Cation Transport in *Escherichia coli*

II. *Intracellular chloride concentration*

STANLEY G. SCHULTZ, NORMAN L. WILSON, and WOLFGANG EPSTEIN

From the Biophysical Laboratory, Harvard Medical School, Boston

ABSTRACT The intracellular Cl concentration in *E. coli* has been studied as a function of the Cl concentration in the growth medium and the age of the bacterial culture. The ratio of extracellular to intracellular Cl concentration is shown to be 3.0 in the logarithmic phase and 1.13 in the stationary phase, both ratios being independent of the extracellular Cl concentration. If it may be assumed that Cl is passively distributed in this organism, these results are consistent with a transmembrane P.D. of 29 mv, interior negative, during the logarithmic phase, and 3 mv, interior negative, in the stationary phase.

Although the transmembrane distribution of Cl has been extensively studied in a variety of animal and plant cells, this problem has received relatively little attention in the case of microorganisms. Conway and Downey have studied the disappearance of Cl salts from the supernatant solution of a thick suspension of yeast and concluded that the yeast cell envelope is virtually impermeable to this anion (1). Using a similar method Mitchell and Moyle have studied the uptake of Cl by *S. aureus* (2).

The present study is concerned with the relationship of the intracellular Cl concentration in *E. coli* to the extracellular Cl concentration and the age of the bacterial culture. The intracellular Cl concentration, in both the logarithmic and late stationary phases of growth, is shown to be a linear function of the extracellular Cl concentration. Transmembrane potential differences (P.D.S) may be calculated from the observed Cl distributions if it is assumed that Cl is passively distributed in *E. coli* and that the intracellular Cl exists in free aqueous solution.

METHODS

Escherichia coli, strain K-12, was used throughout these studies. The organisms were maintained until use by monthly transfers on nutrient agar (Difco) plates and stored at 4°C. The compositions of growth media containing 5, 50, 75, and 100 mM Cl are

given in Table I. It should be noted that media containing 75 and 100 mM Cl were obtained by the addition of NaCl to the 50 mM Cl medium, rather than by the substitution of Cl for PO_4 which would lower the buffer capacity of the medium. The salt solutions and glucose solutions were autoclaved separately, then mixed aseptically. A small inoculum of organisms was introduced into 2 liters of medium contained in 6 liter Florence flasks, and the cultures were grown at 37°C with vigorous shaking. The organisms were harvested during the logarithmic and late stationary phases (48 to 52 hours, cell density approximately 1 mg dry weight per ml) of growth in specially designed cytocrit tubes (3).

The methods of harvesting, extruding, and the determination of the wet and dry weights of bacterial pellets using these cytocrit tubes have been described previously (3). The dried pellets were extracted in 0.5 ml 0.1 M NH_4NO_3 at room temperature.

TABLE I

COMPOSITION OF GROWTH MEDIA

Compound*	5 mM Cl medium	50 mM Cl medium	75 mM Cl medium	100 mM Cl medium
	mM	mM	mM	mM
NaH_2PO_4	22	16	16	16
Na_2HPO_4	40	32	32	32
$(NH_4)_2SO_4$	8	5	5	5
NH_4Cl	0	5	5	5
NaCl	0	40	65	90
KCl	5	5	5	5
Na citrate	5	0	0	0

* All media contained 0.4 mM $MgSO_4$ and 55 mM glucose.

After 48 to 72 hours, 0.25 ml of the extraction solution was dissolved in 0.5 ml of a 1:1 (by volume) mixture of ethanol and acetic acid (4) for the determination of Cl by potentiometric titration (5). The standard deviation of a set of replicate standards was 4 per cent, with 99 per cent of the Cl recovered over the range of 0.5 to 1.0 μmol total Cl and 96 per cent recovered when the total Cl was 0.125 μmol. The total Cl content of the analyzed bacterial extracts ranged between 0.25 and 0.8 μmol. The standard deviation of the intracellular Cl concentration determined in 13 replicate pellets was 15 per cent. This larger error can be attributed to variations in the total Cl in the pellets due to fluctuations in the trapped extracellular space.

The remaining 0.25 ml of extraction solution was diluted appropriately, and the Na and K contents were determined using a Perkin-Elmer (model 52-A) flame photometer. Intracellular Na, K, and Cl concentrations were calculated after correction for trapped medium in the pellet as described previously (3).

For the determination of the intracellular Cl concentration of cells exposed to an extracellular Cl concentration of 25 mM the organisms were grown in the 5 mM Cl medium and then concentrated by centrifugation to give a final cell density of 2 per cent by volume. Sufficient $NaCl^{36}$ was then added to raise the concentration of Cl to 25 mM. The culture was then incubated at 37°C with stirring, and samples

were withdrawn at 30 minute intervals. Small amounts of 1 M tris(hydroxymethyl-aminomethane Sigma) were added periodically to the suspension in order to maintain the pH between 6.95 and 7.05 throughout the experiment. The pellets were extracted as described above, and 0.25 ml of the extraction solution and appropriate dilutions of the supernatant solution were assayed for Cl^{36} either in a flow counter (Nuclear Chicago, model 186) or in a liquid scintillation counter (Packard tri-carb, model 314). The Cl concentration in the supernatant solution was checked by potentiometric titration, and the intracellular Cl concentration was calculated from the values for the Cl^{36} concentration in the intracellular water and the specific activity of Cl^{36} in the extracellular medium. This method was employed because, in the presence of 25 mM Cl, the total pellet Cl was too small for accurate potentiometric determination.

The extraction procedure was tested by comparing the recovery of Cl^{36} following either extraction or complete digestion of pellets harvested from late stationary phase cells exposed to 50 mM Cl. The organisms were grown in the 5 mM Cl medium. After 48 hours of incubation sufficient $NaCl^{36}$ was added to an aliquot of the parent culture to raise the medium Cl concentration to 50 mM. Replicate pellets were harvested after 1 hour of further incubation at 37°C. Four pellets were extracted as described above, and 4 pellets were completely digested in 1 M NaOH. Aliquots of the extraction solution and appropriate dilutions of the extracellular medium were assayed for Cl^{36} in the flow counter. In the case of the digested pellets, 0.25 ml aliquots of the alkaline mixture were assayed in the liquid scintillation counter, and the extracellular Cl^{36} was then determined by adding small aliquots of the medium to the assayed vials and recounting. In this way the Cl^{36} in the pellets and extracellular medium may be compared directly, avoiding the possibility of errors which may arise from quenching by the digestion mixture. Intracellular Cl concentrations were calculated as described above. The average intracellular Cl concentration of the digested pellets was 4 per cent higher than that obtained using the extraction procedure; the difference is not statistically significant.

RESULTS AND DISCUSSION

The intracellular Cl concentrations of cells harvested in the logarithmic and late stationary phases of growth, in the presence of 25, 50, 75, and 100 mM Cl are summarized in Table II and Fig. 1 (the subscripts i and o denote the intracellular and extracellular compartments respectively). The ratios of the intracellular to extracellular concentrations of Na and K, during the logarithmic and late stationary phases are also given in Table II. The Na ratios are in good agreement with those found previously in this laboratory using a somewhat different growth medium and in the presence of lower extracellular Na concentrations (3). Furthermore, the average intracellular K concentration during the logarithmic phase of growth of 231 mM agrees well with the figure of 222 mM reported previously (3). These results indicate that the presence of Cl in the growth medium in varying concentrations does not affect the steady-state distribution of Na or K in this organism.

TABLE II

INTRACELLULAR Cl, Na, AND K CONCENTRATIONS IN E. COLI*

$[Cl]_o$	$[Cl]_i$	$\frac{[Cl]_o}{[Cl]_i}$	$[Na]_o$	$[Na]_i$	$\frac{[Na]_i}{[Na]_o}$	$[K]_o$	$[K]_i$	$\frac{[K]_i}{[K]_o}$
mM	*mM*		*mM*	*mM*		*mM*	*mM*	
				Stationary phase				
25	21.2±0.6 (8)§	1.18	145	177±4 (4)	1.22	5	11.2±1.0 (4)	2.2
50	44.5±2.0 (8)§	1.12	170	188±3 (4)	1.11	5	9.6±1.1 (4)	1.9
75	68.1±3.6 (15)	1.10	145	165±4 (8)	1.14	5	13.0±1.1 (8)	2.6
100	69.9±1.0 (7)	1.43	170	148±1 (4)	0.85	5	28.3±0.5 (4)	5.7
Average‡		1.13			1.16			2.2
				Logarithmic phase				
25	8.3±1.5 (14)§	3.0	145	81±2 (7)	0.56	5	217±5 (7)	43.4
50	17.1±0.9 (27)	2.9	120	70±2 (7)	0.58	5	224±8 (6)	44.8
75	23.7±3.0 (19)	3.2	145	80±4 (6)	0.55	5	227±5 (7)	45.4
100	33.5±1.7 (8)	3.0	170	89±8 (4)	0.52	5	257±1 (4)	51.5
Average		3.0			0.55			46.3

* The errors shown are the standard errors of the mean; the number of determinations are given in parentheses.

‡ These values do not include the data for the 100 mM Cl medium. See text for explanation.

§ $[Cl]_i$ determined using Cl^{36}.

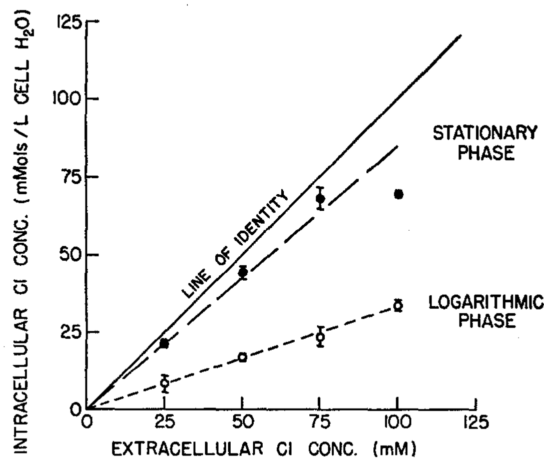

FIGURE 1. The intracellular Cl concentrations during the stationary and logarithmic phases of growth as functions of the Cl concentration in the growth medium.

As is shown in Table II, the stationary phase values of $[Cl]_o/[Cl]_i$, $[Na]_i/[Na]_o$, and $[K]_i/[K]_o$ in the case of the 100 mM Cl medium lie between the average logarithmic and late stationary phase values observed in the cases of the 25, 50, and 75 mM Cl media. These observations suggest that even after 48 hours of incubation, cells grown in the 100 mM Cl medium had not yet achieved the passive ion distribution characteristic of the late stationary phase organism.

In the 25 mM Cl experiments, the intracellular Cl concentrations obtained at 30, 60, and 90 minutes did not differ significantly, implying that Cl was completely exchanged by 30 minutes. This conclusion is supported by the good agreement between the ratios of the extracellular to intracellular Cl concentrations obtained using either Cl^{36} or chemical Cl determinations (the data for the 25 mM Cl experiments represent the averages of the values obtained at the three sampling times).

The Stationary Phase

As is shown in Fig. 1, the intracellular Cl concentration of cells harvested in the late stationary phase (after 48 to 52 hours) is linearly related to the extracellular Cl concentration over the wide range studied. Furthermore, as may be seen in Table II, the average values of $[Cl]_o/[Cl]_i$ and $[Na]_i/[Na]_o$ do not differ significantly. These data are consistent with a passive distribution of Na and Cl across a cell membrane with a transmembrane P.D. of 3 mv, interior negative. A transmembrane P.D. of this magnitude and orientation may be accounted for by a Donnan potential secondary to the non-diffusible polyanions within the cell membrane.

The stationary phase ratios of $[K]_i$ to $[K]_o$ shown in Table II exceed those found for Na, although they are much lower than the $[K]_i/[K]_o$ values seen in the logarithmic phase. It should be noted, however, that the observed deviation in the case of K is the result of an excess intracellular K concentration of only 4 to 5 mMols/liter cell H_2O, a value which represents only 2 per cent of the logarithmic phase intracellular K concentration. Although major binding of intracellular K in this organism is highly unlikely (3) a selective binding of this small amount of K by the fixed anions of the cytoplasm and the cell wall cannot be ruled out and could easily account for this observation. The contribution of this small amount of bound intracellular K to the stationary phase value of $[K]_i/[K]_o$, though considerable when $[K]_o$ is low, would be negligible when $[K]_o$ is high. This explanation is supported by the previous observation that the stationary phase value of $[K]_i/[K]_o$ was 1.05 when $[K]_o$ was 120 mM (3).

The Logarithmic Phase

As is seen in Table II the average $[Cl]_o/[Cl]_i$ ratio in cells harvested during logarithmic growth is 3.0 over the fourfold range studied. Mitchell and Moyle have studied the disappearance of Cl from the supernatant solutions of thick suspensions of *S. aureus* (2) and noted that the Cl equilibrated with only 40 per cent of the total intracellular water. These authors concluded that either Cl was exchanging stoichiometrically across the membrane for some other anion or that the bacterial suspension was not homogeneous and that only 40 per cent of the cocci were permeable to Cl. It should be noted that their results would give a ratio of $[Cl]_o$ to $[Cl]_i$ of 2.5, in good agreement with our data, and could be explained by the passive distribution of Cl in accordance with a transmembrane P.D. of 24 mv, interior negative.

The Cl distribution in *E. coli* during the logarithmic phase cannot be demonstrated to be the result of either an active or a passive transport mechanism in the absence of a direct measurement of the transmembrane P.D. However, as is seen in Table II, the ratios $[Na]_i/[Na]_o$ and $[K]_i/[K]_o$ in the logarithmic phase differ markedly from the value for $[Cl]_o/[Cl]_i$. If one assumes, for each ion, that the intracellular and extracellular ionic activity coefficients are equal, the following equilibrium potentials can readily be calculated: $E_K = 61 \log ([K]_o/[K]_i) = -100$ mv; $E_{Na} = 16$ mv; $E_{Cl} = -29$ mv. One may conclude from these data that active transport processes are responsible for the steady-state distributions of at least two of these ions. Although we cannot rule out the possibility that Cl is actively transported by *E. coli*, the assumption that Cl is distributed passively is supported by the observation that the $[Cl]_o/[Cl]_i$ ratio is constant over a wide range of extracellular Cl concentrations.

Further support for the assumption that Cl is passively distributed in *E. coli* is derived by analogy with other single cell systems similarly characterized by a high intracellular K and low intracellular Na and Cl concentrations. Considerable evidence has accumulated suggesting that Cl is passively distributed in many animal cells including frog skeletal muscle (6), squid giant axon (7, 8), human red blood cells (9, 10), rabbit polymorphonuclear leukocytes (11), sheep myocardium (12), and the Ehrlich mouse ascites tumor cell (13). In all these cells the intracellular Cl concentration is lower than that in the surrounding medium. Active Cl transport has been demonstrated in several secretory tissues such as frog stomach (14, 15), rat small intestine (16), salivary glands (17), and across the vacuolar membranes of the algae *Halicystis ovalis* (18), *Chara* (19), and *Nitellopsis obtusa* (20). The latter cells are characterized by a Cl concentration in the cell sap which exceeds that in the surrounding cytoplasm and in the extracellular medium. It should be noted in this respect that the cell sap is essentially a salt solution

in which Cl is the major anion. Furthermore, active Cl transport has been demonstrated only across the vacuolar membrane; the distribution of Cl across the plasmolemma, separating the cytoplasm from the external medium, is believed to be passive (20).

The present study of the intracellular Cl concentration in *E. coli* was undertaken for the purpose of gaining further insight into the physical forces which influence Na and K transport in this microorganism. The results indicate that, as in the cases of Na and K (3), the intracellular Cl concentration is a function of the age of the bacterial culture. The transmembrane distributions of Cl, Na and K in the late stationary phase strongly suggest that these ions are passively distributed during this period. Two arguments have been presented which supported the assumption that Cl is passively distributed in *E. coli* during the logarithmic phase of growth. If this is the case, the present data indicate that the transmembrane P.D during the logarithmic phase is 29 mv, interior negative, and that both K uptake and Na extrusion are active processes taking place against respective electrochemical potential gradients.

Dr. Schultz is a Given Foundation–National Research Council Fellow in Academic Medicine.
Mr. Wilson, a student at the Harvard Medical School, participated in this work during the tenure of a summer research fellowship.
Dr. Epstein is a Training Fellow of the Basic Science Research Training Program, United States Public Health Service (2G-466) of the Department of Medicine, New York University–Bellevue Medical Center, and is at present on leave of absence from New York University–Bellevue Medical Center.
The authors are grateful to Dr. A. K. Solomon for his encouragement and advice during the course of this study and for his critical reading of the manuscript.
This work was supported in part by the United States Atomic Energy Commission and the National Science Foundation.
Received for publication, March 19, 1962.

BIBLIOGRAPHY

1. CONWAY, E. J., and DOWNEY, M., *Biochem. J.*, 1950, **47**, 347.
2. MITCHELL, P., and MOYLE, J., *J. Gen. Microbiol.*, 1959, **20**, 434.
3. SCHULTZ, S. G., and SOLOMON, A. K., *J. Gen. Physiol.*, 1961, **45**, 355.
4. WHITTEMBURY, G., RAMIREZ, M., FERNANDEZ, J., and MONGE, C., *Acta Physiol. Latinoam.*, 1957, **7**, 76.
5. RAMSAY, J. A., BROWN, R. H. J., and CROGHAN, P. C., *J. Exp. Biol.*, 1955, **32**, 822.
6. ADRIAN, R. H., *J. Physiol.*, 1961, **156**, 623.
7. HODGKIN, A. L., *Biol. Rev.*, 1951, **26**, 339.
8. CALDWELL, P. C., and KEYNES, R. D., *J. Physiol.*, 1960, **154**, 177.
9. SOLOMON, A. K., *J. Gen. Physiol.*, 1952, **36**, 57.
10. SOLOMON, A. K., *Ann. New York Acad. Sc.*, 1958, **75**, 175.
11. WILSON, D. L., and MANERY, J. F., *J. Cell. and Comp. Physiol.*, 1949, **34**, 493.
12. CARMELIET, E. E., *J. Physiol.*, 1961, **156**, 375.
13. HEMPLING, H. G., *J. Gen. Physiol.*, 1958, **41**, 565.

14. HEINZ, E., and DURBIN, R. P., *J. Gen. Physiol.*, 1957, **41**, 101.
15. HOGBEN, C. A. M., *in* Electrolytes in Biological Systems, (A. M. Shanes, editor), American Physiological Society, Washington, D.C., 1955, 176.
16. CURRAN, P. F., and SOLOMON, A. K., *J. Gen. Physiol.*, 1958, **41**, 143.
17. LUNDBERG, A., *Physiol. Rev.*, 1958, **38**, 21.
18. BLOUNT, R. W., and LEVEDAHL, B. H., *Acta Physiol. Scand.*, 1960, **49**, 1.
19. GAFFEY, C. T., and MULLINS, L. J., *J. Physiol.*, 1958, **144**, 505.
20. MACROBBIE, E. A. C., and DAINTY, J., *J. Gen. Physiol.*, 1958, **42**, 335.