J. Membrane Biol. 161, 197–205 (1998)

The Journal of
Membrane
Biology

Characterization of the Erythrocyte Sodium-Lithium Countertransporter: Limitations and Assumptions of Traditional and Kinetic Methodologies

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Received: 10 December 1996/Revised: 2 October 1997

Abstract. The present work examined the key elements featuring in the various methods used to characterize the erythrocyte sodium-lithium countertransport. Effects of medium composition on lithium efflux were investigated in 20 subjects. Mean lithium efflux (mmol Li/l RBC.h) into a 150 mM sodium medium was significantly higher than efflux into a revised sodium-rich medium (149 mM) containing 1 mM Mg (0.335 ± 0.100 *vs.* 0.298 ± 0.085 respectively; $P < 0.03$). Mean lithium efflux into sodium-free media where sodium had been entirely replaced by magnesium, was significantly lower than efflux into a choline-based medium containing only 1 mM magnesium (0.088 ± 0.027 *vs.* 0.109 ± 0.034 respectively; $P = 0.03$). Sodium-lithium countertransport *activity* and the transporter's kinetic profile were measured simultaneously in 35 subjects using traditional choline-based and kinetic methodologies. There was a significant correlation between countertransport activity and maximal rate of turnover (V_{max}) ($r = 0.62$; $P < 0.001$); V_{max} values were consistently greater than their corresponding countertransport activities ($P < 0.001$). On subdividing the subject group into tertiles based on the Michaelis-Menten constant (k_m) values (mM), <75, 75 − 150 and >150, the slopes of the regression lines for each group diminished progressively (0.64, 0.49 and 0.23 respectively), correlations within each group remained significant ($P < 0.001$, $P < 0.001$ and $P < 0.02$). No significant correlation was found between k_m values and countertransport activity ($r = 0.035$; $P =$ NS).

Increasing the number of points representing sodium concentrations within the range 0–150 mM, improved the confidence in the emerging estimates of V_{max} and k_m obtained by linear transformation. Comparison of kinetic data derived using four different analytical methods (two linear transformations, a nonlinear regression and a statistical method), showed no significant differences between the estimates yielded for either V_{max} ($P = 0.88$, NS) or k_m ($P = 0.92$, NS). This study has highlighted the critical roles of assay conditions and derivation techniques used when measuring sodium-lithium countertransport, emphasizing the need for standardization of the methodology.

Key words: Sodium — Lithium — Countertransport — Erythrocyte — Methodology

Introduction

Interest into ouabain-insensitive lithium transport across the erythrocyte membrane was stimulated when it was found to be elevated in hypertension and that the elevation was a genetically linked phenomenon [5]. This observation has since been confirmed on several occasions and interesting associations with disease states other than hypertension have also been reported [18, 20]. As the transport process has been explored however, it has become clear that the method of measurement is critical [13, 14, 18]. Conflicting results from differing laboratories have served to complicate the issue of what an elevated countertransport activity actually represents [6, 13, 21].

The past decade has seen the introduction of several new approaches in the study of sodium-lithium countertransport. Often, new techniques have been applied before they have been fully validated. As a result, methods reported in the literature have ended up as a collection of

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old and new techniques, the combination of which has in some cases been unique to particular groups of investigators [6, 14]. It is not clear whether the different methods employed by different laboratories are responsible for the mass of conflicting data within the literature surrounding the sodium-lithium countertransporter.

The present work has set out to examine critically the key elements of the various techniques currently used to characterize sodium-lithium countertransport. We have focused particularly on (i) variations in media composition, (ii) Michaelis-Menten analysis to compare activity measurements and (iii) definition of the optimum conditions for performing kinetic analysis. The aim has been to define those essential features which, if applied generally, would encourage standardization of the methodology and help generate a clearer definition of the behavior of this membrane transport system.

Materials and Methods

Subjects

A total of 48 (39 male and 9 female, aged 19–35) healthy normotensive white subjects were studied during the course of these investigations. Subjects selected for study were on no medication, had no family history of hypertension or diabetes and had normal glucose tolerance and renal function. The Chelsea and Westminster Hospital Ethics committee granted ethical approval for this study and all subjects gave their informed consent.

Collection and Lithium Loading of Red Blood Cells

Fasting venous blood samples were collected into lithium-heparin tubes. Erythrocytes were washed free of plasma with buffered *washing solution* (in mM): 150 choline chloride; 10 Tris-MOPS, pH 7.4 at 4°C. After washing, 5 ml packed cells were suspended in 25 ml of *loading solution* at 37°C for 3 hr (in mM): 150 LiCl; 10 glucose; 10 Tris-MOPS, pH 7.4 at 37°C. Following incubation the erythrocytes were washed five times with *ouabain washing solution* (in mM): 150 choline chloride; 0.1 ouabain; 10 Tris-MOPS, pH 7.4 at 4°C. Following the final wash, the supernatant was discarded, the cell pellet mixed thoroughly and the haematocrit determined. The intracellular sodium and lithium concentrations were analyzed to ensure that internal lithium levels were sufficient to saturate the internal binding sites of the countertransporter; adequate intracellular lithium concentrations were achieved in all cases; mean ± SD lithium concentration was 9.4 ± 3.4 mM. When ready the erythrocytes were kept on ice at 4°C until used (<2 min).

Influence of Medium Composition

The effects of medium composition on erythrocyte lithium efflux were investigated in 20 subjects. Lithium-loaded erythrocytes were prepared as described above. 200 μl aliquots of packed cells were added to duplicate samples of each of the five separate incubation media (2 ml). Each efflux medium contained glucose (10 mM), ouabain (0.1 mM) and Tris-MOPS (10 mM; pH 7.4 at 37°C), chloride made up the principal anion and each had an osmolality between 292 and 302 mosmol/kg. There were two sodium-rich media (mM) [(i) Na, 150, (ii) Na, 149; Mg, 1] and three sodium-free media [(iii) Mg, 75 (iv) choline 149; Mg, 1, and (v) choline, 149, Mg, 1, bumetanide, 0.02] (Table 1).

Erythrocytes were added to the various incubation media, mixed and then separated into three aliquots per medium. The aliquots were then incubated at 37°C and sampled at 20, 40, and 60 min. After centrifugation at 4°C, the supernatants were analyzed for lithium concentration. Lithium was determined by atomic absorption spectrophotometry [12]. The rate of lithium efflux was calculated using linear regression versus time of the lithium concentrations in each supernatant.

Traditional sodium-lithium countertransport activity was determined by relating lithium efflux into the specific all-sodium medium [(i) Na, 150] and deducting the equivalent flux into sodium-free medium [(iii) Mg, 75]. The *revised* method involved the use of an altered sodium-rich medium [(ii) Na, 149 plus Mg, 1] and deducting the equivalent flux into a sodium-free medium based on choline substitution [(iv) choline, 149 plus Mg, 1]. A further modification involved the addition of 0.02 mM bumetanide to the sodium-free medium [(v) choline, 149; Mg, 1; bumetanide, 0.02] in order to block the operation of the Na-K 2Cl cotransporter functioning in these experiments in the Li-K 2Cl mode. Comparisons of the lithium efflux rates between the respective all-sodium medium [(i) Na, 150] and the revised sodium-rich medium [(ii) Na, 149; Mg, 1] were made using a Students' paired *t*-test. Comparisons of flux rates into the three variants of sodium-free media [(iii) Mg, 75, (iv) choline 149; Mg, 1, and (v) choline, 149; Mg, 1; bumetanide, 0.02] were made using analysis of variance. Differences between the countertransport activity values obtained for the *traditional* and the *revised* methods were measured nonparametrically using a Wilcoxon test.

Comparison of Na^+-Li^+ Countertransport Activity with Maximal Rate of Turnover

Studies were undertaken in 35 subjects where sodium-lithium countertransport activity was measured simultaneously with determination of the kinetic profile of the transporter. Media used were all based on the *revised* constituents with choline serving as the sodium-substitute (*see above*). Sodium-lithium countertransport activity was determined by comparison of lithium efflux measured in media (ii) and (iv). Maximal rate of turnover (V_{max}) and the Michaelis-Menten constant (k_m) were determined following the construction of kinetic response curves using ten different external sodium concentrations over the range of 0–150 mM [0, 5, 10, 20, 40, 60, 80, 100, 125, and 150 mM]. Isotonicity was maintained in each case by inclusion of varying amounts of choline chloride. All efflux media contained (mM); Mg, 1, glucose, 10, ouabain, 0.1, and Tris-MOPS, 10 (pH 7.4 at 37°C).

Values for V_{max} and k_m were calculated via linear regression analysis using the Eadie-Hofstee method. The inter- and intraassay variations in values obtained for V_{max} and k_m were determined on fresh cells from ten subjects on three separate occasions over a period of six months. On each occasion measurements of the kinetics of sodium-lithium countertransport were made in triplicate. Results are expressed as means (SD) or medians with ranges. Differences between sodium-lithium countertransport activity and V_{max} were investigated using a Wilcoxon nonparametric test. Associations between countertransport activity, V_{max} and k_m were assessed by product-moment correlation.

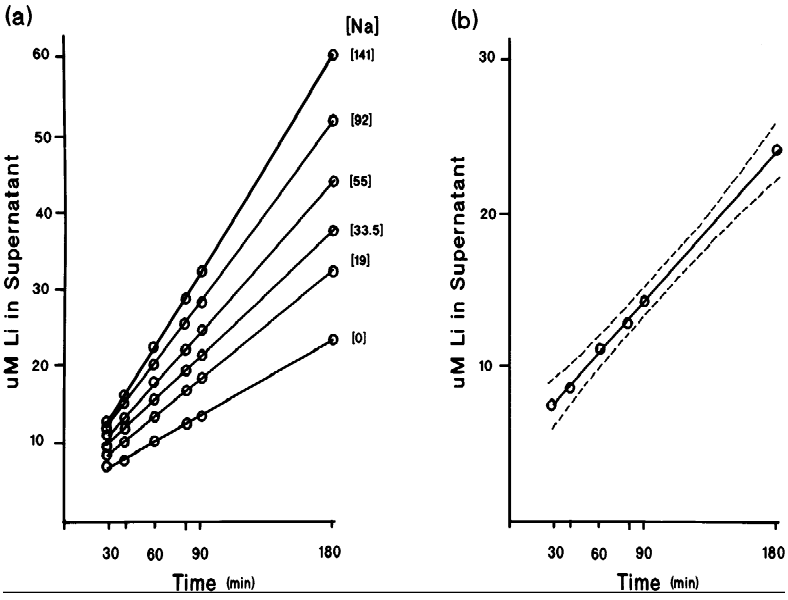

Fig. 1. (*a*) Graph showing measurements of lithium efflux at six different external sodium concentrations [0, 19, 33.5, 55, 92, and 141 mM Na]. (*b*) Graph demonstrating the 95% confidence intervals encompassing 0 mM sodium efflux rate curve. 95% confidence intervals were also determined at the 19 and 141 mM external sodium concentrations (*data not shown*). Data relate to subject DW.

Optimum Number of Data Points for Kinetic Analysis

The way in which the progress curves for kinetic analysis are constructed influences the precision of the emerging estimates of V_{max} and k_m. A balance has to be drawn between having a sufficient number of points and the overall practicability of the assay. Thus employing a five point assay, looking at lithium efflux at five external sodium concentrations, with duplicate samplings, to characterize the kinetics of the countertransporter in one individual yields thirty flux measurements over one hour; a seventeen point assay would yield 102 such measurements in the same time period.

In the section above, a ten point assay was employed over the substrate concentration range of 0–150 mM, the data were then analyzed using the Eadie-Hofstee transformation of the Michaelis-Menten equation. In the present set of experiments, 5, 10 and 17 point assays were undertaken concurrently in 35 subjects.

175 μl packed cells were incubated in external media (2 ml) of varying sodium concentration covering the range 0–150 mM, isotonicity being maintained by inclusion of appropriate amounts of choline chloride, solutions also contained (mM): 1 $MgCl_2$; 10 glucose; 0.1 ouabain; 10 Tris-MOPS, pH 7.4 at 37°C. Five-point assays employed media with the following sodium concentrations: 0, 20, 60, 100 and 150 mM, 10-point assays: 0, 10, 20, 40, 60, 80, 100, 110, 120, and 150 mM, and 17 point assays: 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150 mM.

Two forms of analyses were applied to each of these data sets and compared; first Eadie-Hofstee and second Hanes-Woolf. These transformations were obtained following rearrangement of the standard equation relating V_{max} and k_m to the observed flux rate and external sodium concentration of the incubation media giving:

(i) Eadie-Hofstee regression analysis:

$$\text{Flux rate plotted against } \frac{\text{Flux rate}}{[\text{Na}]_e}$$

where: V_{max} = intercept with the ordinate

k_m = ratio of the intercept of the line on the ordinate and the abscissa

(ii) Hanes-Woolf regression analysis:

$$\frac{[\text{Na}]_e}{\text{Flux rate}} \text{ plotted against } [\text{Na}]_e$$

where: V_{max} = the reciprocal of the slope

k_m = intercept with the abscissa

Computerized calculation of the percentage errors on the regression lines of each of these plots was undertaken using SPSS (SPSS, IL) to yield the mean value for all 35 individuals using both analytical transformations for 5, 10 and 17 point assays.

Influence of Experimental Error on Outcomes of Kinetic Analysis

In the previous section, linear transformations were employed to derive the kinetic constants V_{max} and k_m using different numbers of data points. In the present set of experiments, two alternative forms of kinetic analysis have been used in addition and compared with the two linear transformation methods. These are a nonlinear regression method using the Marquart algorithm [15, 16] in the form of a commercially available program for the IBM personal computer (Enzfitter, Biosoft, UK) which has been used successfully to analyze countertransport kinetics [6] and a statistical derivation of the kinetic parameters [8]. The statistical or Cornish-Bowden analysis was performed using the kinetic efflux data. Curves were derived connecting a point on the *x*-axis representing the substrate concentration with a point on the *y*-axis, which is a derived efflux rate for that substrate concentration. The coordinates of those points where the lines intersected gives estimates of both V_{max} (*y*-value) and k_m (*x*-value). The median of all the estimates obtained was taken to be the overall value of V_{max} and k_m.

In a group of 6 patients, erythrocyte lithium efflux rates were measured individually at six different external sodium concentrations (0, 19, 33.5, 55, 92, and 141 mM). The total length of incubation was

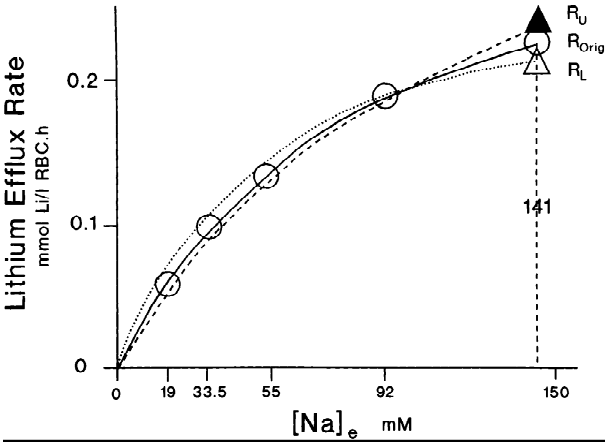

Fig. 2. Diagram showing impact of estimated error obtained at a single external sodium concentration (141 mM). A representative kinetic progress curve ($R_{Orig,m}$) has been constructed from the raw efflux rates measured at each of the six external sodium concentrations shown in Fig. 2 (subject DW). Two additional progress curves have been derived (R_U, – – – and R_L, • • • •), which have incorporated the upper (R_U, ▲) and lower (R_L, △) estimates of error obtained at 141 mM external sodium concentration.

180 min, with samples taken at 30, 40, 60, 80, 90, and 180 min time points [*see* Fig. 1]. The calculated rates of efflux at each of the six external sodium concentrations were then plotted against the respective sodium values to yield a single progress curve (R_{Orig}).

The standard errors and the respective 95% confidence intervals of the slopes of each of the three selected efflux curves were determined, covering the lower (0), mid- (19) and upper (141) ranges of external sodium concentrations (mM) (*see* Fig. 2).

By using these slopes and their confidence intervals, a pair of secondary efflux curves, encompassing the extreme ranges of error, were generated for the three external sodium concentrations. At each of these sodium concentrations (0, 19, and 141 mM Na), two additional sets of kinetic progress curves were constructed, based on (*a*) the upper limit (R_U) and (*b*) the lower limit (R_L) of the confidence interval of the efflux values (*see* Fig. 3).

The progress curve from the original efflux data, plus the three pairs of derived progress curves incorporating the estimated ranges of error, then underwent four different forms of kinetic analysis, linear transformations (Eadie-Hofstee and Hanes-Woolf), a nonlinear regression (Marquart) and a statistical manipulation (Cornish-Bowden), to yield estimates of V_{max} and k_m. For each of these estimates, the percentage variation from the values obtained using the original experimental efflux data, was calculated (Table 4).

Results

Influence of Media Composition

Lithium Efflux Data

Mean (SD) lithium efflux (mmol Li/l RBC.h) into traditional all sodium medium [(i) Na, 150] was significantly higher than the efflux into revised sodium-rich medium containing 1 mM Mg [(ii) Na, 149; Mg, 1] (Table 1, $P < 0.03$). Mean (SD) lithium efflux into traditional sodium-free media where sodium had been entirely replaced by magnesium [(iii) Mg, 75], was significantly lower than the efflux into a choline-based medium containing only 1 mM Mg [(iv) choline, 149; Mg, 1] ($P = 0.03$). Addition of 0.02 mM of bumetanide to the choline-1 mM Mg medium (*v*), was associated with significant lowering of lithium efflux to the same level as that seen in the all Mg medium. The lithium effluxes into these two respective media correlated significantly with one another ($n = 20$; $r = 0.58$; $P < 0.005$).

Sodium-Lithium Countertransport Activity

Activity values, calculated by deducting flux measurements in sodium-free media from sodium-rich media, showed a trend to higher values in experiments employing traditional media [media (i) and (iii)] when compared with responses obtained when choline was substituted for magnesium as the sodium-free cation or moiety [media (ii) and (iv)] (Table, 2). Activity values derived from these two sets of media showed a high degree of correlation ($n = 20$; $r = 0.76$; $P < 0.001$).

Comparison of Sodium-Lithium Countertransport Activity with Maximal Rate of Turnover and Michaelis-Menten Constants

Simultaneous measurement of sodium-lithium countertransport activity, V_{max} and k_m in 35 subjects, showed a non-normal distribution for the countertransport activity (kurtosis = −0.532, skew = 0.351) and k_m (kurtosis = 0.694, skew = 0.956) data, with median [range] values of 0.235 [0.124 − 0.365] mmol Li/l RBC.h and 110 [26 − 289] mM respectively. By contrast the distribution of V_{max} values was normal with a mean (SD) of 0.424 (0.155) mmol Li/l RBC.h. Values obtained for intra- and interassay variation in V_{max} were 5.9 ± 3.9 and 9.8 ± 6.5, respectively, while those for k_m were 9.5 ± 5.5 and 11.1 ± 5.8, respectively.

There was a significant correlation between sodium-lithium countertransport activities and V_{max} values ($r = 0.62$; $P < 0.001$); all data points fell to the right of the line of identity (Fig. 4), indicating that V_{max} values were consistently greater than their corresponding sodium-lithium countertransport activities ($P < 0.001$). Those subjects whose V_{max} values lay furthest away from the line of identity had higher k_m values. On subdividing the subject group into tertiles of k_m (mM), (*a*) <75, (*b*) 75–150 and (*c*) >150, the slopes of the regression lines

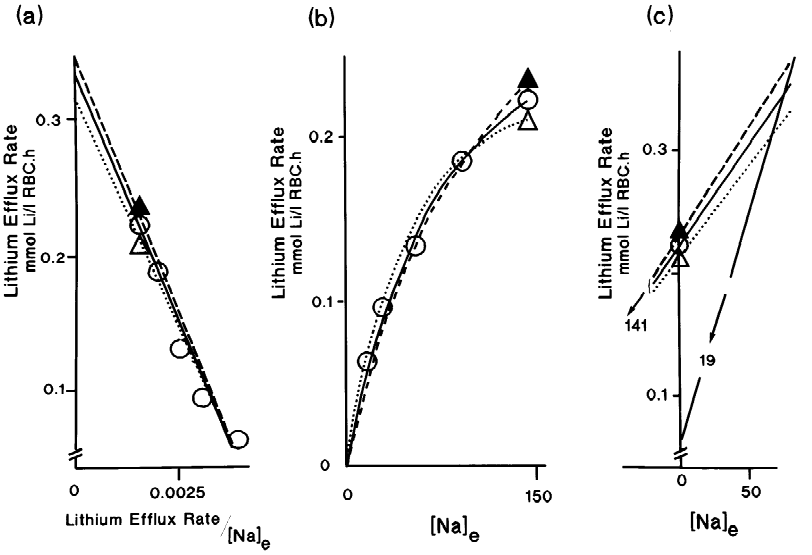

Fig. 3. Data from subject DW has been submitted in its final stages to alternative methods of kinetic analysis. The diagram shows (*a*) one of the two methods of linear transformation employed (Eadie-Hofstee) (*b*) nonlinear regression and (*c*) statistical manipulation (Cornish-Bowden). Data points for all substrate concentrations [19, 33.5, 55, 92, and 141 mM external sodium] are included in figures (*a*) and (*b*). Figure (c) focuses on data relating to one point [141 mM]. Cornish-Bowden manipulation shown in Figure (c) is determined by connecting a point on the *x*-axis that represents the substrate concentration with a point on the *y*-axis, which is a derived efflux rate for that substrate concentration. This line is then extrapolated beyond the *y*-axis. The coordinates of the point where that line intersects the extrapolated curves from other sodium concentrations gives an estimate of both V_{max} (*y* -value) and k_m (*x*-value). The median of all the estimates obtained gives the overall values of V_{max} and k_m. In this diagram, additional to the line representing the original data at Na = 141 mM, two further lines representing those derived using the upper (R_U, ▲) and the lower (R_L, △) estimates of error around this point are shown. The influence of the variations around the 141 mM Na efflux value can be clearly seen by noting the distinct intersections with the line that represents the original efflux data at Na = 19 mM. The coordinates of these separate intersections provide the distinct estimates of V_{max} and k_m.

Table 1. Lithium efflux rates into each incubation medium of different ionic composition

Medium	(*n*)	Ionic content [mM]	Mean ± SD Lithium efflux (mmol Li/I RBC.h)	Statistical significance	
Sodium-rich					
(i) Traditional	20	[Na, 150]	0.335 ± 0.100	$P < 0.03$	
(ii) Revised	20	[Na, 149; Mg, 1]	0.298 ± 0.085		
Sodium-free					
(iii) Traditional Magnesium*	20	[Mg, 75]	0.088 ± 0.027	$P < 0.03$	P = NS
(iv) Revised Choline	20	[choline, 149; Mg, 1]	0.109 ± 0.034	$P < 0.01$	
(v) Choline plus Bumetanide	20	[choline, 149; Mg, 1; bumetanide, 0.02]	0.090 ± 0.048		

All incubation media contained glucose (10 mM), ouabian (0.1 mM) and Tris-MOPS (10 mM; pH 7.4 at 37°C) and had osmolalities between 292 and 302 mosmol/kg. *The traditional magnesium medium also contained 85 mM sucrose.

for each group diminished progressively (0.64, 0.49 and 0.23 respectively), yet the correlations within each group remained significant ($P < 0.001$, $P < 0.001$ and $P < 0.02$ respectively) (Fig. 4). However, k_m values did not display a significant correlation with individual sodium-lithium countertransport activities ($r = 0.035$; P = NS).

Optimum Number of Data Points for Kinetic Analysis

Comparison of the three assay conditions (5, 10 and 17 point assays), showed that increasing the number of points representing sodium concentrations within the range 0–150 mM, improved the confidence in the emerg-

Table 2. Constituent cations of the efflux media used to determine sodium-lithium countertransport activity and the median acitvity values observed

Method	Ionic content [mM]	Median [range] sodium-lithium countertransport activity (mmol Li/l RBC.h)	
Traditional			
(i) Sodium-rich	[Na, 150]	0.246 [0.074 - 0.472]	$P = 0.06$
(iii) Sodium-free	[Mg, 75]		
Revised			
(ii) Sodium-rich	[Na, 149; Mg, 1]	0.189 [0.084 - 0.413]	
(iv) Sodium-free	[Choline, 149; Mg, 1]		

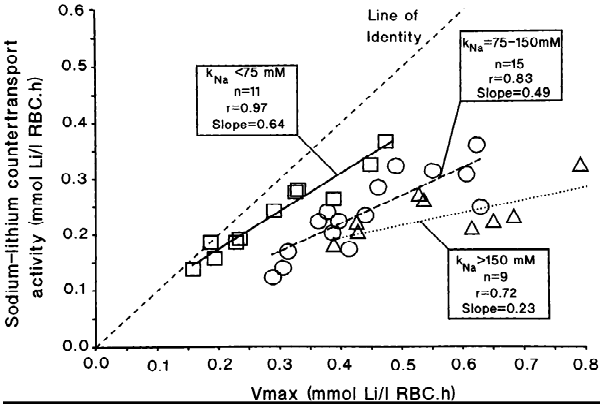

Fig. 4. Graph showing the relationship of activity to derived V_{max} of the countertransporter in 35 healthy subjects. The subjects have been divided into tertiles according to individual k_m values (<75 [□], 75–150 [○] and >150 [△]). The regression lines for *activity vs.* V_{max} are given for each tertile.

ing estimates of V_{max} and k_m by either transformation. The distribution of error around these estimates was lowest in the 17 point assay. With regard to V_{max}, both Eadie-Hofstee and Hanes-Woolf transformations yielded similar error values for each type of assay (Table 3). In the case of k_m however, there was a significant reduction in error in the Hanes-Woolf but not the Eadie-Hofstee transformation as the number of points employed increased from 5 to 17 (Table 3).

Influence of Experimental Error on Outcomes of Kinetic Analysis

The techniques of analysis applied to the data in these studies allowed estimates of error to be incorporated when the various types of transformation were undertaken. The results showed that the variance surrounding the original estimates of V_{max} and k_m (derived from R_{Orig}) was approximately twice as large for k_m as compared

Table 3. Decreasing errors with increasing number of observations used for estimation of V_{max} and k_m

Kinetic parameter	(*n*)	Trans-formation	Percentage error (Mean ± SD)		
			5 point	10 point	17 point
V_{max}	35	EH	13.5 ± 9.0	9.6 ± 7.7	5.7 ± 5.4*
	35	HW	15.1 ± 9.5	11.3 ± 7.5	5.1 ± 5.4*
k_m	35	EH	14.5 ± 6.1	12.4 ± 6.1	10.4 ± 5.0
	35	HW	12.5 ± 5.2	9.1 ± 4.1	5.0 ± 4.8*

EH = Eadie-Hofstee, HW = Hanes-Woolf
* < 0.05 - 5 point versus 17 point

Table 4. Mean estimates of variation (%) about the original estimates of V_{max} and k_m

Method of analysis	V_{max} (%)	k_m (%)
Eadie-Hofstee	5.8 ± 3.7	10.9 ± 7.4
Hanes-Woolf	6.5 ± 3.4	10.9 ± 5.9
Nonlinear regression	4.7 ± 3.0	9.6 ± 5.6
Cornish-Bowden	4.6 ± 2.5	9.5 ± 6.3
Mean	5.4 ± 1.0	10.2 ± 0.8

Data obtained using four different analytical methods in six subjects, the first two being linear transformations (Eadie-Hofstee and Hanes-Woolf) plus nonlinear regression and Cornish-Bowden analysis.

with V_{max} ($P < 0.001$), irrespective of the method of analysis of data used (Table 4).

Comparison of the data subjected to the two types of linear transformation analysis showed that Eadie-Hofstee and Hanes-Woolf analysis yielded similar values of mean (SD) V_{max} ($P = 0.28$; Students paired-*t*) and median [range] k_m values ($P = 0.15$; Wilcoxon) (Table 5). Collective comparison of the results yielded by all four analytical methods using ANOVA showed that there were no significant differences between the estimates yielded of V_{max} ($P = 0.88$; NS; ANOVA) and k_m ($P = 0.92$, NS; Kruskal-Wallis).

Table 5. Individual estimates of V_{max} and k_m for the six different subjects investigated using the original efflux data values

	Linear transformation			
Subject	Eadie-Hofstee		Hanes-Woolf	
	V_{max} (mmol Li/l RBC.h)	k_m (mM)	V_{max} (mmol Li/l RBC.h)	k_m (mM)
1. PW	0.337	116.1	0.361	129.3
2. MH	0.262	63.8	0.256	60.9
3. TI	0.459	88.9	0.468	92.0
4. DW	0.352	151.3	0.349	149.7
5. TT	0.349	87.6	0.363	93.6
6. DP	0.201	50.2	0.198	55.3
Mean ± SD	0.327	88.2*	0.333	92.8*
Median[range]*	± 0.088	[50.2 – 151.3]	± 0.094	[55.3 – 149.7]

Discussion

In the present study, lithium chloride was used to load the erythrocytes for efflux measurements. Lithium carbonate has also been used by others and tends to allow more rapid loading to occur. No significant difference is seen in efflux behavior whether chloride or carbonate salts are used [3, 11].

Our findings have shown that magnesium inhibits lithium efflux when used as a substitute for sodium in sodium-free media. The rates of lithium efflux into magnesium-based solutions were similar in magnitude and correlated with those into bumetanide-containing choline-based media. As bumetanide, at the concentration used, specifically inhibits sodium-potassium cotransport, these results confirm the earlier observation that magnesium reduces that component of lithium leak that is handled by the cotransport mechanism [19]. A small degree of inhibition of lithium efflux occurred with 1 mM magnesium in the sodium-rich media. This suggests that the component of efflux that is influenced by external magnesium is extremely sensitive to this ion. These particular experiments in sodium rich media did not incorporate the addition of bumetanide.

Our data imply that the predominant use of magnesium-based media in the sodium-lithium countertransport assay over the past fifteen years, 90% from 1980 to 1986 and 51% from 1986 to date, has led to a consistent overestimation of the activity of this membrane transporter [14]. Involvement of the sodium-potassium cotransporter, a transport system with its own inherent variation, may be partly responsible for the high degree of interindividual variability in countertransport activity reported within the literature [10]. Even where investigators are aware of the merits of using choline in their sodium-free media, problems may still arise. Thus for example, continued acceptance of the 0.400 mmol Li/l RBC.h ''cutoff'' value first proposed in 1980 as the upper limit of ''normal'' countertransport activity, may not be valid since the 0.400 mmol Li/l RBC.h value was derived originally from experiments using magnesium substitution [5]. It could also be argued that the critical impact of the sodium substituent would be lessened by routine inclusion of bumetanide as an inhibitor of cotransport, analogous to the use of ouabain in blocking sodium-potassium ATP-ase, yet this is not the accepted practice.

The assumption has frequently been made that when sodium-lithium countertransport activity has been determined, it equates with the maximum rate of turnover of the transporter. This presupposes that an external sodium concentration of 150 mM is sufficient to saturate the external binding sites and thus stimulate maximal turnover. The present work employed kinetic progress curves to characterize the countertransporter, as have been used in investigation of the sodium-hydrogen exchanger [7]. It showed that although there is a degree of similarity and a strong correlation between countertransport *activity* and V_{max}, they are not necessarily one and the same; V_{max} values were consistently greater than their corresponding sodium-lithium countertransport activities. Since the internal lithium concentrations were sufficient to ensure saturation of the internal lithium binding sites [4], it is unlikely that the discrepancy between activity and V_{max} was due to intracellular events. When our subject group was divided into tertiles according to individual k_m values, the regression lines for *activity vs.* V_{max} showed that the relationship between these two parameters is dependent on k_m [Fig. 4]. Viewed in this way, k_m, emerged as a critical, although not direct, determinant of measured activity; k_m can be taken to represent a function of the extent to which *activity* fails to represent true V_{max}. Although the difference between activity and V_{max} values was greatest at higher k_m values,

in contrast to what has been reported elsewhere [4], differences between countertransport activity and V_{max} were still present even at the lowest k_m values (<25 mM). Sodium-lithium countertransport activity must therefore be viewed not so much as a biochemical constant but more as a measure of lithium efflux determined under clearly defined conditions. It follows that measuring sodium-lithium countertransport activity without full characterization of the conditions of measurement is confusing and runs the danger of yielding data of limited credibility [1].

Work which has focused on the kinetic characteristics has started to throw light on the relationship between the membrane transporter and disease [14]. In the calculation of V_{max} and k_m, Michaelis-Menten kinetics were assumed and if measurements of the lithium efflux rates used to derive these parameters were made under ideal conditions, there would be no error. Only 2 points would be needed to define the straight line relationship obtained by derivation [17]. Under normal experimental conditions, however, more than two data points are needed to obtain estimates of confidence for V_{max} and k_m. In our study, we attempted to determine how many data points would supply a reasonable degree of confidence for these parameters. The two linear transformations of the Michaelis-Menten equation that were used (Eadie-Hofstee and Hanes-Woolf) both gave similar values for V_{max} and k_m; using both transformations the errors for the respective regression coefficients however, diminished as the number of data points employed increased. A ten point assay emerged as an optimum procedure that balanced practicability against generation of estimates of V_{max} and k_m with minimal error. In addition to linear transformations, it was also possible to subject data to nonlinear regression and statistical analysis. These two additional techniques were used to investigate the degree of robustness of the various analytical methods with respect to the influence of experimental error. Although there were differences between the estimates of V_{max} and k_m obtained using the four different methods of analysis, within the limits of the small number of subjects studied, the overall values for V_{max} and k_m did not differ significantly. Critical review of the results in six subjects using all four transformations (two linear, one nonlinear and a statistical method) showed no significant difference in the magnitude of the errors surrounding the estimates of V_{max} and k_m; though there was a consistent trend to lower error estimates with the statistical Cornish-Bowden method (Table 4).

One limiting factor with the present study could have been that only six efflux media of differing external sodium concentrations (0, 19, 33.5, 55, 92 and 141 mM) were used; increasing this number would be likely to increase the confidence of the emerging estimates of V_{max} and k_m for each of the four transformation techniques. With linear and nonlinear regression, increasing the number of data points to 10 would have resulted in a further 4 data sets being included in the final analysis. With the statistical method however, including four further external sodium concentrations, would have resulted in an extra 26 data sets being incorporated in the final analysis. Whether such data expansion would impact significantly on the relative robustness of this analytical approach compared to the other techniques, has yet to be tested.

The relative advantages of one method of deriving the kinetic constants over another have often been discussed within the literature [2, 8, 9, 15]. The main advantage of using either linear or nonlinear methods in place of statistical analysis appears to be that of convenience [9, 15]. Our experience with use of the statistical method suggests that it may minimize the deleterious effect of random errors and thus lead to more definitive estimates of V_{max} and k_m [8].

In conclusion, the present study has emphasized the critical role of the detailed conditions used in undertaking characterization of sodium-lithium countertransport. These ranged from media composition to the impact of changes upon other neighboring transport systems residing within the cell membrane. Kinetic characterization of the countertransporter offers greater insight into the biological relevance of this transport system. Of the various transformations of kinetic data, the statistical approach appears to offer advantages in diminishing the influence of errors on the derived estimates of V_{max} and k_m.

This research was supported in part by grants from the Joint Trustees of Westminster and Roehampton Hospitals, London and the Lant Trust for Medical Research.

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