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Refined Evaluation of the Exponential Curve Parameters and Initial Exchange Rate Constant for $^{22}Na^+$ Washout in Cultured Human Skin Fibroblasts (42708)

LASZLO HOPP, AKITOSHI NAKAMURA, MINORU KINO, SATORU KURIYAMA, AND ABRAHAM AVIV

Hypertension Research Center, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103

Abstract. A technique is proposed to evaluate the exponential curve parameters and the initial exchange rate constant (k_e^i) for $^{22}Na^+$ washout from cultured human skin fibroblasts. After loading with the isotope, the cells were subjected to cold washing and warming steps. A desaturation curve for $^{22}Na^+$ washout was developed including the activity in the warming medium that corresponded to $t = 0$ min. Using nonlinear regression analysis, a general three exponential function adequately described the $^{22}Na^+$ washout in the time interval of 0–70 min. A back extrapolation was performed to estimate the initial time (t_i; a negative number) when the total activity was present in the cells. The t_i was substituted into the first derivative function of the three exponents to yield the k_e^i. Calculated from the equilibrium distribution of $^{22}Na^+$ and the specific activity of the medium, the concentration of Na^+ (in m*M*; mean ± SD) for fibroblasts of two individuals were 13.3 ± 2.3, $n = 3$, and 19.0 ± 5.2, $n = 4$. This indicates that the washout originated mainly or exclusively from the cellular milieu. Therefore, the k_e^i represents the equilibrium exchange rate constant for Na^+ washout from an inhomogeneous cell-related space. Multiple experiments demonstrated that the k_e^i value for the two subjects were significantly higher than the initial slopes of the washout curves (k_A), a commonly used parameter to characterize Na^+ washout, and significantly lower than the slopes of the fastest exponential components (k_3): $k_e^i = 0.531 \pm 0.017$, $k_A = 0.502 \pm 0.019$, and $k_3 = 0.557 \pm 0.017$ min^{-1} ($n = 3$) for one subject, and $k_e^i = 0.567 \pm 0.065$, $k_A = 0.479 \pm 0.031$, and $k_3 = 0.667 \pm 0.094$ min^{-1} ($n = 6$) for the other subject. The respective equilibrium exchange rates for these cells, namely the products of k_e^i and cellular Na^+ contents, were 1.10 ± 0.16 and 1.19 ± 0.24 nmole/10^5 cells. Using the exponential curve parameters, analytical solutions of a serial model and a parallel model with three compartments were performed. According to these analyses the major portion of the cellular Na^+ comprises a fast exchangeable cellular compartment. The relative size of this compartment (expressed as a fraction of total cellular Na^+ content) for fibroblasts of the two subjects was 96.2 and 89.2% for the serial model and 96.1 and 89.3% according to the parallel model. The proposed technical and analytical approach can be applied to other cellular tracer washout with fast initial exchange rate and multiple exponential compartments, particularly in tissue culture preparations which have a relatively small and simple extracellular space.

Cellular Na^+ regulation plays an important role in a variety of physiological and pathological processes (1–4). Investigations designed to characterize precisely the cellular Na^+ homeostasis have been hampered by the fast and complex washout of Na^+ isotopes from cell and tissue preparations (5, 6). These characteristics of Na^+ washout have contributed to the difficulty in distinguishing between the extra- and intracellular Na^+ tracer. When no attempt is made to remove completely the extracellular isotope before the washout experiment, it must be assumed that the initial, fast washout component entirely represents Na^+ isotope of extracellular origin (2, 5, 7–11). The rapidity of the Na^+ washout, however, may result in a substantial overlap of cellular and extracellular Na^+ exchange during its initial phase. The discrimination between Na^+ isotope from cellular and extracellular origin is further complicated by the existence of more than one cellular compartment for this ion. When a washing procedure is performed at 37°C, before initiation of the washout experiment, substantial amount of cellular radioactivity may be lost (12). This loss occurs mainly from the cellular compartment with the most rapidly exchangeable Na^+ and, thus, the relative contribution of cellular compartments

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with less rapidly exchangeable Na^+ to the estimated washout rate constant value is expected to increase. As a result, underestimation of the washout rate constant is likely to occur when the washing is performed at 37°C. When cold, instead of warm, washing is carried out to minimize cellular loss of the Na^+ isotope before the start of the washout experiments (5, 9, 13), the fastest and critical phase of the isotope washout takes place at an average cellular temperature below 37°C. Such a temperature may retard the initial phase of the washout which is dependent to a large extent on the rate of Na^+ extrusion by the Na^+ pump.

In this article we describe a modified technical and analytical approach to evaluate $^{22}Na^+$ washout from tissue culture preparations. This approach circumvents the aforementioned shortcomings and it facilitates a more precise estimation of the curve parameters of the $^{22}Na^+$ washout and the initial $^{22}Na^+$ exchange rate constant (k_e^i).

Materials and Methods. The experiments were performed *in situ* using serially passed cultured skin fibroblasts (passages 6–7) from two volunteers (R.L. and S.G.). The tissue culture processing was described elsewhere (14). Three days before the experiments cells (8×10^5/well) were inoculated into Nunc-6-well (35-mm diameter) clusters. The growing medium was Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 2 m*M* L-glutamine (95% air–5% CO_2). On the day of the experiments the growing medium was aspirated, and the cells were washed once with 5 ml DMEM containing 10% FBS and loaded in the same medium for 2 hr with $^{22}Na^+$ (25 μCi/ml; Amersham, Arlington Heights, IL) at 37°C. To obtain this high radioactive concentration, a 12.5% dilution of the medium with the $^{22}Na^+$ stock solution was necessary. Choline chloride was used to maintain the isotonicity. The Na^+ concentration in this medium was 135 m*M*. The purpose of the relatively long loading period was to ensure that cells reach equilibrium in the fresh and somewhat diluted medium. After the loading cells were washed three times with 5 ml ice cold 0.1 *M* $MgCl_2$ solution (washing step, approximately 30 sec). Immediately thereafter (t_w), 0.7 ml prewarmed (37°C) washout medium without the isotope was added to the cells (warming step) and aspirated 25–45 sec later (t_0). Subsequently, aliquots (0.7 ml) of fresh medium were added and withdrawn periodically. The loading and washout procedures were performed in 95% air–5% CO_2 at 37°C. The washout medium was identical to the loading medium except that it did not contain $^{22}Na^+$. At the end of each experiment cells were trypsinized. The remaining activity in the cells and the radioactivity of the samples of the washout and warming media were counted in a gamma counter. Samples from two wells were pooled in order to ensure a reasonable reading above the background. The fraction of the activity that remained in the cells at a given time ($A_{c,t}$) was calculated according to

$$A_{c,t} = \frac{\sum A_\infty - \sum A_t}{\sum A_\infty}, \qquad [1]$$

where $\sum A_\infty$ denotes the total activity at initiation of the washout experiment and $\sum A_t$ represents the activity that left the cells within the time period t. The $\sum A_\infty$ was computed by adding in a reverse manner the activities of aspirated aliquots of the washout medium (including the warming solution) and the activity remaining in the cells at the termination of the experiment. The $\sum A_t$ was determined by summing the activities of all the aspirated aliquots up to time t. (The value of $A_{c,t}$ is 1 at t_w and 0 at t_∞.)

The washout data closely fitted the sum of three exponential functions up to 70 min of observation. Best fitness was examined as previously described (15). A three exponential function yielded significantly better fitness than a two exponential function. An additional exponential term did not significantly improve the fitness. The parameters of this function were obtained by nonlinear regression analyses. A logarithmic transformation of the three exponential function was used in order to assure a comparably equal contribution of all observations to the analyses. This transformation gives a higher mathematical weight to the low $A_{c,t}$ values at the end of the curves, whereas the relatively small weight of the initial $A_{c,t}$ values is balanced by frequent sampling (11 observations within the first 10.5 min of the washout and

6 observations within the subsequent 60 min, as shown in Fig. 1). Furthermore, the intercept of the function with the ordinate was fixed according to the t_0 measurement (a_0). Thus, the analyses included five parameters according to

$$\ln A_{c,t} = \ln[a_1 \cdot \ell^{-k_1 \cdot t} + a_2 \cdot \ell^{-k_2 \cdot t} + a_3 \cdot \ell^{-k_3 \cdot t}], \quad [2]$$

where a_{1-3} ($a_3 = a_0 - a_1 - a_2$) are the exponential coefficients and k_{1-3} are the exponential factors. (It is necessary to emphasize that a_0 is less than unity because during the warming step, some activity has already been lost from the cells.) Approximations of the parameters in Eq. [2] were obtained by sequential peeling (16). These values were used as initial estimations for nonlinear regression analyses. The calculations were carried out by an IBM 3033-U computer using the SAS NLIN program package (17). After the parameters were obtained, Eq. [2] was solved for t by a trial and error procedure at the $A_{c,t}$ value of 1. The calculations were stopped when the $A_{c,t}$ value was within the range of 0.995–1.005. This resulted in a less than 1% error in further calculations of the initial $^{22}Na^+$ exchange rate constant (k_e^i). The time calculated (t_i) was substituted into the first derivative of Eq. [2], thereby yielding the k_e^i value

$$-k_e^i := a_1 \cdot k_1 \cdot \ell^{-k_1 \cdot t_i} + a_2 \cdot k_2 \cdot \ell^{-k_2 \cdot t_i} + a_3 \cdot k_3 \cdot \ell^{-k_3 \cdot t_i}. \quad [3]$$

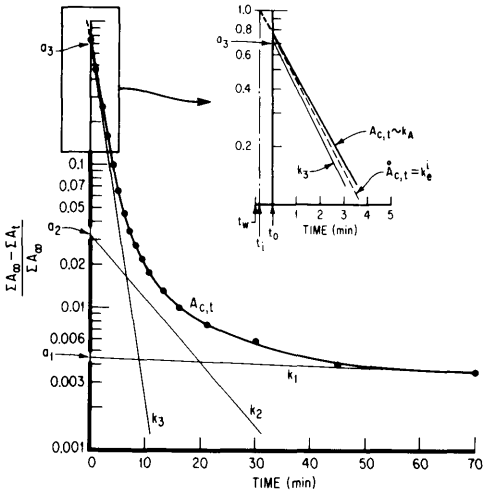

FIG. 1. $^{22}Na^+$ washout from cultured skin fibroblasts of R.L. The points represent single observations. The abscissa is the time of washout in minutes and the ordinate depicts the fraction of $^{22}Na^+$ activity remaining in the cells. The bold line depicts the washout as per Eq. [2] using parameters $k_1 = 0.004 \pm 0.003$; $a_1 = 0.005 \pm 0.001$; $k_2 = 0.103 \pm 0.012$; $a_2 = 0.032 \pm 0.003$; $k_3 = 0.564 \pm 0.009$; $a_3 = a_0 - a_1 - a_2 = 0.725 \pm 0.004$. The straight lines are the three exponential components in Eq. (2). The inset illustrates the back extrapolation from the beginning of the washout experiment to obtain t_i. The first derivative of Eq. (2) at this time point ($\dot{A}_{c,t}$) provides the initial exchange rate constant (k_e^i) whereas $A_{c,t}$ represents the measured data that are approximated by a straight line, the slope of which is k_A. For further details and abbreviations, see text.

Protein was measured by the Lowry method (18). Intracellular water volume was determined according to the equilibrium distribution of 3-*O*-^{14}C-methyl-D-glucoe (Amersham) following 150 min incubation in DMEM with FBS (19). Cell number was determined in a Coulter counter. Statistical analysis utilized Student's paired *t* test. Data are presented as means ± SD.

Results. Using the specific activity (SA) of the medium (cpm/nmole), the amount of the total Na^+ taken up by cells of the two subjects at the end of the 2-hr loading period was as follows: 2.07 ± 0.31 (n = 3) and 2.09 ± 0.39 nmole/10^5 cells (n = 6). When these values were normalized for the intracellular water volume (0.160 ± 0.013 and 0.110 ± 0.022 μl/10^5 cells; n = 4 for both cases), or to protein contents (22.4 ± 0.95 and 21.8 ± 0.60 μg/10^5 cells; n = 4 for both cases), intracellular Na^+ concentrations of 13.3 ± 2.3 and 19.0 ± 5.2 m*M* or 92.0 ± 14.0 and 95 ± 18.1 μmole/g protein were obtained. These values are within the range of the normal intracellular Na^+ levels reported for fibroblasts and other cell types (2, 5, 12, 20–22). Thus, the total activity taken up by the cells appears to represent Na^+ of cellular origin.

A representative $^{22}Na^+$ washout curve of R.L. is shown in Fig. 1. The three exponential curve fitting appears to be quite appropriate in describing the data. The inset to the figure demonstrates the manner by which the k_e^i value was obtained (as described under Materials and Methods) and its relationship in this particular case to the washout data at

the beginning of the experiment ($A_{c,t}$) and to the slope of the fastest exponential component (k_3). The slope of $A_{c,t}$ (k_A) was approximated by linear regression analysis of the semilogarithmic transformation of the data in the apparently linear first 3 min ($k_A = 0.524$ min^{-1}; $r = 0.9996$). The results of three separate experiments on fibroblasts from R.L. and six on fibroblasts from S.G. are summarized in Table I. In the case of R.L., the k_e^i value is significantly lower than the k_3 ($k_3 - k_e^i = 0.027 \pm 0.009$; $P < 0.05$), yet it is 5.5% higher than the k_A ($P < 0.05$). These differences are more pronounced for cells from S.G. ($k_3 - k_e^i = 0.100 \pm 0.041$; $P < 0.01$; and $k_e^i - k_A = 0.088 \pm 0.039$; $P < 0.01$). The k_A value is often used as representative of the Na^+ exchange rate constant in experiments where slower components of the $^{22}Na^+$ washout are disregarded. The k_e^i value and the Na^+ content calculated from the equilibrium $^{22}Na^+$ distribution and the medium specific activity can be used to calculate the equilibrium Na^+ exchange rate (R; Table I).

The three exponents of the $^{22}Na^+$ washout indicate the existence of at least three cell-related compartments for the isotope (for detail of the relationship between experimental washout curves and compartmental analysis, see (24)). Exponential factors (k_{1-3}) and exponential coefficients (A_{1-3}; corrected by means of t_i as described in the footnote to Table I) can be used for the analytical solution of specific compartmental models. Though the precise arrangement of the three cell-related compartments cannot be obtained from the results of these experiments, one can analyze the data according to models of three compartments (Table II) arranged in series (23) or in parallel (24). The steady state pool sizes of the three compartments and intercompartmental rate constants shared by the parallel and serial models are essentially the same. For cells of the two subjects, both models predict an approximate contribution of 96 and 89% of the largest compartment to the total cellular Na^+ content.

TABLE I. PARAMETERS OF THE $^{22}Na^+$ WASHOUTS FROM CULTURED HUMAN SKIN FIBROBLASTS

Parameter	R.L. ($n = 3$)	S.G.[a] ($n = 6$)
k_1 (min^{-1})	0.011 ± 0.008	0.025 ± 0.010
A_1	0.011 ± 0.005	0.022 ± 0.011
k_2 (min^{-1})	0.122 ± 0.024	0.201 ± 0.045
A_2	0.047 ± 0.016	0.179 ± 0.067
k_3 (min^{-1})	0.557 ± 0.017	0.667 ± 0.094
A_3	0.942 ± 0.021	0.798 ± 0.074
k_e^i (min^{-1})	0.531 ± 0.017†	0.567 ± 0.065‡
k_A (min^{-1})	0.502 ± 0.019	0.479 ± 0.031
Na^+ (nmole/10^5 cells)	2.07 ± 0.31	2.09 ± 0.39
R (nmole/10^5 cells/min)	1.10 ± 0.16	1.19 ± 0.24

Note. A_{1-3} values were calculated as $A_x = a_x \cdot e^{-k_x \cdot t_i}$, where a_x and k_x are parameters obtained from Eq. [2] and t_i is calculated as described under Materials and Methods.

[a] Parameters of cells from R.L. and S.G. were obtained by different investigators 1 year apart. This may account for differences in SDs for the two subjects.

†, ‡ Significantly different from both k_3 and k_A at $P < 0.05$ and $P < 0.01$, respectively.

TABLE II. COMPARTMENT PARAMETERS OF CULTURED HUMAN SKIN FIBROBLASTS DERIVED FROM $^{22}Na^+$ WASHOUT DATA

	3 compartments in series		3 compartments in parallel	
	R.L.	S.G.	R.L.	S.G.
M_1	1.99 (96.2)	1.86 (89.2)	1.99 (96.0)	1.86 (89.3)
M_2	0.062 (3.0)	0.178 (8.5)	0.060 (2.9)	0.179 (8.6)
M_3	0.017 (0.8)	0.046 (2.2)	0.023 (1.1)	0.045 (2.0)
$k_{1,0}$	−0.531	−0.568	−0.531	−0.568
$k_{0,1}$	0.552	0.637	0.552	0.637
$k_{2,1}$	0.004	0.020	0.004	0.020
$k_{1,2}$	0.119	0.211	0.123	0.210
$k_{3,2}$	0.003	0.001	0.000	0.000
$k_{2,3}$	0.012	0.004	0.001[a]	0.001[a]
$k_{3,1}$	0.000	0.000	0.001	0.001
$k_{1,3}$	0.000	0.011	0.011	0.025

Note. M_i (nmole/10^5 cells) = steady state compartment mass. The numbers in parentheses indicate the relative compartment size in %. $k_{i,j}$ (min^{-1}) = intercompartmental rate constants from the jth to the ith compartment.

[a] According to the parallel model, parameter $k_{2,3}$ should be zero. Due to the approximate nature of the analytical solution, this parameter is slightly different from zero.

Discussion. In this paper we report several modifications in performing and analyzing $^{22}Na^+$ washout from cultured cells *in situ*.

First, with an ice cold wash, the extracellular isotope is removed without a substantial loss of the intracellular $^{22}Na^+$. Second, a short warming period is provided for the cells to attain the 37°C washout temperature. The radioactivity leaving the cells during this warming step is determined and it serves for the calculation of the starting value (a_0) for the washout measurements (see values on the ordinate at time zero in Fig. 1). Third, when evaluating the data, a back extrapolation is made to a negative time point based on the whole washout curve. At that time point, the entire radioactivity was present within the cells (t_i in the inset to Fig. 1). This time can be best defined as a hypothetical starting point for the $^{22}Na^+$ exchange process at 37°C cellular temperature. It is calculated by assuming that the pattern describing the $^{22}Na^+$ washout from the arbitrary zero time (t_0) to the end of the experiment is representative of washout during the period between the hypothetical starting point at 37°C (t_i) and t_0. In this regard, only several seconds and roughly 20% of the y (ordinate) value are extrapolated based on a 70-min segment of the Na^+ washout curve that describes approximately 80% of the total change in this value. Biologically, this extrapolation serves as a refined estimation of the crucial initial events of the $^{22}Na^+$ exchange process which, in reality, cannot be directly measured because (1) without washing the preparation, the initial events do not represent only cellular processes, and (2) when cold washing is performed, the initial phase of the washout represents cellular processes at an average temperature lower than 37°C. As the majority of Na^+ washout occurs through the active Na^+ pump, temperature is an important factor for the extrusion of the ion.

The washout of Na^+ isotopes for different tissues (7–9, 13, 25) and cultured cell preparations ((8); for review also see (26)) has been previously described by biexponential or triexponential functions. The physiological meaning of these components is not entirely clear. The fast component has been considered to be too large to represent a cellular Na^+ pool. Thus, it was speculated that this component represents the washout of extracellularly trapped Na^+ or Na^+ bound to the external cell membrane. Different researchers have suggested that the slowest (7, 9, 10), the middle component (13, 25), or both (8, 11) represent Na^+ washout from the intracellular compartment(s). Accordingly, the values of the rate constants for Na^+ washout varied within a 10-fold range. Detailed Na^+ washout analyses have been rarely performed in cultured fibroblast preparations. Garay *et al.* (8) reported a two exponential $^{22}Na^+$ washout from primary cultures of rabbit adventitial fibroblasts of which the large, faster component was assumed to be of extracellular origin. In our preparation the cellular Na^+ content, calculated from the total $^{22}Na^+$ taken up during the loading period, is well within the normal range for this variable (2, 5, 12, 20–22). Hence, the largest and fastest of the three exponential components is likely to represent cellular Na^+. This assertion is further substantiated by subsequent studies demonstrating that the exponential factor of the largest component is reduced by ouabain and is increased in a Ca^{2+}-deficient medium (27). The exponential factor of the middle component was also sensitive to ouabain (27). Therefore, it appears to represent a distinct Na^+ space within the cell. The origin of the smallest component is uncertain. A defined cellular space or Na^+ bound to the extracellular matrix must be considered. In the latter case, however, the dissociation of the cation is expected to occur with a faster rate than that inferred from the exponential factor of the smallest component. Thus, it is proposed that like the other two components, this component is also of cellular origin. It must be pointed out, however, that the parameters of the smallest component (k_1, A_1) are based mainly on the tail of the washout curve with the lowest activities. Therefore, these values should be considered only as gross estimations (see high SD). The reason for the discrepancy of the present findings in cultured human fibroblasts with results in cultured rabbit fibroblasts (8) is not clear, but technical (e.g., the use of primary cultured cells vs serially passed cells) and species differences must be considered.

A value of 0.42 min^{-1} was documented for $^{22}Na^+$ exchange rate constant of cultured chick heart nonmuscle cells primarily consisting of fibroblasts (6). In cultured rat fibroblasts, a $^{22}Na^+$ efflux rate constant of 0.32

min^{-1} was found (28). Both values are based on a 6-min apparently monoexponential washout curve corresponding to the k_A value in Table I. There is a relatively good agreement between these data and the k_A values of the present study.

Parameters of tracer washout can be used for analytical solutions of multicompartmental models (23, 24). Because of the complexity of such calculations, inaccuracies in the washout curve parameters may result in errors that are difficult to estimate. Our approach to the evaluation of the fastest and largest exponential component defined by A_3 and k_3 appears more reliable than that of existing methods. Cold washing leads to underestimation of the k_3 whereas warm washing results in a substantial depletion of the fastest component even before starting the washout experiment and, therefore, A_3 will be underestimated. Analyses of the data by a serial and a parallel model (23, 24) demonstrate a large pool containing 89–96% of the total cellular Na^+ content independent of the model chosen. The remaining 4–11% Na^+ is distributed in two compartments of uneven size. The largest pool is likely to represent cytosolic Na^+.

Finally, our cold washing–warming–back extrapolation procedure can be applied to any kind of complex tracer washout with fast initial exchange rate. The first derivative of the washout curve obtained this way may be equated with the equilibrium exchange rate constant of the tracer. If multicompartmental analyses are to be undertaken, curve parameters at t_i can be used to calculate compartmental transport rate constants and compartment sizes.

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