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The Effects of Varying the Cellular and Extracellular Concentrations of Sodium and Potassium Ions on the Uptake of Glycine by Mouse Ascites-Tumour Cells in the Presence and Absence of Sodium Cyanide

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1. Tumour cells were starved to deplete them of ATP and transferred to 0·9mM-glycine in Ringer solutions containing 2mM-sodium cyanide and various Na^+ and K^+ concentrations. The uptake of glycine then usually reached a peak by about 10min. 2. When cellular [Na^+] and extracellular [Na^+] were each about 30m-equiv./l., the maximum amount of glycine absorbed increased between 1·2- and 3·0-fold on lowering extracellular [K^+] from 128 to 10m-equiv./l. 3. When extracellular [Na^+] was 150m-equiv./l., the ratio, R, of the cellular to extracellular glycine concentrations increased progressively, from near 1 to about 9, when cellular [Na^+] was lowered from 120 to 40m-equiv./l. 4. When cellular [Na^+] was almost constant, either at 45 or 70m-equiv./l., R fell about 14-fold when extracellular [Na^+] varied from 150 to 16m-equiv./l. 5. Values of R near 0·2 were found when cellular [Na^+] was about four times as large as extracellular [Na^+]. 6. R fell about threefold when the cells were put with 12mM- instead of 0·9mM-glycine. 7. The results were taken to imply that, under these conditions, the spontaneous movements of both Na^+ and K^+ across the cell membrane, down their respective concentration gradients, served to concentrate the glycine in the tumour cells (Christensen's hypothesis).

Eddy, Mulcahy & Thomson (1967) showed that, in the presence of 2mM-sodium cyanide, starved mouse ascites-tumour cells depleted of cellular Na^+ and suspended in a Ringer solution containing 150m-equiv. of Na^+/l. and 0·9mM-glycine appeared to accumulate concentrations of glycine that were four to five times as large as that in the external medium. The maximum accumulation ratio under these conditions was, however, only about 20% of the value reached during respiration; $[Gly]_2$ in the respiring cells was about 15mM when $[Gly]_1$ was 0·6mM. (The subscript 1 with [Na^+], [K^+] or [Gly] denotes the extracellular solution, subscript 2 the corresponding component contained in the cellular water.) The starved cells were depleted of ATP and studies with various metabolic inhibitors suggested that the accumulation of glycine in the presence of cyanide was independent of ATP. Another significant finding was that the uptake of glycine was much larger when $[Na^+]_1$ was large and $[Na^+]_2$ was small than it was in the converse situation. This result was taken to mean that the presence of a large concentration gradient of Na^+ across the cell membrane stimulated the formation of a glycine concentration gradient of the opposite sense (Eddy & Mulcahy, 1965). Analogous findings were reported by Vidaver (1964*a*,*b*) working with pigeon erythrocytes and by Crane (1964) with intestinal preparations. All these observations are in qualitative agreement with a hypothesis originally formulated by Riggs, Walker & Christensen (1958) and subsequently developed by others (Crane, 1962; Mitchell, 1963; Vidaver, 1964*a*,*b*). According to that hypothesis these systems may accumulate a substrate such as glycine, without the direct intervention of ATP, by making use of the energy inherent in the tendency of the Na^+, and possibly of K^+, to move spontaneously across the cell membrane down their respective concentration gradients. The latter gradients are themselves maintained by the so-called 'sodium pump', which is probably driven by ATP. One would expect that, if the large glycine concentration gradients observed during respiration depended on such a mechanism, glycine gradients of similar magnitude might be temporarily maintained in the presence of cyanide, provided that the appropriate ion gradients were set up between the tumour cells and their environment. The present work was undertaken to test this possibility and to examine further the quantitative aspects of Christensen's hypothesis outlined by Eddy (1968). The following questions were

examined. (1) How did the steady-state values of $[Gly]_2/[Gly]_1$ vary with $[Na^+]_1$, $[Na^+]_2$, $[K^+]_1$ and $[K^+]_2$ in the presence and absence of cyanide? (2) Did both the Na^+ gradient and the K^+ gradient influence the distribution of glycine? (3) Did the experimental observations conform to the expected behaviour (Eddy, 1968) of a glycine-carrier system that interacted with both Na^+ and K^+ though not with ATP?

MATERIALS AND METHODS

These were exactly as described by Eddy *et al.* (1967) and Eddy (1968). The rate of uptake of glycine in the presence of cyanide was rather variable and usually the uptake reached a peak between 5 and 15min. To detect the peak, $[Gly]_2$, $[Na^+]_2$ and $[K^+]_2$ were therefore studied as a routine for about 30min. by using the 'small-sample technique' of Eddy (1968). The [^{14}C]glycine content of a given cell sample was extracted with ethanol and assayed. A parallel cell sample was washed with buffered choline chloride solution and the $[Na^+]_2/[K^+]_2$ ratio determined (Eddy *et al.* 1967). The sum $[Na^+]_2+[K^+]_2$ and the cellular water content were each determined by using the 'large-sample technique' of Eddy (1968). $[Gly]_2$ and the separate values of $[Na^+]_2$ and $[K^+]_2$ were then computed. Working with large cell samples was more difficult than working with small samples, so it was fortunate that both the sum $[Na^+]_2+[K^+]_2$ and the cellular water content could be

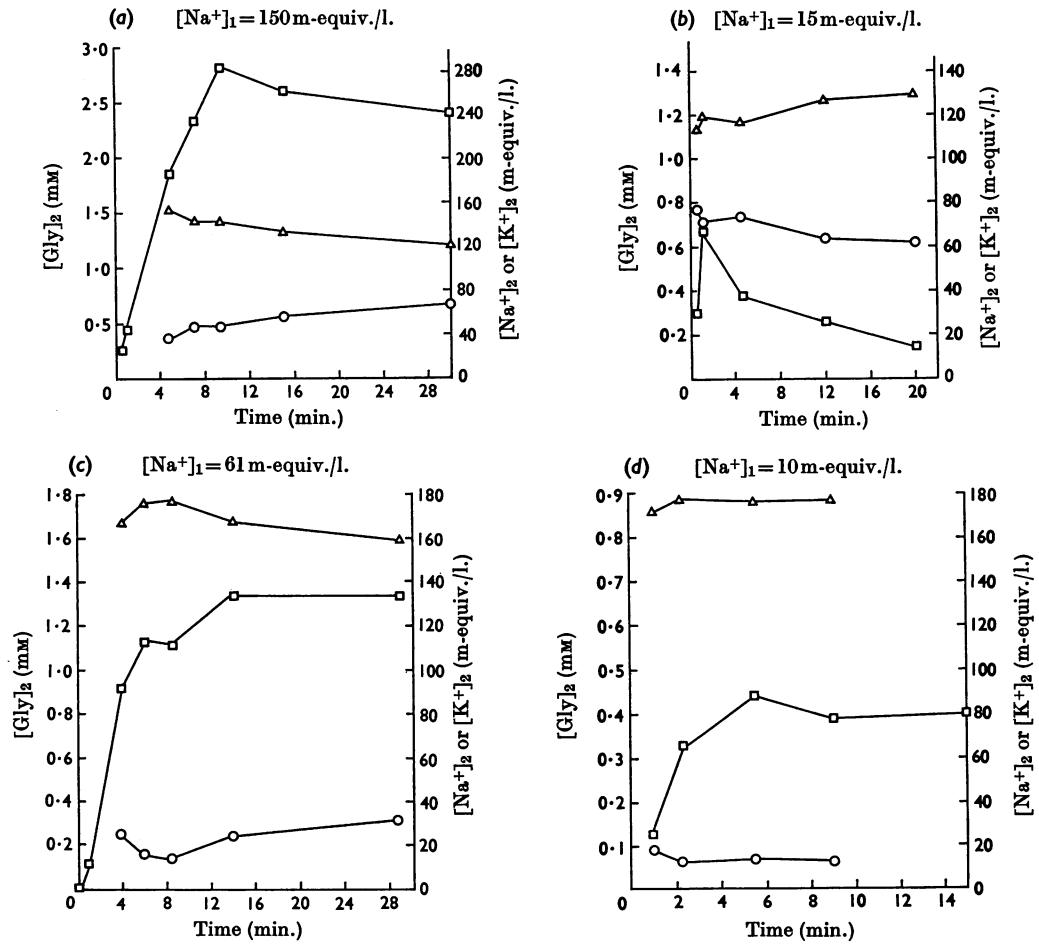

Fig. 1. Uptake of [^{14}C]glycine by the starved tumour cells in the presence of 2mM-NaCN and the concomitant changes in $[Na^+]_2$ and $[K^+]_2$ at various values of $[Na^+]_1$. $[Gly]_1$ was 0·9mM (approx. 10^5counts/min./ml.). □, $[Gly]_2$; ○, $[Na^+]_2$; △, $[K^+]_2$. $[Na^+]_1+[K^+]_1$ was 158m-equiv./l. $[Na^+]_1$ was: (*a*) 150m-equiv./l.; (*b*) 15m-equiv./l.; (*c*) 61m-equiv./l.; (*d*) 10m-equiv./l. The tumour cells were initially starved (see the text) for 25min. in the presence of cyanide with $[Na^+]_1$ about 8m-equiv./l. for (*a*), (*c*) and (*d*); they were starved for 60min. with $[Na^+]_1$ 150m-equiv./l. for (*b*).

assumed to be constant over the first 20min. of the incubation with glycine in the Ringer–cyanide solutions, despite the changes in $[Na^+]_2$, $[K^+]_2$ and $[Gly]_2$. Similar methods were used to find the steady-state values of $[Gly]_2$, $[Na^+]_2$ and $[K^+]_2$ during respiration, when the observations were usually made after the cells had spent 40min. with glycine.

RESULTS

General aspects of the kinetics of glycine uptake in the presence of cyanide. As in the earlier work (Eddy, 1968) the cells were starved for at least 25min. in a Ringer solution containing 2mM-sodium cyanide and were then put with glycine in another Ringer–cyanide solution. Figs. 1(*a*), 1(*b*), 1(*c*) and 1(*d*) show how the [^{14}C]glycine content of the samples of the starved cells typically varied with time when $[Na^+]_1$ was 150, 15, 61 and 10m-equiv./l. respectively. The changes in $[Na^+]_2$ and $[K^+]_2$ are also shown. These depended both on $[Na^+]_1$ and $[K^+]_1$ and on the history of the cells. The cells for Figs. 1(*a*), 1(*c*) and 1(*d*) were depleted of Na^+ during the preliminary starvation for 25min., whereas those for Fig. 1(*b*) had previously accumulated Na^+. Active transport of Na^+ was probably not involved, as the sodium pump appeared to have stopped working under these conditions (Eddy *et al.* 1967). A comparison may be made of Fig. 1(*a*), which shows the behaviour in the presence of cyanide, and curve *A* of Fig. 5(*a*) in Eddy *et al.* (1967), where cyanide and iodoacetate were used together both during the preliminary starvation and in the test with glycine. In general the cells retained the accumulated glycine for longer periods when cyanide alone was used. They also took up Na^+ more slowly from the environment. The rate of loss of glycine in the presence of cyanide was two to three times as fast in certain experiments as is shown in Fig. 1(*a*). The loss phase is examined further below.

The maximum value of $[Gly]_2$ in each of the four situations was computed by using the relevant value of the cellular water content shown in Table 1. $[Gly]_2$ was 2·83mM from Fig. 1(*a*), 1·34mM from Fig. 1(*c*) and 0·40mM from Fig. 1(*d*), the corresponding values of $[Na^+]_1/[Na^+]_2$ being 3·00, 2·86 and 0·83 respectively. The interpretation of Fig. 1(*b*), where $[Na^+]_2 > [Na^+]_1$, is less straightforward. Here less glycine was present in the cellular water than was trapped outside the cells, so $[Gly]_2$ may be considerably in error. The procedure used was to compute $[Gly]_2$ for the samples taken at about 10min., the apparent peak at 1min. shown in Fig. 1(*b*) not being reproducible. On that basis $[Na^+]_1/[Na^+]_2$ was 0·23 when $[Gly]_2$ was 0·28mM. The results leave little doubt that $[Gly]_2 < [Gly]_1$ under these conditions. Comparison with the other values from Fig. 1 shows that, in the transient steady state represented by the peak accumulation, $[Gly]_2$ increased with $[Na^+]_1/[Na^+]_2$, as was previously inferred (Eddy *et al.* 1967).

$[Na^+]_2 + [K^+]_2$ and the cellular water content in the presence of cyanide. Table 1 shows how these two quantities varied with the history of the cells. $[Na^+]_2 + [K^+]_2$ was virtually constant at a weighted mean value of 190·72 ± 1·40 (54) m-equiv./l., except when relatively large amounts of choline chloride were present when the weighted mean was 134·90 ± 1·66 (12) m-equiv./l. The cellular water content at 10min. appeared to be governed more by the ionic environment during the preliminary

Table 1. *Ion and water contents of the starved cells treated with cyanide in various Ringer solutions*

The conditions were similar to those in which glycine uptake was measured. The cells were starved for 25min. in a given Ringer solution with 2mM-NaCN at 37°. They were then transferred for 10min. at 37° to a fresh Ringer solution containing 0·9mM-glycine, 2mM-NaCN and the other constituents shown below. The respective mean values ± S.E.M. of the Na^+, K^+ and water contents of the cells were then measured (see the Materials and Methods section) in the specified numbers of independent experiments.

Preliminary starvation at		Glycine uptake from			Cellular composition		
$[Na^+]_1$ (m-equiv./l.)	$[K^+]_1$ (m-equiv./l.)	$[Na^+]_1$ (m-equiv./l.)	$[K^+]_1$ (m-equiv./l.)	Other additions	$[Na^+]_2+[K^+]_2$ (m-equiv./l.)	Dry wt./cellular water (mg./ml.)	No. of observations
10	150	148	10	—	192·1 ± 1·2	162·9 ± 1·6	28
10	150	148	10	—	186·7 ± 8·2	208·2 ± 10·1	7
10	150	9	149	—	190·5 ± 4·0	194·6 ± 11·0	7
152	8	148	10	—	187·4 ± 4·6	227·9 ± 9·5	6
152	8	9	149	—	192·6 ± 3·7	233·1 ± 10·8	6
10	150	30	10	117mM-Choline chloride	137·7 ± 2·3	205·5 ± 5·4	6
152	8	30	10	117mM-Choline chloride	132·1 ± 2·6	246·5 ± 12·5	6

starvation than by that during the test with glycine, so the values for this parameter in the first five rows of Table 1 were averaged as follows: (1) starvation at 150m-equiv. of K^+/l., weighted mean 175·73 ± 2·56 (54) mg./ml.; (2) starvation at 152m-equiv. of Na^+/l., weighted mean 235·83 ± 5·97 (18) mg./ml. The weighted mean values cited above were used extensively for computing $[Gly]_2$, $[Na^+]_2$ and $[K^+]_2$ in experiments like those illustrated in Fig. 1.

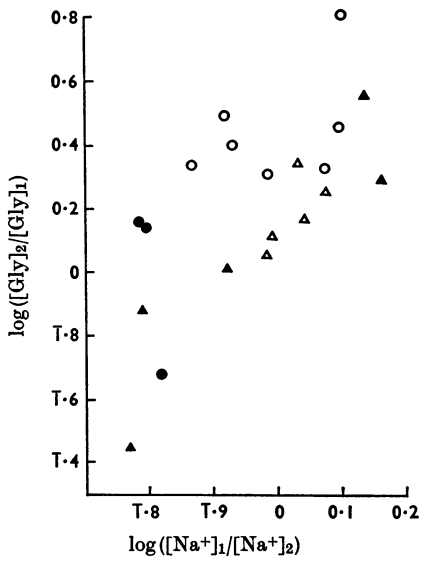

Fig. 2. Attempt to assess the effect of the K^+ gradient on $[Gly]_2/[Gly]_1$ in the transient steady state attained in the presence of cyanide. $\log([Gly]_2/[Gly]_1)$ was determined as a function of $\log([Na^+]_1/[Na^+]_2)$ with the starved cells in two contrasting situations: (1) ○ and ●, 2mM-NaCN, $[K^+]_1$ 10m-equiv./l., $[Na^+]_1$ about 30m-equiv./l., 117mM-choline chloride; (2) △ and ▲, 2mM-NaCN, $[K^+]_1$ 128m-equiv./l., $[Na^+]_1$ about 30m-equiv./l. $[Gly]_1$ was approximately constant at 0·9mM. Depending on the state of the cells $[Na^+]_2$ varied from about 25 to 70m-equiv./l. The value of $[Na^+]_2+[K^+]_2$ is shown in Table 1. The uptake of glycine in the above two situations was compared on ten occasions. ○ and △, Cells initially starved for 25min. with 2mM-NaCN at 150m-equiv. of K^+/l.; ● and ▲, initial starvation with 2mM-NaCN at 150m-equiv. of Na^+/l.

Effect of the K^+ gradient on glycine accumulation in the presence of cyanide. In the experiments illustrated in Fig. 1, as in similar previous work in this Laboratory, $[Na^+]_1$ and $[K^+]_1$ were not independent variables since their sum was constant. The same applied to the pair $[Na^+]_2$ and $[K^+]_2$. Fig. 2 summarizes a series of experiments in which $[Na^+]_1$ and $[K^+]_1$ were independently varied. The sum $[Na^+]_2+[K^+]_2$ was as shown in Table 1. The experimental design is explained in the legend to Fig. 2. It consisted in comparing the maximum transient value of $[Gly]_2/[Gly]_1$ observed with a given cell preparation in two circumstances: (1) with $[K^+]_2 \gg [K^+]_1$ and (2) with $[K^+]_2 \simeq [K^+]_1$. It was not feasible to keep $[Na^+]_1/[Na^+]_2$ constant, and so it was treated as a variable against which $[Gly]_2/[Gly]_1$ was plotted. In practice the double-logarithmic plot shown in Fig. 2 was preferred for two reasons. First, it decreased the scatter of the observations. Secondly, a simple thermodynamic argument shows that, if a constant fraction of the energy inherent in the Na^+ gradient were used to concentrate glycine, $\log([Na^+]_1/[Na^+]_2)$ would vary linearly with $\log([Gly]_2/[Gly]_1)$. Inspection of Fig. 2 suggests that at a given Na^+ gradient $[Gly]_2/[Gly]_1$ usually became somewhat larger when choline ions replaced K^+ outside the cells. A regression analysis of the data is summarized in the first two rows of Table 2. When $[Na^+]_1=[Na^+]_2$ the effect on $\log([Gly]_2/[Gly]_1)$ of virtually removing the K^+ gradient is equal to the change in the value of the coefficient b. The mean change in $\log([Gly]_2/[Gly]_1)$, with 95% confidence limits computed by using the Fisher–Behrens d-statistic, was 0·276 ± 0·208. $[Gly]_2/[Gly]_1$ changed therefore between about 1·2- and 3·0-fold.

Table 2. *Coefficients of the equation* $\log([Gly]_2/[Gly]_1) = a\log([Na^+]_1/[Na^+]_2) + b$

The coefficients were obtained from a linear regression analysis of the data in Figs. 2, 3, 5 and 6 respectively. F = no. of degrees of freedom. The description of the data is explained in the text.

Source of data	Added cyanide	Coefficients ± S.E.M. a	b	F
Fig. 2, transient steady state in the presence of choline chloride	+	1·639 ± 0·598	0·412 ± 0·079	8
Fig. 2, transient steady state in the presence of KCl	+	2·056 ± 0·367	0·136 ± 0·045	8
Fig. 3, transient steady state, all the observations	+	1·257 ± 0·068	0·068 ± 0·023	50
Fig. 3, transient steady state, $[Na^+]_1$ = 150m-equiv./l.	+	1·371 ± 0·224	0·019 ± 0·089	20
Fig. 5, loss phase	+	1·784 ± 0·199	−0·020 ± 0·070	12
Fig. 6, steady state during respiration	−	1·774 ± 0·181	0·211 ± 0·070	21

The situation examined experimentally in Fig. 2 was also studied theoretically. In the light of the observations in Eddy (1968) Christensen's hypothesis was expressed in the following approximate relationship between $[Na^+]_1$, $[Na^+]_2$, $[K^+]_1$, $[K^+]_2$ and the resultant steady-state values of $[Gly]_2/[Gly]_1$:

$$\frac{[Gly]_2}{[Gly]_1} = \frac{[Na^+]_1}{[Na^+]_2} \cdot \frac{(1+\theta[K^+]_2)}{(1+\theta[K^+]_1)} \qquad (1)$$

where θ is a constant. It is then readily shown that the limits inferred above for the effect of the K^+ gradient correspond to the inequality $\theta \leqslant 0{\cdot}025$.

Effect of the Na^+ *gradient on glycine accumulation in the presence of cyanide.* The relation between $[Na^+]_1/[Na^+]_2$ and $[Gly]_2/[Gly]_1$ was investigated in a series of experiments lasting 18 months, a given type of measurement being repeated at intervals over the whole period, so that the results could be combined as shown in Fig. 3. The relevant data in Table 1 were obtained by combining measurements made before and after that period.

(1) $[Na^+]_1$ and $[K^+]_1$ constant, $[Na^+]_2$ and $[K^+]_2$ varying. Repetition of the experiment illustrated in Fig. 1(*a*) gave peak values of $[Gly]_2/[Gly]_1$ ranging from about 1 to 9, with a correspondingly large variation of $[Na^+]_2$ at the peak. Plotting $\log([Gly]_2/[Gly]_1)$ against $\log([Na^+]_1/[Na^+]_2)$ revealed a marked correlation ($r = 0{\cdot}808$, $P < 0{\cdot}001$) between the two variables (▲ in Fig. 3). The actual regression coefficients are shown in row 4 of Table 2. The slope a was not significantly greater than 1 ($P > 0{\cdot}10$).

(2) $[Na^+]_1$ and $[K^+]_1$ varying, $[Na^+]_2$ and $[K^+]_2$ constant. Inspection of the pooled results revealed a set where $[Na^+]_2$ was near 45m-equiv./l. In this set the mean values of $[Gly]_2/[Gly]_1$ (three determinations) were 0·39, 1·05 and 6·29 respectively when $[Na^+]_1$ was 16, 40 and 150m-equiv./l. Similarly, there was a further set where $[Na^+]_2$ was near 70m-equiv./l. Here the mean values of $[Gly]_2/[Gly]_1$ were 0·28, 0·61 and 3·95 respectively when $[Na^+]_1$ was 17, 47 and 150m-equiv./l.

(3) Symmetry of the system. Fig. 3 (■ and ○) shows that $[Gly]_2 < [Gly]_1$ when $[Na^+]_2 > [Na^+]_1$. Moreover, all the observations lie about a straight line ($r = 0{\cdot}934$, $P < 0{\cdot}001$). Reference to row 3 of Table 2 shows that the regression line for the pooled results almost passed through the origin. Its mean slope (coefficient a) was significantly greater than 1 ($P < 0{\cdot}001$). Within the limits set by the considerable scatter of the results, it appears that, if $[Gly]_2/[Gly]_1$ was x at a given value of $[Na^+]_1/[Na^+]_2$, then it was x^{-1} when the direction of the Na^+ gradient was reversed.

(4) Effect of varying $[Na^+]_1$ during the preliminary starvation. The initial starvation was conducted at one of three values of $[Na^+]_1$ (■, ○ and ●, see legend to Fig. 3). The way in which these symbols are distributed over Fig. 3 in the region where $\log([Gly]_2/[Gly]_1) < 0{\cdot}4$ indicates that the principal effect on that ratio of varying $[Na^+]_1$ during the starvation was to vary $[Na^+]_2$ during the uptake of glycine.

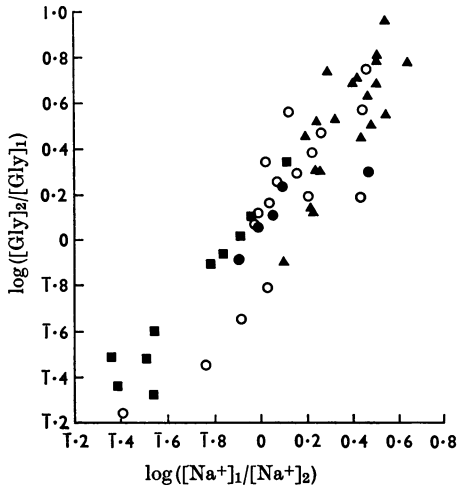

Fig. 3. Plot of $\log([Gly]_2/[Gly]_1)$ as a function of $\log([Na^+]_1/[Na^+]_2)$ in the transient steady state reached when the starved tumour cells were put with 0·9mM-glycine in the presence of 2mM-NaCN. $[Gly]_1$ fell by up to about 30% when $[Gly]_2$ was relatively large. The choice of $[Na^+]_1$ is described in the text. $[K^+]_1$ also varied with $[Na^+]_1 + [K^+]_1$ constant at 160m-equiv./l. $[Na^+]_2 + [K^+]_2$ was constant at about 191m-equiv./l. During the preliminary starvation in the presence of 2mM-NaCN the cells were kept for 25min. at one of three values of $[Na^+]_1$ as follows: ○ and ▲, 10m-equiv. of Na^+/l., 148m-equiv. of K^+/l.; ●, 52m-equiv. of Na^+/l., 106m-equiv. of K^+/l.; ■, 150m-equiv. of Na^+/l., 8m-equiv. of K^+/l.

(5) Application of eqn. (1). Fig. 4 shows certain solutions of eqn. (1), when θ was either 0·01 or 0·05, in two situations. In the first, $[Na^+]_1$ was 150m-equiv./l. and $[Na^+]_2$ varied, whereas in the second, both $[Na^+]_1$ and $[Na^+]_2$ varied. The variation followed the pattern observed in the experiments used for Fig. 3. Fig. 4 shows that when θ was 0·01 the respective lines representing the two situations were fairly close, whereas they were wider apart when θ was 0·05. Thus combining the two types of observations would obviously contribute to the scatter in Fig. 3. Fig. 4 also shows that the value of the coefficient a obtained from the pooled data would tend to increase with θ. In practice, when $[Na^+]_1$ was constant the mean value of b was fairly small, though values as large as 0·2 ($P = 0{\cdot}05$) were feasible (row 4, Table 2). Such considerations suggest that the value of θ ($\leqslant 0{\cdot}025$) originally

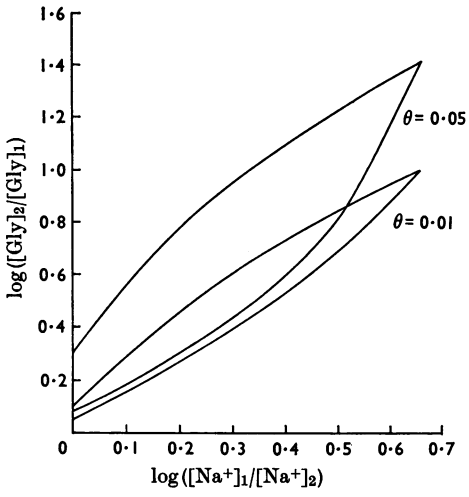

Fig. 4. Relations between $\log([Gly]_2/[Gly]_1)$ and $\log([Na^+]_1/[Na^+]_2)$ expected on the basis of eqn. (1). Two ways of varying $[Na^+]_1/[Na^+]_2$ were studied. With the parameter θ (see the text) constant, the upper line of each pair represents the behaviour expected when $[Na^+]_1$ was 150m-equiv./l., $[K^+]_1$ 8m-equiv./l. and $[Na^+]_2$ varied systematically with $[Na^+]_2+[K^+]_2$ 190m-equiv./l. In the corresponding lower line $[Na^+]_1$ and $[K^+]_1$ also varied with $[Na^+]_1+[K^+]_1$ 160m-equiv./l.

deduced from Fig. 2 is also consistent with the data in Fig. 3 and Table 2. Attempts to infer θ directly by plotting the combined observations in an appropriate form based on eqn. (1) were unsuccessful owing to the scatter of the points.

(6) $[Gly]_2/[Gly]_1$ as a function of $[Gly]_1$. Table 3 shows that on the average $[Gly]_2/[Gly]_1$ was smaller the larger the glycine concentration with which the cells were provided. Eddy (1968) showed that glycine was absorbed from an 11mM solution with the uptake of approx. 1 equiv. of Na^+. The assimilation of glycine itself would therefore contribute to the increase in $[Na^+]_2$ that occurred before the glycine peak was reached. Insofar as the Na^+ gradient was lowered, the maximum value of $[Gly]_2/[Gly]_1$ might therefore be smaller at large than at small values of $[Gly]_1$. The magnitude of the effect was examined in relation to the data in Table 3 as follows. The value of $[Na^+]_1/[Na^+]_2$ when $[Gly]_1$ was 0·9mM and $[Gly]_2$ 3·6mM was estimated to be 2·33 from the data in Table 2; $[Na^+]_2$ was therefore 64m-equiv./l. When $[Gly]_2$ was 16·2mM $[Na^+]_2$ would be expected to be (16·2 − 3·6) m-equiv./l. larger, or about 77m-equiv./l. $[Gly]_2/[Gly]_1$ would then be about 2·4 if eqn. (1) applied. In fact it was 1·3 (Table 3). One possibility is that, whereas the assimilated glycine mixed with all the cellular water, the Na^+ entering as the hypothetical complex ENaGly, as postulated

Table 3. *Maximum uptake of glycine* ($[Gly]_2$) *by the starved cells in the presence of* 2mM-*sodium cyanide as a function of* $[Gly]_1$

A series of measurements like those shown in Fig. 1(*a*) were made and the results pooled. The error in $[Gly]_2$ can be computed directly from the S.E.M. of $[Gly]_2/[Gly]_1$, which is shown in the fourth column.

$[Gly]_1$ (mM)	No. of observations	$[Gly]_2$ (mM)	$[Gly]_2/[Gly]_1$
0·10	1	0·46	4·65
0·50	2	1·36	2·73±0·32
0·95	6	3·58	3·77±0·50
3·27	5	7·49	2·29±0·24
4·98	2	9·61	1·93±0·10
8·70	7	12·53	1·44±0·13
12·12	4	16·24	1·34±0·10

by Eddy (1968), may have mixed with only a portion of the cellular water. The relevant value of $[Na^+]_2$ might then be considerably larger than the average value for the cellular phase. A calculation based on Fig. 3 showed that a compartment representing about 14% of the cellular water would be required to account for the data in Table 3 on that basis.

Relation between $[Na^+]_2$ *and* $[Gly]_2$ *after the peak.* Fig. 5 shows how $[Na^+]_1/[Na^+]_2$ varied with $[Gly]_2/[Gly]_1$ for 40min. after the maximum amount of glycine was absorbed. The corresponding regression coefficients shown in Table 2 are similar to those applying at the peak (compare rows 4 and 5). It therefore seems likely that the rate the cells accumulated Na^+ was a major factor governing the rate at which they lost glycine.

Glycine uptake during respiration. (1) Effect of the Na^+ gradient. The way in which $[Gly]_2/[Gly]_1$ varied with $[Na^+]_1/[Na^+]_2$ in the steady state reached when the respiring cells were put with 0·9mM-glycine for 40min. is shown in Fig. 6, which is based on the additional data in Table 4. A weighted mean value of $[Na^+]_2+[K^+]_2$ of 173·0 ± 1·6 (18) m-equiv./l. was used in calculating $[Na^+]_2$ for the cells from the Ringer solutions without added choline chloride or ouabain, i.e. for most of the points in Fig. 6. The corresponding mean value in the presence of ouabain was 186·4 ± 3·3 (6) m-equiv./l. The appropriate values of the ratio dry wt./cellular water in the simple Ringer solutions were obtained from a regression analysis of the data in the first four rows of Table 4.

In contrast with the situation in Fig. 3, the activity of the sodium pump was an important factor governing $[Na^+]_2$ and $[K^+]_2$ during respiration. $[Gly]_2/[Gly]_1$ fell when $[K^+]_1$ was much smaller than about 5m-equiv./l. (the two lowest ▲

values in Fig. 6), as Riggs *et al.* (1958) first showed. These workers attributed the effect to the concomitant changes in either $[K^+]_2$ or $[Na^+]_2$. Presumably the sodium pump is then governed by

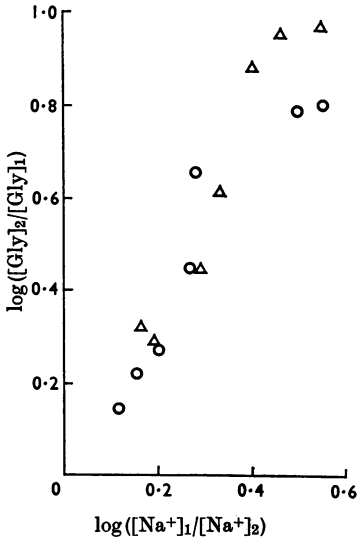

Fig. 5. Relation between $\log([Gly]_2/[Gly]_1)$ and $\log([Na^+]_1/[Na^+]_2)$ after the maximum accumulation of glycine was reached and the cells were losing glycine (see Fig. 1*a*). A series of measurements of these two quantities were made on two separate occasions (△ and ○) between 20 and 60 min. after the starved cells were put with 0·9 mM-glycine in the standard Ringer solution with 2 mM-NaCN.

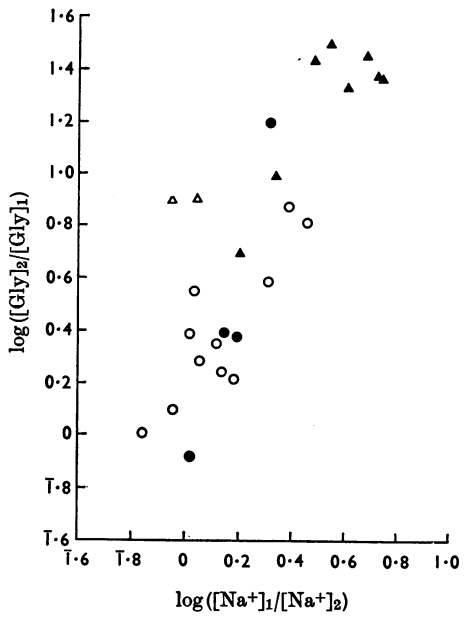

Fig. 6. Relation between the steady-state values of $\log([Gly]_2/[Gly]_1)$ and $\log([Na^+]_1/[Na^+]_2)$ during respiration. The cells were incubated with 0·9 mM-glycine for 40 min., when a steady state appeared to be reached. Each point represents the mean of two determinations of each variable based on the data in Table 4. ▲, $[Na^+]_1$ about 150 m-equiv./l., $[K^+]_1$ either 8 m-equiv./l. or near zero (see the text); ●, the above plus 0·1 mM-ouabain (see the text); ○, $[Na^+]_1+[K^+]_1$ 158 m-equiv./l., with $[Na^+]_1$ at 6, 12, 25, 50 or 75 m-equiv./l.; △, $[Na^+]_1$ 32 m-equiv./l., $[K^+]_1$ 8 m-equiv./l., plus 120 mM-choline chloride.

Table 4. *Ion and water contents of the respiring cells as a function of the ionic composition of the environment from which glycine was absorbed*

After spending 20 min. at 37° in the standard Ringer solution, the cells were kept for 40 min. at 37° with 0·9 mM-glycine in a Ringer solution of the composition shown below. The respective mean values ± S.E.M. of the Na^+, K^+ and water contents of the cells were then measured (see the Materials and Methods section) in the specified numbers of independent experiments.

Ringer solution			Cellular composition		
$[Na^+]_1$ (m-equiv./l.)	$[K^+]_1$ (m-equiv./l.)	Other additions	$[Na^+]_2+[K^+]_2$ (m-equiv./l.)	Dry wt./cellular water (mg./ml.)	No. of observations
9	151	—	171·5 ± 2·4	183·7 ± 6·4	4
53	107	—	175·5 ± 1·0	203·3 ± 6·2	4
128	32	—	174·8 ± 2·4	255·4 ± 3·6	4
152	8	—	171·3 ± 4·5	275·4 ± 8·0	6
32	8	120 mM-Choline chloride	158·4 ± 6·9	313·2 ± 9·9	8
152	8	0·1 mM-Ouabain	180·8 ± 1·7	295·2 ± 7·2	2
160	0	0·1 mM-Ouabain	189·2 ± 5·2	313·8 ± 22·5	4

$[K^+]_1$. In the present work similar effects were also observed in the presence of 0·1 mM-ouabain (the four ● points in Fig. 6), especially when K^+ was omitted from the Ringer solution (the three lower ● points). It is not possible to decide from Fig. 6 whether the sodium pump affected $[Gly]_2/[Gly]_1$ only by regulating $[Na^+]_2$ and $[K^+]_2$. Comparison with Fig. 3, where the sodium pump was apparently not involved, suggests that that was its principal effect, however (see below).

(2) Glycine accumulation in the presence as compared with the absence of cyanide. A regression analysis of the data in Fig. 6 (the measurements involving choline chloride being omitted) ($r = 0{\cdot}906$, $F = 21$, $P < 0{\cdot}001$) gave the results shown in Table 2 (row 6). Application of the Fisher–Behrens d-statistic showed both (1) that the regression coefficient a was larger ($P \simeq 0{\cdot}01$) and (2) that the intercept b was also perhaps larger ($P \simeq 0{\cdot}05$) for the respiring cells than for those with cyanide. The mean value of $\log([Na^+]_1/[Na^+]_2)$ for the cells respiring in the standard Ringer solution was 0·647, with $[Gly]_2/[Gly]_1$ varying from 33·6 to 15·5 (95% confidence limits). The corresponding limits for the system with cyanide were 9·2 to 6·3. Thus the tumour cells appeared to accumulate only about 30% as much glycine in the presence of cyanide as did the respiring cells.

The difference between the two systems may also be expressed as follows. The respiring cells in the standard Ringer solution with $[Na^+]_2$ 34 m-equiv./l. concentrated glycine to the same extent [$\log([Gly]_2/[Gly]_1) = 1{\cdot}41$] as the system with cyanide would be expected to do when $[Na^+]_1$ was 150 m-equiv./l. and $[Na^+]_2$ was between 14 and 21 m-equiv./l. The latter values depend on the assumption that the data in Fig. 3 can be extrapolated over the required range. Now Eddy *et al.* (1967) tentatively concluded that glycine efflux from the respiring cells might be governed by a value of $[Na^+]_2$ that, owing to the operation of the sodium pump, was possibly only half the mean value of $[Na^+]_2$ in the cellular water. Such a factor would not apply when the sodium pump was stopped by cyanide. If that interpretation is correct the peak accumulation of glycine in the transient steady state in the presence of cyanide may be almost as large as the steady-state value maintained during respiration under the influence of the same ionic gradients.

(3) Effect of the K^+ gradient. A number of determinations of $[Gly]_2/[Gly]_1$ were made in which $[Na^+]_1$ and $[Na^+]_2$ were almost equal and the Ringer solution contained 120 mM-choline chloride (△ in Fig. 6). The possible effect of the K^+ gradient was computed by comparing the mean value of $\log([Gly]_2/[Gly]_1)$ under these conditions with the appropriate value of b in Table 2. Replacing choline chloride by potassium chloride was shown in that way to lower $[Gly]_2/[Gly]_1$ between 6·7- and 3·5-fold (95% confidence limits). The corresponding limits for θ in eqn. (1) are about 0·05 and 0·02 respectively.

DISCUSSION

The above observations on the system with cyanide show clearly that the starved tumour cells, depleted of ATP (Eddy *et al.* 1967), accumulated glycine from a 0·9 mM solution in amounts that were closely correlated with the relative magnitudes of $[Na^+]_1$ and $[Na^+]_2$ (Fig. 3). Though the importance both of K^+ and of other factors has to be considered (see below), a likely interpretation is that the peak accumulation represented a transient steady state controlled by $[Na^+]_1$ and $[Na^+]_2$. The tumour cells accumulated glycine when $[Na^+]_1 \gg [Na^+]_2$ and excluded it when $[Na^+]_2 \gg [Na^+]_1$. Vidaver (1964*a*,*b*) described a similar situation in haemolysed and restored pigeon erythrocytes deficient in ATP. He was mainly concerned with the effects of varying $[Na^+]_1$ and $[Na^+]_2$ on the glycine influx and efflux. The chief interest in the present work, on the other hand, was in the relations between $[Na^+]_1$, $[K^+]_1$ etc. and the steady-state values of $[Gly]_2/[Gly]_1$. Vidaver found a maximum value of $[Gly]_2/[Gly]_1$ of 3 for the restored erythrocytes when $[Na^+]_1$ was 140 m-equiv./l. and $[Na^+]_2$ was small (Table 3 of Vidaver, 1964*a*). Comparable values up to 9 were demonstrated in the present work with the tumour cells. As the intact erythrocytes concentrated glycine only about eightfold, however, whereas the tumour cells concentrated it 25-fold, the two types of cells appear to have been similarly affected by the deficiency of ATP.

On what energy source did the accumulation of glycine in the presence of cyanide depend? (1) Eddy *et al.* (1967) considered that ATP was not involved as (*a*) little ATP was available and (*b*) glycine uptake under these conditions was not retarded by various metabolic inhibitors.

(2) Another possibility would be that the glycine simply exchanged with the endogenous amino acids, which are approximately equivalent to a 26 mM-glycine solution (Eddy, 1968). It was not feasible, owing to the considerable experimental errors due to the size of the endogenous pool, to show whether the accumulations represented in Fig. 3 were accompanied by an equivalent increase in the cellular amino acid content. Nevertheless two circumstances make it likely that exchange was not involved. (*a*) Amounts of glycine equivalent to the larger values of $[Gly]_2$ in Fig. 3 (up to 8 mM) were absorbed without being exchanged when $[Gly]_1 > 4$ mM (Eddy, 1968). (*b*) If the equivalent of at least 8 mM-glycine were available for exchange,

it is difficult to see why (i) glycine was excluded from the cells when $[Na^+]_2 > [Na^+]_1$ (Fig. 3) and, more generally, (ii) why the glycine concentration in the steady state appeared to be governed by $[Na^+]_1$ and $[Na^+]_2$. Though the possibility that amino acid exchange occurred is not ruled out, the evidence therefore favours the idea of an alternative source of energy.

(3) A likely alternative source is suggested by Christensen's hypothesis (Riggs *et al.* 1958) that the spontaneous movements of Na^+ or of K^+, or of both ions, across the cell membrane served to concentrate the glycine. Vidaver's (1964*a*,*b*) observations with the pigeon erythrocytes and Crane's (1964) work with intestinal preparations both also support that hypothesis in qualitative terms.

Evidence bearing on the relative roles of Na^+ *and* K^+. In the mathematical model of the carrier cycle considered by Eddy (1968), glycine entered the cells exclusively as the carrier complex ENaGly and the carrier returned to the outer membrane phase as either EK or E. It was decided to ignore, in the first instance, the problem of how a charge balance between the two phases was maintained. Such a system might accumulate glycine even in the complete absence of K^+, though it obviously could not function without Na^+. Support for the model came from the experimental finding that almost 1 equiv. of Na^+ accompanied glycine into the cells and that about 0·6 equiv. of K^+ was expelled from them. The movement of ENa appeared to be characterized by a relatively small velocity constant (k^n) as compared with the movements of E, EK and ENaGly (velocity constants, k^c, k^k and k^{ng} respectively). The conclusion reached was that $k^n/k^c < 0{\cdot}3$, $k^k/k^c > 3$ and $k^{ng}/k^c \ll 1$. Eqn. (1), derived from eqn. (10) of Eddy (1968), is based on the approximation that k^n was actually zero (the parameter $\theta = k^k/76k^c$).

Application of eqn. (1). (1) Provided that $[K^+]_1$, $[K^+]_2$ and θ were each quite small, $[Gly]_2/[Gly]_1$ would be near 1 when $[Na^+]_1$ and $[Na^+]_2$ were both 150 m-equiv./l. This appears to be so in the presence of cyanide, as is shown by the value of b in row 4 of Table 2, and encourages the view that the membrane potential was then small enough to be neglected (cf. Aull, 1967).

(2) Eqn. (1) assumes that the carrier E had the same intrinsic properties in the inner as in the outer membrane phase. The roughly symmetrical distribution of the observations about the origin in Fig. 3 supports this idea.

(3) According to eqn. (1), the slope a of the regression line based on the data in Fig. 3 would be 1·0, unless factors other than the changes in $[Na^+]_1/[Na^+]_2$ contributed. Table 2 shows that the values found from both Figs. 3 and 6 were larger than 1 ($P < 0{\cdot}001$). Other factors were therefore involved. The chief possibilities concern (*a*) the K^+ gradient, (*b*) the membrane potential, (*c*) the intracellular distributions of Na^+ and K^+ and (*d*) the entry of glycine as E2NaGly rather than ENaGly, other evidence of which is lacking, however (Eddy *et al.* 1967; Eddy, 1968).

Various lines of evidence implicate $[K^+]_1$ and $[K^+]_2$. (*a*) $[Gly]_2/[Gly]_1$ increased between 1·2- and 3·0-fold when $[K^+]_2/[K^+]_1$ was increased at least tenfold with $[Na^+]_1/[Na^+]_2$ constant (Fig. 2). θ would then be $\leqslant 0{\cdot}025$, a value compatible with the magnitude of a for the pooled observations in Fig. 3. (*b*) A similar series of experiments with the respiring cells gave an increase between 3·5- and 6·7-fold with θ between about 0·02 and 0·05. (*c*) The carrier appeared to interact with both K^+ and Na^+ (Eddy *et al.* 1967), and glycine uptake was accompanied by a net loss of K^+ (Eddy, 1968). θ was $> 0{\cdot}04$ on the basis of the latter observations. (*d*) The behaviour of the cells in the choline–Ringer solutions showed that, even when $[Na^+]_1$ and $[Na^+]_2$ were ostensibly equal, $[Gly]_2/[Gly]_1$ was between 2 and 4 in the presence of cyanide and about 8 during respiration. All these observations (3*a*–*d*) strongly suggest that factors other than the Na^+ concentration gradient governed $[Gly]_2/[Gly]_1$. They appear to implicate either (1) the distribution of K^+ or (2) a membrane potential that varied with the environmental conditions and, perhaps, depended partly on a K^+ diffusion potential. At present the first possibility seems the more likely one in view of section (*c*) above. However, the various estimates of θ agree only very roughly. Since the value of θ given in section (*c*) above was larger than the value in section (*a*), it seems possible that only a fraction of the K^+ loss induced by glycine (Eddy, 1968) may have actually passed through the glycine carrier.

(4) Riggs *et al.* (1958) observed that the replacement of up to about half of the cellular K^+ by the cations of $\alpha\gamma$-diaminobutyric acid stimulated the uptake of glycine by the ascites-tumour cells, whereas an equivalent replacement by Na^+ lowered the uptake. This was subsequently taken to mean that, of the two alkali ions, only Na^+ directly affected $[Gly]_2/[Gly]_1$ (Wheeler, Inui, Hollenberg, Eavenson & Christensen, 1965; Christensen, 1967). Christensen, Riggs, Fischer & Palatine (1952) earlier showed that the absorption of the diaminobutyrate ion may lower $[Na^+]_2$, which may possibly explain why the uptake of glycine was stimulated. The specific effect of K^+ in this experiment cannot be defined, however, unless (*a*) the exact magnitude of the stimulatory effect is known and (*b*) the variation of $[Na^+]_2$ is taken into account.

(5) Table 3 shows that $[Gly]_2/[Gly]_1$ fell abruptly as $[Gly]_1$ (and $[Gly]_2$) increased. Glycine transport was not then simply uncoupled from ion transport,

as Na^+ was absorbed with glycine from an 11 mM solution (Eddy, 1968) and glycine entry depended on $[Na^+]_1$ (Eddy *et al.* 1967). The fall in $[Gly]_2/[Gly]_1$ was larger than was expected on the basis of eqn. (1), unless the Na^+ absorbed with the glycine was assumed to be confined to about 14% of the cellular water. Another possibility compatible with eqn. (1) is that the uptake of relatively large amounts of glycine was limited by either the availability, or the accumulation, of another ion whose movements accompanied the passage of unequal amounts of Na^+ and K^+ across the cell membrane (the hypothetical counter-ions referred to in Eddy, 1968).

Though the arguments set out above reveal a number of important problems relating to the application of Christensen's hypothesis to the ascites-tumour system, the results taken as a whole appear to support the hypothesis.

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