

A Net Gain of Sodium Ions and a Net Loss of Potassium Ions Accompanying the Uptake of Glycine by Mouse Ascites-Tumour Cells in the Presence of Sodium Cyanide

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1. The tumour cells were starved in a solution lacking Na^+ and then transferred to a Ringer solution containing 2mM-sodium cyanide, 150m-equiv. of Na^+ /l. and 10m-equiv. of K^+ /l. Such cells were depleted of ATP and contained an endogenous pool of various amino acids equivalent to a 26mM solution. 2. At 4min. after the transfer the cellular Na^+ content had increased by about 100% and roughly an equivalent amount of K^+ had left the cells. 3. Under these conditions [^{14}C]glycine was absorbed from an 11mM solution and reached the same cellular concentration by about 4min. The pool size increased by approximately the same amount (ΔGly), so glycine did not simply exchange with the endogenous components. 4. After 4min. with glycine, the cells contained about 20% more Na^+ (ΔNa^+) than the control and about 10% less K^+ (ΔK^+). The mean values of $\Delta\text{Na}^+/\Delta\text{Gly}$ and $\Delta\text{K}^+/\Delta\text{Gly}$ from five experiments were respectively 0.90 ± 0.11 and 0.62 ± 0.11 equiv./mole. 5. A further indication that these two ratios were not equal was that the cells absorbed more water than the movement of glycine itself required. The excess of water was osmotically equivalent to 0.95 ± 0.16 equiv. of solute/mole of glycine absorbed. 6. The variation of $\Delta\text{Na}^+/\Delta\text{Gly}$ with the duration of the incubation was consistent with the stimulated uptake of Na^+ being linked to the actual transport of glycine. The same may apply to the movement of K^+ , though the time-dependence was not examined in that case. 7. The observations were analysed in terms of a model in which both K^+ and Na^+ moved with a glycine-carrier system without ATP being involved. The analysis supported the idea that the spontaneous movements of the ions through the system might concentrate glycine in the cells significantly by purely physical means (Christensen's hypothesis).

The uptake of various amino acids by mouse ascites-tumour cells, respiring their endogenous nutrient reserves, resembles both amino acid and carbohydrate transport in several other mammalian systems in being greatly stimulated by the presence of extracellular Na^+ . The mechanism of the effect, however, is not understood (Heinz, 1967). According to Christensen's hypothesis (Riggs, Walker & Christensen, 1958; Crane, 1964; Vidaver, 1964) amino acid transport in these systems is essentially a physical process in which ATP and similar compounds do not participate directly. The spontaneous movement of Na^+ inwards or of K^+ outwards across the cell membrane is supposed to provide the energy necessary to concentrate the amino acid in the cells. A prediction that has not hitherto been examined experimentally is that glycine uptake should induce these ion movements when ATP is not available. A suitable test system was described by Eddy, Mulcahy & Thomson (1967), who found that preparations of the mouse

tumour cells starved for about 25min., in the presence of 2mM-sodium cyanide in a Ringer solution containing K^+ as the principal cation, had the following properties: (1) they took up glycine at a rate that depended on $[\text{Na}^+]$; (2) they appeared to be depleted of ATP; (3) they failed to accumulate K^+ rapidly, as though the sodium pump had almost stopped. When such cells were suspended in a Ringer solution containing 150m-equiv. of Na^+ /l. they accumulated Na^+ and lost K^+ . The observations described in the present paper demonstrate that both changes were faster in the presence of glycine. An attempt has been made to decide whether (1) the effect was simply a response to the water uptake that usually accompanies glycine uptake, (2) Na^+ and glycine (and, perhaps, K^+) associate with the same carrier to cross the cell membrane, as the above hypothesis requires, or (3) the mere presence, rather than the transport, of the amino acid altered the permeability of the cell membrane to Na^+ and K^+ .

MATERIALS AND METHODS

The assay of Na^+ and K^+ by flame photometry and the measurement of the radioactivity of samples containing [^{14}C]glycine were carried out as described by Eddy *et al.* (1967). Sodium as ^{24}Na was counted in solution by the procedure described for ^{42}K by Eddy *et al.* (1967). When ^{14}C was to be counted in the presence of ^{24}Na the samples were first kept at -10° for 9 days to allow the ^{24}Na to decay. No correction for the presence of ^{14}C in the assay of ^{24}Na was necessary. Sulphur as [^{35}S]sulphate was counted in a similar fashion to ^{14}C .

Determination of cellular amino acids. The method of Rosen (1957) was used. Mrs M. Pennington kindly assisted with these measurements.

Manipulation of the tumour cells. The standard Ringer solution (Umbreit, Burris & Stauffer, 1957) contained 155 m-equiv. of $\text{Na}^+/\text{l.}$, 8 m-equiv. of $\text{K}^+/\text{l.}$ and no added Ca^{2+} or glucose. The cells were harvested, washed and shaken in suspension in the standard Ringer solution in an open flask for 20 min. at 37° . The last step allowed the sodium pump to lower cellular [Na^+] to about 40 m-equiv./l. (Eddy *et al.* 1967). The tumour cells were then collected and dispersed in Na^+ -deficient Ringer solution (final concentrations about 10 m-equiv. of $\text{Na}^+/\text{l.}$ and 148 m-equiv. of $\text{K}^+/\text{l.}$) containing 2 mM-NaCN. The suspension was left in a stoppered flask at 37° for 20 min. to deplete cellular energy reserves. Cellular [Na^+] was then about 20 m-equiv./l.

Flux measurements. (1) Small-sample technique. This was based on the methods described by Eddy *et al.* (1967). The starved and depleted cells (50–100 mg.) were suspended in the standard Ringer solution (10 ml.) containing (a) 2 mM-NaCN; (b) an appropriate glycine concentration up to 12 mM with [^{14}C]glycine at approx. $0.3 \mu\text{C}/\text{ml.}$; (c) approx. $0.05 \mu\text{C}$ of $^{24}\text{NaCl}/\text{ml.}$ Portions (1 ml.) of the suspension were withdrawn at selected intervals up to 20 min. and mixed with ice-cold standard Ringer solution (7 ml.). The cells were collected by centrifugation and surplus liquid was allowed to drain away. They were then extracted with ethanol (2 ml.) overnight at 4° . The extract was assayed for glycine either as ^{14}C or by using the ninhydrin reagent. Alternatively, the cells were suspended in 0.01 N- HNO_3 (10 ml.) and assayed for ^{24}Na . When cellular Na^+ was to be determined, the original 1 ml. samples were mixed instead either (a) with an ice-cold Ringer solution (7 ml.) containing 158 m-equiv. of $\text{K}^+/\text{l.}$ and no Na^+ , or (b) with ice-cold buffered choline chloride solution (7 ml.) (Aull & Hempling, 1963). In either case the cells were recovered and washed once more with the respective cold solution (7 ml.). They were then suspended in 0.01 N- HNO_3 (10 ml.) for at least 3 hr. The suspension was centrifuged at 1000g for 10 min. and the supernatant solution analysed for Na^+ . In estimating both cellular glycine and cellular $^{24}\text{Na}^+$, allowance was made for the presence of these components in the extracellular phase (Eddy *et al.* 1967).

(2) Large-sample technique. The method was to determine the respective water, glycine, Na^+ and K^+ contents of a given cell sample that was sufficiently large both to be weighed and to contain a relatively large amount of cellular as opposed to extracellular water. The starved and depleted cells (80–100 mg. dry wt.) were transferred to a weighed centrifuge tube (about 16 g.), which was held at 37° for 1 min. Up to nine tubes were handled in parallel by initiating a series of incubations at 10 sec. intervals. A

suitable volume (8 ml.) of the standard Ringer solution at 37° , containing 2 mM-NaCN, 12 mM-glycine and, where appropriate, 6 mM-choline chloride, was added to the centrifuge tube. The cells were brought into suspension and the incubation was terminated after a known interval (up to 5 min.) by chilling the tube in an ice bath. One drop of 'carrier-free' [^{35}S]sulphate solution (about $3 \mu\text{C}$) was added. The solution was well mixed and the cells were separated by centrifugation twice as described by Eddy *et al.* (1967). The supernatant solution (A) was kept and the wet cell pellet immediately weighed. It was then dried at 80° for at least 24 hr. and weighed again. The dried pellet was suspended in 0.01 N- HNO_3 for 3 hr. The cell debris was separated by centrifugation at 1000g for 10 min. and the respective Na^+ , K^+ , glycine and ^{35}S contents of the supernatant solution (B) were assayed. The volume of extracellular water in each pellet was estimated from the relative amounts of ^{35}S in the pellet itself (solution B) and the corresponding supernatant solution A. Cellular water was computed as (loss of wt. on drying) – (extracellular water). Cellular dry weight was computed as (wt. of dried pellet) – (estimated wt. of extracellular salts). The respective amounts of Na^+ , K^+ and glycine in the cells were similarly computed.

KINETIC ANALYSIS

Eddy *et al.* (1967) considered a simplified version of the classical carrier model of facilitated substrate diffusion (Wilbrandt & Rosenberg, 1961) in which the total amount of carrier in the hypothetical outer-membrane phase was constant and the carrier, E, interacted with each of the three ligands Na^+ , K^+ and glycine. The initial rate at which glycine entered the cells was measured at various values of [Na^+], [K^+] and [Gly] and the simplified equations were used to determine the various dissociation constants characterizing the system (Table 2 of Eddy *et al.* 1967). It was assumed that: (1) glycine entered the cells exclusively as ENaGly ; (2) such complexes were in equilibrium with the free ligands; (3) K^+ and Na^+ competed for one binding site on E, whereas glycine was bound at another site. Only the two last assumptions are retained in the following more general treatment, which assumes that: (1) glycine crosses the cell membrane as ENaGly , EGly or EKGly ; (2) Na^+ crosses as ENaGly or ENa ; (3) K^+ crosses as EKGly or EK ; (4) E itself crosses the cell membrane. The question might also arise whether other ions (H^+ , OH^- , Cl^- or HCO_3^-) were involved in maintaining electro-neutrality. To simplify the discussion I shall ignore that possibility, however, in the first instance. The complete carrier cycle may then be defined by a series of equations representing the respective rates of movement of the various species. For instance, glycine moves as ENaGly at a rate $k^{\text{ng}}([\text{ENaGly}]_1 - [\text{ENaGly}]_2)$, where k^{ng} is a constant and the subscripts 1 and 2 denote respectively the outer-membrane and inner-membrane phases implicit in the hypothesis. Similarly Na^+ crosses the membrane as ENa at the rate $k^{\text{n}}([\text{ENa}]_1 - [\text{ENa}]_2)$. As there is no net movement of the carrier, the sum of such terms:

$$\begin{aligned} & k^{\text{n}}([\text{ENa}]_1 - [\text{ENa}]_2) + k^{\text{k}}([\text{EK}]_1 - [\text{EK}]_2) \\ & + k^{\text{c}}([\text{E}]_1 - [\text{E}]_2) + k^{\text{ng}}([\text{ENaGly}]_1 - [\text{ENaGly}]_2) \\ & + k^{\text{kg}}([\text{EKGly}]_1 - [\text{EKGly}]_2) + k^{\text{g}}([\text{EGly}]_1 - [\text{EGly}]_2) \\ & = 0 \end{aligned} \quad (1)$$

The six superscripts in eqn. (1) distinguish the six velocity constants for the movements of the respective carrier species in brackets.

Steady-state relationships. When glycine has reached a steady state:

$$k^{ng}([E\text{NaGly}]_1 - [E\text{NaGly}]_2) + k^{kg}([EK\text{Gly}]_1 - [EK\text{Gly}]_2) + k^g([EGly]_1 - [EGly]_2) = 0 \quad (2)$$

Let:

$$\begin{aligned} k_1 &= [E]_1[Na^+]_1/[E\text{Na}]_1, & k_2 &= [E]_1[K^+]_1/[EK]_1, \\ k_3 &= [E]_1[\text{Gly}]_1/[EGly]_1, & k_4 &= [E\text{Na}]_1[\text{Gly}]_1/[E\text{NaGly}]_1, \\ k_5 &= [EGly]_1[Na^+]_1/[E\text{NaGly}]_1, \\ k_6 &= [EK]_1[\text{Gly}]_1/[EK\text{Gly}]_1, \text{ and} \\ k_7 &= [EGly]_1[K^+]_1/[EK\text{Gly}]_1 \end{aligned} \quad (3)$$

Let the same set of dissociation constants apply in the inner-membrane phase. Substitution of eqns. (3) into both eqns. (1) and (2) gives:

$$\frac{[\text{Gly}]_2}{[\text{Gly}]_1} = \frac{k^n \cdot \frac{[Na^+]_2}{k_1} + k^k \cdot \frac{[K^+]_2}{k_2} + k^c \cdot k^{ng} \cdot \frac{[Na^+]_1}{k_1 k_4} + k^{kg} \cdot \frac{[K^+]_1}{k_2 k_6} + \frac{k^g}{k_3}}{k^n \cdot \frac{[Na^+]_1}{k_1} + k^k \cdot \frac{[K^+]_1}{k_2} + k^c \cdot k^{ng} \cdot \frac{[Na^+]_2}{k_1 k_4} + k^{kg} \cdot \frac{[K^+]_2}{k_2 k_6} + \frac{k^g}{k_3}} \quad (4)$$

A special case of this equation ($k^n = k^{kg} = k^k = 0$) may be obtained from an expression for the amino acid flux derived by Curran, Schultz, Chez & Fuisz (1967). Eqn. A7 of their paper, with $J_A = 0$ and the assumption of carrier symmetry, leads to the similar conclusion that $[\text{Gly}]_2/[\text{Gly}]_1$ is then independent of $[\text{Gly}]_1$ when the other variables are constant. The same result may be inferred from the geometry of Fig. 7 of Crane, Forstner & Eichholz (1965). The latter authors appear to hold the contrary view that the substrate ratio would be smaller the higher the substrate concentration.

Relative movements of Na^+ , K^+ and glycine (simplified case). Preparations of the starved tumour cells depleted of Na^+ are allowed to accumulate Na^+ for a few minutes, in exchange for cellular K^+ , from the standard Ringers solution containing NaCN. Glycine is added to one series of preparations, and another series serve as controls. The following quantities are measured: (1) the glycine uptake (ΔGly); (2) the extra uptake of Na^+ in the presence of glycine (ΔNa^+); (3) the corresponding uptake of K^+ (ΔK^+); (4) the differential water uptake ($\Delta\text{H}_2\text{O}$). The problem is to relate ΔGly , ΔK^+ etc. to the various parameters of eqn. (4). The system is not now in a steady state with respect to glycine. Consider the special case where $k^g = k^{kg} = 0$. Let e , the total amount of carrier, be defined by:

$$e = [E]_1 + [EGly]_1 + [E\text{Na}]_1 + [EK]_1 + [E\text{NaGly}]_1 + [EK\text{Gly}]_1 + [E]_2 + [EGly]_2 + [E\text{Na}]_2 + [EK]_2 + [E\text{NaGly}]_2 + [EK\text{Gly}]_2 \quad (5)$$

Combination with eqns. (3) gives:

$$e = [E]_1 \left(1 + \frac{[Na^+]_1}{k_1} + \frac{[K^+]_1}{k_2} + \frac{[\text{Gly}]_1}{k_3} + \frac{[Na^+]_1[\text{Gly}]_1}{k_1 k_4} + \frac{[K^+]_1[\text{Gly}]_1}{k_2 k_6} \right) + [E]_2 \left(1 + \frac{[Na^+]_2}{k_1} + \frac{[K^+]_2}{k_2} + \frac{[\text{Gly}]_2}{k_3} + \frac{[Na^+]_2[\text{Gly}]_2}{k_1 k_4} + \frac{[K^+]_2[\text{Gly}]_2}{k_2 k_6} \right) \quad (6)$$

Also, the counterpart of eqn. (1) applies, namely:

$$0 = [E]_1 \left(k^n \cdot \frac{[Na^+]_1}{k_1} + k^k \cdot \frac{[K^+]_1}{k_2} + k^c + k^{ng} \cdot \frac{[Na^+]_1[\text{Gly}]_1}{k_1 k_4} \right) - [E]_2 \left(k^n \cdot \frac{[Na^+]_2}{k_1} + k^k \cdot \frac{[K^+]_2}{k_2} + k^c + k^{ng} \cdot \frac{[Na^+]_2[\text{Gly}]_2}{k_1 k_4} \right) \quad (7)$$

Eqns. (6) and (7) can be solved for $[E]_1$ and $[E]_2$ when all the k values are known. The ion fluxes are then computed at selected values of $[Na^+]_1$, $[K^+]_1$, $[\text{Gly}]_1$ etc. Thus the net uptake rate of Na^+ , \bar{Na}^+_g , can be written as:

$$\bar{Na}^+_g = \frac{k^{ng}}{k^c} ([E\text{NaGly}]_1 - [E\text{NaGly}]_2) + \frac{k^n}{k^c} ([E\text{Na}]_1 - [E\text{Na}]_2)$$

With a similar notation:

$$\bar{K}^+_g = \frac{k^k}{k^c} ([EK]_1 - [EK]_2)$$

and:

$$\bar{\text{Gly}} = \frac{k^{ng}}{k^c} ([E\text{NaGly}]_1 - [E\text{NaGly}]_2)$$

Eqns. (6) and (7) also apply when $[\text{Gly}]_1 = [\text{Gly}]_2 = 0$ and then give the quantities \bar{Na}^+_{+0} and \bar{K}^+_{+0} .

The hypothesis accordingly predicts that:

$$\frac{\Delta\text{Na}^+}{\Delta\text{Gly}} = \frac{\bar{Na}^+_g - \bar{Na}^+_{+0}}{\bar{\text{Gly}}} \quad (8)$$

and also that:

$$\frac{\Delta\text{K}^+}{\Delta\text{Gly}} = \frac{\bar{K}^+_g - \bar{K}^+_{+0}}{\bar{\text{Gly}}} \quad (9)$$

Eqns. (8) and (9) would only hold when ΔGly , ΔK^+ and ΔNa^+ were a valid measure of the rates of movement of the respective species.

Other factors relevant to the application of eqns. (8) and (9).

(1) Work with C. Hogg (unpublished) supports the earlier suggestion (Eddy *et al.* 1967) that both k^g and k^{kg} of eqn. (4) were small enough to be neglected and that simple diffusion of glycine into the cells was relatively unimportant. In effect, glycine only entered the cells as ENaGly.

(2) The idea that the free carrier E was mobile is consistent with the fact that glycine efflux occurred as rapidly

at low values of $[Na^+]_1$ and $[K^+]_1$ as into the standard Ringer solution (Eddy *et al.* 1967).

(3) Under the conditions in which ΔNa^+ and ΔK^+ were measured (Table 2), $[K^+]_2$, $[Na^+]_2$ and $[Gly]_2$ varied with time. As the integrated forms of both eqns. (8) and (9) are rather complex, $[K^+]_2$, $[Na^+]_2$ and $[Gly]_2$ were assumed constant at the average values given in Table 3. Similarly, the observed values of ΔNa^+ , ΔK^+ and ΔGly were used as a measure of the differential rates defined by eqns. (8) and (9).

(4) Eqns. (4), (8) and (9) take no account of the, as yet unidentified, counter-ions that would be required to maintain electro-neutrality when unequal numbers of Na^+ and K^+ crossed the membrane in opposite directions. The effect of the counter-ion distribution on $[Gly]_2/[Gly]_1$ might be quite small, especially if either Cl^- or HCO_3^- were involved. The distribution of the latter ions between the cellular and extracellular phases might be expected to keep near an equilibrium determined by the electrical potential difference across the cell membrane.

(5) The effect of the membrane potential on the ionic activities in the two phases was ignored. So also was its effect on the movement of free E, an aspect related to factor (4) above.

RESULTS

Net uptake of glycine in the presence of cyanide. Eddy *et al.* (1967) found that when cellular $[Na^+]$ was about 50m-equiv./l. the starved tumour cells concentrated $[^{14}C]$ glycine about fivefold from a 0.8mM solution in the standard Ringer containing 2mM-sodium cyanide. In contrast, when cellular $[Na^+]$ was approx. 100m-equiv./l. glycine merely entered the cells without being concentrated. Further work has shown that, before being exposed to glycine, such cells contained a fairly constant amount of ninhydrin-positive material soluble in ethanol $[0.147 \pm 0.008 \text{ S.E.M. (17) } \mu\text{mole/mg. dry wt. of cells, calculated as glycine}]$. This is more than the amount of glycine assimilated under these conditions. The reactive compounds exhibited the chromatographic behaviour of the various common amino acids. They represent a $25.7 \pm 1.3 \text{ S.E.M. (17) mM}$ solution, if they are indeed uncombined and freely dissolved in the cellular water. The amino acids were detected in the freshly harvested tumour cells by Christensen & Riggs (1952).

Fig. 1(a) compares the two methods of measuring the glycine uptake. The starved tumour cells were placed in 8.2mM-glycine and 2mM-sodium cyanide. They eventually accumulated 57.0 $\mu\text{m-equiv.}$ of ninhydrin-positive material/mg. dry wt. of cells, more than the control without added glycine. Uptake of $[^{14}C]$ glycine followed a similar time-course and reached a maximum of 58.5 $\mu\text{m-equiv./mg.}$ (about 10 $\mu\text{equiv./ml.}$ of cell water). Fig. 1(b) shows that less than 10% of the total ninhydrin-positive material escaped from the control cells over the same period, a result that was confirmed in other similar experiments. The low rate of loss, together

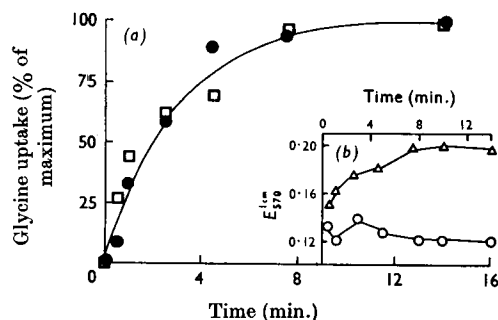


Fig. 1. Comparison of the net uptake of $[^{14}C]$ glycine at 37° and the change in the amino acid content of the cells assayed with the ninhydrin reagent. One portion of the starved cells was placed in 8.2mM-glycine in the standard Ringer containing 2mM-NaCN. The other portion served as a control and was kept without glycine. Both suspensions were sampled at intervals and the cells assayed for $[^{14}C]$ -glycine and for amino acid content (see the Materials and Methods section). The net uptake determined by both methods was the same within the experimental errors (see the text). Fig. 1(a) shows the progress of the uptake: \square , ninhydrin method; \bullet , isotopic method. Fig. 1(b) compares the extinctions (mean E_{270}^{1cm} from two determinations, corrected for the reagent blank) observed after samples (0.25ml.) of the ethanolic extract of the cells had been allowed to react with ninhydrin: Δ , glycine added; \circ , control.

with the fact that the net uptake of glycine equalled the uptake measured with ^{14}C (Figs. 1a and 1b), suggests that most of the glycine was not taken up by exchanging with the endogenous amino acids.

In an attempt to deplete the natural pool of amino acids, cellular $[Na^+]$ was allowed to increase to about 100m-equiv./l. while the tumour cells were suspended in a relatively large volume of the standard Ringer solution with 2mM-sodium cyanide for 30min. The additional cellular Na^+ did not stimulate the expected loss of the cellular amino acids to the environment. The same amount of ethanol-soluble material reacting with ninhydrin was present as in a control preparation kept at 155m-equiv. of K^+ /l. The increased ability to accumulate glycine from the standard Ringer-cyanide solution, which was observed at relatively low values of cellular $[Na^+]$ (Eddy *et al.* 1967), was not therefore simply due to changes in the amounts of the endogenous amino acids present in the system.

Effect of glycine on $^{24}Na^+$ uptake. The $^{24}Na^+$ entered the tumour cells more rapidly in the presence of 12mM-glycine than in the absence of the amino acid (Fig. 2). The influx of $^{24}Na^+$, in the absence of added glycine, was fast for about 2min. and slower for the next 4min. Similar behaviour was observed in two other experiments.

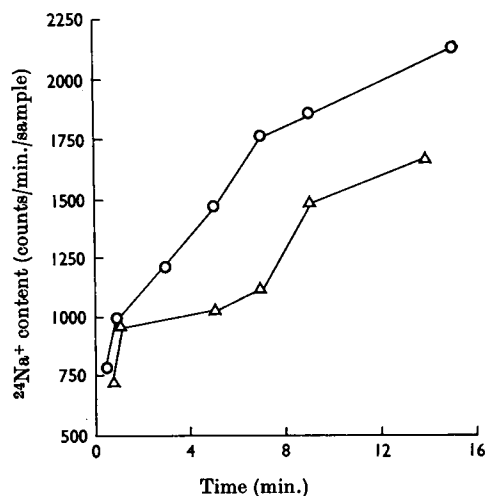


Fig. 2. Uptake of $^{24}\text{Na}^+$ as a function of time in the presence and absence of added glycine. The starved cells (about 6mg./ml.) were suspended in the standard Ringer solution at 37° containing about $0.05\mu\text{C}$ of $^{24}\text{Na}^+$ /ml. and 2mM-NaCN, either with 12mM-glycine (○) or without it (△). The small-sample technique was used (see the Materials and Methods section). Portions (1ml.) were withdrawn, processed and the cells assayed for $^{24}\text{Na}^+$. The radioactivity (about 500 counts/min./sample) due to the residual medium trapped in the cell pellet is included in the values illustrated.

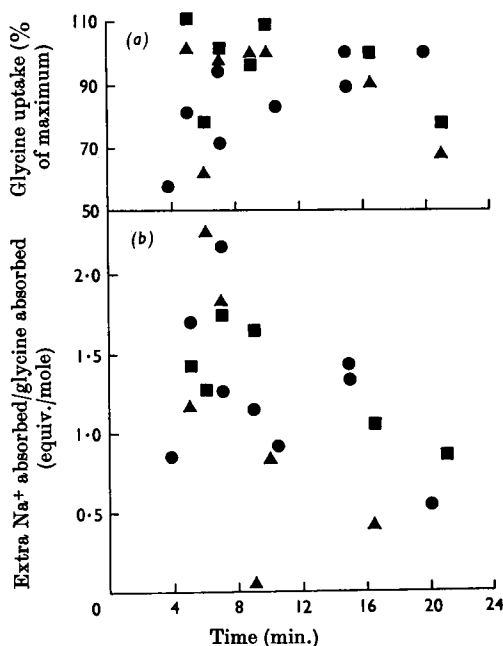


Fig. 4. Ratio of extra equivalents of Na^+ absorbed to moles of $[^{14}\text{C}]$ glycine absorbed ($\Delta\text{Na}^+/\Delta\text{Gly}$ measured with $^{24}\text{Na}^+$) as a function of the incubation time (b). The systems illustrated in Fig. 2 with $[\text{Gly}]$ 4mM, 8mM and 12mM respectively (△, ■ and ●) were sampled at various times. The corresponding uptake of glycine is shown in (a) as a percentage of the maximum uptake that occurred at that glycine concentration. The cellular $[^{14}\text{C}]$ glycine content usually decreased after about 15min. Similar values of ΔGly were found when glycine was assayed with the ninhydrin reagent.

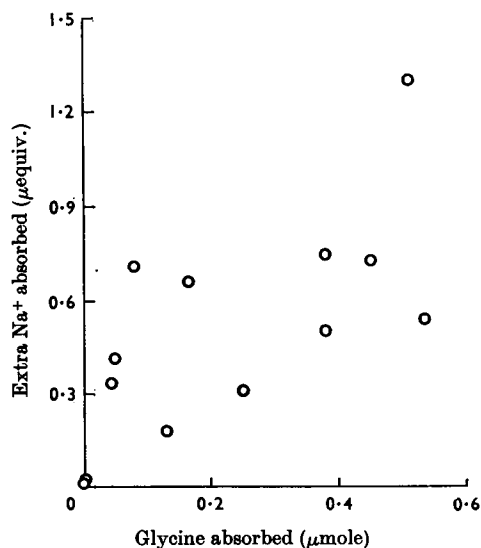


Fig. 3. Extra uptake of $^{24}\text{Na}^+$ at 6 min. as a function of the amount of $[^{14}\text{C}]$ glycine absorbed by the cells (approx. 6mg. dry wt.) from various extracellular concentrations (1mM-, 4mM-, 8mM- or 12mM-glycine) in the Ringer-cyanide solution. Conditions were as in Fig. 2.

(a) Variation with $[\text{Gly}]$. A series of measurements like those illustrated in Fig. 2 was made with $[\text{Gly}]$ at 1mM, 4mM, 8mM or 12mM. Glycine uptake at each value of $[\text{Gly}]$ almost reached a peak by 6 min., the height of the peak varying with $[\text{Gly}]$. Fig. 3 shows that the extra uptake of Na^+ , measured as $^{24}\text{Na}^+$, tended to increase with the amount of glycine entering the cells. The linear correlation coefficient was 0.698 ($P \approx 0.01$). The slope of the linear regression line of the extra uptake of Na^+ on glycine uptake was 1.24 ± 0.38 (s.e.m.), so the stoichiometry of the relationship was rather uncertain. In any case, the additional uptake of $^{24}\text{Na}^+$ was correlated both with the glycine uptake and with $[\text{Gly}]$ itself. The presence of glycine, either inside or outside the cells, rather than the transport of the amino acid, accordingly might be the factor governing the influx of Na^+ .

(b) Variation with time. Some light on the latter problem was thrown by an analysis of the ratio

(extra $^{24}\text{Na}^+$ uptake/glycine uptake) at various times. [Gly] was either 4mM, 8mM or 12mM. Fig. 4(a) shows that by about 6min. at least 75% of the eventual uptake had occurred. Fig. 4(b) shows how the above ratio varied up to about 20min. The negative correlation coefficient with respect to time was 0.455; the correlation is just significant ($P < 0.05$). If the mere presence of glycine, either inside or outside the cells, stimulated the uptake of Na^+ the ratio (extra $^{24}\text{Na}^+$ uptake/glycine uptake) would increase once glycine had stopped accumulating. The opposite trend was in fact observed, so there are no grounds for accepting that interpretation. The results are consistent with the alternative that a component of the Na^+ influx was closely associated with the glycine influx.

The apparent decrease in the ratio (extra $^{24}\text{Na}^+$ uptake/glycine uptake) with time may (1) be fortuitous. If the effect is real, however, it may mean (2) that a fixed amount of $^{24}\text{Na}^+$ was eventually taken up both in the presence and the absence of glycine. A different interpretation (3) is that the influx of Na^+ , determined as $^{24}\text{Na}^+$ and occurring via the complex ENaGly discussed in the Kinetic Analysis section, may have initially exceeded the net uptake of Na^+ through the same system, owing to the recycling of both ligands. On that view, once the isotope had equilibrated with the cells, the uptake calculated from the cellular $^{24}\text{Na}^+$ content would equal the net Na^+ uptake. The

results do not clearly distinguish between these three possibilities.

Effect of glycine on the net uptake of Na^+ , K^+ and water. (a) Na^+ uptake. The observations shown in Table 1 demonstrate that, on nine out of ten occasions, the addition of glycine increased the cellular Na^+ content by a small amount as compared with the controls without added glycine. The mean value \pm s.e.m. of $\Delta\text{Na}^+/\Delta\text{Gly}$ (the Na^+ increment/mole of glycine absorbed), when [Gly] was 3mM or greater, was 0.837 ± 0.154 (9) equiv./mole, which differs significantly from zero ($P < 0.001$, Student's *t* test).

In the last three experiments illustrated in Table 1, a number of samples were taken at different times. $\Delta\text{Na}^+/\Delta\text{Gly} \pm$ s.e.m. was 0.89 ± 0.73 (2), 1.24 ± 0.34 (5), 1.68 ± 0.25 (3) and 1.68 ± 0.12 (2) at 3, 6, 10 and 17min. respectively. The apparent failure of $\Delta\text{Na}^+/\Delta\text{Gly}$ to increase significantly after the first 5min., when most of the glycine was absorbed, is consistent with the view expressed above that the extra Na^+ entered the cells with the glycine.

Cellular Na^+ was also determined as the difference between total Na^+ and extracellular Na^+ rather than directly on washed cell samples as was done for Table 1 (column 1, Table 2). [Gly] was about 11mM and both cellular K^+ and cellular water content were determined as well. Comparison of Tables 1 and 2 shows that the two methods in fact led to rather similar estimates of the mean $\Delta\text{Na}^+/\Delta\text{Gly}$.

Table 1. *Extra Na^+ found in tumour cells that had taken up a certain amount of glycine*

The starved tumour cells were suspended for a fixed period (4 to 7 min., depending on the experiment) with 2mM-NaCN in the standard Ringer solution. Extracellular [Gly] was zero (the controls) or as shown below. The small-sample technique was used (see the Materials and Methods section). The cells were washed with a Ringer solution containing 158m-equiv. of $\text{K}^+/\text{l.}$ in Expts. 1, 2 and 3. They were washed with choline chloride solution in Expts. 4, 5 and 6 (see the Materials and Methods section) and the cellular ion content was assumed to be constant at $1.17 \mu\text{equiv./mg. dry wt. of cells}$ (cf. Eddy *et al.* 1967 and Table 2). Glycine was determined as ^{14}C . ΔNa^+ was the mean cellular Na^+ content/mg. dry wt. of cells in the system with glycine, at the end of the incubation, less the corresponding Na^+ content of the controls. These were initiated and incubated in parallel. Each experiment was set up in duplicate and the results were averaged. The Na^+ content of a given cell extract was determined on three separate occasions and the results were averaged.

Expt. no.	[Gly] (mM)	$10^2 \times \Delta\text{Gly}$ ($\mu\text{moles/mg. dry wt. of cells}$)	$10^2 \times \Delta\text{Na}^+$ ($\mu\text{equiv./mg. dry wt. of cells}$)	Na^+ as % of cellular Na^+	$\Delta\text{Na}^+/\Delta\text{Gly}$
1	3.0	3.33	3.51	14	1.05
	8.1	6.48	6.42	26	0.99
2	1.0	1.66	0.00	0	0.00
	3.8	4.64	2.50	15	0.52
	12.2	8.49	4.60	28	0.54
3	12.2	9.90	0.90	4	0.10
	12.2	7.30	2.80	13	0.38
4	9.3	4.54	5.92	14	1.30
5	10.5	9.58	12.51	27	1.30
6	10.1	6.74	9.17	18	1.36

Table 2. *Effect of glycine on relative changes in the content of cellular Na⁺, K⁺ and water*

The starved tumour cells were suspended for a fixed period (3 to 5 min. depending on the experiment) with 2 mM-NaCN in the standard Ringer solution. The large-sample technique was used (see the Materials and Methods section). ΔNa^+ (\pm S.E.M.) was the cellular Na⁺ content/mg. dry wt. of cells in the test system (with the specific additions) minus the Na⁺ content of the cells in the controls. The cells were sampled at the end of the incubation. ΔK^+ , $\Delta\text{H}_2\text{O}$ and ΔGly were similarly defined. ΔGly was determined with the ninhydrin reagent. $\Delta\text{H}_2\text{O}$ was computed relative to the cellular water content of the controls. As the differences to be detected were small the cell suspensions were prepared in triplicate in a given experiment. The mean results from the stated number of independent experiments were then averaged. Likewise, the ion assays on a given solution were repeated on three occasions and the results averaged.

Addition	11 mM-Glycine	6 mM-Choline chloride	11 mM-Glycine+ 6 mM-choline chloride
No. of expts.	5	3	5
ΔNa^+ (m μ equiv./mg.)	75.1 \pm 8.9	-20.5 \pm 10.9	13.4 \pm 11.7
ΔK^+ (m μ equiv./mg.)	-52.7 \pm 9.2	-6.4 \pm 2.0	-87.6 \pm 24.0
ΔGly (m μ moles/mg.)	84.7 \pm 5.7		60.2 \pm 3.8
$\Delta\text{Na}^+/\Delta\text{Gly}$	0.898 \pm 0.112		0.214 \pm 0.220
$\Delta\text{K}^+/\Delta\text{Gly}$	-0.622 \pm 0.109		-1.534 \pm 0.498
$\Delta\text{H}_2\text{O}$ (μ l./ml. of cell water)	50 \pm 7	-34 \pm 5	-76 \pm 8

ΔGly and its S.E.M. A more detailed analysis of the original observations suggested the following conclusions: (1) the variation in the behaviour of replicate cell suspensions had a bigger effect on the S.E.M. than the errors involved in assaying Na⁺, K⁺ and glycine; (2) the presence of glycine caused no systematic errors in the determinations of either (a) the extracellular water, or (b) the cellular dry wt. Such errors would affect each of the ratios in Table 2.

(b) K⁺ uptake. Table 2 (column 1) further shows that glycine caused K⁺ to be displaced from the cells ($P < 0.005$). The mean value of $\Delta\text{K}^+/\Delta\text{Gly}$ did not differ significantly from the mean value of $\Delta\text{Na}^+/\Delta\text{Gly}$ ($P > 0.1$).

(c) Water uptake. The tumour cells with 11 mM-glycine contained more water than the control suspensions without glycine ($P < 0.001$). The extra water, 0.050 ± 0.007 ml./ml. of cellular water in the controls, corresponded to the movement of 16.35 ± 2.29 (S.E.M.) (5) osmotic equivalents of substrate/ml. of cellular water. The mean difference between the cellular and the extracellular values of [Gly] was 3.00 ± 0.34 (5) mM. The remainder, 13.35 ± 2.31 osmotic equivalents ($P < 0.001$), corresponded to $\Delta\text{H}_2\text{O}/\Delta\text{Gly}$ being 0.946 ± 0.164 (5) osmotic equivalent/mole of glycine absorbed.

(d) Role of osmotic factors. Christensen & Riggs (1952) were the first to observe that cellular [Na⁺] tended to increase and cellular [K⁺] to decrease when glycine was absorbed by the respiring tumour cells. Tryptophan was later shown to induce similar ion movements. These appeared to follow, rather than to accompany, the uptake of the amino acid (Table 8 of Riggs, Coyne & Christensen, 1954), which might mean that the net ion movements were due to the cells swelling (cf. Schultz & Zalusky, 1964). In the present work, the ionic composition

of the cells was accordingly examined after the expulsion of cellular water brought about by the addition of 6 mM-choline chloride. The latter increased the osmotic pressure to about the same extent as glycine initially increased it (column 1, Table 2). Table 2 (column 2) shows that the cells then lost roughly the expected amount of water relative to the controls without choline chloride. Small amounts of Na⁺ and K⁺ were apparently also displaced. It is clear that the effect of glycine on the ion movements was not simply a response to the initial osmotic gradient. When glycine was taken up in the presence of 6 mM-choline chloride (column 3, Table 2) the cells lost about twice as much water as they did with choline chloride alone. The explanation is not known. A significant decrease in cellular K⁺ ($P < 0.05$) took place relative to the controls. Also, the Na⁺ content increased slightly, though not significantly. Thus the presence of choline chloride, which reversed the direction of the net movement of water relative to the controls, appeared to lower $\Delta\text{Na}^+/\Delta\text{Gly}$ ($P \approx 0.025$). This was perhaps partly because choline chloride displaced Na⁺ from the cells (column 2).

At least three factors were expected to influence $\Delta\text{H}_2\text{O}$: (1) cellular [Gly] was usually about 2 mM larger than the value outside, but only a small fraction of $\Delta\text{H}_2\text{O}$ is accounted for on that basis; (2) some of the tubes contained choline chloride; (3) a net uptake of Na⁺ and a net loss of K⁺ occurred. The water uptake, $\Delta\text{H}_2\text{O}^*$, due to the last component was computed as that part of $\Delta\text{H}_2\text{O}$ not due to the other two factors. The osmotic equivalent of $\Delta\text{H}_2\text{O}^*$ in μ equiv./ml. of cellular water in the controls was then plotted against the corresponding value of $\Delta\text{Na}^+ + \Delta\text{K}^+$ expressed in similar units. Fig. 5 shows that these two quantities were

roughly linearly related ($r=0.880$; $P=0.001$). As ΔNa^+ and ΔK^+ separately showed less correlation with $\Delta\text{H}_2\text{O}^*$, the results in Fig. 5 appear to support the following conclusions: (1) the system was

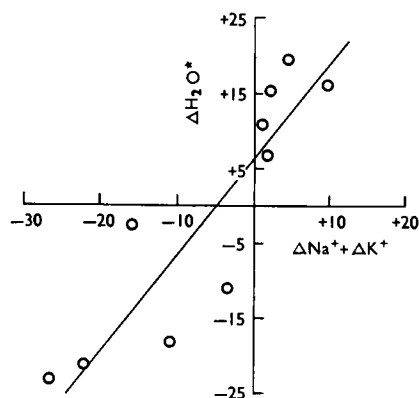


Fig. 5. Variation of $\Delta\text{H}_2\text{O}^*$ with $\Delta\text{Na}^+ + \Delta\text{K}^+$ (see the text) (the relation of the ion movements to the movement of water). $\Delta\text{H}_2\text{O}^*$ ($\mu\text{equiv. of solute/ml. of cellular water}$) was computed as the extra water found in the cells when glycine was taken up less the expected water movement due both to (a) the small glycine gradient and, where appropriate, (b) the presence of choline chloride. $\Delta\text{Na}^+ + \Delta\text{K}^+$ ($\mu\text{equiv./ml. of cellular water}$) represented the additional net movement of cations stimulated by glycine. Both quantities were expressed as osmotic equivalents of solute apparently entering unit volume of the cellular water in the controls (see the text).

probably near osmotic equilibrium; (2) the net movements of Na^+ and K^+ were not necessarily stoichiometrically related; (3) the ion movements were not induced by water entering the cells under the influence of the small glycine concentration gradient.

The slope of the regression line of $\Delta\text{H}_2\text{O}^*$ on $\Delta\text{Na}^+ + \Delta\text{K}^+$ was about 1 (1.194 ± 0.228). The slope would be 1.0 if no osmotically active particle other than glycine moved with Na^+ and K^+ . Another ion would presumably be involved in order to maintain electro-neutrality. In a buffered system, either H^+ or OH^- might serve that role without affecting the slope. Further examination raised the question whether it was justifiable to combine the observations from columns 1 and 3 of Table 2. (1) The mean value of $\Delta\text{H}_2\text{O}^*$ from the five determinations in the absence of choline chloride corresponded to a slope of at least 2, whereas the mean value from all the ten observations was nearer 1; the larger slope would be expected when either Cl^- or HCO_3^- served as the counter-ions. (2) The regression line in Fig. 5 failed to pass through the origin ($P=0.05$). These difficulties, which appear to involve the hypothetical counter-ion, remain unresolved.

Estimates of the relative velocity coefficients. The observed values of $\Delta\text{Na}^+/\Delta\text{Gly}$ and $\Delta\text{K}^+/\Delta\text{Gly}$ (column 2, Table 2) were arbitrarily assumed to range from 0.7 to 1.0 and from 0.4 to 0.85 respectively. Table 3 shows how $\Delta\text{Na}^+/\Delta\text{Gly}$ and $\Delta\text{K}^+/\Delta\text{Gly}$ computed from eqns. (8) and (9) varied with the relative magnitudes of k^n/k^c , k^k/k^c and k^{ng}/k^c

Table 3. Trial solutions of eqns. (8) and (9) for comparison with data in Table 2

The relative velocity coefficients were assigned the values shown below. $\Delta\text{Na}^+/\Delta\text{Gly}$ and $\Delta\text{K}^+/\Delta\text{Gly}$ were then computed at the following average values of the other variables: $[\text{Na}^+]_1$, 150m-equiv./l.; $[\text{Na}^+]_2$, 81m-equiv./l.; $[\text{K}^+]_1$, 10m-equiv./l.; $[\text{K}^+]_2$, 110m-equiv./l.; $[\text{Gly}]_1$, 11mm; $[\text{Gly}]_2$, 5.5mm. The various dissociation constants were assumed to have the values shown in Table 2 of Eddy *et al.* (1967).

Case no.	k^n/k^c	k^k/k^c	k^{ng}/k^c	$\Delta\text{Na}^+/\Delta\text{Gly}$ (equiv./mole)	$\Delta\text{K}^+/\Delta\text{Gly}$ (equiv./mole)	$[\text{Gly}]_2/[\text{Gly}]_1$
1	1	1	1	-0.358	+0.511	3.8
2	1	1	20	-0.036	+0.213	3.8
3	1	3	20	+0.116	+0.085	7.5
4	0.3	1	1	+0.247	+0.264	7.2
5	0.3	3	20	+0.583	-0.226	14.5
6	0.3	3	1	+0.405	+0.062	14.5
7	0.3	20	1	+0.665	-0.391	44.8
8	0.1	3	20	+0.836	-0.399	20.6
9	0.1	1	1	+0.683	+0.083	10.4
10	0.1	1	0.05	-0.393	+2.789	10.4
11	0	3	20	+1.000	-0.512	26.2
12	0	20	1	+1.000	-0.711	59.2
13	0	3	0.05	+1.000	+2.563	26.2
14	0	1	1	+1.000	-0.059	13.7
15	0	20	20	+1.000	-0.890	59.2
16	0	3	1	+1.000	-0.366	26.2
17	0	1	20	+1.000	-0.163	13.7
18	0	20	0.05	+1.000	+3.188	59.2

at the selected values of $[\text{Na}^+]_1$, $[\text{Na}^+]_2$ etc. It should be noted how the stoichiometrical ratios changed sign in certain cases. For instance, glycine stimulated a net loss of Na^+ and a gain of K^+ relative to the control in case 1, whereas the reverse situation held in case 8. Inspection of Table 3 shows that the two ratios were in the observed ranges when, very approximately, $k^n/k^c < 0.3$, $k^k/k^c > 3$ and $k^{ng}/k^c \leq 1$.

Predictions from eqn. (4). Eqn. (4) reduces to the following relatively simple form when $k^{kg} = k^g = 0$:

$$\frac{[\text{Gly}]_2}{[\text{Gly}]_1} = \frac{[\text{Na}^+]_1}{[\text{Na}^+]_2} \cdot \frac{1 + \frac{k^n}{k^c} \cdot \frac{[\text{Na}^+]_2}{k_1} + \frac{k^k}{k^c} \cdot \frac{[\text{K}^+]_2}{k_2}}{1 + \frac{k^n}{k^c} \cdot \frac{[\text{Na}^+]_1}{k_1} + \frac{k^k}{k^c} \cdot \frac{[\text{K}^+]_1}{k_2}} \quad (10)$$

The predicted glycine concentration ratio is then independent of the values both of k^{ng} and $[\text{Gly}]_1$ when the other variables are constant. Also, when $k^k \gg$ both k^n and k^c :

$$\frac{[\text{Gly}]_2}{[\text{Gly}]_1} = \frac{[\text{Na}^+]_1}{[\text{Na}^+]_2} \cdot \frac{[\text{K}^+]_2}{[\text{K}^+]_1}$$

$\Delta\text{Na}^+/\Delta\text{Gly}$ and $\Delta\text{K}^+/\Delta\text{Gly}$ are then both 1.

A situation of special interest entailed suspending the respiring cells in a Ringer solution with $[\text{Na}^+]_1$ 150m-equiv./l., $[\text{K}^+]_1$ 10m-equiv./l. and $[\text{Gly}]_1$ 0.9mm. A series of observations showed that $[\text{Na}^+]_2$ became steady at about 30m-equiv./l., with $[\text{K}^+]_2$ about 160m-equiv./l. $[\text{Gly}]_2/[\text{Gly}]_1$ was about 25. The last column in Table 3 shows how $[\text{Gly}]_2/[\text{Gly}]_1$ as computed from eqn. (10) varied with the magnitudes of the relative velocity coefficients. Clearly, quite substantial gradients of glycine concentration were predicted when these coefficients had the values deduced above. The values of k^k/k^c at which $[\text{Gly}]_2/[\text{Gly}]_1$ was 25 when k^n/k^c was either (a) zero or (b) 0.3 were also computed. They were respectively 2.8 and 6.7.

The result of applying Christensen's hypothesis to the data in Table 2 may be summarized as follows: (1) if the carrier moved with both ions $[\text{Gly}]_2/[\text{Gly}]_1$ during respiration in the standard Ringer would be at least about 10 and perhaps larger than the ratio observed; (2) if only Na^+ and not K^+ were associated directly with the actual transport of glycine $[\text{Gly}]_2/[\text{Gly}]_1$ would probably not be greater than 5 and might be smaller. The predicted ratio would be no larger if E2NaGly , rather than ENaGly , were assumed to be involved, for Na^+ would then leak faster through the system when $\Delta\text{Na}^+/\Delta\text{Gly}$ was near 1.

DISCUSSION

Origin of the effects of glycine on the ion movements. The above observations provide a convincing

demonstration that, in the presence of cyanide, glycine stimulated both the net uptake of Na^+ and the net loss of K^+ by the tumour cells. Various interpretations may be suggested in the light of the recent literature.

(1) Eddy *et al.* (1967) showed that cyanide greatly restricted the formation of ATP under these conditions. Glycine transport might then compete with the sodium pump for the limited amount of ATP available, relatively less Na^+ being expelled from the cells during glycine transport (cf. Newey & Smyth, 1964). There are two reasons for rejecting that interpretation: (a) the postulated competition would be expected to affect Na^+ efflux rather than Na^+ influx, whereas in fact both Na^+ influx (Fig. 2) and the net uptake of Na^+ (Tables 1 and 2) were larger with glycine present; (b) the uptake of glycine, in the conditions described in Table 2, was not inhibited by various compounds that would have further depleted the cells of ATP (Eddy *et al.* 1967). Thus ATP was probably not involved directly and the postulated competition seems unlikely. It will be observed that the direction of the ion gradients across the cell membrane would have permitted the movements of both ions to be stimulated by glycine without ATP being involved.

(2) The ion movements were not associated with glycine entry as such, but with the subsequent movement of water. A similar problem was raised by Schultz & Zalusky (1964) in relation to glucose absorption in the rabbit ileum. As the glycine concentration gradient in the present experiments was eventually quite small, the point at issue is whether the water entered the cells mainly because unequal amounts of Na^+ and K^+ crossed the cell membrane. Alternatively, the flow of water might have induced the ion movements. The latter possibility applied to the observations in column 1 of Table 2 requires that an uptake of water that stimulated the entry of Na^+ would also stimulate K^+ efflux. Such behaviour seems unlikely and was not indicated by the result of adding choline chloride to the system (Table 2, column 2). It seems much more likely that the ion movements governed the corresponding water movements rather than the converse.

(3) The presence of glycine rather than its transport into the cell stimulated the uptake of Na^+ . An argument against this possibility was considered in connexion with Fig. 4 and Table 1. Further evidence seems desirable. The similar problem that arises with K^+ was not resolved experimentally and also needs examining.

(4) Na^+ influx and K^+ efflux were both coupled to glycine influx. All the observations appear to be consistent with that interpretation. The stoichiometry is defined by eqns. (8) and (9). As Barry,

Smyth & Wright (1965) have done for the relation of Na^+ transfer to hexose transfer in the rat jejunum, it is important to ask whether the results support the notion of a fixed relationship between the ion and the amino acid fluxes that is implicit in the carrier model.

Carrier stoichiometry problem. A rigorous analysis is ruled out by the magnitude of the experimental errors. These stem from the fact that Na^+ uptake, for instance, was usually stimulated no more than about 20% by glycine. A clear distinction should be made between (1) the ratio of Na^+ to glycine in the carrier complex (1 for ENaGly) and (2) the apparent stoichiometry of the associated fluxes ($\Delta\text{Na}^+/\Delta\text{Gly}$), a complex quantity defined by eqn. (8). Table 3 shows that $\Delta\text{Na}^+/\Delta\text{Gly} \leq 1$ and may even be negative when glycine enters the cells exclusively as ENaGly . The ratio might conceivably exceed 1 in the more complex situation where EGly was also mobile and $[\text{Gly}]_2 > [\text{Gly}]_1$. ΔNa^+ depends, not simply on the Na^+ flux through the carrier, but rather on the difference between the number of Na^+ ions passing through the carrier in the presence and in the absence of glycine. Only when k^a/k^c is relatively small and Na^+ cannot leak through the system in the absence of glycine does $\Delta\text{Na}^+/\Delta\text{Gly}$ provide a direct measure of the composition of the carrier complex. The application of eqn. (8) involved a number of other assumptions and approximations outlined in the Kinetic Analysis section. The same assumptions might not apply, of course, either to the behaviour of the tumour cells with other amino acids or to glycine transport in other tissues.

The influx ratio defined by $\Delta^{24}\text{Na}^+/\Delta\text{Gly}$ is influenced by similar factors. It might exceed the true carrier stoichiometrical ratio if glycine recycled through the system and only the net uptake of glycine were recorded (see Fig. 4 and the text.)

In attempting to explain the Na^+ -dependent exchange processes that take place between cellular and extracellular amino acids in pigeon erythrocytes, Wheeler & Christensen (1967) put forward arguments that imply that the uptake ratio based on $\Delta^{24}\text{Na}^+/\Delta\text{Gly}$ might be greater than 1 for another reason. In their scheme: (a) the translocation of the substrate S as NaES is not the rate-limiting step, as was assumed for eqns. (8) and (9); (b) S and Na^+ are translocated exclusively as NaES . Wheeler & Christensen (1967) point out that condition (a) 'removes the necessity for the stoichiometry of the associated fluxes of Na^+ and the amino acids to correspond to the composition of the ternary complex'. Though this factor would not be involved in the measurements of the net displacements of Na^+ and glycine shown in Tables 1 and 2, it complicates the interpretation of the measure-

ments with $^{24}\text{Na}^+$. No difficulties arise in this connexion from the use of $[^{14}\text{C}]\text{glycine}$, which seemed to be absorbed without exchanging with the endogenous amino acids (Fig. 1). The merits of these rival assumptions about the rate-limiting steps need further examination.

Previous attempts to determine the carrier stoichiometrical ratio for Na^+ were made with pigeon erythrocytes (E2NaGly ; Vidaver, 1964; Wheeler & Christensen, 1967) and the rabbit ileum (ENaAla ; Curran *et al.* 1967). In each case the conditions were such that ATP might have been involved in the ion movements induced by the amino acid. Also, it was assumed that Na^+ did not leak through the amino acid carrier when the amino acid was omitted. Table 3 shows that this assumption was approximately, though not perhaps exactly, true for the ascites cells when the carrier complex was ENaGly . Of course, E2NaGly might function with an appropriately larger rate of leakage of Na^+ in the controls. The entry kinetics for the ascites system provide no evidence, however, for the involvement of E2NaGly (Inui & Christensen, 1966; Eddy *et al.* 1967), though such a complex may be involved in the pigeon erythrocytes. The analysis based on Table 3 indicated that leakage of K^+ in the controls was not negligible. The relative rates of glycine uptake and of the turnover of K^+ by the tumour cells (Eddy *et al.* 1967) mean that an appreciable fraction of the K^+ leaving the cells in the absence of glycine probably passed through E.

Wheeler & Christensen (1967) observed that whereas Na^+ entered preparations of pigeon erythrocytes more rapidly when glycine was present, cellular $[\text{Na}^+]$ did not increase because Na^+ efflux was also stimulated. Cellular $[\text{K}^+]$ was also constant. Depending on the conditions, a variable fraction of the glycine appeared to be absorbed by exchanging with the endogenous amino acids. This phenomenon did not occur in the present work, though a large pool of endogenous amino acids was maintained in the tumour cells. The system behaved as though these amino acids played no part in the uptake of glycine in the presence of cyanide. It would be interesting to know whether the same holds during respiration. Earlier work by Christensen & Riggs (1952) suggests that some exchange may then occur. These workers found that cellular $[\text{Na}^+]$ increased and cellular $[\text{K}^+]$ decreased when the respiring tumour cells absorbed relatively large amounts of glycine (Christensen & Riggs, 1952) or tryptophan (Riggs *et al.* 1954) during several hours. The ionic displacements appeared to occur after most of the tryptophan had accumulated in the cells. The stoichiometric ratios with respect to glycine were usually near 0.1 for either ion. The parts played by the sodium pump,

by energy depletion and by water movements, as well as the extent to which amino acid exchange occurred, would all have to be assessed before their work can be regarded as conflicting with the ratios shown in Table 3. Similar problems arise in connexion with the work of Hempling & Hare (1961, 1964).

A possible objection to the notion of a fixed stoicheiometry is found in Fig. 5, which shows that the variation in $\Delta\text{Na}^+ + \Delta\text{K}^+$ was probably not just due to experimental errors. This result may mean that the relative numbers of Na^+ ions, K^+ ions and glycine molecules associating with E were not constant. It is feasible that the relative amounts of Na^+ and K^+ that moved through other channels across the cell membrane, however, may have varied, especially when choline chloride was present (see the text with Table 2). Further work is needed to resolve this important issue.

Role of ATP. The chief possibilities, in the light of the above discussion, are as follows.

Hypothesis 1a. This is Christensen's hypothesis (Riggs *et al.* 1958). The spontaneous movements of either Na^+ or K^+ (or both ions) down their respective concentration gradients provide the energy needed to concentrate the glycine. The coupling with the amino acid movements is essentially a physical process (eqn. 4).

Hypothesis 1b. An alternative coupling mechanism might involve a chemical intermediate, possibly with the formation of a phosphorylated carrier protein (see Garrahan & Glynn, 1966; Cockrell, Harris & Pressman, 1967) that was subsequently hydrolysed as glycine entered the cell. There are no grounds at present for preferring hypothesis 1b to hypothesis 1a and only the latter is considered further.

Hypothesis 2. Czky (1963) suggested that Na^+ was involved, not in the entry of the solute glycine, but in a subsequent reaction in which osmotic work was done during the hydrolysis of ATP (cf. Johnstone & Scholefield, 1965).

Hypothesis 3. Eddy & Mulcahy (1964) envisaged an ATP-dependent glycine pump in which ENaGly crossed the cell membrane spontaneously and ENa was then expelled in a reaction that hydrolysed ATP. The pump might stop when deprived of ATP. Alternatively, the entry of glycine, which might still involve Na^+ , would simply be uncoupled from the expulsion of ENa and ENa would return to the outer-membrane phase spontaneously.

Each of the above hypotheses is consistent with the observed effects of $[\text{Na}^+]_1$, $[\text{K}^+]_1$ and $[\text{Gly}]_1$ on the rate of glycine uptake by the respiring tumour cells. Hypotheses 1 and 3, though not hypothesis 2, would also be consistent with the sense of the glycine gradient being reversed when the Na^+ gradient was reversed (cf. Crane, 1964). The most important

differences between the three hypotheses concern the behaviour expected when the cells are depleted of ATP. According to hypothesis 2 the uptake of glycine would not then either (a) depend on $[\text{Na}^+]_1$, as it is found to do (Eddy *et al.* 1967), or (b) induce both a net uptake of Na^+ and a net efflux of K^+ , as recorded in Table 3. Hypothesis 2 can probably be rejected therefore. Hypotheses 1 and 3 are both compatible with the observed requirement for Na^+ in the presence of cyanide and, also, with the observed influx of $^{24}\text{Na}^+$ induced by glycine under these conditions. Of the three, only hypothesis 1, however, would explain why the net movements of either or both ion species down their own concentration gradients became faster when glycine was transported. The present findings thus favour hypothesis 1 in the form where both ions move through the glycine-carrier system. The assumption that other channels are not involved in the induced movements of K^+ and Na^+ needs further experimental backing. If it is correct the magnitudes of $\Delta\text{Na}^+/\Delta\text{Gly}$ and $\Delta\text{K}^+/\Delta\text{Gly}$ would indicate that the ion gradients that are normally set up across the cell membrane during respiration would make $[\text{Gly}]_2/[\text{Gly}]_1$ quite large even when ATP was lacking (Table 3). The same result is likely on general thermodynamic grounds, given that the system contains an apparatus for tightly coupling the movements of both ions to that of glycine. Confirmatory evidence has appeared in a preliminary report (Eddy & Mulcahy, 1965). It leads to the important question how far the glycine gradients found with the respiring cells are reproduced in the presence of cyanide on the lines predicted by eqn. (4).

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