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A Sodium Ion Concentration Gradient Formed during the Absorption of Glycine by Mouse Ascites-Tumour Cells

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1. To deplete them of ATP the tumour cells were starved at 37° in a Ringer solution containing 33m-equiv. of Na^+/l., 131m-equiv. of Li^+/l., 2mM-sodium cyanide and 0·1mM-ouabain. The cellular content of K^+ was largely replaced by Li^+, but cellular [Na^+] remained near 33m-equiv./l. 2. The addition of 12mM-glycine to the system caused cellular [Na^+] to increase, during the next 4min., by about 4m-equiv./l., so that it slightly exceeded extracellular [Na^+]. This occurred in parallel with the absorption of glycine. 3. The cellular K^+ content fell by an amount representing about 10% of the amount of Na^+ absorbed. 4. The results provide a clear demonstration that the flow of glycine into the cells is linked to a parallel movement of Na^+; K^+ appears to play a facultative role in the carrier system, whereas Li^+ is almost inert. 5. The effects produced by glycine were not reproduced by L-arabinose.

When preparations of mouse ascites-tumour cells were depleted of both ATP and cellular Na^+ and then suspended in a Ringer solution containing 1mM-glycine and 150m-equiv. of Na^+/l., the cells accumulated glycine at concentrations up to nine times the value in the extracellular phase. A series of such observations supported the view that the steady-state distribution of glycine between the cells and their environment was governed by the corresponding distribution of Na^+ and K^+ (Eddy, 1968*b*). An attempt was made in the present work (1) to demonstrate the converse relation and (2) to compare the effects of glycine and of L-arabinose on the system. For this purpose, the tumour cells were depleted of ATP while the cellular [Na^+] was brought to about 32m-equiv./l., in conditions where Li^+ largely replaced the original cellular content of K^+. The cells were then put with either 12mM-glycine, or 12mM-L-arabinose, in a solution containing 33m-equiv. of Na^+/l. and 131m-equiv. of Li^+/l. When glycine flowed into the cells, Na^+ started to accumulate there and, eventually, cellular [Na^+] exceeded extracellular [Na^+]; L-arabinose, which is probably not concentrated by the tumour cells (Kolber & Le Fevre, 1967), failed to produce this effect. The experiment is feasible because Li^+ is almost inert towards the glycine transport system (Eddy & Hogg, 1969).

MATERIALS AND METHODS

These were as described by Eddy (1968*a*) and Eddy & Hogg (1969). The alkali metal ions Na^+, K^+ and Li^+ were determined in an Eppendorf flame photometer (Eppendorf Gerätebau Netheler und Heinz G.m.b.H., Hamburg, Germany). Cl^- was determined polarographically at a half-wave potential of +0·325v by means of a model PO4 Polariter (Radiometer A/S, Copenhagen, Denmark). Dr J. Hodge of Wythenshawe Hospital kindly checked selected results of the Cl^- assay in an amperometric titration with Ag^+.

Replacement of cellular K^+ *by* Li^+ *during starvation in the presence of cyanide.* The tumour cells (about 12ml. packed volume) were collected and washed in the usual way except that the standard Ringer solution was replaced by one containing 32m-equiv. of Na^+/l., 131m-equiv. of Li^+/l. and no added K^+. After these operations (about 30min.), the cells were suspended in a similar Ringer solution containing 0·1mM-ouabain. The mixture (120ml.) was shaken for 20min. at 37°. The cells were collected and transferred to a similar solution (final vol. 121ml.) for a further 10min. at 37°. Then 2mM-NaCN was added, as a neutralized solution in the above Ringer solution (final vol. 125ml.). The suspension was shaken in a closed flask at 37° for 12min. and mixed, and three portions (40ml. each) were quickly transferred at 37° to identical closed conical flasks (100ml.) for 3min. further. The main part of the experiment was then started by three operators working in parallel. Each added one portion (40ml.) of the cell suspension at 37° to a flask (100ml.) containing 5μc of $^{24}Na^+$ and the Ringer solution. The Ringer solution in two of the flasks contained sufficient glycine or L-arabinose, as required, to give a 12mM solution finally. The third flask served as a control. Each operator then took seven samples, at standard intervals, during the next 6min. Each sample (5ml.) was collected in a weighed chilled centrifuge tube held in an ice bath. The samples were processed exactly as described by Eddy (1968*a*). As in the earlier work, the addition of $^{35}SO_4^{2-}$ served to mark the extracellular phase. The respective cellular dry weight (50–70mg.), cellular water and the corresponding contents of Na^+, K^+, Li^+, Cl^- and ninhydrin-positive amino acids

were assayed in each sample. The composition of the extracellular phase was similarly determined.

RESULTS

Preliminary work showed that when the tumour cells were kept in a Ringer solution containing 32m-equiv. of Na^+/l. and 131m-equiv. of Li^+/l. without added K^+, cellular [K^+] fell whereas cellular [Li^+] increased. The exchange was much faster in the presence of either 0·1mM-ouabain or of 2mM-sodium cyanide, presumably because the sodium pump was then unable to reabsorb the K^+ that had left the cells. In the procedure described in the Materials and Methods section, cellular [K^+] fell to about 10m-equiv./l. during about 45min., 2mM-sodium cyanide being present in the system during the last 15min. The glycine influx rate at 1mM-glycine then showed a marked dependence on [Na^+] despite the rise in cellular [Li^+]. The recommended procedure involves four related factors: (1) by analogy with previous work (Eddy & Hogg, 1969), the cells would eventually be depleted of ATP; (2) the sodium pump would stop; (3) cellular K^+ was largely, but not entirely, replaced by Li^+; (4) the respective Li^+, Na^+ and Cl^- contents of the cells reached fairly steady values (see below).

Comparison of the ionic movements induced by L-*arabinose and glycine.* After the preliminary treatments, referred to above, the preparation of tumour cells was divided into three portions, 12mM-glycine being added to one, 12mM-L-arabinose to the second, whereas the third served as a control. The upper part of Fig. 1 shows how cellular [Na^+] varied in three such experiments, in each of which the same trends were apparent. Whereas cellular [Na^+] was practically constant in the control (●), it eventually exceeded the control values by $3{\cdot}80 \pm 0{\cdot}37$m-equiv./l. (S.E.M.; $P < 0{\cdot}001$) in the last four sample pairs with glycine (■) and was smaller than the control values by $1{\cdot}42 \pm 0{\cdot}31$m-equiv./l. ($P = 0{\cdot}001$) in the last six sample pairs with L-arabinose (▲). When all the samples were considered from the three experiments, cellular [Na^+] for a given sample was significantly correlated with the respective amount of glycine the cells had absorbed ($r = 0{\cdot}794$, $n = 23$, $P < 0{\cdot}001$). The movements of the Na^+ and of glycine thus appeared to be closely linked. The point is reinforced by the circumstance that, in the presence of glycine, [Na^+] tended to become distinctly larger than the comparable values found not only in the control (32·5m-equiv./l.), but also (1) in the extracellular phase (33·5m-equiv./l.) and (2) in the presence of L-arabinose (31m-equiv./l.). L-Arabinose is known to penetrate the ascites-tumour cells, although without being concentrated in the cellular water relative to the extracellular phase (Kolber & Le Fevre, 1967). The lower part of Fig. 1 shows that changes in cellular [K^+], opposite in sign and about 20% of the magnitude of the changes in [Na^+], were induced by both glycine and L-arabinose.

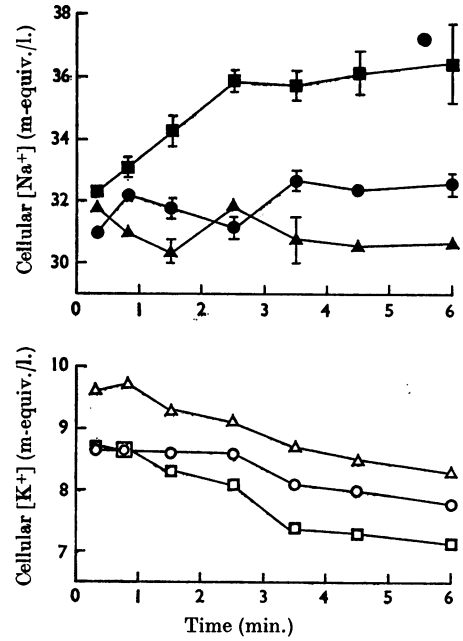

Fig. 1. Changes in cellular [Na^+] and cellular [K^+] after the addition of either 12mM-glycine or 12mM-L-arabinose to the system. The starved tumour cells were first equilibrated in a Ringer solution that eventually contained 33·5m-equiv. of Na^+/l., 131m-equiv. of Li^+/l. and about 1m-equiv. of K^+/l., 0·1mM-ouabain and 2mM-NaCN (see the Materials and Methods section). Glycine was added to one portion of the suspension, L-arabinose to another, and the third portion served as a control. Seven samples were taken from each portion at the indicated times and assayed for glycine, Na^+, K^+ etc. Observations from three experiments were pooled, in one of which arabinose was not tested. The mean values of cellular [Na^+] and [K^+] are shown, the bar in the upper diagram indicating the S.E.M., which in certain instances was smaller than the symbol. Arabinose present: ▲, [Na^+]; △, [K^+]. Glycine present: ■, [Na^+]; □, [K^+]. Control: ●, [Na^+]; ○, [K^+].

Stoicheiometry of the ion movements. The net movements of Na^+ induced by glycine and arabinose are shown in Fig. 2 as a function of time. They are compared with (*a*) the extra amounts of $^{24}Na^+$ retained by the samples and (*b*) the amount of glycine absorbed. As averaged over the last four sample pairs from each of the three experiments, the extra amount of K^+ displaced ($\pm$S.E.M.) during glycine absorption was $2{\cdot}7 \pm 0{\cdot}9$n-equiv./mg. dry wt. of cells ($P \simeq 0{\cdot}01$), or only about 10% of the extra Na^+ absorbed. Similarly a mean increase in

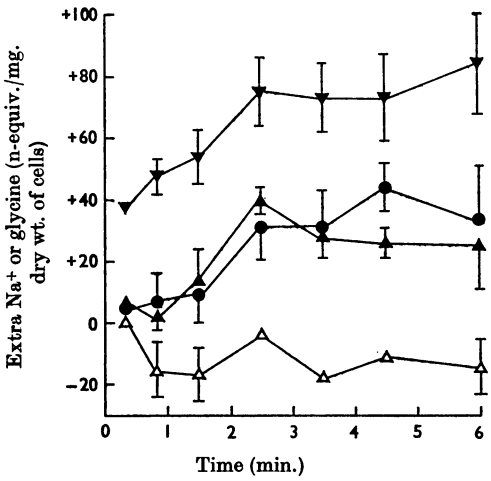

Fig. 2. Changes in the cellular contents of Na^+, glycine and $^{24}Na^+$, brought about by glycine or L-arabinose, in the three experiments illustrated in Fig. 1. The amount of each component in the test system minus the corresponding amount in the control are given, ± S.E.M. ▼, Cellular glycine. Na^+ increment: with glycine, ▲; with arabinose, △. $^{24}Na^+$ increment: with glycine, ●.

cellular K^+ content of 2·1 ± 0·5n-equiv./mg. ($P \simeq 0{\cdot}001$) accompanied the mean loss of 13·2 ± 2·3n-equiv. of Na^+/mg. ($P < 0{\cdot}001$) in the six sample pairs taken, in each of the two experiments, after the addition of L-arabinose. [K^+] was affected more because the cells appeared to lose water (see below).

Na^+ and glycine. Fig. 2 shows that the uptake of glycine reached a peak at very roughly the same time as the uptake of Na^+. Thus about 40n-equiv. of glycine/mg. were absorbed during the first 0·3min. and a further 30–40n-equiv./mg. during the next 2min., together with a roughly equivalent amount of Na^+. Hence the stoicheiometry of the associated fluxes during the slower process resembled the value of 0·90 ± 0·11 (5) found in earlier work (Eddy, 1968*a*) in which Li^+ was absent and extracellular [Na^+] was 150m-equiv./l. The rates of uptake of glycine with Na^+ were also comparable in the two series of experiments. The rapid initial absorption of glycine had not been detected previously, however, and remains unexplained. It can hardly be the result of gross cellular damage, as measurement of the extracellular space by means of $^{35}SO_4^{2-}$ ions gave values near 20% for each sample, whether glycine or arabinose was present or absent. Averaging of the four pairs of observations on the samples withdrawn after 2min. (Fig. 2) showed that the concentration of the absorbed glycine in the cellular water was then 13·3 ± 0·5mM, which is near 12mM, the concentration outside the cells. As the ratio cellular [Na^+]/extracellular [Na^+] was almost 1, and both cellular and extracellular [K^+] were relatively small, the eventual distribution of glycine was consistent with eqn. (1) of Eddy (1968*b*) based on the ion-gradient hypothesis. The initial rapid uptake of glycine may possibly have occurred by an Na^+-independent route. This might have happened during the initial shock to which the tumour cells were subjected when they were transferred to the hyperosmotic glycine solution. Alternatively, the exposure to cyanide and Li^+ may have damaged the glycine-carrier system in a certain fraction of the cells, so that the dependence on Na^+ was partially lost. Further evidence on this question is needed.

$^{24}Na^+$ uptake. The time-course followed the pattern observed previously at 150m-equiv. of Na^+/l. (Eddy, 1968*a*) in which initially a portion of the cellular Na^+ exchanged much more rapidly than the remainder. In the present work 100n-equiv./mg. of the cellular Na^+ content of 180n-equiv./mg. exchanged during 20sec. and a further 30n-equiv./mg. at a steady rate for the next 5·5min. (not shown). Fig. 2 shows that the absorption of $^{24}Na^+$ and the net movement of Na^+ took place at roughly the same rate. Once glycine absorption had reached a peak, entry and efflux of the amino acid via the carrier complex ENaGly (see Eddy, 1968*a*) would continue. Nevertheless, the presence of glycine after 2·5min. failed to stimulate the exchange between extracellular $^{24}Na^+$ and the remaining portion (about 70n-equiv./mg.) of the cellular Na^+, which exchanged slowly. The latter was probably physically segregated, therefore, from the extra Na^+ that entered with the glycine. Other observations showed that arabinose retarded the rapid initial rate of exchange with $^{24}Na^+$. Thus the Na^+ apparently lost from the cells after the addition of arabinose seemed to be mainly in the category that exchanged rapidly with $^{24}Na^+$. The work of Kolber & Le Fevre (1967) suggests that the tumour cells would have almost equilibrated with the added arabinose by about 1min.

Cellular Cl^-. The mean Cl^- content of the cells in the first three sample pairs (Fig. 2) increased by 0·4 ± 3·3n-equiv./mg. (S.E.M.) in the presence of glycine. It increased by 24 ± 9·9n-equiv./mg. ($P \simeq 0{\cdot}05$), or the equivalent of 4·2 ± 1·7m-osmoles/ml. of cellular water, in the last four sample pairs. Arabinose caused a mean loss of 26 ± 7n-equiv. of Cl^-/mg. in the seven sample pairs.

Cellular Li^+. Cellular [Li^+] was fairly constant in a given experiment, at a value that varied on different occasions from 140 to 180m-equiv./l. No significant trends were detected after the addition of either of the two substrates.

Cellular water. As averaged over the seven sample pairs from each of the two experiments, this fell in the presence of arabinose by 41 ± 4 µl./ml. of

cellular water, a value equivalent to the movement out of the cells of about 13m-osmoles of substrate/ml. of cellular water. If half of this were Cl^-, 37n-equiv. of the latter/mg. would leave the cells. The measured Cl^- loss in the seven sample pairs was possibly as large, being 26 ± 7n-equiv./mg. As the mean loss of Na^+ was only $11{\cdot}1 \pm 2{\cdot}7$n-equiv./mg. and the mean gain of K^+ was $2{\cdot}4 \pm 0{\cdot}4$n-equiv./mg., we must either suppose (1) that between about 20 and 30n-equiv. of Li^+/mg. left the cells, a quantity that would be difficult to detect, or (2) that arabinose penetrated only a fraction of the cellular water.

The mean increase in the cellular water content brought about by glycine in the last four sample pairs from each of the three experiments was 25 ± 7 μl./ml. of cellular water. This corresponds to the uptake of $8{\cdot}1 \pm 2{\cdot}3$m-osmoles of substrate/ml. of cellular water. As $5{\cdot}2 \pm 0{\cdot}5$m-osmoles of Na^+ were absorbed with the glycine and $0{\cdot}5 \pm 0{\cdot}1$m-osmole of K^+ was released, $4{\cdot}7 \pm 0{\cdot}5$m-osmoles of Cl^- would be required to maintain electroneutrality. The water movement expected on that basis corresponds to $9{\cdot}4 \pm 1{\cdot}0$m-osmoles, in rough agreement with the observed value, with the measured uptake of Cl^- and with previous observations (Eddy, 1968*a*).

DISCUSSION

Coupled movements of Na^+ *and glycine.* The present observations reinforce my previous conclusion (Eddy, 1968*a*) that, when the tumour cells absorb glycine, by the Na^+-dependent route, approx. 1 extra equiv. of Na^+ is simultaneously absorbed. The presence of cyanide ensured that the cellular ATP content was probably small in both series of experiments. In this series the action of the sodium pump was also restricted by ouabain. It seems unlikely that metabolism was involved in the transport of either Na^+ or of glycine in these circumstances (Eddy, 1968*a*,*b*; Eddy & Hogg, 1969). An unexplained aspect of the results is the rapid initial uptake of glycine into the cells that took place by the time the first set of samples was collected (Fig. 2). Possible interpretations were considered above. The subsequent slower uptake of glycine occurred at the rate expected on the basis of the earlier work.

The direct connexion between the movements of the Na^+ and of glycine itself is made specially clear by Figs. 1 and 2. When glycine was omitted cellular $[Na^+]$ was virtually constant and almost equalled extracellular $[Na^+]$. When glycine, as opposed to L-arabinose, was absorbed, cellular $[Na^+]$ rose steadily and eventually exceeded extracellular $[Na^+]$ by a small but significant margin. Thus the spontaneous movement of glycine into the cells tended to set up a concentration gradient of Na^+ in the opposite sense to the original gradient of glycine concentration. L-Arabinose, a compound that is not known to be concentrated by the tumour cells, produced no increase in the Na^+ content of the cells. On the contrary, the latter quantity fell slightly (Fig. 2), possibly in response to the osmotic stress caused by the addition of 12mM-arabinose.

The effect of glycine absorption on the cellular Na^+ content was to be expected on the basis of the particular form of the ion-gradient hypothesis of amino acid transport that appears to apply to the tumour-cell system (Eddy, 1968*a*,*b*). The hypothesis envisages that the Na^+ is a co-substrate (Riggs, Walker & Christensen, 1958) of a glycine-carrier system (E) that facilitates the simultaneous passage across the cell membrane of both ligands as the complex ENaGly. The species ENa and EGly each fail to traverse the membrane at a significant rate, though they are supposed to be formed at its two surfaces in accordance with the classical concepts of carrier kinetics. In other words, there is strict coupling between the movements of the two ligands through the carrier into the cell interior. Recent work suggests that the analogous coupling between the transport of Na^+ and 3-*O*-methylglucose in the rabbit ileum (Goldner, Schultz & Curran, 1969) is as strict as the coupling with glycine in the tumour-cell system, whereas transport of Na^+ and amino acids is less strictly coupled in the intestinal preparations (Curran, Schultz, Chez & Fuisz, 1967). The behaviour with glycine stands in sharp contrast with that with L-arabinose. The latter appears to penetrate the cells without interacting with Na^+ and, probably for that reason, without being concentrated there.

Glycine uptake was earlier shown to stimulate Na^+ uptake when both species were flowing down their respective concentration gradients into the tumour cells (Eddy, 1968*a*). The present observations provide a stricter test of the association between the fluxes because a negligible net uptake of the Na^+ into the cells occurred unless glycine was added. In the absence of glycine, of metabolic energy and, apparently, of significant net movements of other ionic species, cellular $[Na^+]$ was almost constant and equal to extracellular $[Na^+]$. Hence the system appeared to be near equilibrium with respect to the movement of Na^+. This was despite the indication, from the magnitude of the cellular $[Cl^-]$/extracellular $[Cl^-]$ ratio (about 0·6), that the intracellular electrical potential was slightly negative relative to the extracellular phase. Therefore, on the view that the Na^+ was initially equilibrated between the two phases, work was done when cellular $[Na^+]$ increased in the presence of glycine. The spontaneous movement of glycine into the cells presumably provided the energy required.

Facultative role of K^+. When cellular $[K^+]$ was

initially about 160m-equiv./l., the uptake of 1 equiv. of glycine expelled about 0·6 equiv. of K^+ from the cells (Eddy, 1968*a*). In the present work cellular [K^+] was near 10m-equiv./l. and only about 0·1equiv. of K^+ was expelled. Thus K^+ was probably not a necessary component of the system. It is emphasized that the facultative role of K^+ does not preclude a significant degree of coupling, through the carrier system, between the flow of glycine and that of K^+ (Eddy, 1968*a*,*b*; Eddy & Hogg, 1969).

The nature of the third ionic species involved in maintaining electroneutrality during the carrier cycle has not been established. In agreement with previous work (Eddy, 1968*a*), the study of the cellular water content indicated that when the cells took up glycine some other type of osmotically active particle besides Na^+ was also absorbed. The Cl^- assays indicate, but do not prove, that the particles in question may be Cl^-. Whether these interact directly with the carrier system remains an open question.

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