Biochimica et Biophysica Acta, 509 (1978) 148—158

BBA 78001

USE OF SALICYLIC ACID TO MEASURE THE APPARENT INTRACELLULAR pH IN THE EHRLICH ASCITES-TUMOR CELL AND *ESCHERICHIA COLI*

JAVIER GARCIA-SANCHO * and ANA SANCHEZ *

Department of Biological Chemistry, The University of Michigan, Ann Arbor, Mich. 48109 (U.S.A.) and Departamento de Bioquímica, Facultad de Medicina, Santander (Spain)

(Received August 4th, 1977)
(Revised manuscript received December 20th, 1977)

Summary

The distribution of salicylic acid between the intracellular and extracellular phases has been used to estimate the intracellular pH in the Ehrlich cell and *Escherichia coli*. The validity of the method was established by: (i) comparison of the results obtained with salicylic acid with those obtained with 5,5-dimethyloxazolidine-2,4-dione; (ii) by following changes of the apparent intracellular pH under circumstances in which such changes are predictable, e.g., the addition of weak acids or proton conductors to the incubation medium during incubation at acidic pH; (iii) by comparison of the apparent intracellular pH changes with the uptake of H^+ by the cells estimated from the changes of the medium pH. Optimal results are obtained with this indicator when the extracellular pH is below 5.5, because in this case the indicator is to a sufficient extent in its penetrating form, so that its movement can reflect intracellular pH changes occurring in less than 30 s. When the intracellular pH falls below 5.2 measurable binding of salicylic acid to the intracellular material of the Ehrlich cell takes place, but above this pH no binding has been found.

The Ehrlich cell and cells of *Escherichia coli* behaved similarly under various experimental circumstances tested, but striking differences were found in the inherent permeability of the membrane to H^+ and in the changes in this parameter by lowering the temperature to 2°C.

* Present address: Departamento de Fisiología y Bioquímica, Facultad de Medicina, Valladolid, Spain.

Abbreviation: DMO, 5,5-dimethyloxazolidine-2,4-dione.

Introduction

The problem of measuring intracellular pH (pH_i) was given a practical answer by Waddell and Butler in 1959 [1] through the measurement of the distribution of the weak acid 5,5-dimethyloxazolidine-2,4-dione (DMO). The principle is that the plasma membrane must be passively permeable to the undissociated form of the weak acid (or the uncharged form of a weak base), but impermeable to the charged form. If these criteria are met, the distribution ratio reached at the steady state will be a function of both the intracellular and the extracellular pH-values. Besides DMO, other indicators have been used [2—4]. A detailed review of the theory and limitations of such methods has been published by Waddell and Bates [5].

In the course of studies on amino acid transport we needed to measure pH_i values below 6 and to monitor rapid pH_i changes. The distribution ratio of DMO would asymptotically approach 1 as the pH_i falls below its pK' (6.15) and give no precision. Of a series of weak acids examined we found salicylic acid (pK'_{a_1} = 3.0) the most promising. Its second dissociable group, the hydroxyl group in position 2, has a pK'_a of 13; hence, it was always protonated and not a factor in the change of distribution with pH. On the other hand, the fast equilibration of salicylic acid between the intracellular and the extracellular phases suggested that it could be especially suitable for monitoring the movements of H^+ across the cell membrane occurring in short time intervals.

In the present paper we describe the use of salicylic acid for the measurement of apparent intracellular pH in the Ehrlich cell and in *E. coli* under various experimental conditions. Striking differences were found in the permeability of plasma membrane of both cell types and in its modifications by lowering the temperature to 2°C.

Materials and Methods

Chemicals. 2-^{14}C-labeled 5,5-dimethyloxazolidine-2,4-dione (10.1 Ci/mol) and [7-^{14}C] salicylic acid (60 Ci/mol) were purchased from New England Nuclear Corporation; [6,6'-(*n*)-^{3}H]sucrose (3.6 Ci/mol) from Amersham/Searle Corporation. All other products were analytical grade reagents.

Analytical procedures. Radioactivity was measured by liquid scintillation counting [6]. Unlabeled salicylic acid was occasionally determined from its spontaneous fluorescence. Total cell water was estimated from the loss of weight by desiccation at 100°C to constant weight, and extracellular space was measured as sucrose space [7].

Epxeriments with the Ehrlich cell. Methods of collecting and handling the Ehrlich cell were as described by Inui and Christensen [8]. Test periods were started by adding 0.3 ml of a 50% cell suspension in 0.15 M NaCl to 3 ml of incubation medium at 37°C containing tracer amounts of ^{14}C-labeled DMO or [^{14}C]-labeled salicylic acid. In a few experiments 30 μM unlabeled salicyclic acid was added to the medium and determined fluorometrically. The incubations were terminated in two different ways. In experiments performed at pH 6.55 and with incubation periods longer than 5 min, the cells were separated by centrifugation at 2500 rev./min for 2 min. In experiments

intended to monitor changes of pH during brief incubation periods, the cell suspension was mixed with 10 ml of ice-cold 0.15 M NaCl solution prior to the centrifugation step. This procedure was introduced in order to stop the H^+ movements at the moment of the cold dilution. The fact that diluting aliquots of the same cell suspension with ice-cold media buffered at different pH values did not modify the value estimated for pH_i showed that H^+ movements were in fact terminated by the ice-cold dilution procedure. Under these conditions, the addition of the cold diluent is followed by a redistribution of salicylic acid between the intracellular and the extracellular phases. However, when the pH of the cold-diluted medium was below 5.5 a new equilibrium distribution was reached before the cells were sedimented by centrifugation. This point was proved by the fact that cells incubated with [^{14}C]salicylic acid and then diluted with cold medium containing no salicylic acid showed the same final ^{14}C distribution ratio than cells incubated in medium containing no indicator and which were then diluted with cold medium containing [^{14}C]salicylic acid.

The distribution of salicylic acid obtained after ice-cold dilution tended to be somewhat higher than the one reached without that procedure, but the values calculated for pH_i in both circumstances bore a linear relationship defined by the equation: $pH_{\bar{s}} = 0.8\ pH_{\bar{c}} + 1$ (12 pairs of values ranging between 5.8 and 8; $r = 0.995$), where the subscripts $\bar{c}$ and $\bar{s}$ indicate that the cold dilution procedure has or has not been applied, respectively. Values of pH_i obtained using the cold dilution procedure were corrected according to the above equation.

After centrifugation, the supernatant solutions were decanted, and the cell pellets weighed and extracted with 1 ml of 0.6 M perchloric acid. pH_i values were calculated from the distribution ratio reached by salicylic acid [2].

The incubation medium was modified Krebs-Ringer phosphate solution containing only 0.5 mM $CaCl_2$. In experiments below pH 6, ϵ-aminocaproate was used as buffer instead of phosphate. This buffer (pK' 4.5) has been shown to have no significant effect on the cell water and K^+ distribution, or on amino acid transport in the Ehrlich cell (unpublished results by the authors with H.N. Christensen).

Binding of salicylic acid by the Ehrlich cell. For these experiments, cells were incubated with [^{14}C]salicylic acid for 5 min at pH 4.6. The cells were then centrifuged down and the pellet lysed, either by freezing-thawing or sonication. Aliquots of 0.5 ml of lysate containing 20—40 mg dry wt. were dialyzed for 20—40 h at 4°C against 10—20 ml of a solution containing (mM): KCl, 100/NaCl, 17/KH_2PO_4, 1.2/$CaCl_2$, 0.5/phosphate or ϵ-aminocaproate buffer, 20, at different pH values. Radioactivity was determined at the end of the experiment and referred to the water volume inside or outside the dialysis bag. Above pH 5.2 no measurable binding of salicylic acid to non-dialyzable material was observed. At pH 4.8 an apparent binding coefficient (equiv. of salicylic acid bound per kg dry wt. of non-dialyzable material/equiv. salicylic acid per kg of dialysis medium) of 2.4 ± 0.3 (mean ± S.E.) was obtained. That indicates that, if binding were to distort values of pH_i calculated from salicylic acid distribution, a value 0.25 units higher would be estimated when the real pH was 4.8. At intracellular pH above 5.2 where binding was negligible no error would be introduced due to this factor.

· *Experiments with E. coli.* A methionine-requiring strain of *E. coli* K12 CS7 was used for these experiments. Bacteria were grown in minimal medium C [9] supplemented with 0.25 mg/ml of L-methionine. The cells were harvested at the late exponential period (10 min centrifugation at 20 000 × *g* and 2°C), washed with 5% sucrose solution and resuspended in a solution of 50 mM KCl/ 20 mM choline chloride/10 mM potassium phosphate buffer, pH 7.3, at 10 mg (wet wt.)/ml. Aliquots were used to determine total water content of the cell pellet and sucrose space.

The incubations were carried out at room temperature and at pH 4.33, except when otherwise indicated. They were started by adding 1 ml of medium (45 mM KCl/10 mM KH_2PO_4/20 mM ϵ-aminocaproate and tracer amounts of both [^{14}C]salicylic acid and [^{3}H]sucrose) to 0.2 ml of the 10 mg/ml cell suspension. The incubation was terminated by filtration through Millipore filters.

The distribution of salicylic acid was calculated from the ^{14}C content of the filters and the filtrates. Corrections for the extracellular [^{14}C]salicylic acid retained in the filters was carried out on the basis of [^{3}H]sucrose counts. The amount of intracellular water contained in each filter was calculated from the determinations of total water and sucrose space in the initial 10 mg/ml cell suspension.

Uptake of H^+ by cells. For these experiments 2 ml of medium without buffer, pH approx. 7.3, containing 10 (Ehrlich cell) or 40 (*E. coli*) mg (wet wt.) of cells were quickly mixed with 20 μl of a solution of 0.1 M ϵ-aminocaproate · HCl to give the suspension a pH of 4.3. The time course of the pH changes of the cellular suspension was recorded, and the uptake of H^+ calculated as the amount of H^+ that disappeared from the medium.

Results

The main objective of the experiments to be described below was to test if salicylic acid could be used for the measurement of pH_i. For these purposes, the pH_i was estimated from the salicylic acid distribution ratio under the following circumstances: (i) simultaneously using other known pH probes, e.g., DMO; (ii) in situations in which changes of pH_i could be predicted, e.g., incubation in acidic medium, and modifying the cell membrane permeability to H^+ by different additions to the medium; (iii) with simultaneous measurement of H^+ uptake calculated from the changes of the pH of the medium. In these experiments, in addition to the behavior of salicylic acid as a pH indicator, important differences in the behavior of the Ehrlich cell and *E. coli* could be evidenced.

Comparison of the intracellular pH values calculated from the simultaneous distribution of DMO and salicylic acid. In these experiments, the Ehrlich cells were incubated at pH 6.55 with both tracer amounts of [^{14}C]DMO and 30 μM salicylic acid during periods ranging between 6 and 30 min. The apparent pH_i values calculated from the distribution of both indicators differed only in (mean ± S.E.) 0.07 ± 0.01 pH units, DMO always being the indicator giving higher pH_i estimates.

Changes of the apparent intracellular pH upon incubation in acidic medium. Fig. 1, left, shows that pH_i, as estimated with [^{14}C]salicylic acid, fell quickly

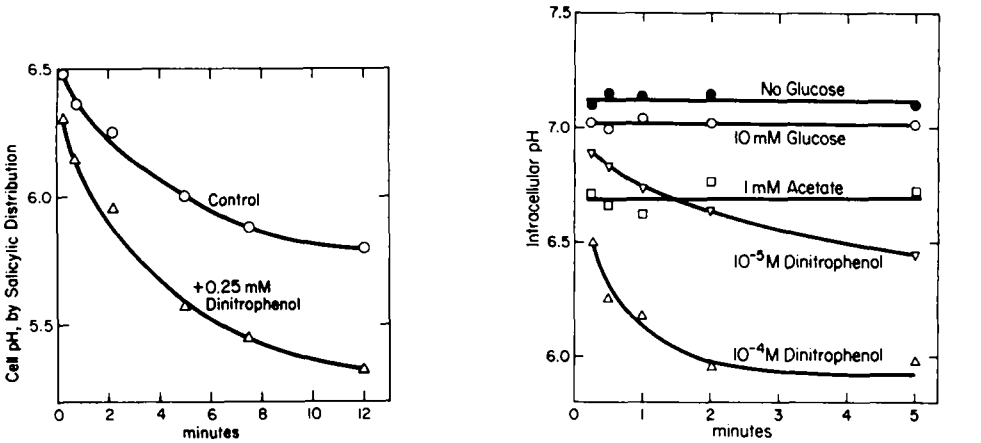

Fig. 1. Time course of the cellular pH changes of the Ehrlich cell incubated at pH 4.5 (left) or of *E. coli* incubated at pH 4.33 (right). In the case of *E. coli*, the medium contained 10 mM glucose, except for the experiments represented by the upper line.

when the Ehrlich cell was incubated in medium at pH 4.5, the fall being faster during the initial period of incubation. The addition of 0.25 mM dinitrophenol strongly accelerated the pH_i decrease. The addition to the medium of the sodium salts of cacodylic, acetic or fluoroacetic acids accelerated the decrease in pH_i observed on incubation of the Ehrlich cell at pH 4.5 (Table I). Note that the lower the pK' of the weak acid, the greater the fall of pH_i. The effect of these agents was just as large when they were present only during the interval after cold dilution. Dinitrophenol caused an effect much greater than any of these three, and its effect differed also in being partially sensitive to the temperature.

In contrast with the results found with the Ehrlich cell, pH_i of *E. coli* did not fall in response to a lowered extracellular pH if no additions were made to

TABLE I

EFFECTS OF THE ADDITION OF SOME WEAK ACIDS TO THE INCUBATION MEDIUM OR TO THE ICE-COLD DILUENT ON THE INTRACELLULAR pH OF THE EHRLICH CELL

Cells were incubated for 2 min in medium containing 40 mM ϵ-aminocaproate buffer at pH 4.6, the ice-cold dilution procedure being performed as described in Methods. The compounds indicated in the first column were introduced into the incubation medium as the sodium salt at a concentration of 1.7 mM (pH_i values illustrated in Column A) or into the ice-cold diluent at a concentration of 0.5 mM (pH_i values illustrated in column B). Note that in both cases the concentration of the compound in the final cold cell suspension is the same, namely 0.4 mM.

Added compound	pK'_a	Apparent intracellular pH	
		A	B
None		6.48	6.51
Cacodylate	6.2	6.46	6.46
Acetate	4.8	6.20	6.18
Fluoroacetate	2.7	6.16	
2,4-Dinitrophenolate	4.0	5.18	5.68

the medium (Fig. 1, right). The addition of glucose produced a small but noticeable decrease of pH_i. The addition of potassium acetate or dinitrophenolate produced a stronger fall of pH_i. In the first case, the fall appeared quickly and was maintained, while with dinitrophenol the decline of pH_i was progressively established.

A further study of the effects of various concentrations of potassium acetate, salicylate or dinitrophenolate on the distribution reached by [^{14}C]-salicylic acid in *E. coli* after 1-min incubations at pH 4.33 is shown in Fig. 2. For the first two, the relation between the distribution ratio of [^{14}C]salicylic acid and the extracellular concentration of the weak acid, both in logarithmic scale, presented a linear portion. This kind of relationship can be predicted between the values of pH_i and the extracellular concentration of a weak acid which penetrates the cell membrane in its undissociated form and then dissociates H^+ at the intracellular side. In the case of dinitrophenol the fall of [^{14}C]salicylic acid distribution ratio was much stronger, and its relation to the extracellular concentration of dinitrophenol was curvilinear.

Correlation between changes of the apparent intracellular pH and H^+ uptake by the cells. The uptake of H^+ from the medium and changes of pH_i were closely correlated in both the Ehrlich cell and *E. coli* (Fig. 3, left and right). In the case of *E. coli* the membrane permeability to H^+ had to be increased by adding acetate, salicylate or dinitrophenolate in order to obtain measurable uptake of H^+ and changes of pH_i. In both cell types dinitrophenol did not modify the slope of the line defining the relation of pH_i change to uptake of H^+, but the fall of pH_i was greater relative to the amount of H^+ taken up.

By measuring the uptake of H^+ from the changes of the pH of the medium, a value variable from 0.05 to 0.2 mequiv./kg intracellular water per min was estimated for the permeability coefficient for H^+ uptake by *E. coli* at external

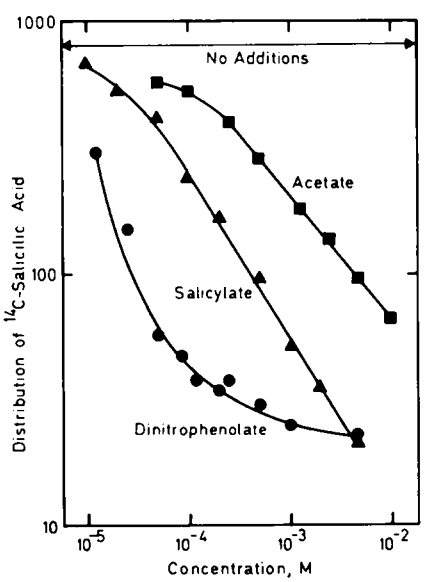

Fig. 2. Effects of different concentrations of acetate, salicylate or dinitrophenolate added to the medium on the distribution ratio reached by [^{14}C]salicylic acid in *E. coli* incubated at pH 4.33 during 1 min.

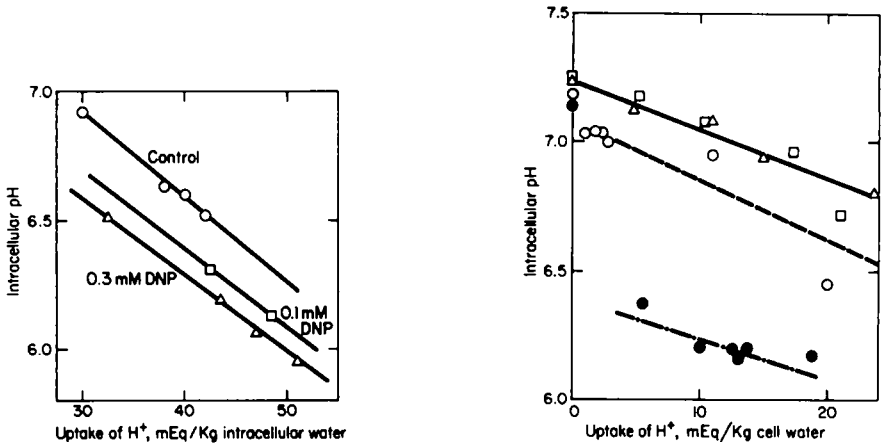

Fig. 3. Correlation between the apparent intracellular pH and the uptake of H^+ by the Ehrlich cell (left) or by *E. coli* (right). In the case of *E. coli*, the medium contained various concentrations of acetate (△———△), salicylate (□———□), 10^{-5} M dinitrophenol (○———○) or 10^{-4} M dinitrophenol (●———●). DNP, 2,4-dinitrophenol.

pH values between 4.3 and 4.6. In parallel experiments with the Ehrlich cell, also at 25°C, a value of 4—6 mequiv./kg intracellular water per min was obtained.

Dinitrophenol increased the uptake of H^+ by *E. coli* (Fig. 4), the effect presenting a time course consistent with the one shown for the changes of pH_i (Fig. 1, right). The final amount of H^+ taken up at the steady state depended on the concentration of dinitrophenol used (Fig. 4), as did the final value of pH_i (Fig. 1).

Effects of temperature on the distribution reached by [^{14}C]salicylic acid. A decrease of temperature from 37 to 0°C moderately increased the distribution

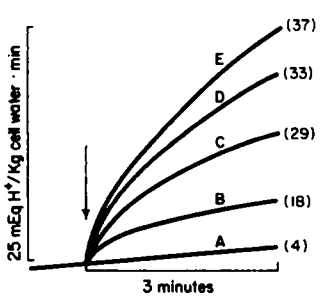

Fig. 4. Effects of 2,4-dinitrophenol on the uptake of H^+ by *E. coli*. The records of the changes of the external pH of five experiments carried out with the same cell suspension have been inverted and superimposed, A, B, C, D and E contained 0, 20, 50, 75 and 100 μM dinitrophenol, respectively. The arrow marks the addition of dinitrophenol. Numbers in parentheses at the right hand side represent the uptake of H^+ after 15 min of incubation; at this time a plateau had been reached, at least for A, B and C. The pH of the medium at zero time was 4.75.

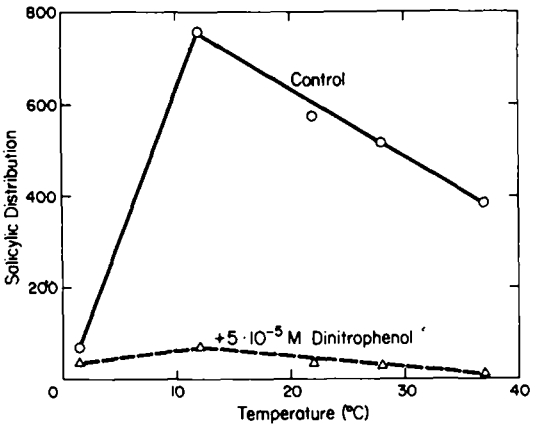

Fig. 5. Effects of temperature on the distribution ratio reached by [^{14}C]salicylic acid in *E. coli* incubated at pH 4.33 for 1 min.

ratio reached by [^{14}C]salicylic acid in the Ehrlich cell, as described in Methods, in relation to the ice-cold dilution procedure. In bacteria, two contrary effects were observed, each one taking place at a different temperature range (Fig. 5). A decrease of temperature from 37 to 12°C moderately increased salicylic acid distribution. Below 12°C the direction of change reversed itself and a tremendous decrease of salicylic acid distribution took place, suggesting a massive down-hill uptake of H^+ from the medium. Table II shows that H^+ taken up after an incubation at 2°C and pH 4.33 can be partially re-extruded in a subsequent incubation at the same pH and 25°C. On the other hand, most of H^+ taken up during an incubation at 2°C and pH 4.33 is lost in a subsequent incubation at the same temperature and pH 7.1 (compare the two last lines in Table II), suggesting that bacterial membrane becomes largely permeable to H^+ at 2°C.

TABLE II

EFFECTS OF INCUBATION AT 2°C AT DIFFERENT pH VALUES ON THE DISTRIBUTION OF SALICYLIC ACID IN *E. COLI*

The rapid pH changes were managed by adding ε-aminocaproate or potassium phosphate buffers, more or less acidified. Cells were allowed to warm for 1.5 min before the incubation at 25°C was started. Every datum is the mean of two experiments.

Incubation at 2°C	Incubation at 25°C	Final salicylic acid distribution	Intracellular pH
None	8 min at pH 4.33	798	7.23
5 min at pH 4.33	None	112	6.38
5 min at pH 4.33	8 min at pH 4.33	280	6.78
5 min at pH 4.33 + 5 min at pH 7.1	8 min at pH 4.33	478	7.01

Discussion

Our findings support the validity of salicylic acid as a pH probe. Similar estimates of pH_i were obtained with salicylic acid and DMO at pH ranges where both indicators could be used. At lower pH values, where other known indicators were not usable, salicylic acid distribution changed in the way predicted for a pH probe. Weak acids, which penetrate the cell membrane in their undissociated form and then liberate H^+ at the intracellular side, lowered the apparent pH_i, the effects being related to their pK'_a values and no modified by temperature (Fig. 2 and Table I). In contrast, dinitrophenol, a known proton conductor which acts in a catalytic way [10,11] had an ability to lower pH_i which was greater than expected from its pK'_a value, and its effect was sensitive to temperature (Fig. 2 and Table I). The time course of the effects of simple weak acids and dinitrophenol on pH_i was different (Fig. 1, right), as expected from their different action mechanisms.

The validity of the salicylic acid method was also supported by the close correlation found between pH_i estimates and uptake of H^+ (Fig. 3). However, dinitrophenol increased the pH_i drop obtained with a given amount of H^+ taken up by the cells. This result could be expected if dinitrophenol, in addition to acting as a proton conductor, increases the rate of production of acidic metabolites as suggested by results obtained in the Ehrlich cell by Poole et al. [12]. Similarly, an increase of the rate of production of acidic metabolites following the combustion of glucose [13] would explain the drop of pH_i produced by this sugar in *E. coli* (Fig. 1, right).

Binding of salicylic acid by intracellular material could induce serious errors in calculation of pH_i. We find that salicylic acid is not bound by the Ehrlich cell at pH_i values above 5.2. Hence, binding could not have influenced the results presented here, but it should be taken in account when measuring pH_i values below 5.2. In the case of *E. coli*, the influence of binding seems to be dismissed by the way in which the distribution of [^{14}C]salicylic acid was modified by the addition of cold salicylate to the medium (Fig. 2). Binding at the lower pH_i values would have made the obtained relation curvilinear at its lower end.

The estimated pH_i value may be a weighed average value if H^+ should not reach the same concentration in all the cellular compartments. This fact, together with the impossibility of comparison of the values of the apparent pH_i with the real ones, measured with an H^+ electrode, leads us to define the pH_i values obtained with the salicylic acid method as operational ones. These values are suitable, however, to measure the effects of experimental circumstances on pH_i if the opportune controls for comparison are also performed.

A great advantage of salicylic acid over other indicators used to estimate pH_i is its rapidity in reaching an equilibrium distribution across the cell membrane, which allows us to monitor changes of pH_i following a rapid time course. The best results in short term experiments should be obtained at external pH values below 5.5, because only in these circumstances is the indicator to a sufficient extent in its penetrating form to reach an equilibrium distribution in less than 0.5 min. On the other hand, the lower the external pH, the higher the distribution ratio reached will be [2], thus minimizing the necessary corrections for the

amount of indicator retained in the extracellular space, and hence increasing the precision of the method.

The distribution of salicylic acid was somewhat dependent on temperature. Cooling led to an increase of the distribution ratio reached, in both the Ehrlich cell and in *E. coli* above 12°C. Several factors could contribute to this effect, including asymmetric effects of temperature on standard electro-chemical potentials of H^+, salicylic acid and salicylate at each side of the membrane. Eivdence for different thermodynamic properties for the intracellular and the extracellular solvent has been reported for skeletal muscle [4].

The Ehrlich cell and *E. coli* differed largely in their permeability to H^+, the bacterial membrane being much less permeable. On the other hand, cooling below 12°C decreased the membrane permeability to H^+ in the Ehrlich cell (see Methods) but increased it tremendously in bacteria (Fig. 5); the phenomenon was reversible on rewarming the cells. Differences have also been reported for the effects of temperature on the permeability to amino acids in both cell types. Cells of *E. coli* loose most of previously accumulated amino acids during a washing with medium cooled below 8°C [14], while the decrease of temperature to 0°C prevents the loss of previously accummulated substrate in the Ehrlich cell. It has recently been reported that lysosomal membrane, like we find in bacterial membrane, is rendered permeable to H^+ by lowering the temperature from 25 to 0°C, and the possible role of a thermal transition in membrane lipids in this process has been discussed [15].

Our results suggest the operation of an active H^+-extruding mechanism in the membrane of *E. coli*, responsible for the reextrusion against gradient at 25°C of H^+ taken up at 2°C (Table II), and of the fact that different steady state levels of net H^+ uptake (Fig. 4) or pH_i (Fig. 1) are observed with different concentrations of dinitrophenol. The operation of this H^+ pump could not be evidenced in the experiments carried out with the Ehrlich cell.

Acknowledgements

We are deeply indebted to Prof. H.N. Christensen for his continuous advise during the course of this work and for helpful suggestions on the preparation of the manuscript. Support from the Institute of Child Health and Human Development (Grant HD01233) of the National Institutes of Health, U.S.P.H.S., is gratefully acknowledged. J.G.-S. was supported by the Fundación Juan March, Madrid, Spain, and A.S. by the Ministerio de Educación y Ciencia, Madrid, Spain.

References

1 Waddell, W.J. and Butler, T.C. (1959) J. Clin. Invest. 38, 720—729
2 Rottemberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54—63
3 Johnson, R.G. and Scarpa, A. (1976) J. Biol. Chem. 251, 2189—2191
4 Hannan, H.F. and Wiggins, P.M. (1976) Biochim. Biophys. Acta 428, 205—222
5 Waddell, W.J. and Bates, R.G. (1969) Physiol. Rev. 49, 285—329
6 Pal, P.R. and Christensen, H.N. (1961) J. Biol. Chem. 236, 894—897
7 Messineo, L. and Musarra, E. (1972) Int. J. Biochem. 3, 691—699
8 Inui, Y. and Christensen, H.N. (1966) J. Gen. Physiol. 50, 203—224
9 Davis, B.D. and Mingioli, E.S. (1950) J. Bacteriol. 60, 17—28

10 Mitchell, P. and Moyle, J. (1967) Biochem. J. 104, 588—600
11 Hopfer, V., Lehninger, A.L. and Thompson, T.E. (1968) Proc.Natl. Acad. Sci. U.S. 59, 484—490
12 Poole, D.T., Butler, T.C. and Williams, M.E. (1972) Biochim. Biophys. Acta 266, 463—470
13 Poole, D.T. (1967) J. Biol. Chem. 242, 3731—3736
14 Piperno, J.R. and Oxender, D.L. (1968) J. Biol. Chem. 243, 5914—5920
15 Reijngoud, D.-J. and Tager, J.M. (1975) FEBS Lett. 54, 76—79