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Relative magnitudes of the rate constants associated with monensin-mediated H^+, Na^+ and K^+ translocations across phospholipid vesicular membranes

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Abstract

Monensin (Mon)-mediated decay of the pH difference (ΔpH) across soyabean phospholipid vesicular membrane has been studied as a function of K^+ and Na^+ ion concentrations. In these experiments, the ΔpH was created using temperature jump, and ionic strength was regulated at 0.3 using CsCl. Rate constants associated with the translocation of Mon–H, Mon–K and Mon–Na have been estimated (without making any assumptions) from an analysis of the ΔpH decay data. These estimates contradict the claim made in the literature (E. Nachliel, Y. Finkelstein, M. Gutman, Biochim. Biophys. Acta, 1285 (1996) 131–145) that the translocation rate constants of the three above-mentioned species are significantly different. Our observations on the changes in ΔpH decay rate on adding carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) also suggest that the dominant barrier to the ΔpH decay process is not the 'polar region' of the membrane. Therefore, the differences in the electric dipole moments of Mon–H, Mon–K and Mon–Na are unlikely to cause large differences in their translocation rate constants. © 1998 Elsevier Science B.V.

Keywords: Monensin; Ion transport; Proton transport; Temperature jump

1. Introduction

The importance of monensin (Mon) as a tool for biochemical research has motivated several workers to study the mechanism of monensin-mediated H^+ and M^+ ($M^+ = Na^+$, K^+, Li^+) transport across phospholipid bilayer membrane using a variety of techniques [1–5]. The conclusions of one such recent work [5] appear controversial. Nachliel et al. [5] monitored the transient electrical currents across black lipid membranes after creating a 'proton pulse' on one side of the membrane by the photodissociation of H^+ from pyranine using a laser pulse. From an analysis of the data, they concluded that the rate constant associated with the translocation of Mon–H across the membrane ($= k_1$) is nearly 40-times that associated with Mon–K ($= k_2$) and nearly 5-times that associated with Mon–Na, even though all the three species are electrically neutral. In contrast with these conclusions, the ΔpH decay data [4] (ΔpH represents pH difference across membrane) had shown

Abbreviations: ΔpH, pH difference across vesicular membrane; SBPL, soyabean phospholipid; τ, ΔpH relaxation time; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ACES, *N*-(acetamido)-2-aminoethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; lip, lipid; Mon, monensin; Mon–M, M^+ bound monensin; Mon–H, H^+ bound monensin; $[Mon]_0$, monensin concentration in vesicle solutions; b_i, internal buffer capacity of vesicle solutions; *T*-jump, temperature jump

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that k_1 is not very much different from k_2. Nachliel et al. [5] have explained this discrepancy by implying that the analysis of the ΔpH decay data in Ref. [4] is based on two incorrect assumptions: (a) $k_1 = k_2$ and (b) the species Mon–H–M^+ is a 'dead-end product', not participating in the ΔpH decay. Nachliel et al. [5] have attributed the large differences in translocation rate constants of Mon–H, Mon–K and Mon–Na to differences in the electric dipole moments of the three species and the consequent differences in their interaction with the membrane bilayer [5]. Such a conclusion implies that (i) even for the electroneutral species the main barrier for the translocation is located in the 'polar region' of the membrane bilayer, and (ii) by modifying the 'polar region', the monensin-mediated ion-transport rates can be altered by large factors. If valid, these are important conclusions contributing to the understanding of the details of the ion-transport mechanism. However, they contradict earlier beliefs [2–4]. Therefore, these conclusions need confirmation from independent estimates of k_1 and k_2, determined using other techniques and without making unjustifiable assumptions. Eqs. (1)–(8) of Ref. [4] show that such estimates can be obtained from the dependence of ΔpH relaxation times, τ, on the alkali metal ion concentrations [Na^+] or [K^+] as shown in the following. Such experiments (unlike in the previous work [4]) require the ionic strength to be regulated to a constant value by adding metal ions which show negligible binding with monensin. In the present work, we have used Cs^+ ions for this purpose and have carried out experiments at 0.3 ionic strength.

2. Theory

The dependence of τ (= relaxation time associated with monensin mediated ΔpH decay) on [M^+] (= [K^+] or [Na^+]) can be written as follows, using Eq. (1) of Ref. [4].

$$\tau = F\left(A/[M^+] + B[M^+] + C\right) \quad (1)$$

F can be written as follows in terms of the lipid concentration (= [lip]) and concentration of monensin (= $[Mon]_0$) in vesicle solution volume.

$$F = (b_i/\ln 10)\left(1.05[lip]/[Mon]_0\right) \quad (2)$$

since monensin concentration in SBPL vesicular membrane = 0.95 $[Mon]_0$/[lip] M [4]. F varies with pH, since the internal buffer capacity b_i varies with pH (Eq. (2) of Ref. [4]).

A, B and C given by the following expressions are independent of [M^+].

$$A = (1/k_2)\{K_M + (K_M/K_H)[H^+]\} \quad (3)$$

$$B = (1/k_1)\{1/K_{HM} + (K_H/K_M)/[H^+]\} \quad (4)$$

$$C = (1/k_2)\{1 + (K_M/K_H)([H^+]/K_{HM})\} + (1/k_1)\{1 + K_H/[H^+]\} \quad (5)$$

where k_1 and k_2 are the rate constants associated with the translocations of Mon–H and Mon–M, respectively, across the vesicular membrane, and K_H, K_M and K_{HM} the apparent dissociation constants [4] associated with the following equilibria:

$$\text{Mon-H} \rightleftharpoons \text{Mon}^- + H^+,\ K_H$$

$$\text{Mon-M} \rightleftharpoons \text{Mon}^- + M^+,\ K_M$$

$$\text{Mon-H-M}^+ \rightleftharpoons \text{Mon-H} + M^+,\ K_{HM} \quad (6)$$

Eqs. (3)–(5) show that the pH dependences of A and C can be used to determine K_H, k_1, k_2, K_M and K_{HM} without making any assumptions about the relative magnitudes of k_1 and k_2 or about the dissociation constants. If K_H is known, the other above-mentioned parameters can also be determined from the pH dependences of B and C.

In principle, the species Mon–H–M^+ can transport H^+ and M^+ by translocation with rate constant k_i. The translocation of this species can be included in the rate equations by replacing k_1 by k_1^* in Eqs. (3)–(5) where,

$$k_1^* = k_1\{1 + (k_i/k_1)([M^+]/K_{HM})\} \quad (7)$$

If the second term of Eq. (7) is prominent, it will not be possible to fit the τ data to Eq. (1) with A, B and C independent of [M^+]. The τ data given below show that the second term of Eq. (7) is not prominent. Therefore, $k_i \ll k_1,\ k_2$. Such a conclusion has the support of Parsegian's calculation [6], which shows that the energy required for the translocation of charged species across the membrane is much higher than that for the translocation of electroneutral species.

3. Materials and methods

Soyabean phospholipid (SBPL) vesicles with 2 mM pyranine inside, buffer and salt concentration conditions as given in the figure legends were prepared from asolectin (Sigma) by the procedure similar to that used in our earlier works [7]. HCl was used to adjust the pH of *N*-(acetamido)-2-aminoethanesulfonic acid (ACES) + tris(hydroxymethyl)aminomethane (TRIS) buffer mixtures used in the experiments. In the present experiments, the total alkali-metal-ion concentration ($[K^+] + [Cs^+]$ or $[Na^+] + [Cs^+]$) was maintained at 0.3 M by adding appropriate amounts of CsCl (obtained from SISCO). Microlitre amounts of 3 mM monensin (Sigma) in ethanol and 10 mM or 40 mM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) in ethanol were added to vesicle solutions before the temperature jump (*T*-jump) experiments at 24°C. The details about the ΔpH decay experiments and the measurements of relaxation times τ have been given in our earlier works [4,7].

4. Results

For the ΔpH decay in liposomes, it is necessary to abolish the electric potential across the membrane generated by the H^+ transport [8]. In Mon-mediated ΔpH decay, this is achieved by the transport of a suitable alkali metal ion (such as K^+ or Na^+) by Mon. Representative experiments showed that, in the absence of K^+, Na^+ or Li^+ in SBPL vesicle solutions, ΔpH relaxation could not be observed within the upper time limit (< 10 s) of our instrument, even on adding Cs^+ ions up to 0.3 M. Such an observation implies negligible compensating charge flux because of negligible binding of Cs^+ ions to Mon. Therefore, we have used Cs^+ ions to regulate the ionic strength in our experiments on the dependence of τ on $[K^+]$ or $[Na^+]$.

4.1. Dependence of τ on $[K^+]$ and $[Na^+]$

In the experiments, where K^+ transport provided the compensating charge flux for ΔpH decay, the magnitude of τ/F (Eq. (1)) was dominantly determined by $(A/[K^+] + C)$ since the translocation of Mon–K dominantly limited the rate of ΔpH decay [4]. Fig. 1(a) shows representative ΔpH relaxation traces obtained under such conditions for two different values of $[K^+]$ (but keeping $[K^+] + [Cs^+] = 0.3$ M) at pH ~7.2. On the other hand, when the translocation of Mon–H dominantly limited the rate of ΔpH decay, as in the experiments with Na^+ [4], τ/F was dominantly determined by $(B[Na^+] + C)$. Increase in τ with increase in $[Na^+]$ can be seen in the representative ΔpH relaxation traces obtained at pH ~7.2 (Fig. 1(b)).

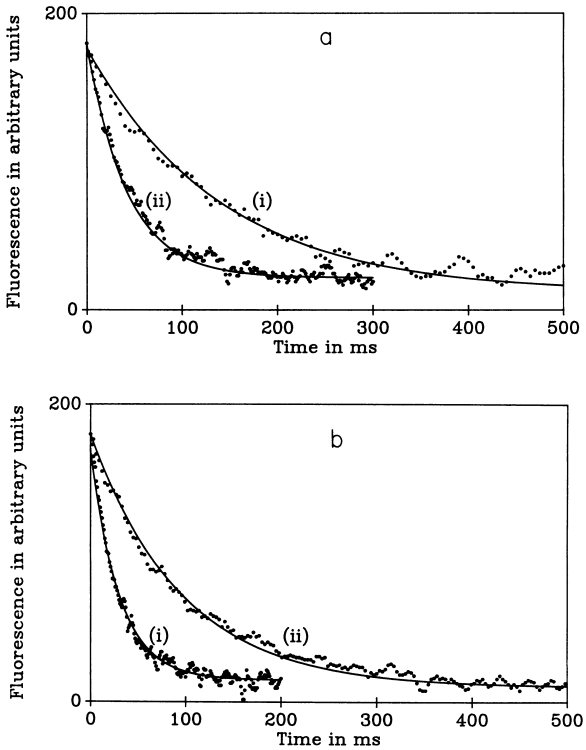

Fig. 1. Monensin-mediated ΔpH relaxation traces observed in the experiments using (a) (i) $[K^+] = 18$ mM, $[Cs^+] = 282$ mM and (ii) $[K^+] = 198$ mM, $[Cs^+] = 102$ mM; and (b) (i) $[Na^+] = 48$ mM, $[Cs^+] = 252$ mM and (ii) $[Na^+] = 220$ mM, $[Cs^+] = 80$ mM. Other conditions were, 0.25 M ACES + 0.25 mM TRIS buffer mixture inside and 10 mM ACES + 15 mM TRIS buffer mixture outside vesicles at pH 7.2, $[lip] = 4.7$ mM and $[Mon]_0 = 2.4\,\mu$M. Solid lines are simulated exponentials with time constants τ as (i) 140 ms, (ii) 42 ms in (a) and (i) 30 ms, (ii) 95 ms in (b).

The variations of τ with $1/[K^+]$ and $[Na^+]$ observed in a set of experiments in the pH range 6.2–7.7 are shown in Fig. 2(a–f) and Fig. 3(a–f), respectively. The slopes of the linear regions of these plots can be identified with *FA* and *FB* (of Eq. (1)), respectively. The intercepts obtained from an extrapolation of the linear regions of Figs. 2 and 3 can be

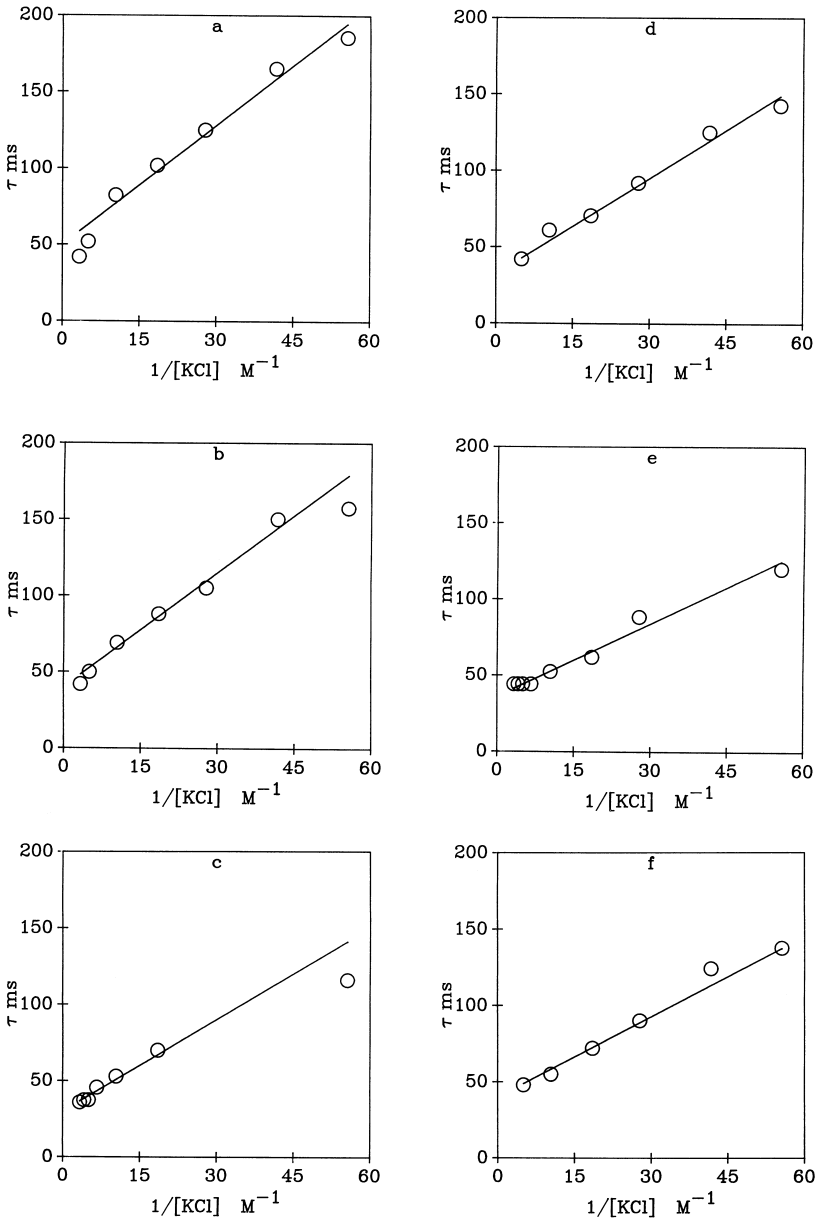

Fig. 2. The dependence of τ on $1/[K^+]$ observed in SBPL vesicles at pH (a) 6.2, (b) 6.5, (c) 7.0, (d) 7.2, (e) 7.5 and (f) 7.7, keeping [KCl] + [CsCl] = 0.3 M. Other experimental conditions were, [lip] = 5 mM, $[Mon]_0 = 2.5\,\mu M$, inside buffer 0.25 mM ACES + 0.25 mM TRIS and outside buffer 10 mM ACES + 15 mM TRIS. The slopes *AF* in units of $M \times 10^{-3}$ s, intercepts *CF* in units of 10^{-3} s (estimated from the linear regions of plots) and *F* (calculated using Eq. (2)) are (a) 2.6, 50, 11.1; (b) 2.5, 40, 17.2; (c) 2.0, 30, 26.8; (d) 2.1, 32, 29.5; (e) 1.6, 36, 31.2; and (f) 1.75, 40, 30.8.

identified with *FC*. Since *F* can be calculated from a knowledge of experimental conditions (Eq. (2)), Figs. 2 and 3 can be used to obtain estimates of *A*, *B* and *C* as functions of pH. These estimates (Figs. 4 and 5) show the theoretically predicted pH dependences (Eqs. (3)–(5)):

Fig. 4(a) shows that *A* increases linearly with $[H^+]$:

$$A = a[H^+] + d_1 \quad (8)$$

Identifying Eq. (8) with Eq. (3), we can write:

$$a = (1/k_2)(K_M/K_H);\ d_1 = (K_M/k_2) \quad (9)$$

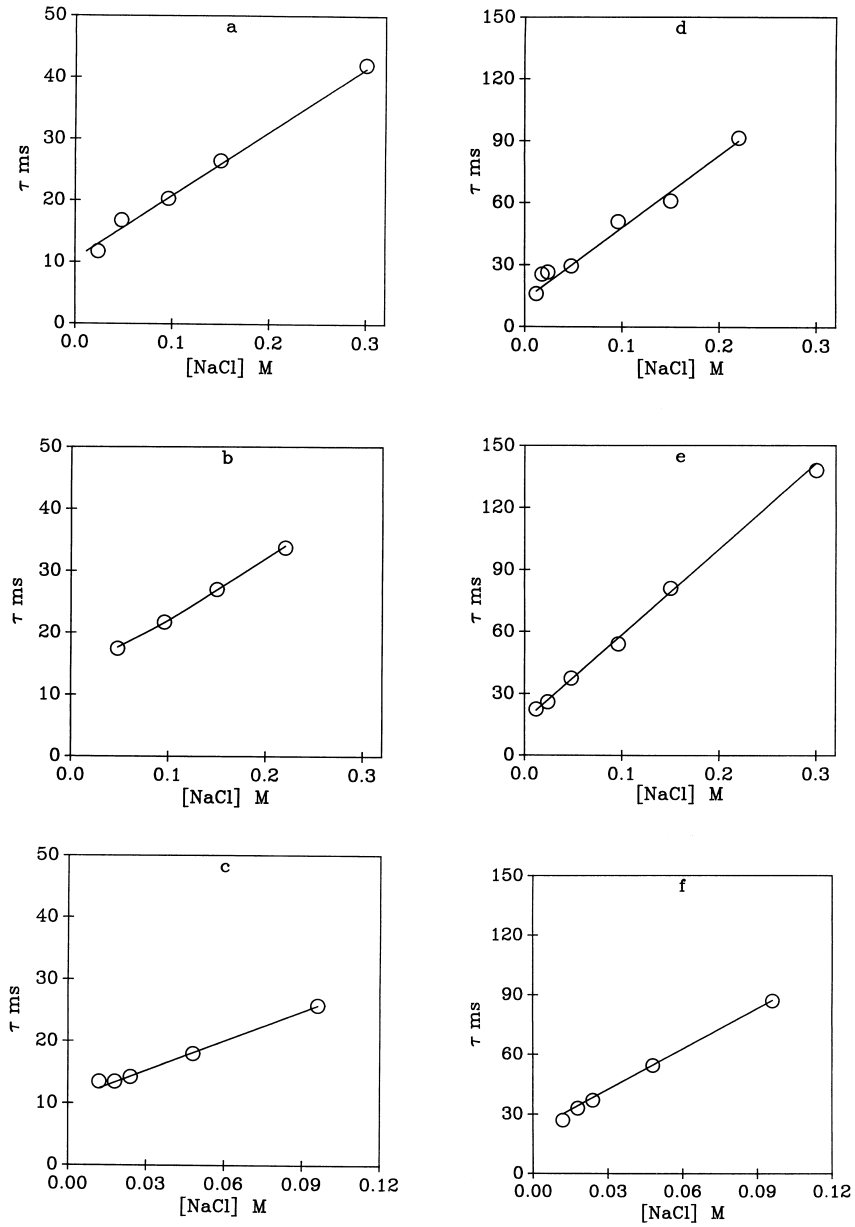

Fig. 3. The dependence of τ on $[Na^+]$ observed in SBPL vesicles at pH (a) 6.5, (b) 6.72, (c) 7.0, (d) 7.22, (e) 7.48, and (f) 7.65, keeping [NaCl] + [CsCl] = 0.3 M. Other experimental conditions were, [lip] = 5 mM, $[Mon]_0 = 2.5\,\mu M$, inside buffer 0.25 mM ACES + 0.25 mM TRIS and outside buffer 10 mM ACES + 15 mM TRIS. The slopes *BF* in units of $M^{-1} \times 10^{-3}$ s, intercepts *CF* in units of 10^{-3} s (estimated from the linear regions of plots) and *F* (calculated using Eq. (2)) are (a) 103, 10.5, 17.2; (b) 105, 10.5, 21.9; (c) 159, 10.5, 26.8; (d) 350, 13, 29.5; (e) 415, 17, 31.2; and (f) 680, 22, 31.1.

Fig. 5(a) shows that B increases linearly with $1/[H^+]$:

$$B = b/[H^+] + d_2 \quad (10)$$

Comparing Eq. (10) with Eq. (4), we can write:

$$b = (1/k_1)(K_H/K_M);\ d_2 = (1/k_1)(1/K_{HM}) \quad (11)$$

Eq. (5) shows that C should increase linearly with a pH-dependent function $f(H^+)$.

$$C = f(H^+) + r \quad (12)$$

$$f(H^+) = p([H^+]/K_H) + q(K_H/[H^+]) \quad (13)$$

$$p = (1/k_2)(K_M/K_{HM});\ q = 1/k_1;\ r = 1/k_1 + 1/k_2 \quad (14)$$

Eqs. (12) and (13) show that C is a linear function of the two variables $[H^+]/K_H$ and $K_H/[H^+]$. Therefore, the parameters p, q and r occurring in these equations can be determined by the general linear least-squares fit procedure [9]. Fig. 4(b) and Fig. 5(b) show that the p and q thus determined (and given in Table 1) make the C against $p([H^+]/K_H) + q(K_H/[H^+])$ plots linear as expected.

From a and d_1 an estimate of K_H can be obtained using Eq. (9). This estimate can be used along with either a, d_1, p, q, r or b, d_2, p, q, r to get estimates of rate constants and dissociation constants occurring in Eqs. (3)–(5), without making any assumption (see footnote of Table 1). Such estimates and the parameters, determined from one set of τ data and used in the calculations of solid lines of Figs. 2–5, are given in Table 1.

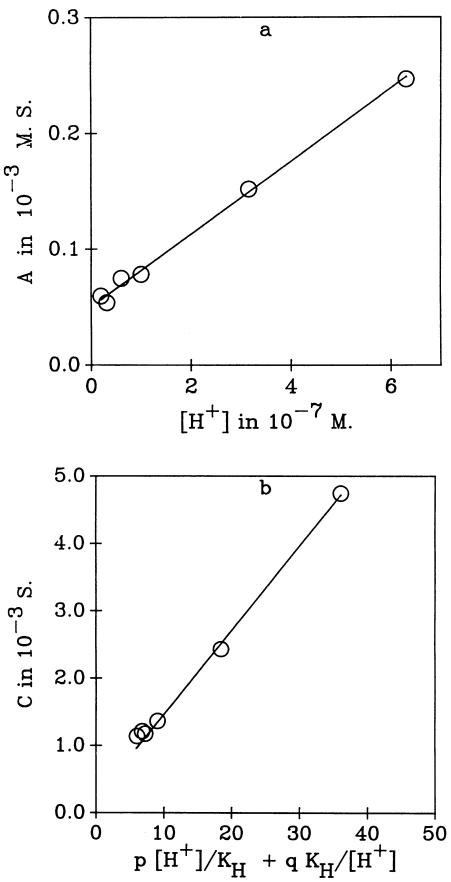

Fig. 4. Linear dependences of the parameters (a) A on $[H^+]$ and (b) C on $p[H^+] + q/[H^+]$ (in units where $q = 1$). The parameters p and q which make the plot (b) linear were determined as described in the text (Eqs. (12)–(14)). A and C used in these plots were estimated from the τ data given in Fig. 2. The solid lines were calculated using Eqs. (8) and (12).

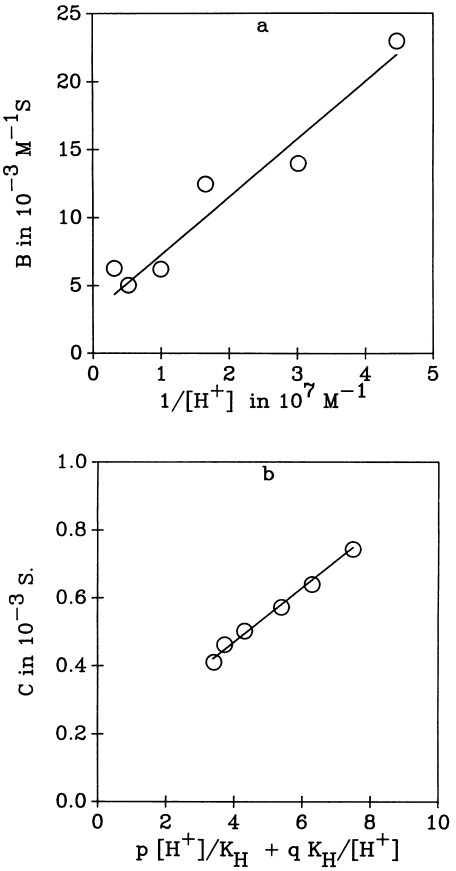

Fig. 5. Linear dependences of the parameters (a) B on $1/[H^+]$ and (b) C on $p[H^+] + q/[H^+]$ (in units where $q = 1$). The parameters p and q which make the plot (b) linear were estimated as described in the text (Eqs. (12)–(14)). B and C used in these plots were estimated from the τ data given in Fig. 3. The solid lines were calculated using Eqs. (10) and (12).

4.2. Changes in τ on adding carbonyl cyanide m-chlorophenylhydrazone (CCCP) to vesicle solutions

CCCP added to vesicle solutions is mainly partitioned to the membrane phase and can affect the magnitude of τ by

- contributing to the internal buffer capacity b_i,
- disturbing the membrane order [7], and
- participating in the proton transport cycle [10].

Even though CCCP is a potent proton carrier, for it to

Table 1
Parameters determined from the analysis of Figs. 4 and 5. Dissociation and rate constants estimated using the parameters are given as footnote

Parameter	Metal-ion transported by monensin	
	K^+	Na^+
a	3.15×10^{2} s	
d_1	5×10^{-5} M s	
b		4.3×10^{-10} s
d_2		3×10^{-3} M^{-1} s
p	$(1.05\pm0.05)\times10^{-3}$ s	$(2.1\pm0.15)\times10^{-4}$ s
q	$(1.15\pm0.2)\times10^{-4}$ s	$(7.5\pm0.5)\times10^{-5}$ s
r	$(3\pm1)\times10^{-4}$ s	$(1.5\pm0.2)\times10^{-4}$ s

From the parameters for K^+: $pK_H = 6.8$ ($K_H = d_1/a$); $k_1 = (9\pm2)\times10^3$ s^{-1} ($=1/q$); $k_2 = (7\pm3.8)\times10^3$ s^{-1} (using $1/k_2 = (r - 1/k_1)$); $K_M = 0.35$ M (using $K_M = ak_2K_H$); $K_{HM} = 0.05$ M (using $K_{HM} = K_M/(p\ k_2)$).
From the parameters for Na^+: $k_1 = (13.5\pm1)\times10^3$ s^{-1} ($=1/q$); $k_2 = (14.5\pm3.5)\times10^3$ s^{-1} (using $1/k_2 = (r - 1/k_1)$); $K_M = 0.027$ M (using $K_M = K_H/(bk_1)$); $K_{HM} = 0.025$ M (using $K_{HM} = 1/(d_2\ k_1)$).

contribute to ΔpH decay in liposomes there must be back transport of $CCCP^-$ and an efficient mechanism for associated compensating charge flux of alkali metal ions. Such a mechanism is absent in our system. Also the translocation rate constants of ionic species, such as that of $CCCP^-$, are small compared to those of Mon–M or Mon–H (see Ref. [10]). Because of these two factors, CCCP makes negligible contributions to the ΔpH decay in our experiments. Since the pK of CCCP in the membrane is $\sim$6.5 [7], at pH $\sim$7.5 it will be mainly in the anionic form, $CCCP^-$, and can be expected to be preferentially localised in, and disturb the 'polar region' of the membrane. On decreasing the pH, the concentration of the electrically neutral form CCCPH (which can disturb the non-polar region also) increases at the expense of $CCCP^-$. Thus, if the main 'barrier' to the transport of Mon–H and Mon–M is located in the 'polar region' of the bilayer, τ/b_i is expected to change by a larger factor on adding a given amount of CCCP under higher pH conditions. On the other hand, if this 'barrier' is in the 'non-polar region', τ/b_i is expected to change by a larger factor at lower pH conditions.

In representative experiments on 5 mM SBPL vesicle solutions containing 2 μM monensin and $[K^+]$ = 100 mM, when the concentration of CCCP in vesicle solutions $[CCCP]_0$ was increased from 0 to 270 μM, τ increased from 50 to 80 ms at pH 7.5. In a similar experiment with $[Na^+] = 48$ mM, τ changed from 40 to 50 ms. Therefore, since b_i increases from 3.4×10^{-2} to 4.7×10^{-2} M at pH = 7.5 for such an addition of CCCP, we can say that τ/b_i changes negligibly by the presence of $CCCP^-$ in the 'polar region' of the membrane. With similar additions of CCCP at pH 6.8, even though b_i changed from 2.5×10^{-2} to 7×10^{-2} M, τ changed from 57 to 72 ms in the experiments with 100 mM KCl, and from 18 to 25 ms in the experiments with 48 mM NaCl. This corresponds to a decrease of τ/b_i by a factor of 2, presumably because of the presence of CCCPH at sufficiently large concentration in the 'non-polar region' of the membrane at this pH. Our earlier data [7] (obtained upto pH $\sim$6.2) had also shown such a trend. In view of the arguments given here, we can conclude that the observed changes in τ on adding CCCP to vesicle solutions favour the hypothesis that the main barrier to the translocation of Mon–H and Mon–M is in the 'non-polar region'.

5. Discussion

5.1. Translocation rate and dissociation constants

Eqs. (12)–(14) predict the following: p estimated from the analysis of data should depend on the choice of metal ion since it depends on the metal-ion dissociation constants. Furthermore, the estimate of q depends on k_1 associated with Mon–H and, hence, should not depend on the metal ion chosen for providing the compensating flux. The p and q (Table 1) estimated from the set of data given in this paper show good agreement with such a prediction (within the limits of error attributable to small differences in vesicle preparations), thereby increasing the confidence in our analysis. If the estimates of translocation rate constants given by Nachliel et al. [5] are correct then r $(= 1/k_1 + 1/k_2)$ should increase by a factor of $\sim$10 when the metal ion is changed from Na^+ to K^+. This is not observed (see Table 1).

At first sight, our estimates of metal-ion dissociation constants appear to be orders of magnitude larger than those determined in solutions [11]. This is because our estimates correspond to apparent dissociation constants determined using the concentrations of

ions in the aqueous medium and the monensin concentration in the membrane. The determinations in solutions were by using ion and monensin concentrations in the same phase.

The apparent dissociation and translocation rate constants detemined in the present work (Table 1) are close to those determined earlier [4] and are different from the estimates given by Nachliel et al. [5]. For example, our estimates of K_M are nearly 10-times more than those given in Ref. [5]. Most importantly, the translocation rate constants k_1 associated with Mon–H and k_2 associated with both, Mon–Na and Mon–K obtained in the present work (without assuming $k_1 = k_2$) have similar magnitudes. In our earlier work, we had argued [4] that the observed τ data are not consistent with significantly differring values for k_1 and k_2. The present work confirms such a statement and contradicts the conclusions given in Ref. [5].

Our data has also shown that the translocation of the species Mon–H–M^+ is not a dominant process for the rate limiting steps of H^+/M^+ translocations responsible for the ΔpH decay. However, it is not possible from our data to say whether the species Mon–H–M^+ is involved in the faster step of H^+/M^+ exchange at the aqueous medium–membrane interface.

5.2. Cation specificity

The cation specificity inferred from our estimates of K_M (Table 1) are consistent with those reported from other types of studies [11]. The cation specificity can be understood in terms of a steric factor in the conformation of deprotonated monensin, which is more favourable to the binding of Na^+ ions that are smaller in size compared to K^+. Such a hypothesis predicts the binding constants for Cs^+ ions to be smaller than those for K^+ ions. Our observations mentioned in Section 4 are consistent with this prediction. Furthermore, protonation of monensin could abolish the steric factor responsible for the cation specificity, by changing the conformation of monensin. Our estimates of K_{HM} are consistent with this expectation. The relative magnitudes of K_M and K_{HM} for a given metal ion are expected to be determined by a combination of steric factors (which may be unfavourable to the binding of a metal ion to monensin) and electrostatic interactions. Presumably, our estimates reflect the importance of steric factors in the metal-ion binding to monensin.

5.3. Reliability of our estimates

The parameters given in Table 1 have been determined with the help of equations given above and using the data given in Figs. 2 and 3. In the linear plots of Figs. 2 and 3, the magnitude of variation of τ used in the determination of slopes is comparable to the corresponding intercepts on the τ-axis. Therefore, the errors in the estimates of A and B determined from the slopes and C determined from the intercepts are of similar magnitude. We can also see from the data that these estimates do not have large uncertainties. The expected dependence of these parameters on pH seen in Figs. 4 and 5 adds to the confidence in our estimates. The estimates of errors in translocation rate constants k_1 and k_2 are given in Table 1 along with the errors in p, q and r. Because of the compounding of errors, the errors in the estimates of K_{HM} are larger. Differences in vesicle preparations also contribute to the errors in the estimates of rate constants, since the expressions used by us give the translocation rate constants only when SBPL vesicle sizes are close to those assumed in Ref. [7] (Eq. (A-11)). The difference in the estimates of k_1 from vesicles containing K^+ and vesicles containing Na^+ could be attributed to differences in vesicle preparations.

5.4. Location of the dominant barrier to the translocations

The large differences in the translocation rate constants of the membrane permeable species Mon–H, Mon–Na and Mon–K inferred in Ref. [5] have been attributed to the differences in their electric dipole moments and the consequent differences in their interaction with the membrane [5]. Such an explanation implies that the main barrier to their translocation is located in the ‘polar regions’ of the bilayer, where we expect larger interaction between the electric dipole moments of the membrane permeable species and the membrane. Our identification of the ‘nonpolar region’ as the main barrier region from the observed changes in τ on adding CCCP is contrary to such an inference.

Independent evidence for the aforementioned conclusion comes from the work of Riddell and Armugam [12] on monensin-mediated Li^+ ion transport across phospholipid bilayer membranes. Using the nuclear-magnetisation transfer technique, they found that changing the nature of the 'polar region' (by changing the constituents of the lipid) does not change the rates of monensin-mediated metal-ion transport by large amounts (see Fig. 4 of Ref. [12]). This behaviour is similar to that observed on τ by us on adding $CCCP^-$, in our attempt to alter the 'polar region' of the membrane (described here).

5.5. Possible sources of errors in the analysis of electrical transients

As shown here, it is not possible to reconcile the conclusions of Ref. [5] with those inferred from the ΔpH decay studies (Ref. [4] and present work). We have discussed below, three possible sources of error in the analysis of electrical transients observed following a 'proton pulse' [5,13] which could have caused such differences.'

1. In the experiments of Nachliel et al. [5,13], ΔpH is produced by photodissociation of the protons from pyranine on one side ('1') of the membrane. Thus, in these experiments (at the temperature, the pressure and the time scale of observation) the concentrations of protonated and deprotonated species deviate from equilibrium even within side '1'. Therefore, two types of processes contribute to the decay of proton pulse generated in these experiments: (i) The fast proton recombination reactions restoring the equilibrium within '1' and (ii) the slower monensin-mediated translocations across the membrane bringing about an equilibrium between side '1' and side '2'. Since the former process is very much faster than the latter, the information content about the 'translocation process' in the amplitude of the proton pulse decay is very small. This could lead to larger errors in the estimates of translocation rate constants even when the errors in the parameters associated with the faster processes are small. In the *T*-jump experiments, the ΔpH is created by the fast proton exchange reaction equilibria at the 'new temperature' on both the sides '1' and '2' of the membrane [14]. Since the 'new temperature' can be taken to be constant during the short period of observation, the decay of ΔpH can be considered to be only by the process (ii). Thus, the translocations across the membrane constitute the information content of the *T*-jump relaxation traces. The above-mentioned difference is also responsible for the differences in the time scales used in the two experiments.
2. In the figures of Ref. [5], the agreement between the computed and observed electrical transients is poor at longer times after the proton pulse. This could contribute to the uncertainty in the estimates of rate constants. Base line drifts of the amplifier outputs also contribute to the errors in the analysis of transients. For example, the computed transients corresponding to different estimates of translocation rate constants shown in Fig. 4(E and F) of Ref. [5] can be made to agree with the observed transients by suitably multiplying the amplitudes and adjusting the base line.
3. It is possible that some of the assumptions used in the analysis of data in Refs. [5,13] are not strictly valid. For example, the analysis based on Scheme 2 of Ref. [5] would be valid only for a single-membrane capacitor with electrically conducting membrane surfaces. A rigorous treatment would require us to consider the membrane capacitor as made up of several tiny capacitors in parallel, some of them having monensin and others not having monensin in the time scale of observation ($< 200\ \mu s$). We may note that the electrical resistance between these tiny capacitors is non-negligible since the surface concentration of monensin species is small and the lateral diffusions of monensin and electrically charged species is relatively slow. In this situation, the observed transients can be expected to have a component from the tiny membrane capacitors not having monensin (similar to that shown in the inset of Fig. 1 of Ref. [5]) in addition to that predicted by Scheme 2. Furthermore, the Scheme 2, given in Ref. [5], does not include all the relevant reactions, such as that between Mon–H–M^+ and the deprotonated pyranine. Also, the pK of pyranine used in Ref. [5] (~ 7.70) differs from that determined by us and that given in the literature by others (~ 7.22) [15]. (Ground state pK rather than the excited state pK is relevant when discussing proton-exchange reac-

tions after the laser induced proton pulse.) In addition, the possibility of contributions to current transients from motions of charged species in the membrane under the action of membrane potential (generated following a proton pulse), are not included in the analysis given in Ref. [5].

References

[1] R. Sandeaux, J. Sandeaux, G. Gavach, B. Brun, Biochim. Biophys. Acta 684 (1982) 127–132.
[2] F.G. Riddell, M.K. Hayer, Biochim. Biophys. Acta 817 (1985) 313–317.
[3] F.G. Riddell, S. Armugam, B.G. Cox, Biochim. Biophys. Acta 944 (1988) 279–284.
[4] B.S. Prabhananda, M.H. Kombrabail, Biochim. Biophys. Acta 1106 (1992) 171–177.
[5] E. Nachliel, Y. Finkelstein, M. Gutman, Biochim. Biophys. Acta 1285 (1996) 131–145.
[6] A. Parsegian, Nature 221 (1969) 844–846.
[7] B.S. Prabhananda, M.H. Kombrabail, Biochim. Biophys. Acta 1235 (1995) 323–335.
[8] P.J.F. Henderson, J.D. McGivan, J.B. Chappell, Biochem. J. 111 (1969) 521–535.
[9] W.H. Press, S.A. Teukolsky, W.T. Vetterling, B.P. Flannery, Numerical Recipes in Fortran, For Cambridge University Press Foundation Books, New Delhi, 1996, pp. 665–675.
[10] B.S. Prabhananda, M.H. Kombrabail, Biochim. Biophys. Acta 1323 (1997) 137–144.
[11] W.K. Lutz, H.K. Wipf, W. Simon, Helv. Chim. Acta 53 (1970) 1741–1746.
[12] F.G. Riddell, S. Armugam, Biochim. Biophys. Acta 945 (1988) 65–72.
[13] M. Gutman, E. Nachliel, Electrochim. Acta 34 (1989) 1801–1805.
[14] B.S. Prabhananda, M.M. Ugrankar, Biochim. Biophys. Acta 1070 (1991) 481–491.
[15] K. Kano, J.H. Fendler, Biochim. Biophys. Acta 509 (1978) 289–299.