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The Effects of Sodium Ions and Potassium Ions on Glycine Uptake by Mouse Ascites-Tumour Cells in the Presence and Absence of Selected Metabolic Inhibitors

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1. The initial rate, v, of glycine uptake by ascites-tumour cells respiring their endogenous nutrient reserves was studied as a function of the respective extracellular concentrations of glycine, Na^+ and K^+. With the extracellular concentration of $Na^+ + K^+$ constant at 158m-equiv./l. and that of glycine either 4 or 12mM, v tended to zero as the extracellular concentration of Na^+ approached zero. Glycine appeared to enter the cells as a ternary complex with a carrier and Na^+. K^+ competed with Na^+ for one of the carrier sites, whereas glycine was bound at a second site. The values of the five relevant binding constants showed that the two sites interacted. 2. The glycine uptake rate at various extracellular concentrations of glycine and Na^+ was scarcely affected by starving the cells for 30min. in the presence of 2mM-sodium cyanide provided that cellular Na^+ and K^+ contents were kept at the normal values. When the cells took up Na^+, however, v decreased approximately threefold. 3. When their Na^+ content was relatively small and the extracellular concentration of Na^+ was large, the starved cells accumulated glycine in the presence of cyanide for about 15min. Glycine then tended to leave the cells. An average of about 5μmoles of glycine/ml. of cell water was taken up from a 1mM solution, representing about 20% of the accumulation observed during respiration. Studies with fluoride, 2,4-dinitrophenol and other metabolic inhibitors supported the view that ATP and similar compounds were not implicated. The relation between the transient accumulation of glycine that occurred in these circumstances and the normal mode of active transport was not established.

The kinetics of amino acid transport in mouse ascites-tumour cells have been extensively investigated since Christensen & Riggs (1952) demonstrated that these cells may concentrate various neutral amino acids such as glycine about 15-fold. The net uptake of the amino acid can be represented as the outcome of an entry process exhibiting saturation kinetics as the concentration of amino acid outside the cells varies and an exit process whose rate varies linearly with the cellular amino acid concentration (Heinz & Mariani, 1957; Johnstone & Scholefield, 1959; Tenenhouse & Quastel, 1960; Scholefield, 1961; Jacquez, 1961; Oxender & Christensen, 1963; Kromphardt, 1963). The present need to re-examine this aspect of the kinetics arises from the circumstance that Na^+ and K^+ now appear to be implicated and presumably interact with a carrier system that also binds selected amino acids (reviewed by Quastel, 1965). Thus the accumulation of a given amino acid in various tissues was found to be greatest when, on the one hand, the extracellular phase contained a relatively high concentration of Na^+ and a certain low concentration of K^+ (Christensen, Riggs, Fischer & Palatine, 1952; Riggs, Walker & Christensen, 1958; Tenenhouse & Quastel, 1960; Kromphardt, Grobecker, Ring & Heinz, 1963; Fox, Thier, Rosenberg & Segal, 1964; Bégin & Scholefield, 1964; Johnstone & Scholefield, 1965; Kuchler & Marlowe-Kuchler, 1965; Inui & Christensen, 1966) and when, on the other hand, the cells contained a relatively large amount of K^+ as opposed to Na^+ (Riggs *et al.* 1958; Vidaver, 1964*a*,*b*,*c*). The principal aim of the present investigation was to formulate a quantitative theory of amino acid transport in the tumour cells in terms of the distribution of Na^+ and K^+ across the cell membrane. Before this could be attempted, however, it appeared to be necessary to answer the following questions, which form the basis of the present paper.

(1) Can the effects of Na^+ and K^+ on the glycine

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entry kinetics be represented by one of the conventional mathematical models of the formation of enzyme–substrate complexes? (2) A model having been formulated, would the presence of metabolic inhibitors such as sodium cyanide or 2,4-dinitrophenol affect its parameters? Various workers have shown in this connexion that such inhibitors lower the rate of uptake of a given amino acid (Heinz, 1957; Heinz & Mariani, 1957; Helmreich & Kipnis, 1962; Rosenberg, Berman & Segal, 1963; Kromphardt, 1963; Jacquez & Sherman, 1965). As the cells accumulate Na^+ and lose K^+ in these circumstances, the question also arises whether amino acid influx is partly governed by the cellular concentrations of Na^+ and K^+. (3) Can the normal difference between the amino acid influx and efflux kinetics be attributed (cf. Riggs *et al.* 1958) to the fact that the concentrations of both Na^+ and K^+ on the two sides respectively of the cell membrane differ markedly? (4) When energy metabolism is restricted in the presence of various metabolic inhibitors, do the cells accumulate glycine at all when steps are taken to keep the cellular concentrations of Na^+ and K^+ near to the values found during active metabolism? This possibility was first examined by Vidaver (1964*b*,*c*), with pigeon erythrocytes.

The relevance of the new findings to the general problem of formulating a quantitative theory of amino acid transport is also discussed.

MATERIALS AND METHODS

Ringer solutions were prepared (Umbreit, Burris & Stauffer, 1957) by mixing 154mM-NaCl, 154mM-KCl, 154mM-$MgSO_4$ and 0·1M-sodium phosphate buffer, pH 7·4. KCl replaced NaCl and K_2HPO_4 replaced Na_2HPO_4 as required. The so-called 'standard Ringer' solution contained 155m-equiv. of Na^+/l. and 8m-equiv. of K^+/l. Other compounds such as NaCN or unlabelled glycine were usually added as stock solutions in the Ringer. An aqueous 30mM solution of [^{14}C]glycine (10μc/ml.) was used (1 vol./30 vol. of final solution). The counting equipment recorded about 10^5 counts/min./ml. of final solution. Solution of oligomycin and antimycin was facilitated by the presence of 1% (v/v) ethanol.

Preliminary manipulation of the cells. A strain of ascites-lymphosarcoma cells (strain LS) was obtained in 1963 from Mr Porteus of the Christie Hospital, Manchester. It was subsequently maintained by intraperitoneal transplantation in male mice (strain C_3H) in the presence of penicillin (1500units/animal) and streptomycin (1·5mg./animal). Cells for experimental work were usually harvested 7 or 8 days after inoculation. They were separated at room temperature at about 1000*g* from the ascitic fluid (MSE Minor centrifuge; Measuring and Scientific Equipment Ltd., London, S.W. 1). The packed cells (1 vol.) were quickly suspended in the standard Ringer solution (10 vol.) at 37°, collected by centrifugation and again suspended in warm Ringer solution (usually the standard Ringer solution) (15 vol.). The cell suspensions were shaken for 20min. (100 oscillations/min., 3cm. throw) in a water bath at 37°. The cells were separated by centrifugation at 1000*g* for 2min., the supernatant solution was discarded and each pellet of cells was centrifuged for a further 30sec. to separate adhering liquid.

Rate of uptake of glycine. (*a*) Initial rate. A given pellet of cells (30–60mg. dry wt.; see above) was kept in its centrifuge tube and suspended at 37°, with the aid of a glass rod, in the appropriate Ringer solution (6–10ml.) containing radioactive glycine. After 1min. the whole suspension (1 vol.) was mixed with ice-cold Ringer solution (4 vol.) and chilled for up to 20min. on ice. Three samples (each 7ml.) were later taken. It was found convenient to make up to nine consecutive measurements of the initial rate over about 7min. with a given preparation of cells.

(*b*) Net uptake. The cell suspension was prepared in a conical flask (25ml. capacity) and shaken at 100 oscillations/min. at 37°. When uptake depended on respiration, the flask was open and contained about 3mg. dry wt. of cells/ml. Otherwise it was closed and up to 12mg. dry wt./ml. was used. Samples (1ml.) were mixed with ice-cold Ringer solution (7ml.) and stored on ice. The cells from 27 samples were washed and recovered in about 45min. as follows.

(*c*) Processing the samples. Batches of six samples at a time were centrifuged at about 1000*g* (MSE Minor centrifuge, unrefrigerated). The centrifuge was turning for not more than 1·5min. and the cells were immediately returned to the ice bath. The supernatant solutions (A) were decanted and retained, and the cells at once centrifuged for a further 0·5min. Free liquid was poured away and the inverted tubes were drained over filter paper for about 15min. The cells were suspended in ethanol (2ml.) overnight at 4°. Duplicate portions (0·4ml.) of the ethanolic extracts were transferred to planchets, dried and assayed for radioactivity. Samples (0·1ml.) of the supernatant solutions (A) were similarly assayed.

'Extracellular space' for glycine applicable to the cells packed in the centrifuge tubes. Method 1. Glycine uptake at 20sec. and 80sec. was measured and the uptake at zero time determined by linear extrapolation. The mean extracellular volume (±S.E.M.) found in this way was 9·1±1·3 (15) ml./g. dry wt. of packed cells.

Method 2. The apparent uptake of glycine determined at 0° corresponded to a mean extracellular volume of 4·9±0·4 (8) ml./g. dry wt. of cells.

Calculation of the rate of uptake of glycine. The difference was found between (1) the radioactivity in counts/min. of the ethanolic extract of the cell sample taken at 1min. and (2) the radioactivity corresponding to the extracellular space for glycine. The latter quantity was determined by method 2 in obtaining the results shown in Table 2. Method 1 was used where metabolic inhibitors were present. The difference in counting rate was converted into μmoles of glycine by dividing by the specific radioactivity of the glycine. The uptake of glycine/min./mg. of cells was then calculated. It appeared to be almost linear with time for the first 1·5min. when the extracellular concentration of Na^+ was either 150 or 20m-equiv./l., the K^+ concentration being 8 and 140m-equiv./l. respectively.

Rate of glycine efflux. The cell preparation (about 5mg. dry wt./ml. of suspension in the standard Ringer solution) was first allowed to accumulate labelled glycine (from a selected concentration between 0·1 and 2mM) for 30min.

Table 1. *Sum of the concentrations of* Na^+ *and* K^+ *in the ascites-tumour cells packed in haematocrit tubes under conditions where the cellular water content varied*

The methods employed are described in the text and involved the cells' spending between 30 and 60 min. in a Ringer solution of the specified composition either at 25° or at 0°. Values are given as means ± S.E.M., with the numbers of observations in parentheses.

Suspension medium	Temp.	Cell water (μl./mg. dry wt. of cells)	Sum of concns. of Na^+ and K^+ (m-equiv./l. of cell water)
Standard Ringer soln. (153 m-equiv. of Na^+/l. + 8 m-equiv. of K^+/l.)	0°	3·74 ± 0·06 (4)	197 ± 2 (4)
	25	3·47 ± 0 03 (12)	206 ± 2 (12)
Standard Ringer soln. + 2 mM-NaCN	0	4·99 ± 0·01 (4)	193 ± 1 (4)
	25	3·75 ± 0·10 (4)	213 ± 1 (4)
Ringer soln. (5 m-equiv. of Na^+/l. + 153 m-equiv. of K^+/l.) + 2 mM-NaCN	25	4·47 ± 0·04 (4)	208 ± 1 (4)

(the cellular glycine concentration ranged from 1 to 20 mM). The warm cell suspension was centrifuged at about 1000*g* for 1 min., the supernatant was discarded and residual drops of liquid were removed with absorbent paper. The cells (about 10–20 mg.) were immediately suspended with a glass rod in a suitable Ringer solution (usually 9 ml. of the standard Ringer solution) at 37°. The suspension was shaken at 37° and samples (1 ml.) were withdrawn at about 1 min. intervals for 4 min. for processing (see above). Both a first-order rate constant and the mean efflux rate during the first minute were calculated from a plot of the logarithm of the amount of tracer in the cells against time.

Water content of the cells. The packed-cell volume was determined in standard haematocrit tubes centrifuged at 4000*g* for 30 min. The mean value (± S.E.M.) of the extracellular space measured with [^{32}S]sulphate appeared to be larger than Heinz & Mariani (1957) found and was 0·590 ± 0·015 (21) ml./ml. of cell volume. Though the volume of the cells varied considerably with the conditions (Table 1), the sum of the cellular Na^+ and K^+ contents was fairly constant at 206 ± 2 (28) (S.E.M.) m-equiv./l. of cell water.

The values given in Table 1 for the water content of the cells at 25° were used in converting the amount of glycine taken up by a given weight of cells into the corresponding concentration in the cellular water. When the cells were transferred from one type of Ringer solution to another, the values in Table 1 appropriate to the second solution were assumed to apply immediately.

Determination of cellular Na^+ *and* K^+. When the cells were packed in haematocrit tubes in the standard Ringer solution at 25° they contained 0·726 ± 0·009 (12) (S.E.M.) μequiv. of $Na^+ + K^+$/mg. dry wt. of cells. Washing the cells at 0° twice with 20 vol. of iso-osmotic buffered choline chloride solution, pH 7·4 (Aull & Hempling, 1963), decreased the ion content to 0·540 ± 0·035 (10) μequiv./mg. dry wt. without changing, however, the relative amounts of Na^+ and K^+. The routine method used to determine the cellular concentrations of Na^+ and K^+ in a given cell sample was based on this finding.

The sample (1 ml. containing about 10 mg. dry wt. of cells) was mixed with ice-cold buffered choline chloride solution (7 ml.) and the cells were separated by centrifugation. They were then washed once with a similar solution (7 ml.) and recovered, and the cell pellet was drained by inverting the centrifuge tube over filter paper for about 10 min. Distilled water (10 ml.) was added, then about 0·05 ml. of 2 N-HNO_3. The cell suspension was kept away from dust overnight at room temperature. Cellular debris was separated by centrifugation for 10 min. at about 1000*g* and the supernatant solution analysed for Na^+ and K^+ by using an EEL flame photometer (Evans Electroselenium Ltd., Halstead, Essex). The S.E.M. from six determinations of the sum of the amounts of Na^+ and K^+ in the cells was usually about 6%. Having determined the ratio of the amounts of Na^+ and K^+ in the washed cells, the concentrations present in the unwashed cells were calculated on the basis that the sum of their concentrations was 206 m-equiv./l. of cell water (Table 1).

Assay of radioactivity. Planchets carrying ^{14}C were counted by using a windowless scintillation counter (type SCA9; Panax Ltd., Redhill, Surrey) and conventional scaling equipment. The counting efficiency was about 20%. The count rate was usually at least ten times the background. About 10^3 counts/sample were recorded.

Potassium as ^{42}K was counted in solution, with similar precautions, by using an M6 tube (20th Century Electronics Ltd., New Addington, Surrey).

Miscellaneous. The method of Barker & Summerson (1941) was used to determine lactic acid and that of Weinstein & Hempling (1964) to determine ATP. Conventional manometric techniques were employed (Umbreit *et al.* 1957).

Chemicals. Compounds of A.R. quality were used wherever these were available. Antimycin was from the Kijowa Fermentation Industry Co. Ltd., Tokyo, Japan. Oligomycin was a gift from Dr G. D. Greville.

KINETIC ANALYSIS

The uptake of glycine appeared to be both stimulated by the presence of Na^+ in the medium surrounding the cells and inhibited by K^+. Let there be one site on a carrier molecule, E, where the amino acid can be bound and a second site binding Na^+. The glycine concentration in the cell is supposed to be negligible compared with that outside. A constant amount, *e*, of E is supposed to be available on the outside of the cell membrane for combination with Na^+ and glycine. Thus *e* is independent of the rate of entry of glycine.

Case 1. The formation of a complex (ENaGly) between

E, Na^+ and a glycine molecule from the external medium may lead to that glycine molecule being released inside the cell. Formation of the corresponding complex with K^+, however, fails to lead to that result. K^+ and Na^+ compete for the same anionic site on E. Let the carrier come to equilibrium with each of the three ligands in the following series of reversible reactions:

$$E + Na^+ = ENa \quad (1)$$

$$E + K^+ = EK \quad (2)$$

$$E + \text{glycine} = EGly \quad (3)$$

$$ENa + \text{glycine} = ENaGly \quad (4)$$

$$EGly + Na^+ = ENaGly \quad (5)$$

$$EK + \text{glycine} = EKGly \quad (6)$$

$$EGly + K^+ = EKGly \quad (7)$$

The corresponding equilibrium dissociation constants are $k_1=[E][Na^+]/[ENa]$, $k_2=[E][K^+]/[EK]$ etc. up to $k_7=[EGly][K^+]/[EKGly]$, the use of the square brackets denoting the activities of the relevant species at equilibrium. As $k_1k_4=k_3k_5$ and $k_2k_6=k_3k_7$, only five of the seven constants are independent.

Let the rate of entry of glycine, v, be $k[ENaGly]$, where k is a constant. Writing $[Na^+]$, $[K^+]$ and [Gly] respectively for the extracellular concentrations of Na^+, K^+ and glycine, and assuming the activity coefficients to be unity, it is then readily shown that:

$$v = \frac{ke}{k_1k_4} \cdot \frac{[Na^+][Gly]}{1+\frac{[Na^+]}{k_1}+\frac{[K^+]}{k_2}+[Gly]\left(\frac{1}{k_3}+\frac{[Na^+]}{k_1k_4}+\frac{[K^+]}{k_3k_7}\right)} \quad (8)$$

When [Gly] and $[K^+]$ are constant, $1/v$ is linearly related to $1/[Na^+]$. Moreover, when $[Na^+]$ and $[K^+]$ are constant, a similar relation holds between $1/v$ and 1/[Gly]. When [Gly] is constant and $[Na^+]$ and $[K^+]$ both vary with their sum constant at about 0·16equiv./l., eqn. (8) takes the form:

$$v = \frac{ke}{k_1k_4} \cdot \frac{[Gly]}{\frac{1}{[Na^+]}\left(1+\frac{0{\cdot}16}{k_2}+\frac{[Gly]}{k_3}+[Gly]\frac{0{\cdot}16}{k_3k_7}\right)+\frac{1}{k_1}-\frac{1}{k_2}+\frac{[Gly]}{k_1k_4}-\frac{[Gly]}{k_3k_7}} \quad (9)$$

Thus $1/v$ is linearly related to $1/[Na^+]$ in these circumstances.

A technique due to Florini & Vestling (1957) for analysing the kinetics of enzyme reactions involving two substrates was used to determine the constants k_1, k_2 etc. up to k_7. When $[K^+]$ is constant the lines representing $1/v$ against 1/[Gly] for different values of $[Na^+]$ intersect at a certain point. With $[K^+]=0$ intersection occurs where $[Gly]=-k_3$. Similarly, when $1/v$ is plotted against $1/[Na^+]$ and $[K^+]=0$ intersection occurs where $[Na^+]=-k_1$. Also, the intercept on the $1/v$ axis, for a given value of [Gly], is equal to $(1+k_4/[Gly])/ke$. Accordingly, by plotting 1/[Gly] against the corresponding value of the intercept, k_4 was derived by finding the value of 1/[Gly] where the intercept would be zero. This occurs where $[Gly]=-k_4$. Next, k_5 was determined as k_1k_4/k_3. The remaining constants were determined by using eqn. (9) as applied to measurements made in the presence of K^+ with $[Na^+]+[K^+]$ constant. The corresponding point of intersection when $1/v$ is plotted against $1/[Na^+]$ occurs where $-[Na^+]=(1+0{\cdot}16/k_2)/(1/k_1-1/k_2)$, from which expression k_2 was calculated. Similarly, the common point in the corresponding plot of $1/v$ against 1/[Gly] occurs where $-[Gly]=k_3(1+0{\cdot}16/k_2)/(1+0{\cdot}16/k_7)$, from which expression k_7 was calculated. Finally, k_6 was derived as k_3k_7/k_2.

The values of the parameters obtained by combining the first series of observations made at $[K^+]=0$ with the second made at $[Na^+]+[K^+]=0{\cdot}16$equiv./l. were checked by using them to calculate v from eqn. (9) at various values of [Gly], $[Na^+]$ and $[K^+]$ where the experimental values of v were well established.

Case 2. Crane, Forstner & Eicholz (1965) have suggested that both the Na^+ and K^+ forms of the substrate–carrier complex may serve to move the substrate into the cell. It can readily be shown by extending the above equations that $1/v$ would then be a linear function of 1/[Gly] with $[Na^+]$ and $[K^+]$ each constant. With [Gly] constant and $[Na^+]+[K^+]$ constant (1) $1/v$ would not, however, be linearly related to $1/[Na^+]$ and (2) nor would v tend to zero as $[Na^+]$ was progressively lowered. Neither of the latter circumstances was encountered in the present work.

Case 3. Let K^+ be regarded as a non-competitive inhibitor of the role of Na^+, the carrier possessing three binding sites. Then 12 equations represent combinations respectively of E and Na^+, E and K^+, E and glycine, ENa and glycine, EK and glycine, EGly and Na^+, EGly and K^+, EKGly and Na^+, ENa and K^+, EK and Na^+ and, finally, ENaK and glycine. The 12 dissociation constants are numbered in that order, and seven of them are independent. The following expression for the rate, v, is found and depends on the assumption that the rate is proportional to the concentration of ENaGly [type IIa in Dixon & Webb's (1964) classification of enzyme inhibitors]:

$$v = \frac{ke}{k_1k_4} \cdot \frac{[Na^+][Gly]}{1+\frac{[Na^+]}{k_1}+\frac{[K^+]}{k_2}+\frac{[Na^+][K^+]}{k_1k_{10}}+[Gly]\left(\frac{1}{k_3}+\frac{[Na^+]}{k_1k_4}+\frac{[K^+]}{k_2k_5}+\frac{[Na^+][K^+]}{k_1k_4k_8}\right)} \quad (10)$$

Whereas $1/v$ would then be a linear function of 1/[Gly] when $[Na^+]$ and $[K^+]$ were constant, $1/v$ would not vary linearly with $1/[Na^+]$ when $[K^+]$ was simultaneously varied and both $[Na^+]+[K^+]$ and [Gly] were constant.

RESULTS

In agreement with the literature (see the introduction) increasing $[Na^+]$ and lowering $[K^+]$ increased the rate of uptake of glycine from either a 4mM or 12mM solution (Fig. 1). The results suggest that not more than about 5% of the uptake at 150m-equiv. of Na^+/l. was independent of extracellular Na^+. Two conclusions follow. First, diffusion of glycine into the cells was probably not

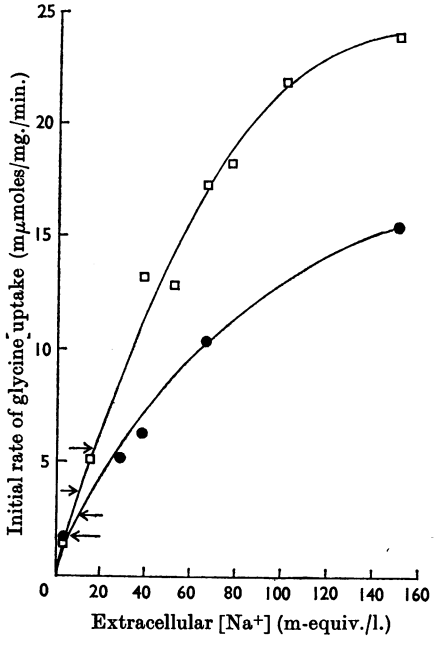

Fig. 1. Initial rate of glycine uptake at 37° as a function of [Na+] outside the ascites-tumour cells. Na+ was replaced by K+ at a constant osmotic pressure. □, 12mM-Glycine; ●, 4mM-glycine. The results from seven experiments were combined, each point being the mean of at least three independent measurements. The two pairs of arrows represent respectively the ranges in which the efflux rate fell when the cellular glycine concentration was 4mM and 12mM (see the text).

an important factor under these conditions. Secondly, K+ was probably not a partial substitute for Na+ (i.e. case 2 in the Kinetic Analysis section would not apply). That K+ interacted with the system, however, soon became apparent when the initial rate of glycine uptake, v, was studied as a function of both [Gly] and [Na+] under the two conditions considered in case 1 in the Kinetic Analysis section. These are: (1) where [K+] was almost zero (8m-equiv./l.), addition of choline chloride serving to maintain the osmotic pressure; (2) where the sum [K+]+[Na+] was 160m-equiv./l., so that [K+] increased as [Na+] decreased. Fig. 2(a) shows how $1/v$ varied with 1/[Gly] in the latter instance at five selected values of [Na+]. Fig. 2(b) shows a similar plot of data obtained with choline as the substituting ion. Clearly K+ inhibited glycine uptake. It will be observed that K_m for glycine was almost constant in Fig. 2(b) and varied more in Fig. 2(a). $V_{max.}$ also varied with [Na+]. The rate data were replotted as in Figs. 3(a) and 3(b) and further analysed by the procedure described in the Materials and Methods section.

The four sets of lines representing $1/v$ against 1/[Na+] or 1/[Gly] respectively were extrapolated towards the regions of negative reciprocal concentrations and positions found where the rate was roughly independent of the third variable. Whereas in Fig. 2 such a point of intersection ('cross-over point') gives a measure of the hypothetical glycine concentration where the rate would be independent of [Na+], in Fig. 3 the rate then becomes independent of [Gly]. As this is just the situation predicted by

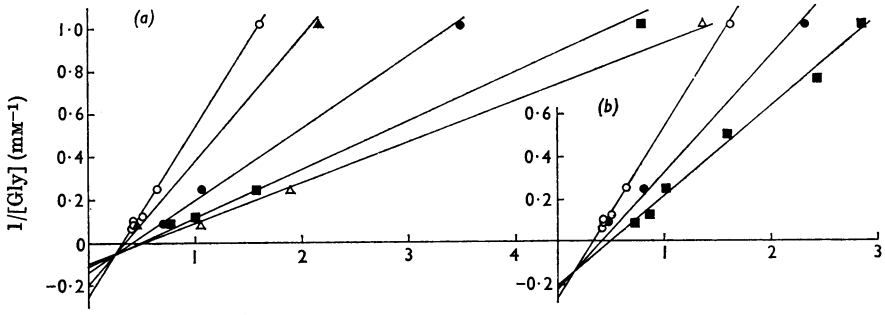

Fig. 2. (a) Plots of $1/v$ against 1/[Gly] at various values of [Na+] with [Na+]+[K+] constant. The results of 20 experiments were combined. Each point represents the mean of from three to ten independent series of measurements of the rates at 37° with different cell preparations (see the Materials and Methods section). The following data illustrate the variations encountered: with 1mM-glycine and 150m-equiv. of Na+/l. v was 6·2±0·5 (20) (S.E.M.) mμmoles/mg./min.; at 36m-equiv. of Na+/l. v was 1·9±0·2 (7) mμmoles/mg./min. Ion concentrations (m-equiv./l.) were: ○, [Na+] 150, [K+] 8; ▲, [Na+] 101, [K+] 58; ●, [Na+] 54, [K+] 104; ■, [Na+] 36, [K+] 120; △, [Na+] 28, [K+] 130. (b) Plots of $1/v$ against 1/[Gly] at various values of [Na+] with [K+] constant at 8m-equiv./l. and choline added as the replacement ion. A given symbol represents the same value of [Na+] as in Fig. 2(a). The results of nine experiments were combined. Each point represents the mean of from two to four independent series of measurements of the rates at 37°.

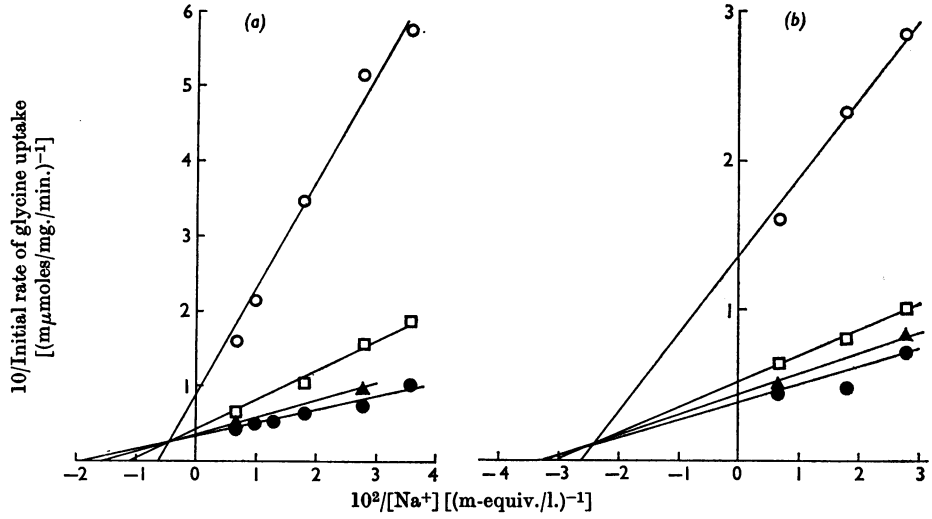

Fig. 3. (*a*) Plots of $1/v$ against $1/[Na^+]$ for various values of [Gly] with $[K^+]$ as replacement ion. The Figure shows the data in Fig. 2(*a*) plotted differently. Glycine concentrations: ○, 1mM; □, 4mM; ▲, 8mM; ●, 12mM. (*b*) Plots of $1/v$ against $1/[Na^+]$ for various values of [Gly] with choline as replacement ion. The Figure shows the data in Fig. 2(*b*) plotted differently.

Table 2. *Parameters of eqn.* (8)

Two series of rate measurements with $[Na^+]$ and [Gly] as independent variables were analysed (see the Materials and Methods section). In one series $[K^+]$ was 8m-equiv./l. and in the second series $[Na^+]+[K^+]$ was constant at 158m-equiv./l. As a first approximation, the various parameters were derived on the assumption that $[K^+]$ was zero in the first series. A correction for the presence of 8m-equiv. of K^+/l. was then made by using the method of successive approximations.

Parameter	Ligand bound	Values from present work with glycine (mM or m-equiv./l.)
k_1	Na^+	37·5
k_2	K^+	76
k_3	Amino acid	6·4
k_4	Na^+, then amino acid	3·5
k_5	Amino acid, then Na^+	21
k_6	K^+, then amino acid	92
k_7	Amino acid, then K^+	approx. 1000
$ke = V_{max.}$ with $[Na^+]$ and [Gly] both infinite		34mμmoles/mg./min.*

* In agreement with eqn. (8), the same value was obtained whether K^+ or choline was the replacement ion. The method used involved first plotting $1/v$ against $1/[Na^+]$ and then plotting the intercepts on the $1/v$ axis against 1/[Gly].

eqn. (8), the analysis was carried further to estimate the various parameters of that equation (Table 2).

Comparison between k_1 and k_5, on the one hand, and k_3 and k_4, on the other hand, shows that both the affinity for glycine and that for Na^+ was affected by prior attachment of the other ligand. A more marked interaction between the two binding sites occurred for the pair K^+ and glycine. Thus the affinity for glycine was at least 14-fold smaller when K^+ was already bound than when either Na^+ had become bound or that site was unoccupied.

The fraction of the total amount of carrier present as ENaGly when [Gly] was 50mM and $[Na^+]$ was 160m-equiv./l. was computed to be 0·82. The corresponding value for EKGly when $[K^+]$ was 160m-equiv./l. was only 0·10. Now eqn. (8) involves the assumption that glycine failed to move into the cell as EKGly. This might be either because (1) the value of k appropriate to the passage of EKGly was small or (2) EKGly itself was small. As EKGly appears to be relatively small, the magnitude of k for EKGly is indeterminate.

Entry kinetics in the presence of sodium cyanide

(*a*) *Constant amounts of* Na^+ *and* K^+ *in the cells.* When the cells were poisoned with sodium cyanide for 30min. under circumstances where their Na^+ and K^+ contents remained roughly at the values characteristic of the respiring cells, no appreciable change in the various kinetic parameters shown in Table 3 took place. Thus the requirement for Na^+ in the extracellular phase was found in both

Table 3. *Parameters expressing the variation of the rate of uptake of glycine with extracellular concentrations of glycine and* Na^+ *in the presence of 2mM-sodium cyanide as opposed to its absence, the cellular* Na^+ *content being roughly constant*

The significance of the 'cross-over points' is discussed in the text. They were found from double-reciprocal plots of $1/v$ against $1/[Na^+]$ and $1/[Gly]$ respectively. When NaCN was used, the cells were incubated with that compound for 25min. in a Ringer solution containing 32m-equiv. of Na^+/l. and 127m-equiv. of K^+/l. The concentration of Na^+ in the cells was then about 40m-equiv./l. The initial rate of glycine uptake was determined in a Ringer solution containing 2mM-NaCN and selected concentrations of glycine (1, 4, 8 or 12mM) and of Na^+ (32 or 150m-equiv./l.), the sum $[Na^+]+[K^+]$ being 158m-equiv./l. The results of two independent series of measurements were combined and are compared below with the data in Figs. 2 and 3, where the cellular Na^+ content was about 35m-equiv./l.

	NaCN present	NaCN absent
$V_{max.}$ ($[Na^+]=\infty$; $[Gly]=\infty$) (mμmoles/mg./min.)	36	34
'Cross-over point' from $1/v$ against $1/[Gly]$ when $-[Gly]$ was (mM):	25	17·5
'Cross-over point' from $1/v$ against $1/[Na^+]$ when $-[Na^+]$ was (m-equiv./l.):	370	230
Mean rate (± S.E.M.) of glycine uptake from the standard Ringer soln. (1mM-glycine) (mμmoles/mg./min.)	5·0±0·6 (15)	6·2±0·5 (24)

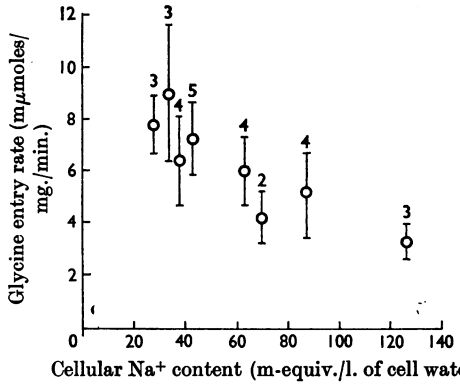

Fig. 4. Effect of varying the Na^+ content of the ascites-tumour cells on the initial rate of glycine uptake from a Ringer solution containing 151m-equiv. of Na^+/l. and 8m-equiv. of K^+/l., 2mM-NaCN and 1mM-glycine. The cellular Na^+ content was varied by first standing the cells for 30min. at 37° with 2mM-NaCN at a selected concentration of Na^+ and with sufficient K^+ to make the sum of their concentrations 160m-equiv./l. The concentrations of Na^+ used for this purpose were 5, 30, 60 and 150m-equiv./l. The mean uptake rate (± S.E.M.) observed in the stated number of independent measurements is plotted against the mean Na^+ content of the cells.

situations and so also, probably, was the inhibitory effect of K^+.

(*b*) *Varying amounts of* Na^+ *and* K^+ *in the cells.* The cellular Na^+ content was varied, as explained in the legend to Fig. 4, during the initial period of 30min. that the cells spent with sodium cyanide. Fig. 4 shows that the rate of uptake of glycine from the standard Ringer solution was then correlated ($r=-0·59$; $P=0·001$) with the cellular ion content.

(*c*) *Time as a variable.* Provided that the cellular Na^+ content was constant at about 40m-equiv./l., the cells took up glycine from a 1mM solution at about the same rate after 30min. as after 60min. with sodium cyanide [6·6±0·8 (3) mμmoles/mg./min. as opposed to 5·2±0·9 (3) mμmoles/mg./min.]. On the other hand, when the cells accumulated Na^+ for 60min., so that the cellular Na^+ content was about 110m-equivl/l., the uptake rate was 1·2± 0·3 (3) mμmoles/mg./min. Comparison with Fig. 4 suggests that the rate at 60min. was lower than it would have been at 30min. at the same Na^+ concentration ($P<0·01$). Thus the effects associated with the cellular ions seemed to increase with time.

(*d*) *Effects of other metabolic inhibitors.* Cell preparations were incubated for 30min. with 2mM-sodium cyanide plus a second metabolic inhibitor. A parallel cell suspension containing 2mM-sodium cyanide served as a control. The cellular Na^+ content was constant at about 40m-equiv./l. The uptake rate from 1mM-glycine in the standard Ringer solution was then measured, cyanide being present in the control (I) and both inhibitors in the test series (II). When the second inhibitor was 0·8μg. of oligomycin/mg. of cells (two experiments) or 0·5mM-2,4-dinitrophenol (three experiments) the rate was the same in (I) and (II). With 10mM-sodium fluoride the rate was about 34% (three experiments) lower than (I) showed, whereas with 1mM-sodium iodoacetate the rate was about 55% (three experiments) lower.

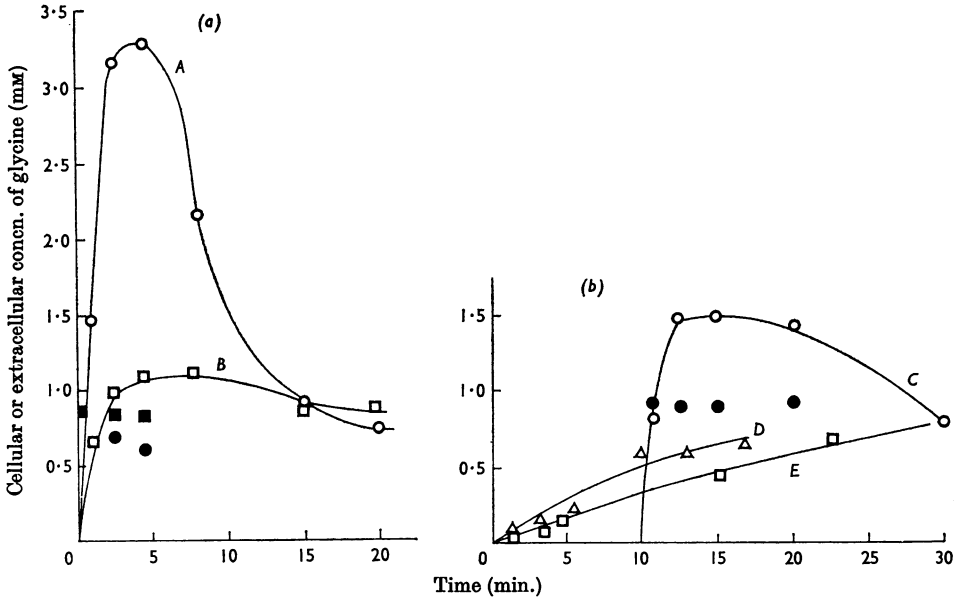

Fig. 5. Effect of allowing the cells to accumulate Na^+ on their ability subsequently to take up glycine in the presence of 2mM-NaCN and 1mM-sodium iodoacetate. During the preincubation period, the cells stood at 37° with the two metabolic inhibitors for 25min., either at 150m-equiv. of Na^+/l. and 8m-equiv. of K^+/l. or at 32m-equiv. of Na^+/l. and 126m-equiv. of K^+/l. They were next transferred to a Ringer solution of the specified composition containing 1mM-glycine plus the inhibitors at 37°. The glycine content of the cells was determined at intervals (see the Materials and Methods section). (*a*) *A*, Preincubation at 32m-equiv. of Na^+/l., uptake at 150m-equiv. of Na^+/l.; ●, glycine concentration outside the cells. *B*, Preincubation and uptake both at 150m-equiv. of Na^+/l.; ■, glycine concentration outside the cells. (*b*) *C*, Preincubation at 32m-equiv. of Na^+/l., uptake at 150m-equiv. of Na^+/l.; in contrast with *A*, the glycine was added to the cells 10min. after they were first suspended at the higher concentration of Na^+. *D*, Preincubation at 32m-equiv. of Na^+/l., uptake at approx. 5m-equiv. of Na^+/l. and 155m-equiv. of K^+/l. *E*, Preincubation at 150m-equiv. of Na^+/l., uptake at approx. 5m-equiv. of Na^+/l. and 155m-equiv. of K^+/l. ●, Glycine concentration outside the cells in *C*, *D* and *E*. Open symbols show the cellular glycine concentration.

Ion gradients and the accumulation of glycine in the presence of the metabolic inhibitors

Cell preparations were kept at 37° for 25min. with 2mM-sodium cyanide and 1mM-sodium iodoacetate, one series (*A*) at 32m-equiv. of Na^+/l. and 126m-equiv. of K^+/l. (when the cellular Na^+ content remained at about 40m-equiv./l.), and another series (*B*) in the standard Ringer solution (when the cellular Na^+ content became about 90m-equiv./l.). The cells were in each case then transferred to the standard Ringer solution containing labelled glycine and the inhibitors. Fig. 5(*a*) shows that, for a short period, the cells from series *A* accumulated glycine up to a concentration equivalent to about 5·5 times that outside, whereas those from series *B* accumulated glycine up to a concentration about 1·4 times that outside. Further aspects are shown in Fig. 5(*b*). The cells for series *C* were maintained initially as for series *A* in Fig. 5(*a*) and were tested with glycine after standing in the Ringer solution for 10min. Little glycine then accumulated in the cells. This suggested that the factors responsible for the accumulation dissipated whether or not glycine itself was present. The ionic composition of the cells appeared to be involved, moreover, as their Na^+ content approximately doubled during that 10min.

(*a*) *Relation with cellular* Na^+. The latter point is illustrated further in Table 4, which includes observations on the glycine content of cells that were initially allowed to accumulate Na^+ and were subsequently transferred to medium of low [Na^+] before being tested with glycine. The glycine content was better correlated with the Na^+ content than with the length of time spent in the preliminary treatments of the cells.

Other observations in Fig. 5(*b*) show how glycine was taken up with relatively low [Na^+] outside the cells. The cells were first kept either at 32m-equiv.

Table 4. *Effects of varying* (1) *the duration of the treatment with* 2mM-*sodium cyanide, either with or without* 1mM-*sodium iodoacetate present, and* (2) *the cellular* Na^+ *content on the ability of the ascites-tumour cells subsequently to accumulate glycine*

The cells were kept at 37° for the specified period, with one or both of the metabolic inhibitors, either in a Ringer solution containing 32m-equiv. of Na^+/l. and 127m-equiv. of K^+/l. or in one containing 151m-equiv. of Na^+/l. and 8m-equiv. of K^+/l. They were then transferred to a Ringer solution containing 152m-equiv. of Na^+/l. and 8m-equiv. of K^+/l., 1mM-glycine and either cyanide or both of the inhibitors. Glycine uptake in these circumstances increased up to a maximum during the next 10min. and then started to decrease (see series *A* in Fig. 5*a*). The Na^+ content of the cells was determined at the peak. The ratio of the maximum concentration of glycine accumulating in the cells to the concentration of glycine outside the cells at that time is shown below as a function of the other variables.

Preincubation	Test and preincubation			
Extracellular $[Na^+]$ (m-equiv./l.) and time	Cyanide	Iodoacetate	Maximum glycine accumulation ratio	Cellular Na^+ content (m-equiv./l.)
151 (for 30min.)	+	−	2·5±0·3 (3)	80
151 (for 60min.)	+	−	1·2±0·2 (3)	120
32 (for 30min.)	+	−	4·9±0·6 (2)	55
32 (for 60min.)	+	−	5·5±0·5 (2)	50
151 (for 30min.) then either to:	+	−		
32 (for 20min.) or to:	+	−	4·0±0·0 (2)	65
32 (for 30min.)	+	−	2·8±0·7 (2)	60
32 (for 30min.)	+	+	3·5±0·3 (2)	82
32 (for 60min.)	+	+	1·3±0·4 (2)	102

of Na^+/l. (series *D*) or in the standard Ringer solution (series *E*). In neither instance did the glycine concentration in the cells become greater than that outside them.

The results shown in Fig. 5 and Table 4 demonstrate that it was not sufficient merely to maintain a low concentration of Na^+ in the cells for glycine to accumulate there. The presence of substantial ion gradients across the cell membrane appeared to be necessary.

(*b*) *Glycine accumulation in the presence of other metabolic inhibitors.* Table 5 shows that in the presence of both fluoride and cyanide the uptake of glycine by cells depleted of Na^+ averaged 13% less than the uptake in the control series containing only cyanide as the inhibitor. As the fluoride ions had ample time to take effect, the accumulation of glycine was probably independent of glycolysis.

Neither did sodium azide, antimycin or 2,4-dinitrophenol have much effect on the accumulation of glycine in these circumstances. None of the above compounds significantly altered the ionic composition of the cells at the stage where their glycine content reached a maximum. The presence of oligomycin lowered glycine accumulation to a variable extent, and in two out of the five tests illustrated in Table 5 no inhibition occurred. The interpretation is complicated by the fact that in the one instance where glycine accumulation was lowered by 82% (1) the overall ion content of the cells was only about half that of the cells in the control and (2) they contained about 45% more Na^+ than the control cells. Possibly the cells were partially lysed by oligomycin and to different extents on the different occasions (cf. Glynn, 1963).

Glycine efflux

(*a*) *Comparison with the entry kinetics.* Fig. 6 shows how the reciprocal of the efflux rate varied with the reciprocal of the cellular glycine concentration. Both $V_{max.}$ and K_m appear to be very large, as would be expected on the basis of earlier reports (see the introduction). Fig. 1 (arrows) compares the glycine influx rates from 4mM and 12mM solutions respectively with the efflux rates from the same cellular glycine concentration. Glycine entered the cells from a 4mM solution containing 5–10m-equiv. of Na^+/l. at about the same rate as efflux occurred from a 4mM solution. The corresponding values with 12mM-glycine were 9–14m-equiv. of Na^+/l. Both pairs of values are significantly lower than the mean cellular concentration of Na^+ from which efflux ostensibly occurred, which was 32±3 (8) (S.E.M.) m-equiv./l. The influx rates from 5 and 14m-equiv. of Na^+/l. were calculated from eqn. (9) by using the data in Table 2. The corresponding double-reciprocal plots are shown in Fig. 6 (broken lines). These show that the efflux rate varied with the glycine concentration between

Table 5. *Glycine uptake by the ascites-tumour cells in the presence of 2mM-sodium cyanide and a second metabolic inhibitor measured after 30min. spent with both compounds at a low concentration of* Na^+

The cells serving as controls were incubated at 37° in a Ringer solution containing 5m-equiv. of Na^+/l. and 153m-equiv. of K^+/l. and 2mM-NaCN for 25min. The test series comprised similar suspensions containing cyanide and the compound being tested. The cells were then transferred either to the standard Ringer solution with 1mM-glycine and 2mM-NaCN (the control) or to a similar solution also containing the test compound. The glycine content of the cells increased for up to about 10min. and then decreased. The ratio of the cellular to extracellular glycine concentration at the peak was determined in each instance. The maximum accumulation ratio for the test suspension divided by the corresponding ratio for the control is shown as a percentage.

Compound added with NaCN		No. of paired observations	Glycine accumulation ratio (% of control)
10mM-NaF		3	87±5
0·1mM-NaN_3		2	95±5
0·06mM-Dinitrophenol		2	109±9
0·5mM-Dinitrophenol		2	100
0·01mM-Antimycin		2	95±6
Oligomycin			
(μg./ml.)	(μg./mg. of cells)		
3	0·4	1	100
5	0·5	1	79
5	0·65	1	49
10	2·8	1	18
10	0·8	1	120

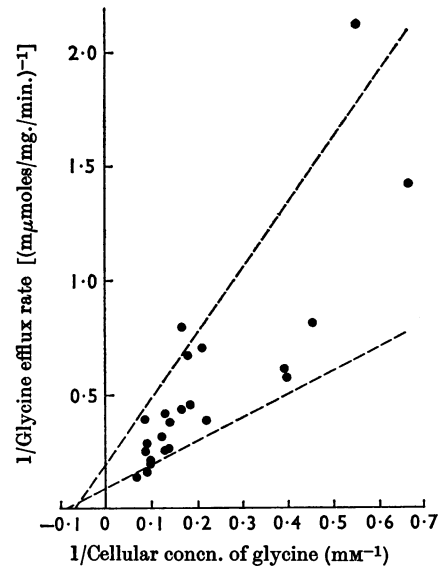

Fig. 6. Double-reciprocal plot showing how glycine efflux varied with the cellular glycine concentration. The broken lines show how the reciprocal rate would be expected to vary with 1/[Gly] (see the text) if, for a given value of [Gly], efflux occurred at the same rate as glycine influx at 5m-equiv. of Na^+/l. (upper line) and 14m-equiv. of Na^+/l. (lower line).

the limits corresponding to the influx rates at 14 and 5m-equiv. of Na^+/l. The rates at the lower glycine concentrations show a tendency to correspond better to the values expected at the lower than at the higher $[Na^+]$ value. It accordingly seems possible that the efflux kinetics resemble the entry kinetics under circumstances where $[Na^+]$ varies between the above limits.

(*b*) *Effects of extracellular* K^+ *and* Na^+. In agreement with earlier work (Christensen *et al.* 1952; Tenenhouse & Quastel, 1960) omitting K^+ from the standard Ringer solution lowered the net uptake of glycine from a 1mM solution to 38±3% (5) (S.E.M.) of the uptake observed with the standard Ringer solution itself. Though the initial rate of glycine uptake was the same whether $[K^+]$ was 0·4 or 5m-equiv./l., glycine efflux was stimulated by about 50% at the lower $[K^+]$ value (Table 6). Other observations not shown in Table 6 indicated that increasing $[K^+]$ from 5 to 50m-equiv./l. failed to influence the efflux rate. Efflux was also slightly stimulated by lowering $[Na^+]$ (Table 6).

(*c*) *Effect of ouabain.* Table 7 shows that glycine efflux was stimulated on average by about 60% by 0·1mM-ouabain (series 1) unless $[Na^+]$ was lowered from 151 to 32m-equiv./l. (series 2), when the drug had no effect on the efflux rate. This behaviour recalls the observations by Gonda & Quastel (1962), who found that efflux of various amino acids from rat brain-cortex slices was accelerated by ouabain.

Ouabain scarcely influenced the Na^+ and K^+ contents of the cells during the 4min. over which glycine efflux was measured (Fig. 7*a*). A similar conclusion held when $[K^+]$ was lowered to 0·4m-equiv./l. (Fig. 7*b*).

(*d*) *Effects of 2mM-sodium cyanide and 1mM-sodium iodoacetate.* When these compounds were present in the standard Ringer solution glycine uptake from a 1mM solution followed the same course for about 10min. as was observed in their absence. The presence of the inhibitors then resulted in glycine starting to leave the cells, as Johnstone & Scholefield (1959) first described. The cells were separated at that stage and transferred to the standard Ringer solution containing the two metabolic inhibitors. The initial rate of glycine

Table 6. *Effects of varying the extracellular concentrations of* Na^+ *and* K^+ *on glycine efflux*

The cells were kept at 37° in the standard Ringer solution for 20min. with 1mM-glycine, when the cellular glycine concentration increased to about 10mM. One portion of the cells was then transferred to the standard Ringer solution and a second portion to a solution of the composition specified, the glycine efflux rate being determined in both suspensions (see the Materials and Methods section).

	Ringer solution				
Series	$[Na^+]$ (m-equiv./l.)	$[K^+]$ (m-equiv./l.)	First-order rate constant of glycine efflux	No. of paired observations	Significance of difference
1	151	5	0.125 ± 0.015	5	$P < 0.02$
	151	0·4	0.187 ± 0.015		
2	151	5	0.091 ± 0.015	3	$P \simeq 1$
	42	5	0.106 ± 0.021		
3	151	5	0.153 ± 0.013	3	$P < 0.05$
	11	5	0.197 ± 0.009		

Table 7. *Effect of ouabain on glycine efflux*

The cells were kept at 37° in the standard Ringer solution for 20min. with 1mM-glycine, when the cellular glycine concentration increased to about 10mM. The glycine efflux rate was then measured both in the presence and absence of ouabain, either at 151 or at 32m-equiv. of Na^+/l. (see the Materials and Methods section).

	Ringer solution					
Series	$[Na^+]$ (m-equiv./l.)	$[K^+]$ (m-equiv./l.	Concn. of ouabain (mM)	First-order rate constant of glycine efflux	No. of paired observations	Significance of difference
1	151	5	0	0.087 ± 0.016	5	$P \simeq 0.05$
	151	5	0·1	0.142 ± 0.018		
2	32	5	0	0.100 ± 0.007	4	$P \simeq 1$
	32	5	0·1	0.108 ± 0.010		

efflux corresponded to a first-order rate constant ($\pm$S.E.M.) of 0.232 ± 0.013 (5), the rate being about 70% faster than was exhibited by control preparations without the metabolic inhibitors. The concentration of Na^+ in the cells when glycine efflux was measured was approximately three times as large in the presence of the two inhibitors as in their absence.

The rate of efflux of glycine in the presence of the inhibitors was the same whether $[K^+]$ was 5 or 0·4m-equiv./l. (three experiments) and whether 0·1mM-ouabain was present or absent (two experiments).

Effect of 2mM-sodium cyanide on K^+ *influx*

Uptake of $^{42}K^+$ by the respiring cells reached a steady state during about 40min., the initial rate ($\pm$S.E.M.) being 17.1 ± 2.5 (5) mμequiv./mg. dry wt. of cells/min. Contrary to a report by Hempling & Hare (1961) the presence of 2mM-glycine failed to influence the uptake of $^{42}K^+$ (five experiments). Uptake of $^{42}K^+$ from the standard Ringer solution containing 2mM-sodium cyanide occurred at a comparable rate for about 10min., then slowed markedly, by which time the Na^+ content of the cells had increased about 100%. Cell preparations incubated for 30min. with 2mM-sodium cyanide in a Ringer solution containing 5m-equiv. of Na^+/l. and 155m-equiv. of K^+/l. and then transferred to the standard Ringer solution accumulated 3.1 ± 0.2 (5) mμequiv. of K^+/mg./min. The rate was unchanged in the presence either of 1mM-glycine (seven experiments) or of 0·1mM-ouabain (two experiments). The latter observation suggests that active transport of $^{42}K^+$ had stopped.

Miscellaneous effects of sodium cyanide

(*a*) *On protein synthesis.* Johnstone & Scholefield's (1959) observation that sodium cyanide almost immediately stopped incorporation of glycine into cellular protein was confirmed. No incorporation occurred under the conditions used for series *A* in Fig. 5(*a*) (four experiments).

(*b*) *On respiration and glycolysis.* Cell preparations

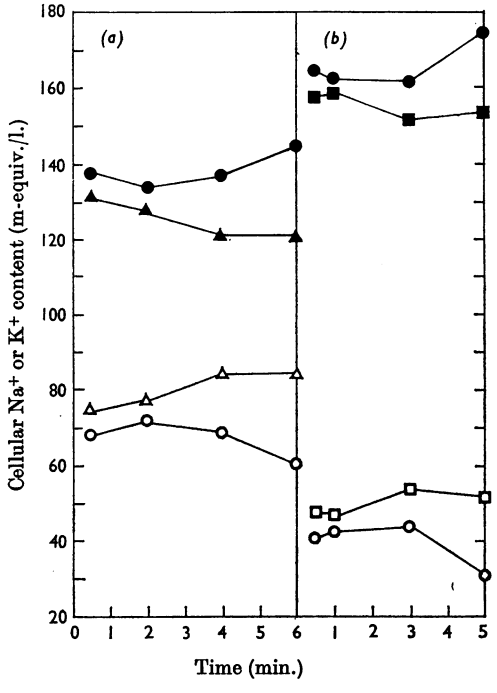

Fig. 7. Effects of 0·1 mM-ouabain and of K^+ deprivation respectively on the Na^+ and K^+ contents of the cells over the period in which glycine efflux was measured (see Tables 6 and 7). The cells accumulated glycine from the standard Ringer solution for 30 min., cellular glycine then being about 10 mM. One half of the preparation (the control) was transferred to the standard Ringer solution (155 m-equiv. of Na^+/l. and 5 m-equiv. of K^+/l.) without glycine and the other half to a similar solution containing either 0·1 mM-ouabain (*a*) or 0·4 m-equiv. of K^+/l. (*b*). The suspensions were sampled at intervals and the Na^+ and K^+ contents of the cells determined. The observations were made in one of three experiments, which give similar results. ○, Na^+, control; ●, K^+, control; △, Na^+, ouabain; ▲, K^+, ouabain; □, Na^+, low extracellular [K^+]; ■, K^+, low extracellular [K^+].

incubated in the standard Ringer solution for 20 min. with 2 mM-sodium cyanide respired for the next 20 min. in the presence of 1 mM-glycine and 2 mM-sodium cyanide $1·08 \pm 0·13$ (5) (S.E.M.) mμ-moles of oxygen/min./mg. dry wt. of cells and produced $0·44 \pm 0·13$ (4) mμmole of lactic acid/min./mg. (A similar low rate was observed in the absence of cyanide.) Endogenous respiration consumed $5·58 \pm 0·10$ (5) mμmoles of oxygen/min./mg. The concentration of ATP (±S.E.M.) in the cells 1–2 min. after these were suspended in the presence of glycine under the conditions used for series *A* of Fig. 5(*a*) was $0·144 \pm 0·019$ (4) mM. Control preparations provided with glucose contained $2·5 \pm 0·1$ (3) mM-ATP.

DISCUSSION

General aspects of the problem. The main issue for discussion is the bearing of the experimental findings on the problem of formulating a quantitative theory of glycine transport. Most theories of carrier-mediated substrate transport regard the outer and inner surfaces of the cell membrane as distinct phases (reviewed by Rosenberg & Wilbrandt, 1963). The carrier is supposed to form a stoicheiometrical complex with the substrate. Glycine would enter the cells as follows (the carrier cycle): step 1, carrier molecules in the outer membrane phase form a complex with glycine in a reversible reaction that comes to equilibrium; step 2, a small fraction of the complexes cross to (or become rearranged into) the inner membrane phase; step 3, some of the complexes then dissociate in equilibrating with intracellular glycine (binding constants at that stage may differ from those at step 1); step 4, free carrier molecules return to the outer membrane phase to enter the cycle again. ATP might be involved at one or more of the steps, an aspect that need not be considered further in the first instance.

The mathematical analysis of the carrier cycle when the carrier itself can interact with Na^+, K^+ and glycine in both phases may assume a relatively simple form in the steady state (A. A. Eddy, unpublished work). The more complex relations that hold when the cells are either accumulating or losing glycine can be simplified on the lines indicated in case 1 in the Kinetic Analysis section. There it was assumed that the total amount of carrier, *e*, in the outer membrane phase was independent of [Gly], [Na^+] and [K^+]. Possible terms involving the cellular ions then disappear. A similar assumption was made by Vidaver (1964*a*) for pigeon erythrocytes and by Inui & Christensen (1966) for the ascites system. The data in Fig. 4 imply that, in the presence of cyanide at least, the carrier interacts with the cellular ions in the inner membrane phase, so the assumption is not valid in general. The errors involved cannot yet be assessed, however. Meanwhile, we shall assume that Table 2 provides an approximate measure of the binding constants in step 1.

Vidaver (1964*a*) concluded that 2 Na^+ ions were associated/mol. of glycine in the carrier complex formed in the erythrocyte system and that K^+ was inert. Inui & Christensen (1966) postulate 1 Na^+ ion/mol. of glycine for the ascites system and assume, without demonstrating the point, that K^+ is unimportant (cf. Wheeler, Inui, Hollenberg, Eavenson & Christensen, 1965). The present observations are also consistent with a 1:1 ratio (or, of course, with a 2:1 ratio if 1 Na^+ ion were very firmly bound). Fig. 2 clearly demonstrates that K^+

is not inert but interferes with the role of Na^+. Further experimental proof of their competitive relationship is being sought. It is noteworthy that K^+ also inhibits amino acid transport in the intestine *in vitro* (Nathans, Tapley & Ross, 1960).

Fig. 1 is interpreted as showing that glycine only enters the cells as ENaGly. Inui & Christensen (1966) found that some glycine was taken up by their tumour cells when [Na^+] was ostensibly zero and [K^+] was 26m-equiv./l., a kinetic component that they identified with the so-called 'non-saturable' uptake exhibited with various α-amino acids (Christensen & Liang, 1966). The explanation of the apparent discrepancy between their work and ours is uncertain as the comparable observations in Fig. 1 were made both with a different cell line and at a relatively high K^+ concentration.

Effects associated with cellular Na^+ *and* K^+. None of the present observations shows clearly whether cellular Na^+ and K^+ act at steps 3 and 4 when the system is respiring, a problem that is discussed further below. Nevertheless, the observations in Figs. 4 and 5 and in Table 4 indicate that one or both of these cellular ions interact with the carrier system in the presence of cyanide. The most likely interpretation of Fig. 5 would seem to be that a relatively high cellular Na^+ content accompanied by a low K^+ content stimulates glycine efflux, as was first proposed by Riggs *et al.* (1958). Whether the parameters shown in Table 2 also apply to glycine efflux in these circumstances obviously requires further investigation. A similar interpretation may be used with Fig. 4. The smaller uptake at the higher Na^+ content may be simply the result of some of the glycine returning to the extracellular phase. Another important possibility concerns step 3. In the nomenclature of the Kinetic Analysis section, EK might move faster than ENa, the glycine influx rate being partly governed by the rate of return of the carrier. No decision can yet be made between these two interpretations of Fig. 4. What seems to be clearly established is that the fall in the glycine entry rate in the presence of cyanide is governed by the ionic composition of the cells, rather than directly by the supply of ATP as others have suggested (see the introduction for references).

Problem of the efflux kinetics during respiration. Comparison of Tables 6 and 7 and Fig. 7 shows that, though both K^+ deprivation and ouabain affected the glycine efflux rate during respiration, both factors had relatively little effect on the ionic composition of the cells over the same period. Analogous findings with ouabain were reported by Bittner & Heinz (1963) for the ascites system and by Kostyo & Schmidt (1963) for rat diaphragm preparations. The circumstances in which the effects in Tables 6 and 7 occurred suggest that during respiration there was a fairly close connexion between glycine efflux and active ion transport. The sodium pump in these cells is probably based on an Na^+-plus-K^+-activated adenosine triphosphatase, which would be retarded by ouabain, or by lowering the extracellular K^+ concentration, or in the presence of the metabolic inhibitors (Grobecker, Kromphardt, Mariani & Heinz, 1963). The sodium pump might be directly involved in moving glycine into the cell (cf. Csáky, 1963*a*,*b*; Eddy & Mulcahy, 1964; Glynn, 1964) or it might indirectly affect glycine efflux by controlling the concentrations of Na^+ and K^+ in certain outer regions of the cell. The calculations associated with Fig. 6 suggest that an Na^+ concentration of about 14m-equiv./l. may control efflux from a 10mM solution, if the efflux and influx of glycine are assumed to be equivalent processes. This value would presumably have to almost double to account for the stimulated glycine efflux rate with ouabain. Both the observed rate of K^+ uptake and the data in Fig. 7 would be consistent with this happening in, say, 20% of the cell volume, during the first minute of the measurements made either in the presence of ouabain or at the low extracellular K^+ concentration. These calculations show it would be premature to reject the idea that glycine efflux from the respiring cells is controlled by cellular Na^+ and K^+.

Role of ATP. The results suggest that the uptake of glycine can occur without the concomitant hydrolysis of ATP. If 0·5mol. of ATP were required to move 1mol. of glycine into the cells, then about 50% of the ATP formed during endogenous respiration (6mol. of ATP/mol. of oxygen) would be used in maintaining the maximum rate of glycine uptake (34mμmoles/mg./min.). It is accordingly all the more striking that roughly the same maximum rate was found in the presence of sodium cyanide, for the cells then contained only small amounts of ATP (about 0·6mμmole/mg.) and respiration and glycolysis together would probably have produced less than about 7mμmoles of ATP/mg./min. With both the latter values in mind, the important fact that various powerful inhibitors of energy metabolism failed to inhibit glycine uptake in the presence of cyanide (Fig. 5) strongly indicates that such accumulation was independent of ATP and similar compounds. It therefore seems unlikely that the various effects of Na^+ and K^+ referred to above are specifically involved in the utilization of ATP by an amino acid pump as Csáky (1963*a*,*b*) and Johnstone & Scholefield (1965) have suggested.

The circumstances in which glycine accumulated in the cells treated with sodium cyanide thus seem superficially to conform to Christensen's hypothesis (Riggs *et al.* 1958) that substrate gradients across the cell membrane can be induced by a counterflow of Na^+ and K^+, a conception that has already received some support from studies in other systems

(Vidaver, 1964*b*,*c*; Crane, 1964). One important question that arises is how far the observed accumulation can be accounted for by using the data in Table 2.

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