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H^+, K^+, and Na^+ transport across phospholipid vesicular membrane by the combined action of proton uncoupler 2,4-dinitrophenol and valinomycin

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Abstract

The decay of the pH difference (ΔpH) across soyabean phospholipid vesicular membrane (created by temperature jump), by the combined action of valinomycin and 2,4-dinitrophenol (DNP) has been monitored with the help of fluorescence from pyranine entrapped inside the vesicles under a variety of concentration conditions. The results suggest the following for the pH region of our interest (pH ~ 6 to pH ~ 8): (i) The rate limiting step in the proton transport cycle is not the transport of proton as DNPH, but the back transpsort of DNP^- and the alkali metal ion M^+ as Val-M^+-DNP^- across the membrane. The rate constant associated with the transport of the ternary complex has been estimated to be $\sim 1.5 \cdot 10^3\ s^{-1}$. (ii) The dissociation constant of the ternary complex Val-M^+-DNP^- in the membrane are ~ 1 mM for $M^+ = K^+$ and ~ 0.001 mM for $M^+ = Na^+$. (iii) The reduction in the cation selectivity of valinomycin on complexing with DNP^- is much more than that observed with the anionic form of carbonyl cyanide m-chlorophenylhydrazone (CCCP). The results also provide a verification of a corollary of Mitchell's hypothesis: an experimental strategy which enhances the ΔpH decay rate should also be a strategy for the efficient uncoupling of oxidative and photophosphorylation.

Keywords: Membrane transport; Ion transport; Phospholipid vesicle; Proton uncoupler; Valinomycin

1. Introduction

The chemiosmotic hypothesis of Mitchell [1] predicts that the dissipation of H^+ gradient across membrane (ΔpH) uncouples oxidative phosphorylation and photo-phosphorylation. A corollary of this hypothesis is that experimental strategies which enhance the ΔpH decay rate should enhance the uncoupling also. The uncoupling of photophosphorylation by the protonophore 2,4-dinitrophenol (DNP) is weak in chloroplasts [2,3]. The uncoupling by the K^+ ionophore valinomycin (VAL) is also weak [4]. Yet the combination of DNP and VAL causes full uncoupling in chloroplasts [5,6]. Therefore, the corollary mentioned above will get support if we see an enhancement in the ΔpH decay rate by the combined action of DNP and VAL in biological membranes. Experimental observation of such an enhancement in model membranes to confirm the corollary of Mitchell's hypothesis and elucidation of the mechanism of enhancement involved were the primary objectives of the present work.

Two different mechanisms suggested in the literature, can explain the combined action of VAL and DNP. In the mechanism of Karlish et al. [6] the uncoupling is the result of increase in the permeability of membrane to H^+ by DNP coupled with an increase in the permeability of membrane to K^+ by VAL. The second mechanism is similar to that suggested by Yamaguchi and Anraku [7] to explain the enhancement of proton uptake by the combined action of VAL and SF6847 (= 3,5-di-*tert*-butyl-4 hydroxybenzylidenemalononitrile): The electroneutral ternary complex valinomycin-K^+-SF^- (SF^- = anionic form of SF6847) is a direct intermediate facilitating the rate limiting step of K^+ and anion transport in the H^+ uptake cycle. (A similar ternary complex had been invoked to explain the enhance-

Abbreviations: Aces, *N*-(acetamido)-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholine)ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Lip, lipid; SBPL, soyabean phospholipid; VAL, valinomycin; Val, neutral valinomycin; Val-M^+, metal ion bound valinomycin; SF6847, 3,5-di-*tert*-butyl-4 hydroxybenzylidenemalononitrile; SF^-, anionic form of SF6847; DNP, 2,4-dinitrophenol.

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ments of H^+ uptake [7] and the ΔpH decay rate [8,9] by the combined action of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and VAL.) However, when compared to CCCP or SF6847, DNP is a less potent uncoupler [10] and is partitioned to the membrane to a lesser extent in vesicle solutions. Therefore, it is not proper to prefer a mechanism involving the VAL-DNP ternary complex and reject the mechanism of Karlish et al. [6] without evidence for the ternary complex Val-K^+-DNP^- and evidence for the involvement of the ternary complex in limiting the rate of ΔpH decay. Yoshikawa and Terada [11] have inferred the formation of the ternary complex Val-K^+-DNP^- from ^{1}H NMR studies on the partitioning of the components of the complex between an organic solvent and water. Studies have shown that the binding site of K^+ on VAL-picrate like anion complex is different from that of Na^+ [12] even in solutions. Thus, the magnitudes of the interaction between M^+ and anion in Val-M^+-anion should change when M^+ is changed from K^+ to Na^+ [13]. Some indications of the validity of such a hypothesis had been obtained from the observed reduction in the cation specificity of VAL in our work with VAL and CCCP [9]. Since the phenolic oxygen of DNP^- can approach more closer to M^+ than the anilino nitrogen of $CCCP^-$, structural considerations suggest a more prominent reduction in the cation specificity when VAL has DNP^- bound to it rather than when $CCCP^-$ bound to it. Verification of this prediction was also an objective of the present work.

In the present paper, we have tested the prediction of the corollary of Mitchell's hypothesis by making observations on the enhancement of ΔpH decay rate by the combined action of VAL and DNP. Furthermore, we have used the following criterion to test the validity of the two mechanisms (mentioned above) after identifying the rate limiting step of ΔpH decay to be that associated with alkali metal ion (M^+) transport which provides the compensating charge flux: If the mechanism of Karlisch et al [6] is valid the rate of M^+ transport across the membrane (and hence the ΔpH decay rate) should decrease by a factor of $\sim$ 3400 at [MCl] = 100 mM (estimated using the metal ion dissociation constants of Val-M^+ [9]) when M^+ is changed from K^+ to Na^+ under similar concentration conditions. However, the decrease is expected to become considerably less prominent in the mechanism of Yamaguchi and Anraku [7] in view of the expected decrease in cation specificity of VAL when DNP^- is bound to it. Soyabean phospholipid (SBPL) vesicles [14] were used as model membranes for such studies. Temperature jump (T-jump) [15] was used to create a pH difference across the vesicular membrane (ΔpH) within $\sim$ 5 μs for studying the kinetics of ΔpH decay as described earlier [16–18].

2. Materials and methods

Preparation of SBPL vesicles from asolectin (Sigma) in aqueous medium containing KCl or NaCl, 2 mM pyranine trapped inside and having different buffers inside and outside at pH and concentrations mentioned in the figure legends, has been described elsewhere [15,16]. *N*-(Acetamido)-2-aminoethanesulfonic acid (Aces), tris- (hydroxymethyl)aminomethane (Tris) and 2-(*N*-morpholine)ethanesulfonic acid (Mes) (Sigma), potassium dihydrogen phosphate (Ranabaxy Lab.) and potassium acetate (S.D. Fine Chem.) were used in the preparation of buffers. DNP and VAL (Sigma) were used to prepare stock solutions of 100 mM DNP and 10 mM VAL in ethanol. Microlitre amounts of the stock solutions were added with vortex mixing to vary the concentrations [DNP] and [Val] in vesicle solutions.

The ΔpH relaxation times (τ) were determined using the T-jump instrument of our earlier works and following the procedure described elsewhere [9,17,18]. At least four T-jump relaxation traces observed at $\sim$ 25°C on the same sample were used to obtain τ.

2.1. Apparent partition coefficients P

The apparent partition coefficient P (= average [DNP] in vesicular membrane/[DNP] in the aqueous medium) with [KCl] = 100 mM or 300 mM were determined at different pH as follows. A 7.4 mM DNP aqueous solution with 20 mM acetate buffer, 12.5 mM Mes buffer and [KCl] = 100 mM (or 500 mM) at specific pH conditions were diluted by a factor of 2 using SBPL vesicles in the eluent (0.25 mM Aces and 100 mM KCl at pH 7). These mixtures were allowed to stand for one hour and their pH was measured. Such mixtures in our experiments had $\sim$ 5 mM SBPL. By using DIAFLO ultrafiltration membrane filters (YM 30 obtained from Amicon) 0.06 ml of the aqueous part of the above mixture was extracted and added to 3 ml of 25 mM Aces at pH 7. The optical densities d_1 of these solutions at 360 nm or 400 nm (determined using a Cary 17D spectrophotometer) are proportional to C_{wm}, the concentration of DNP in the aqueous part when SBPL in solutions is 5 mM. Similar experiments, using the suspension medium alone for the dilution gave optical densities d_2 proportional to the concentration of DNP C_{w0} (= $[DNP]_0$) in the absence of SBPL in solutions. If V_e and V_m are the total volume and membrane volume, respectively, in vesicle solutions (with $V_e \gg V_m$), then:

$$V_e C_{w0} = V_e C_{wm} + V_m C_m \quad (1)$$

where C_m is the average concentration of DNP in the membrane. The apparent partition coefficient P is therefore obtained using Eq. (2).

$$P = C_m/C_{wm} = \{(d_2 - d_1)/d_1\}\{V_e/V_m\}. \quad (2)$$

The concentration in the membrane C_m can be expressed in terms of P and the concentration in vesicle solutions C_{w0} as follows:

$$C_m = C_{w0} P/(1 + PV_m/V_e). \quad (3)$$

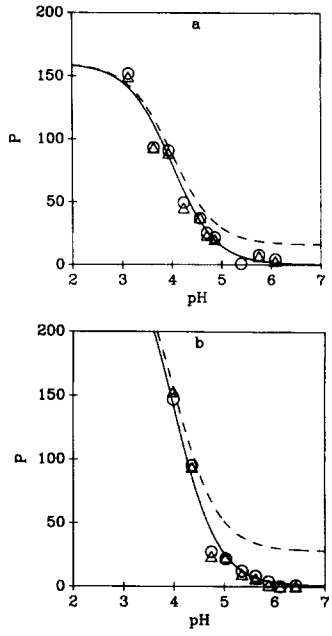

Fig. 1. Dependence of apparent partition coefficient P of DNP on pH in 5.1 mM SBPL vesicle solutions with (a) [KCl] = 100 mM and (b) [KCl] = 300 mM. The data points were obtained using optical densities at 360 nm ○ and 400 nm △ and using Eq. (2). The solutions had 3.7 mM DNP, 10 mM acetate, 6.25 mM Mes and 0.125 mM Aces. Solid lines were calculated using Eq. (A6) with $[DNPH]_m/[DNPH]_w = 160$ and 280 in (a) and (b) respectively. $pK^*_{Hm} = 6.8$ and $pK^*_{Hw} = 4$ were used in these calculations. Broken line was calculated using $pK^*_{Hm} = 5$ and $pK^*_{Hw} = 4$.

Using the dimensions of SBPL vesicles given by Grzesiek and Dencher [19] we can write $V_m \sim$ twice the volume of the aqueous medium inside vesicles (V_i). $V_e/V_i = 2250$ for 1 mM SBPL vesicle solutions [17]. Therefore, for our 5 mM SBPL vesicle solutions we can use $V_e/V_m = 225$ in Eqs. (2) and (3) and determine P. (Eq. (A6) given in the Appendix shows that P depends on pH).

3. Results and discussion

3.1. pH dependence of P and the apparent pK of DNP in SBPL vesicular membrane

P estimated using Eq. (2) and following the procedure given in the previous section are plotted in Fig. 1 as a function of pH for [KCl] = 100 mM and 300 mM. The decrease of P with increase in pH (Fig. 1) suggests that partitioning of DNPH to the SBPL membrane is much more than that of DNP^-: average $[DNPH]_m$ and average $[DNP^-]_m$ in the membrane are respectively $\sim$ 160 times and 0.25 times $[DNPH]_w$ and $[DNP^-]_w$ in the aqueous medium containing 100 mM KCl. Fig. 1 also shows that on increasing [KCl] to 300 mM, the partitioning of both DNPH and DNP^- to membrane increase by a factor of $\sim$ 1.8. Therefore, the terms involving the ion paired species in Eqs. (A5) and (A6) may not be important. The change in P on changing [KCl] is presumably due to associated changes in the membrane. The above estimates of P are smaller than those determined for spin labelled DNP in egg phosphatidylcholine suspensions ($\sim$ 2000 for the protonated form and $\sim$ 475 for the anionic form) [20]. We can expect a difference in the two estimates of P since chemical modification of DNP by spin labelling can change P.

Eqs. (A1) and (A6) with $pK^*_{Hw} = 4$ (close to that given in the literature [10]) account for the pH dependence of optical densities of DNP aqueous solutions at 360 nm and 400 nm. We also note that Eq. (A6) can account for the dependence of P on pH (Fig. 1) if $pK^*_{Hm} > 6$. (For example, use of $pK_{Hm^*} \sim 5$ gives the broken line of Fig. 1 which shows deviations from data beyond the limits of experimental errors.) It is difficult to get better estimates of pK^*_{Hm} from the data in Fig. 1 since $[DNP^-]_m$ is small even at high pH. In our calculations (solid lines in figures) we have chosen $pK^*_{Hm} = 6.8$ obtained by fitting the pH dependence of the partition of DNP between n-octanol and water reported in the literature [21] to Eq. (A6). Possible variations of K^*_{Hm} and K^*_{Hw} with [KCl] have not been included in such calculations since small variations of these parameters cause only negligible changes in P (see Eq. (A6)) and the errors in the experimentally determined P are substantial. Thus, in the pH range 6–8 of our T-jump experiments (i) in the membrane $[DNPH]/[DNP^-] > 0.1$ even though it is < 0.01 in the aqueous medium; (ii) the amount of uncomplexed DNP in the membrane is only a small fraction of the DNP in the aqueous medium (and hence can be taken to be nearly a constant in our VAL concentration range even if VAL-DNP complexes are formed in the membrane).

3.2. Identification of the rate limiting species from kinetic data

The participation of $Val\text{-}M^+\text{-}DNP^-$ complex in the membrane in the rate limiting step can be inferred from kinetic data (Figs. 2–5) since the ΔpH decay rate ($1/\tau$) is proportional to the concentration of such a complex in the membrane as shown below:

(a) At small concentrations, we can expect $[Val\text{-}K^+\text{-}DNP^-]_m$ to increase linearly with $[Val]_0$ or $[DNP]_0$. Therefore, the observed near linear increase of $1/\tau$ with both $[Val]_0$ and $[DNP]_0$ seen in the pH range 6–8 show that $1/\tau$ is proportional to $[Val\text{-}K^+\text{-}DNP^-]_m$ in Figs. 2 and 3. Similarly $1/\tau$ in Fig. 5 is proportional to $[Val\text{-}Na^+\text{-}DNP^-]_m$.

(b) The concentration of uncomplexed VAL in the membrane is expected to decrease with increase in $[DNP]_0$ because of the formation of $Val\text{-}M^+\text{-}DNP^-$. Therefore, since $[Val]_0 \ll [DNP]_0$, $1/\tau$ should tend towards a saturating value at higher $[DNP]_0$, if $1/\tau$ is proportional to $[Val\text{-}M^+\text{-}DNP^-]_m$. Even though this is not seen prominently, tendency towards such a behaviour could be dis-

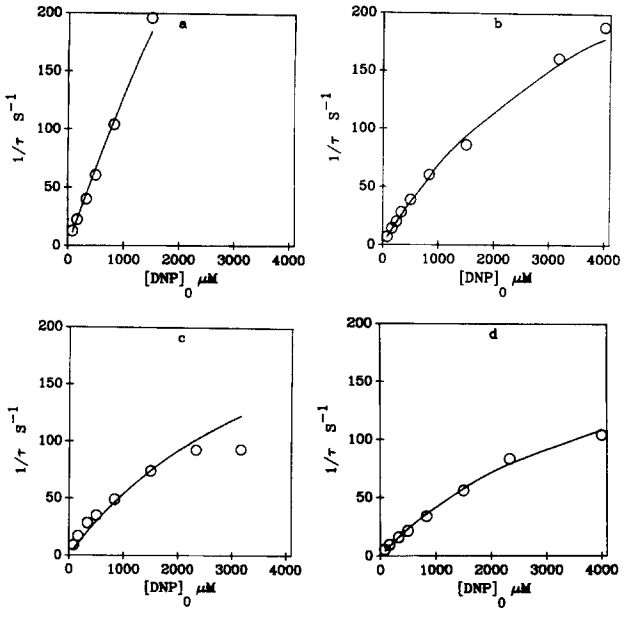

Fig. 2. Variation of ΔpH decay rate $1/\tau$ on $[DNP]_0$ in 5.1 mM SBPL vesicle solutions at pH = (a) 6.05, (b) 6.95, (c) 7.5, (d) 7.8, keeping $[Val]_0 = 17$ μM. [KCl] = 100 mM, buffer inside vesicles 2 mM phosphate, outside vesicles 25 mM ACES in (a)-(c) and 25 mM Tris with $[Val]_0 = 13$ μM in (d). Solid lines were calculated using Eq. (4) with parameters determined as described and given in Section 3.3.

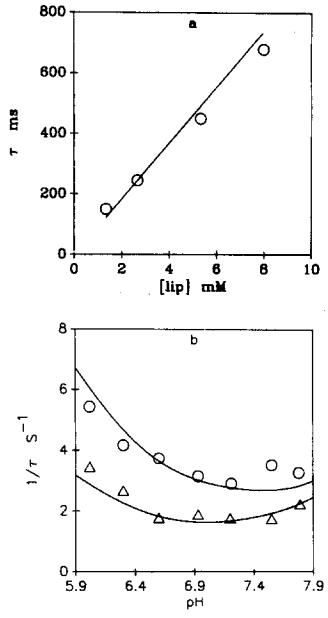

Fig. 4. Dependence of (a) τ on lipid concentration with $[Val]_0 = 2.5$ μM, $[DNP]_0 = 200$ μM, inside vesicles 2 mM phosphate and (b) $1/\tau$ on pH with $[Val]_0 = 4$ μM, $[DNP]_0 = 200$ μM and inside vesicles 2 mM phopsphate ○ and 50 mM phosphate △. Outside vesicles 25 mM Aces. [KCl] = 100 mM, [lip] = 5.1 mM. Solid lines were calculated using Eq. (4) with parameters given in the text (Section 3.3).

cerned in Fig. 2b–2d. The small deviations from linear behaviour at high [Val] seen in Fig. 3 are presumably associated with changes in membrane order [9] and increase in P of DNP coming from the increase in charged species [Val-K^+] (and its counter ion) in the membrane.

(c) If [lip] (= lipid concentration) is increased keeping $[DNP]_0$ and $[Val]_0$ constants, in view of the magnitude of P in our pH range we should expect $[DNP]_m$ to remain nearly a constant and $[Val]_m$ to decrease linearly with [lip].

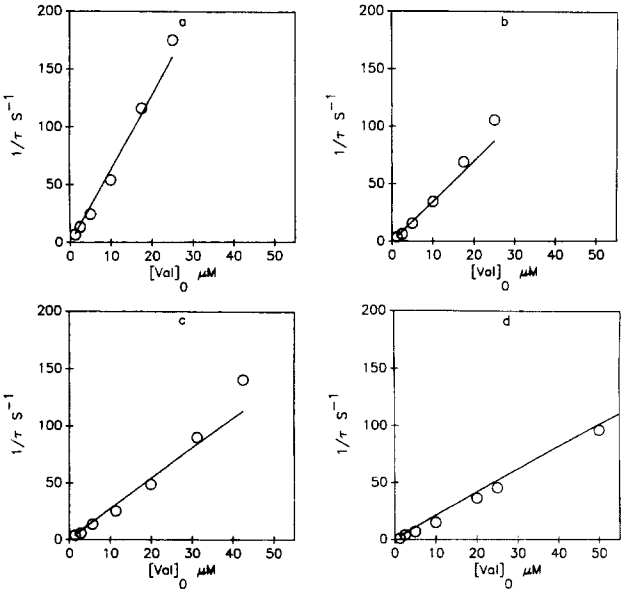

Fig. 3. Variation of $1/\tau$ on $[Val]_0$ in 5.1 mM SBPL vesicle solutions at pH = (a) 6.05, (b) 6.95, (c) 7.5, (d) 7.8 keeping $[DNP]_0 = 830$ μM. [KCl] = 100 mM, buffer inside vesicles 2 mM phosphate, outside 25 mM Aces in (a)–(c) and 25 mM Tris in (d). Solid lines were calculated using Eq. (4) with parameters given in the text (Section 3.3).

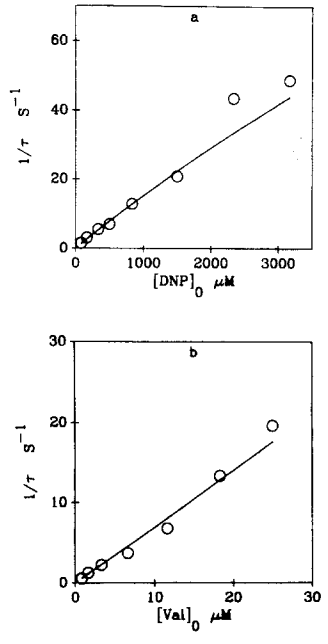

Fig. 5. Dependence of $1/\tau$ on (a) $[DNP]_0$ keeping $[Val]_0 = 17$ μM and (b) on [Val] keeping $[DNP]_0 = 830$ μM in 5.3 mM SBPL vesicle solutions containing 100 mM NaCl. Inside vesicles 2 mM phosphate, outside 25 mM Aces, pH 7. Solid lines were calculated using Eq. (4) and parameters given in the text (Section 3.3).

Therefore, if $1/\tau$ depends on $[Val\text{-}K^+\text{-}DNP^-]_m$, τ should increase linearly with increase in [lip]. Fig. 4a shows such a behaviour.

(d) In our experiments, τ depends on internal buffer capacity (b_i) also, in addition to the concentration of the rate limiting species [17]. If the variation of b_i with pH alone was responsible for the dependence of τ on pH [17], $1/\tau$ should have decreased by a factor of ~ 3.7 on changing the pH from 6 to 7.5. Fig. 4b shows that the observed variation is less than this factor. This discrepancy can be accounted for if $1/\tau$ is proportional to $[Val\text{-}K^+\text{-}DNP^-]_m$. Decrease of K_M^* [9] and conversion of DNPH into DNP^- increase $[Val\text{-}K^+\text{-}DNP^-]_m$ with increase in pH and contribute to the above mentioned deviation.

(e) The dependence of $1/\tau$ on the choice of alkali metal ion (Figs. 2, 3 and 5) shows that the metal ion is also bound in the rate limiting species as in $Val\text{-}M^+\text{-}DNP^-$.

(f) K^+ binding to uncomplexed VAL is ~ 17000 times more selective than Na^+ binding [22]. Therefore, if $[Val\text{-}M^+]$ limits the rate, $1/\tau$ should decrease by a factor of $[Val\text{-}K^+]_m/[Val\text{-}Na^+]_m$ (~ 3400 at [MCl] = 100 mM using the estimates of metal ion binding constants given in Ref. [9]) on changing M^+ from K^+ to Na^+. On the other hand if the species $Val\text{-}M^+\text{-}DNP^-$ is formed and is the rate limiting species of ΔpH decay, $1/\tau$ should decrease by a factor $[Val\text{-}K^+\text{-}DNP^-]_m/[Val\text{-}Na^+\text{-}DNP^-]_m$ on changing the alkali metal ion from K^+ to Na^+. This factor can be different from $[Val\text{-}K^+]_m/[Val\text{-}Na^+]_m$ because of the following reason: From X-ray crystallographic studies, binding site of K^+ on VAL has been found to be different from that of Na^+ [12]. This observation is also consistent with the inferences using NMR [23]. Therefore, if an anion such as DNP^- is already bound to VAL, the Coulombic interaction between DNP^- and K^+ will be different from that between DNP^- and Na^+ on VAL. Because of this additional factor the ion selectivities of $Val\text{-}DNP^-$ and Val will be different. Experimental data at similar concentration conditions show that $1/\tau$ in vesicle solutions containing 100 mM NaCl (Fig. 5) is only ~ 4 times smaller than in vesicle solutions containing 100 mM KCl at pH ~ 7 (Fig. 2b and Fig. 3b). The fact that $1/\tau$ depends on the choice of M^+ confirms that the rate limiting step involves alkali metal ion transport. The reduction in ion selectivity seen from the data is expected if Na^+ binding site on VAL is closer to the DNP^- binding site when compared to that of K^+ in $Val\text{-}M^+\text{-}DNP^-$ ($M^+ = Na^+$ or K^+).

(g) In the ΔpH decay mechanism the transfer of DNPH or DNP^- from the aqueous medium to the membrane or vice-versa and H^+ transfer at the interface are not rate limiting steps. If these were the rate limiting steps $1/\tau$ should have increased with [lip] and buffer concentration, a behaviour not seen in the data of Fig. 4

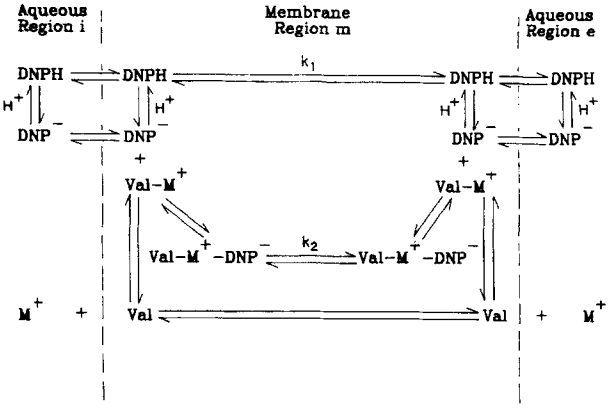

Fig. 6. Suggested mechanism for the ΔpH decay by VAL-DNP combination.

3.3. Analysis of τ data in terms of the mechanism of ΔpH decay

The results and inferences mentioned above are qualitatively similar to that observed by the combined action of VAL and CCCP [9]. However, unlike CCCP, only a small fraction of the DNP added to vesicle solutions is partitioned to the membrane. Therefore, the mechanism for the ΔpH decay by H^+/M^+ transport across the membrane by the combined action of VAL and DNP can be suggested to be that given in Fig. 6. In this mechanism, after a fast H^+ exchange at the interface, H^+ is translocated across the membrane as DNPH. To facilitate its back transport Val is also translocated in a fast step. After the release of H^+ at the second interface, DNP^- and the ion providing the compensating charge, M^+, are translocated in a direction opposite to that of H^+ transport as $Val\text{-}M^+\text{-}DNP^-$. In the present experiments the corrections associated with changes in membrane order at the highest $[Val]_0$ and $[DNP]_0$ (determined from the observed negligible change in nigericin mediated ΔpH decay rate) are negligible within the limits of experimental error. Thus, the expression for $1/\tau$ can be written similar to Eq. (4) of Ref. [9]. In our experiments the buffer capacity in volume V_e external to vesicles ($= b_e$) is such that $b_i V_i \ll b_e V_e$. Also, the small terms in the denominator of $1/\tau$ (Eq. (4) of Ref. [9]) can be neglected since the concentrations of DNP and VAL in the membrane are sufficiently small. Therefore, since, $PV_m/V_e \ll 1$ and $[DNP]_0 \sim C_{w0}$ in our experiments, using Eqs. (3) and (A8) we can write,

$$1/\tau = (\ln 10) k_2 [Val - M^+ - DNP^-]/b_i$$
$$= (\ln 10) k_2 [VAL]_m [DNP]_0 P$$
$$/\{b_i (K_0 + [DNP]_0 P)\} \quad (4)$$

where $K_0 = K_1 (1 + K_M/[K^+])$ at high pH (Eqs. (A3) and (A9)) and b_i is given by Eq. (A10). Using the estimates of K_M determined in our earlier work ($= 25$ mM for $M^+ = K^+$) [9] and using the τ against $1/\{P[DNP]_0\}$ linear plots, k_2 ($\sim 1.5 \cdot 10^3$ s^{-1}) and K_1 (~ 1 mM) can be estimated from the data in Fig. 2b–d. We have used the above estimate of k_2 to analyse the data

obtained with NaCl also, since the sizes of Val-K^+-DNP^- and Val-Na^+-DNP^- can be expected to be similar. Using $K_M = 425$ M for $M^+ = Na^+$, the data of Fig. 5 could be fitted by choosing $K_1 = 0.001$ mM for the dissociation constant of Val-Na^+-DNP^-. The solid lines given in the figures have been calculated using the above estimates.

We also note the following: (a) If the transfer of DNP between the aqueous medium and membrane is much faster than the ΔpH decay we have to use $\Delta[DNP_f]_{il} = \{V_i/(V_e + V_i)\}\ \Delta[DNP]_0$ in equations similar to (A13) of Ref. [9]. This does not alter the expression for $1/\tau$. On the other hand, if such a transfer is too slow we again get expressions similar to those given in Ref. [9]. Therefore, our data are not adequate to distinguish between the above mentioned two situations. (b) The pK of DNP in aqueous medium (~ 4) is considerably less than the pH in our experiments. Nevertheless our data shows that proton transport as DNPH across the membrane is not limiting the rate (unlike the high valinomycin concentration data, with CCCP which has p$K \sim 6$ in water). This can be understood by noting that the apparent pK of DNP in the membrane has a much higher value (~ 6.8) in the membrane. (c) The extent of reduction in cation selectivity of VAL, with DNP^- as the bound anion is considerably more than that with $CCCP^-$ [9]. This is correlated with the fact that the reduction in K_1 on changing the metal ion from K^+ to Na^+ is more when the anion is DNP^- than when the anion is $CCCP^-$. We also note that the results given above are consistent with the corollary of Mitchell's hypothesis.

Appendix A

The equilibrium concentrations of protonated form of DNP, $[DNPH]_w$, deprotonated form, $[DNP^-]_w$ and the ion paired complex $[DNP^-{:}M^+]_w$ in the aqueous medium can be used to define the dissociation constants K_{Hw} and K_{IPw} for the following equilibria. (M^+ = alkali metal ion in the vesicle solutions.)

$$DNPH \rightleftharpoons DNP^- + H^+,\ K_{Hw} = [DNP^-]_w[H^+]/[DNPH]_w$$

$$DNP^-{:}M^+ \rightleftharpoons DNP^- + M^+,\ K_{IPw} = [DNP^-]_w[M^+]/[DNP^-{:}M^+]_w \quad (A1)$$

The apparent dissociation constants K_{Hm}, K_{IPm} are similarly defined using $[H^+]$ and $[M^+]$ in the aqueous medium and carrier concentrations in the membrane. Similarly the apparent dissociation constant K_M^* is also defined (with subscript 'm' indicating concentration in the membrane).

$$Val\text{-}M^+ \rightleftharpoons Val + M^+,\ K_M^* = [Val]_m[M^+]/[Val\text{-}M^+]_m \quad (A2)$$

In SBPL vesicles, experiments have shown that K_M^* is pH dependent [9].

$$K_M^* = K_M(1 + [H^+]/K_{LH}) \quad (A3)$$

where $K_M = 25$ mM for $M^+ = K^+$ and 425 M for $M^+ = Na^+$. p$K_{LH} = 6.6$ [9].

Since VAL is predominantly in the membrane its concentration in the membrane, $[VAL]_m$ can be related to the total VAL concentration, $[Val]_0$ in the SBPL vesicle solutions [17]:

$$[VAL]_m = 0.95[Val]_0/[lip]M \quad (A4)$$

where [lip] = lipid concentration in the solution. However, DNP will be mainly in the aqueous medium. The concentration of DNP in the vesicular membrane C_m can be related to its total concentration in vesicle solutions C_0 using Eq. (3) and the apparent partition coefficient P where,

$$P = \{[DNPH]_m + [DNP^-]_m + [DNP^-{:}M^+]_m\} / \{[DNPH]_w + [DNP^-]_w + [DNP^-{:}M^+]_w\} \quad (A5)$$

Therefore, the pH dependence of P can be expressed as follows.

$$P = \{[DNPH]_m/[DNPH]_w\}\{(1 + K_{Hm}^*/[H^+])/(1 + K_{Hw}^*/[H^+])\}$$

$$K_{Hm}^* = K_{Hm}\{1 + [M^+]/K_{IPm}\};$$

$$K_{Hw}^* = K_{Hw}\{1 + [M^+]/K_{IPw}\} \quad (A6)$$

If K_1 is the dissociation constant of the ternary complex Val-M^+-DNP^- in the membrane,

$$Val\text{-}M^+ - DNP^- \rightleftharpoons Val\text{-}M^+ + DNP^-$$

$$K_1 = [Val\text{-}M^+]_m[DNP^-]_m/[Val\text{-}M^+\text{-}DNP^-]_m \quad (A7)$$

In the pH region of our interest P is small. Also, in our experiments $[Val]_0 \ll C_0$ and the changes in the concentration of DNP due to the formation of ternary complex can be neglected. Therefore, we can write,

$$[Val\text{-}M^+\text{-}DNP^-]_m = [VAL]_m C_m/\{K_0 + C_m\} \quad (A8)$$

$$K_0 = K_1(1 + K_M^*/[M^+])(1 + [H^+]/K_{Hm}^*) \quad (A9)$$

where C_m is the concentration of the uncomplexed DNP in the membrane determined using C_0 ($\approx [DNP]_0$) and P in Eq. (3).

The internal buffer capacity b_i of SBPL vesicles is given by

$$b_i = (\ln 10)\left\{\Sigma C_j\left\{K_{Hj}[H^+]/\left(K_{Hj} + [H^+]\right)^2\right\}\right\} \quad (A10)$$

where C_1 and K_{H1} are the concentration and dissociation constant of the buffer entrapped inside vesicles and $C_2 = 30$ mM, $K_{H2} = 10^{-6.9}$ M; $C_3 = 45$ mM, $K_{H3} = 10^{-7.8}$ M from the endogeneous groups in SBPL vesicles [9,17]. C_n and K_{Hn} for $n > 3$ are associated with pyranine and DNP entrapped inside and the DNP in the inner layer of the vesicle which are in fast H^+ exchange equilibria with the aqueous medium inside vesicles.

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