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Some novel aspects of the relationship between the amino acid gradient and the sodium electrochemical gradient in mouse ascites tumour cells

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Accumulation of 2-aminoisobutyrate by mouse ascites tumour cells was studied in circumstances where nigericin reversed the normal direction of the Na^+ concentration gradient. The membrane potential ($\Delta\psi$) was assayed using oxonol V as a voltage-sensitive probe. The amino acid gradient ($\Delta\mu^A$) that formed did not significantly exceed the likely magnitude of the Na^+ electrochemical gradient when this was in the range 2–6 kJ mol^{-1}. When $\Delta\bar{\mu}^{Na}$ increased up to 11 kJ mol^{-1}, $\Delta\mu^A$ was almost constant at 7–8 kJ mol^{-1}. The observations indicate that when $\Delta\psi$ is large changes in cellular [Na^+] in the range 16–80 mM scarcely affect $\Delta\mu^A$.

1. Introduction

The molecular details of the coupling between the flow of co-substrate (Na^+ or H^+) and that of substrate (S) through symport mechanisms have yet to be defined [1,2]. Our present understanding of the factors that are likely to govern solute accumulation through a symport (see fig. 1) is mainly based on kinetic analysis of (1) the relationships between the two flows and (2) their dual dependence on the *cis* and *trans* values of the parameters [S] and [Na^+]. One widely discussed possibility is that a constant n equivalents of co-substrate accompany the substrate through the carrier protein in either direction [3]. When thermodynamic equilibrium is reached the electrochemical gradient of the solute ($\Delta\bar{\mu}^S$) and that of the co-substrate ($\Delta\bar{\mu}^{Na}$), acting across the plasma membrane, are related by the equation

$$\bar{n} + \Delta\bar{\mu}^S/\Delta\bar{\mu}^{Na} = 0 \qquad (1)$$

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Clearly, the larger the n, the greater is the value of $\Delta\bar{\mu}^S$ at a given value of $\Delta\bar{\mu}^{Na}$. Indeed, a number of examples are known in which one, two or three co-substrate ions are involved [4,5]. In most real systems, as illustrated in fig. 1, the solute S is likely to traverse the cell membrane through not only the symport but also at least one other carrier-mediated pathway (see, e.g., ref. 6). The value of $\Delta\bar{\mu}^S$ is then smaller than would be predicted on the basis of eq. 1, to an extent that can be estimated when the first-order rate constant defining the leak pathway is known [7].

An alternative description of symport mechanisms is based on the formalism of irreversible thermodynamics (see ref. 8). The maximum solute gradient ($\Delta\bar{\mu}^S$) formed at 'static head' is given by

$$\left(\partial J^{Na}/\partial J^S\right)_{\Delta\bar{\mu}^{Na}} + \left(\Delta\bar{\mu}^S/\Delta\bar{\mu}^{Na}\right)_{J_0^S} = 0 \qquad (2)$$

In this equation the notion of a fixed stoichiometry n is replaced by that of a potentially variable differential flow ratio. The latter defines the way in which the two flows J^S and J^{Na} change relative to one another when, for instance, $\Delta\bar{\mu}^S$ is varied with $\Delta\bar{\mu}^{Na}$ being maintained constant. Variable

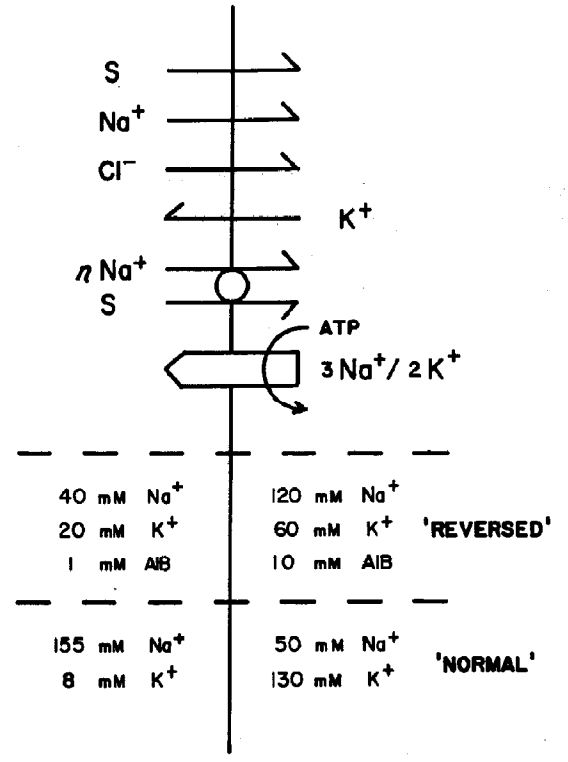

Fig. 1. General ionic conditions prevailing in the mouse ascites tumour cells when these are suspended either (1) in a standard Ringer solution or (2) in the presence of nigericin, which leads to inversion of the normal distribution of Na^+. In both cases a considerable concentration gradient of 2-aminoisobutyrate develops. The amino acid symport absorbs n equivalents of Na^+ per amino acid (S) equivalent. A leak pathway for S is also present.

stoichiometry in this sense is the result of 'slippage' in the system. It arises when the ratio of the number of molecules of co-substrate to that of substrate traversing the carrier together has more than one integral value [5]. In effect, the apparent value of n may vary with the absolute concentrations of Na^+ or S and with $\Delta\bar{\mu}^{Na}$ itself.

Another aspect of co-substrate stoichiometry concerns the kinetic order exhibited in the form of the equations describing the dependence of J^S and J^{Na} on [S] or [Na^+]. Thus, in the example of glycine or methionine transport by mouse ascites tumour cells, first-order kinetics are shown with respect to both solutes. A plausible model is one in which the first step in solute translocation involves random binding of the two ligands to the carrier [E] to form the complex ENaS. The absence of second-order terms in the rate equation is attributed to the relative slowness of the translocation steps as compared with the binding and release of the two ligands [5].

Studies in three laboratories, in which a voltage-sensitive dye was used to assay the membrane potential ($\Delta\psi$), showed that accumulation of glycine, methionine or 2-aminoisobutyrate is roughly consistent with the version of eq. 1 in which $n = 1$. In general, $\Delta\mu^A$ was about 1.8 kJ mol^{-1} larger than $\Delta\bar{\mu}^{Na}$ when the latter varied in the range from about 1 to 9 kJ mol^{-1}. While the activity coefficients for each of the ligands were assumed to be 1 for that purpose a value near 0.5 may be more appropriate for cellular Na^+ [5,9]. As the validity of the dye technique has been questioned (see references in ref. 9), it is reassuring that a recent study with conventional microelectrodes gave estimates of $\Delta\psi$ for the tumour cells in the same range, from -50 to -60 mV, as was inferred using voltage-sensitive dyes [5,10,11].

2. The 'reversed gradient' experiment: a critical test of the gradient hypothesis

A major inconsistency with predictions based on eq. 1 was inferred by Agnes Heinz and her colleagues on the basis of experiments in which nigericin was used to reverse the natural distribution of Na^+ across the plasma membrane of mouse ascites tumour cells [12]. The circumstances of this ingenious experiment are illustrated in fig. 1. We have confirmed the main finding, namely, that despite the accumulation of Na^+ in the cellular compartment, the tumour cells concentrate the amino acid to almost the same extent as that in the absence of nigericin [13]. Reference to fig. 1 leads to two predictions if eq. 1 is to apply. (1) Provided $n = 1$ the membrane potential, $\Delta\psi$, would approach -90 mV. (2) $\Delta\psi$ is expected to exceed $\Delta\psi^K$, the equilibrium potential for K^+. The earlier claim that $\Delta\psi$ and $\Delta\psi^K$ are in fact similar in magnitude [12] in the presence of nigericin was based on evidence that is probably unsound [13]. In order to test these two predic-

tions, we have now studied the magnitude of $\Delta\psi$ with the aid of the voltage-sensitive fluorescent probe oxonol V, a dye which has been used extensively for this purpose, usually by monitoring $A_{630-590}$ [14].

3. Qualitative observations with oxonol V

In the procedure adopted membrane depolarization leads to a decrease in the fluorescence excited at 577 nm and recorded at 630 nm (fig. 2). The amount of dye employed was shown to impair neither the accumulation of 2-aminoisobutyrate, during energy metabolism, nor the maintenance of the normal distribution of Na^+ and K^+ between tumour cells and suspension medium. Fig. 2 illustrates the following points. (1) Addition of gramicidin or valinomycin caused no gross artifactual fluorescence (fig. 2a). (2) Valinomycin hyperpolarized the tumour cells in the presence of ouabain, the membrane potential and fluorescence being progressively lowered as extracellular K^+ was raised (fig. 2b). (3) K^+ caused a similar decrease in fluorescence in the absence of valinomycin (fig. 2c). This behaviour is consistent with that described in ref. 10 but at variance with the data in ref. 14. (4) Addition of glycine depolarized the tumour cells (fig. 2d).

Fig. 3 establishes a number of related points. (5) The addition of K^+ to cell preparations that had previously accumulated Na^+ actuated the sodium pump, a response that was reversed by the addition of ouabain (fig. 3a). (6) No response to glycine was observed in the presence of gramicidin (fig. 3c–f). (7) The latter compound hyperpolarized preparations depolarized by glycine (fig. 3e and f).

All the above observations are consistent with the known actions of the ionophores in raising or lowering the membrane potential. We accordingly constructed a calibration curve relating $\Delta\psi^K$ in the presence of valinomycin to the prevailing fluorescence intensity. The latter quantity was measured relative to the arbitrary zero defined by addition of gramicidin and 0.13 M KCl. When we combined 39 titrations, like the four illustrated in fig. 2, the relationship between relative fluorescence intensity and $\Delta\psi$ proved to be curvilinear rather than linear (fig. 4). The calibration thus allowed $\Delta\psi$ to be estimated directly from the dye

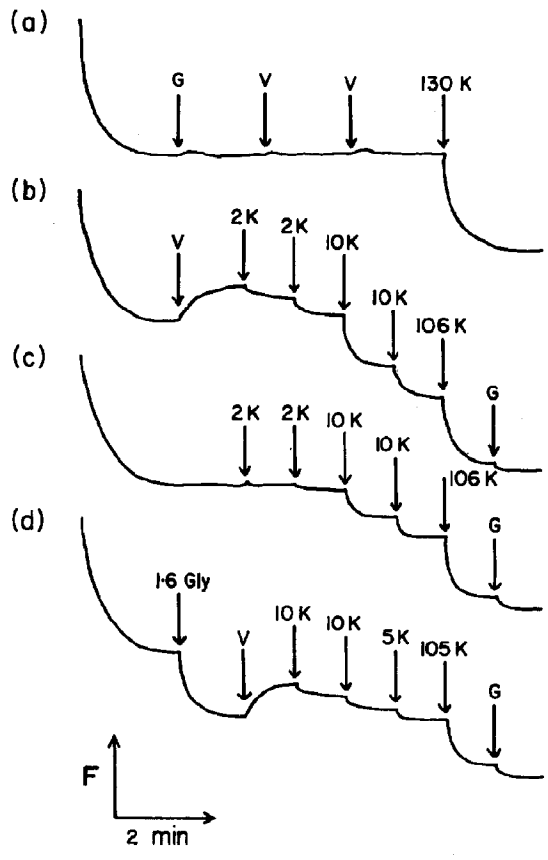

Fig. 2. Traces illustrating the time course of the changes in fluorescence exhibited by suspensions of the tumour cells in solutions of diverse composition. Assays were performed generally described as in ref. 15, fluorescence being excited at 577 nm and recorded at 630 nm. The tumour cells (1.2 mg cellular dry weight) were suspended in the selected Ringer solution (3 ml) containing 8 μg of the dye oxonol V. Extracellular Na^+ was replaced by choline or K^+ as specified, the sum of their concentrations being 163 mM. Traces: (a) The Ringer solution contained 32 mM K^+, 10 μg gramicidin, and two portions of 10 μg valinomycin were added at the indicated times. Finally, KCl (0.1 ml) was added to a final concentration of 130 mM. (b) The Ringer solution contained 163 mM Na^+ and 1 mM ouabain. Valinomycin (V) and gramicidin (G) were added as shown. The KCl concentration was increased by 2, 10 or 106 mM at the indicated times. (c) Similar to trace b but without valinomycin. (d) Similar traces to b and c but initially made 1.6 mM with respect to [glycine].

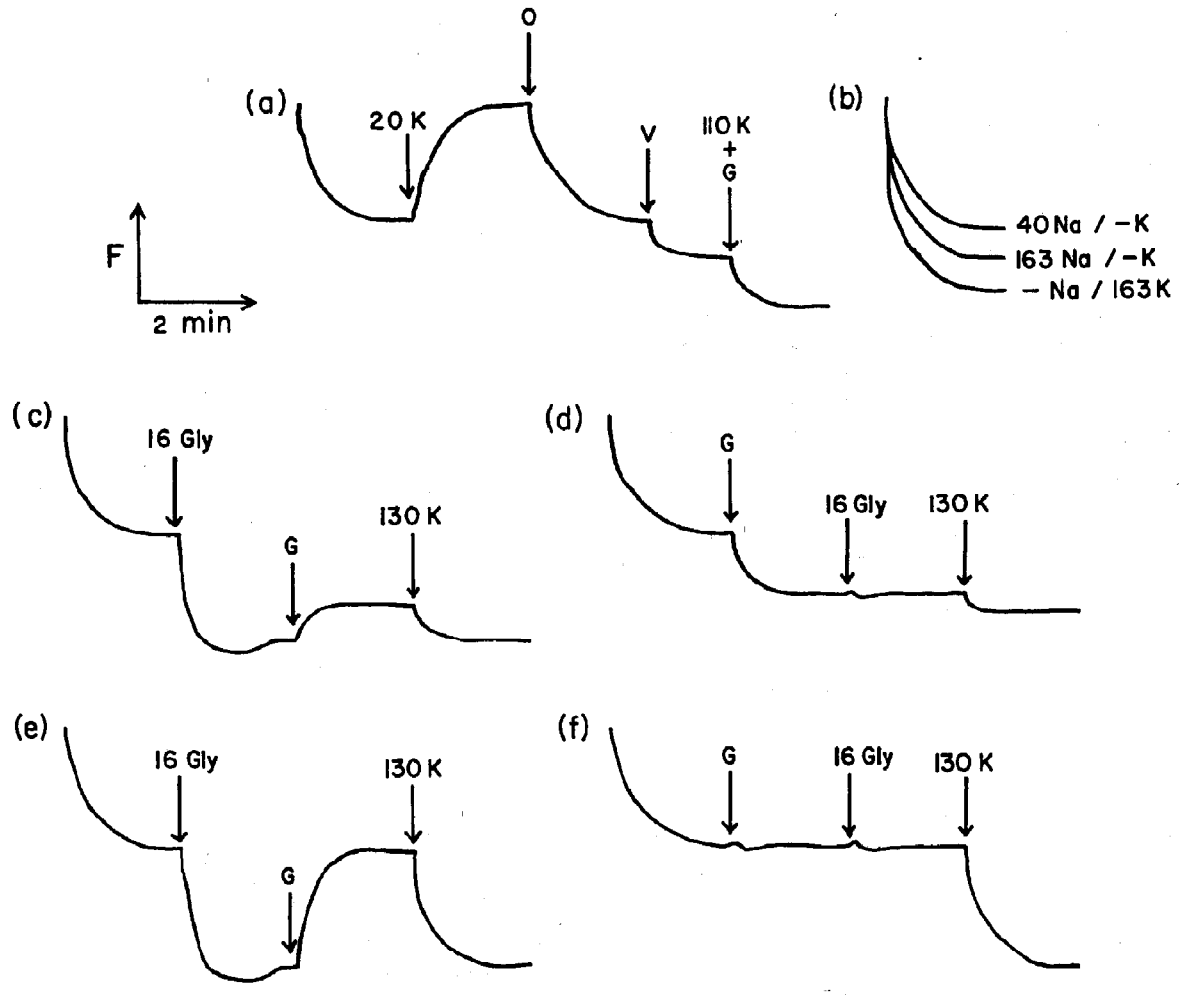

Fig. 3. Effects of ouabain or uptake of glycine on the fluorescence signal in the presence of oxonol V. Procedure as in the legend to fig. 2. Traces: (a) The Ringer solution contained 163 mM Na^+. At the indicated time 20 mM K^+ was added, then 2 mM ouabain, 10 μg valinomycin (V) and, finally, 10 μg gramicidin (G) with 110 mM K^+ as KCl. (b) A composite trace. The three Ringer solutions each contained 2 mM ouabain and 40 mM Na^+, 163 mM Na^+ or 163 mM K^+, respectively. (c) The Ringer solution contained 160 mM Na^+ and 2 mM ouabain; 16 mM glycine (Gly), 10 μg gramicidin (G) or 130 mM K^+ were added. (d). Similar to trace c except that the order of the additions was changed. (e) The Ringer solution contained 40 mM Na^+ and 2 mM ouabain. Glycine, gramicidin and 130 mM K^+ were added in turn. (f) Similar to trace e, but with gramicidin added before glycine.

signals that a given cell suspension produced, without the necessity of titrating the suspension with K^+ and valinomycin.

4. Magnitude of $\Delta\psi$ in the presence of nigericin

The presence of nigericin, in amounts increasing in the range 0.3–3 μg/mg cellular dry weight, progressively lowered the amino acid accumulation ratio below about 25-fold. It raised cellular [Na^+], as did the presence of 2 mM ouabain [13]. Fig. 4 illustrates these general relationships, as well as the magnitude of the relative fluorescence intensity observed in each of 32 cell preparations in the Ringer solution containing about 45 mM Na^+ and 18 mM K^+. Fig. 5 shows how $\Delta\psi$ and $\Delta\psi^K$ were related.

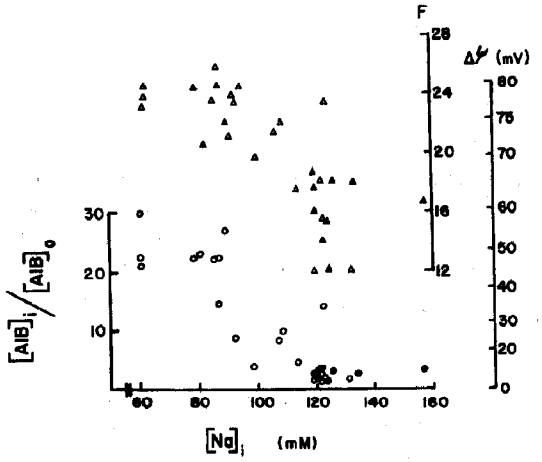

Fig. 4. Accumulation of 2-aminoisobutyrate in the presence of different amounts of nigericin as a function of both (1) the steady level of cellular [Na^+] established in the cell suspension and (2) the magnitude of the signal exhibited in the presence of oxonol V. The main cell suspensions (6 mg dry weight ml^{-1}) were incubated for 40–60 min and samples withdrawn for assay of (1) the relative fluorescence intensity and (2) the cellular contents of 2-amino[^{14}C]isobutyrate, Na^+, K^+ and water [13]. (1) Fluorescence intensity (△, ▲) was assayed relative to a working zero represented by the fluorescence intensity observed when gramicidin (3 μg) and 130 mM KCl were added. The sample (0.2 ml, containing 1.2 mg cellular dry weight) was transferred to the cuvette of the fluorimeter to give a cell suspension containing all the components of the main Ringer solution. The fluorescence intensity reached a steady value in about 2 min, the magnitude of which relative to the working zero was then established by the addition of gramicidin and KCl in turn. The main Ringer solution contained 40–50 mM Na^+, 18 mM K^+, 0.4 mM 2-amino[^{14}C]isobutyrate, 18 mM lactate and a selected amount of nigericin in the range 0.3–3 μg/mg cell dry weight [13]. 2 mM ouabain was present (▲) or absent (△). Conversion of the relative fluorescence intensity into a value for $\Delta\psi$ was based on the scale represented on the right-hand ordinate (see text). (2) Other assays were conducted as described in ref. 13. The amino acid distribution was determined in either the presence (●) or absence (○) of ouabain.

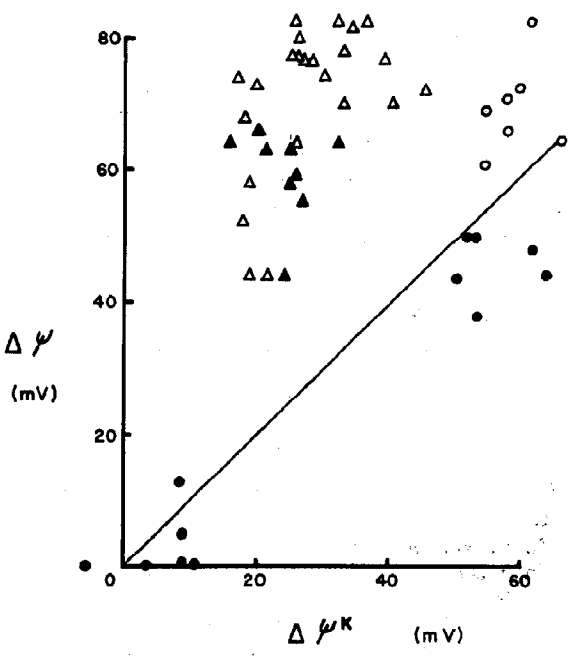

Fig. 5. Comparison of the magnitude of $\Delta\psi$ with the prevailing magnitude of $\Delta\psi^K$. One set of observations refers to the 32 cell suspensions assayed in connection with fig. 4. Several of these contained 2 mM ouabain (▲), the remainder did not (△). Six control suspensions lacking nigericin were studied (○). A further independent set of controls contained 2 mM ouabain, 40 mM Na^+ and 20 mM K^+ (●) without lactate; the lower set (●) contained 40 mM Na^+ and 120 mM K^+ to depolarize the cell suspension.

The mean value (±S.D.) of $\Delta\psi$ for the tumour cells suspended in the selected Ringer solution in the presence of 0.4 mM 2-aminoisobutyrate (controls) was 69.7 ± 7.1 mV. The presence of nigericin at amounts that increased cellular [Na^+] from 3- to 5-fold caused only a small increase in $\Delta\psi$ up to a mean value of 76.9 ± 4.4 mV (17). Thus, the largest effect of nigericin on the magnitude of $\Delta\bar{\mu}^{Na}$ was due to the change in $[Na^+]_o/[Na^+]_i$ from 3.60 ± 0.96 (7) in the controls to 0.64 ± 0.15 (17) in the presence of nigericin. Fig. 5 shows very clearly that the prevailing membrane potential somewhat exceeded $\Delta\psi^K$ in the absence of nigericin and considerably outweighed $\Delta\psi^K$ in its presence. While the second of the above predictions was therefore confirmed, the tumour cells were evidently not hyperpolarized up to 90 mV. The implications of this behaviour in relation to eq. 1 were next examined.

5. Comparison of $\Delta\mu^A$ and $\Delta\bar{\mu}^{Na}$

Fig. 6 combines all the observations made using the Ringer solution containing 40–50 mM Na^+. Clearly, $\Delta\mu^A$ was not directly proportional to $\Delta\bar{\mu}^{Na}$. Indeed, large changes in the latter parame-

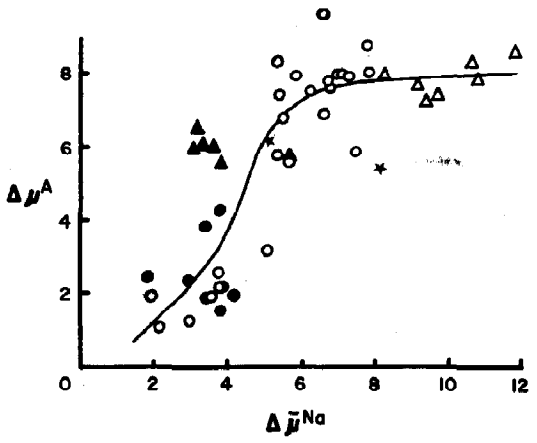

Fig. 6. Comparison of the magnitudes of $\Delta\mu^A$ and $\Delta\bar{\mu}^{Na}$ observed in the cell suspensions containing 2-aminoisobutyrate and different amounts of nigericin. The gradients are represented in units of kJ mol^{-1}. The observations comprise the 32 assays made in the presence of nigericin (○) or nigericin and ouabain (●). The controls (△) contained neither compound. The above sets involved the Ringer solution containing 18 mM lactate, 40–50 mM Na^+ and 18 mM K^+. Lactate was omitted from a further set of controls in which 2 mM ouabain was present (▲). Two sets of observations (★) with methionine are included from ref. 18. The smaller value of $\Delta\bar{\mu}^{Na}$ was obtained in the presence of 30 mM Na^+, 8 mM K^+ and glucose, $\Delta\psi$ being 57 mV. The larger value of $\Delta\bar{\mu}^{Na}$ was observed in the presence of 80 mM Na^+ and 150 mM K^+, the tumour cells being hyperpolarized to 90 mV.

ter, over the range 6–12 kJ mol^{-1}, had little effect on the magnitude of $\Delta\mu^A$. Raising the nigericin concentration above about 2 μg mg^{-1} led to a marked fall in $\Delta\mu^A$, especially in the presence of 2 mM ouabain. A regression analysis of the 32 observations involving nigericin (fig. 6; ○, ●) showed that

$$\Delta\mu^A = (1.449 \pm 0.120)\Delta\bar{\mu}^{Na} - 2.039 \pm 0.614 \quad (3)$$

with a correlation coefficient of 0.911. The slope therefore significantly exceeded the value of 1. Comparison with the further series (fig. 6, ▲) in which $\Delta\bar{\mu}^{Na}$ was in the range 3–4 indicates that the presence of nigericin had a marked effect on the relationship between $\Delta\mu^A$ and $\Delta\bar{\mu}^{Na}$. It will be noted that $\Delta\psi$ in the latter group of preparations was smaller than $\Delta\psi^K$.

6. Origin of $\Delta\psi$

The observations shown in fig. 5 demonstrate that the magnitude of $\Delta\psi$ exceeded that of $\Delta\psi^K$ in the absence of nigericin unless ouabain was present. Furthermore, (1) nigericin caused a modest hyperpolarization and (2) $\Delta\psi$ exceeded $\Delta\psi^K$ even in the presence of ouabain. We attribute cellular hyperpolarization to the action of the sodium pump which more than compensates for the natural permeability of the tumour cells to K^+ [15]. Whether the sodium pump of these mouse tumour cells is completely inhibited by ouabain under these conditions is not known. If inhibition is partial the residual activity may account for the difference between $\Delta\psi$ and $\Delta\psi^K$ (see ref. 11).

7. The stoichiometry of the amino acid symport

Inspection of fig. 6 suggests that, when allowance is made for the possibility that the effective activity coefficient for cellular [Na^+] may be about 0.5 rather than 1 [15], the magnitude of the amino acid gradient was not greater than that of Na^+. To that extent the present observations are consistent with the extensive earlier work discussed in refs. 5 and 15 in which various ionic regimes were employed and energy metabolism was maintained by glycolysis. Whether the use of lactate as substrate in the present type of preparation is relevant to a discussion of the efficiency of coupling to $\Delta\bar{\mu}^{Na}$ is not known. As an explanation of eq. 3, we tentatively suggest that the coupling stoichiometry in the sense of eq. 1 remains at 1 but that a variable loss of coupling between the substrate and co-substrate gradients occurs in certain circumstances. The relevant circumstances include (1) large values of $\Delta\bar{\mu}^{Na}$ when cellular Na^+ is relatively small and $\Delta\psi$ relatively large. They also include (2) the presence of ouabain or the greater concentrations of nigericin that caused cellular Na^+ to accumulate at the expense of cellular K^+ while $\Delta\psi$ remained relatively large. Our earlier observations made with methionine in which $\Delta\psi$ was 90 mV are illustrated in fig. 6 and seem consistent with this description [18]. Our working hypothesis thus focusses attention on the

magnitude of $\Delta\psi$ rather than a possible function for cellular $[K^+]$ (see refs. 16 and 17).

8. Working hypothesis

A possible mechanism of partial uncoupling of the substrate gradient from that of the co-substrate is to suppose that, when $\Delta\psi$ is relatively large, amino acid efflux occurs both through the leak pathway that is known to exist [15] and partially via a binary complex of the carrier (E^-) with the amino acid, rather than as the species E^-Na^+S required for tight coupling of the two gradients. This hypothesis may account qualitatively in a simple, albeit speculative, fashion for the present finding that nigericin raised cellular $[Na^+]$ several fold but had little effect on amino acid accumulation, even though only a small increase in $\Delta\psi$ occurred.

The hypothesis emphasizes the possibility that the extent to which slippage occurs by the above-described mechanism depends on the relative magnitudes of the rate constants governing translocation of E, ES and ENaS. Because the latter species carries one more positive charge than the other two, their relative rates of translocation depend on the absolute magnitude of $\Delta\psi$.

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