringer solution, corresponding to an E_{Cl^-} of —26 mV. In 15 experiments E_{Cl^-} ranged between —20 and —29 mV. This value is inconsistent with reported membrane potentials[2,6] of —11 to —12 mV. However, microelectrode measurements in relation to this study[7,8] resulted in potentials of about —24 mV, consistent with a passive distribution of Cl^-.

In the literature, non-exchangeable Cl^- has been reported in kidney cortex[12] (*cf.* ref. 13), connective tissue[14], skeletal muscle from crayfish and lobster[15], and vascular smooth muscle[16] (*cf.* ref. 17). Sequestered Cl^- in intestinal smooth muscle is discussed by GOODFORD[18] (*cf.* ref. 19). In human red blood cells complete exchangeability of cell Cl^- with $^{36}Cl^-$ is indicated by the combined data of COTLOVE[9] and WIETH[20].

In the present study, Cl^- in Ehrlich cells is shown to be completely exchangeable with $^{36}Cl^-$ and with NO_3^- without there being any evidence of compartmentation. However, deproteinization is indispensable for determination of the true Cl^- content.

ACKNOWLEDGEMENTS

For assistance in computer programming Mr. Bjørn Andersen Nexø is gratefully acknowledged.

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