The influence of amino acids and antimetabolites on glycine retention by Ehrlich ascites carcinoma cells

R. M. Johnstone* and P. G. Scholefield†

(McGill-Montreal General Hospital Research Institute, 3619 University Street, Montreal, P.Q., Canada)

The transport of biologically important substances across cell membranes has been studied in a wide variety of systems. For example, transport of ions into and out of cells has long formed an integral part of studies in physiology (8), and it has been known since 1913 from the studies of Van Slyke and Meyer (20) that amino acids given to an animal appear in the tissues at concentrations which are higher than those of the surrounding medium. The transport of sugars and amino acids from the mucosal to the serosal side of the small intestine in in vitro preparations has also been well established (6, 7, 18).

The demonstration of the capacity of isolated tumor cells to transport actively such substances as ions, sugars, and amino acids (3, 5, 12) has provided the means of more precise studies of this general phenomenon. Thus, the results of Christensen, Riggs, Fischer, and Palatine (3) show that glycine may be concentrated in vitro by Ehrlich ascites carcinoma cells to the extent of more than twelvefold. Later results, obtained by Heinz and Mariani (10) using radioactive glycine, showed that there were possibly three separate reactions involved in the establishment of a steady state concentration of glycine within the ascites cells.

1. A specific mechanism for the transport of glycine (or other amino acids) across the cell membrane.

2. A nonspecific influx and efflux dependent only on the difference between the concentration of amino acid in the medium and that in the cell.

3. An exchange of glycine in the cell with that of the medium without any net flow of glycine, the exchange reaction occurring, according to Heinz and Mariani (10), at a rate which is 5–6 times as great as that of the rate of actual transport.

Many workers have shown that compounds such as dinitrophenol, which interfere with the availability of energy in the form of adenosine triphosphate (ATP), also interfere with the capacity of Ehrlich ascites cells to take up or to transport amino acids (2, 10). However, it was felt that the information obtained from this approach could well be supplemented by a study of the kinetics of interaction of glycine with other amino acids and with amino acid analogs. Christensen et al. (3) and Tenenhouse and Quastel (17) have already shown that certain amino acids inhibit the transport of other amino acids into Ehrlich ascites cells. Cohen and Monod (4), in their studies of the displacement of thiogalactosides in E. coli, concluded that such displacement is competitive and Tenenhouse and Quastel (17) have reached a similar conclusion with reference to amino acid interactions during the process of transport into Ehrlich ascites cells. It has also been shown by Riggs, Coyne, and Christensen (14) that amino acids, previously concentrated, may be displaced from Ehrlich ascites cells on addition of other amino acids. In the studies made by Riggs et al. (14), the amino acids were added at concentrations of 30–60 mmolar, and the final analyses were made 2 or 3 hours later. A more detailed investigation of this phenomenon was undertaken to obtain information concerning the factors controlling the extent of glycine retention under various experimental conditions.

Studies by Wiseman and Ghadially (19), in which RD3 carcinoma cells were used, and by Birt and Hird (1), in which carrot slices were utilized, showed that methionine is not well concentrated by these preparations but that it is an effective inhibitor of the concentration of other amino acids such as glycine. In the present work, therefore, methionine has been used extensively in studies of glycine retention by Ehrlich ascites cells. Further, the effects of the antimetabolites studied on the incorporation of glycine into the
alcohol-insoluble material (mainly into the protein) has been determined in an attempt to estimate the specificity and nature of the effect of the inhibitors. In many cases the influence of added glucose was also investigated to assess the relative effects of glycolysis and its consequences, such as glycolytic ATP production, on the processes of uptake and incorporation of glycine.

**MATERIALS AND METHODS**

**Ascites cells.**—The Ehrlich ascites cells were harvested from CF1 mice 6 or 7 days after intraperitoneal transplantation. After a twenty-fold dilution with ice-cold isotonic saline, the suspension was centrifuged for 20 seconds at maximum speed in the six-place head of the International Clinical Model centrifuge. The supernatant, containing most of the blood elements, was decanted and the procedure repeated twice. The packed-cell volume was then determined by centrifugation of the loosely packed cells for 2 minutes, and finally the cells were resuspended in sufficient calcium-free Krebs-Ringer solution to yield a 1:12 dilution. Ten ml. of such a suspension was used per 30 ml. incubation medium.

The S-37 mouse tumor was obtained originally from the Roscoe B. Jackson Memorial Laboratories and the Ehrlich ascites carcinoma from Dr. J. S. Colter. Both were maintained in the solid form by weekly subcutaneous injections and in the liquid form by weekly intraperitoneal injections. At first there was difficulty in growing the S-37 tumor in an ascitic form in CF1 mice, but it took readily in DBA mice, and the ascitic tumor thus obtained took readily in CF1 mice. In the experiments reported here the S-37 ascites were harvested from DBA mice.

**Incubation technic.**—The incubation medium was a calcium-free Krebs-Ringer phosphate solution. The cells were allowed to incubate in the medium for 7 min. at 37°C, and then sufficient 60 mM glycine-1-C\(^14\) added to yield a final concentration of 2 mM. Approximately 10 \(\mu\)c. was added to each 30 ml. of incubation medium. The incubations were performed in Erlenmeyer flasks shaken aerobically in a Research Specialties Company shaker at 37°C. The rate of shaking varied according to the size of the flasks employed. Thus, a shaking rate of 100/min. was needed with 25-ml. capacity flasks, but, to avoid clumping of the cells, this speed was reduced to 60 per minute when 200-ml. capacity flasks were employed. Samples (2 ml.) were removed for analysis at intervals as required. Inhibitors were usually added after 30 min. of incubation in the solid form whenever possible or as a neutral solution with a total volume of not more than 2 per cent of the remaining incubation mixture.

**Alcohol-soluble glycine.**—The 2-ml. samples were added directly to 3 ml. ice-cold calcium-free Krebs-Ringer solution, centrifuged, the supernatant removed, and the cells resuspended in a further 5 ml. ice-cold calcium-free Krebs-Ringer solution. The suspension was again centrifuged, the supernatant removed, and the interior of the tubes dried with paper tissues. To the washed cells was then added 2 ml. 95 per cent ethanol, and a minimum period of 30 minutes was allowed for completion of the extraction of the alcohol-soluble materials. After centrifugation, an aliquot (300 \(\mu\)l.) of the supernatant was plated on aluminum discs and the radioactivity assayed. A Geiger-Muller thin end-window tube and Baird Atomic "Abacus" Scaler were employed. No correction for self-absorption was necessary.

**"Alcohol-insoluble" glycine.**—The precipitate remaining after extraction with 95 per cent ethanol was washed twice with 5-ml. aliquots of 95 per cent ethanol and once with 5 ml. acetone. The residue was suspended in approximately 0.2 ml. acetone and quantitatively transferred with washings to aluminum discs. The precipitate was caused to adhere to the plates by roughening the surface and by addition of 0.2 ml. 50:50 (v/v) acetone: water just before complete evaporation of the acetone. The plates were then dried by means of a heat lamp. The dry weight did not exceed 1.5 mg./sq cm and was quite consistent among plates in any given experiment. The radioactivity of the plates was therefore measured as such, and no attempt was made to apply any correction for self-absorption. Control experiments indicated that at least 85 per cent of the radioactivity was present in the protein and that the specific activity (counts/min/mg protein) was not appreciably altered on isolation of the protein by standard methods.

**RESULTS**

**The effects of methionine, leucine, and valine.**—The results reported by Christensen et al. (3) show that glycine entry into Ehrlich ascites carcinoma cells may be decreased by addition to the medium of other amino acids. Further, the results of Riggs et al. (14) concerning the interaction of glycine, tryptophan, and diaminobutyric acid show that previously concentrated amino acids may be displaced by other acids. The present results show that the addition of many other amino acids leads to an efflux of glycine from cells which are in equilibrium with a glycine-containing medium. Thus, on addition of sufficient methionine to yield
a final concentration of 5 mM, the glycine content of cells in equilibrium with a medium containing 2 mM glycine fell by approximately 50 per cent as shown in Chart 1. This figure also shows that the addition of leucine and valine under similar experimental conditions had little effect on the glycine content of the cells. This result is in contrast to those of Christensen et al. (3), who showed that under similar experimental conditions valine inhibited glycine uptake by 35 per cent and leucine inhibited glycine uptake by 45 per cent.

The inhibitory effect of methionine might be the result of a direct effect on the transport of glycine or an indirect effect such as, for example, might be obtained through a disturbance of the supply of energy. In the experiments quoted in Chart 1 glucose was therefore added 30 minutes after the addition of methionine, leucine, or valine to supply glycolytic energy. The effects of these three amino acids on the glycine level were not influenced on addition of glucose, and in all subsequent experiments no reversal or potentiation of the effects of added amino acids or their analogs was ever observed on addition of glucose. The inhibitory effects of the amino acids such as methionine were therefore probably due to a direct effect of these amino acids on some phase of glycine transport.

After free glycine was extracted from the cells by 95 per cent ethanol, the insoluble material was washed, plated, and the radioactivity counted as described under “Methods and Materials” to obtain a measure of the glycine incorporation. It is apparent from the results presented in Table 1 that, in the experiment reported in Chart 1, none of the amino acids examined had any effect on glycine incorporation into the alcohol-insoluble fraction. However, a significant stimulation was observed on addition of glucose to each of the three experimental vessels. Such stimulations of glycine incorporation frequently occurred, even in the absence of the second amino acid, but could never be shown to be associated with the reversal of an inhibitory effect on glycine incorporation produced by an amino acid or an amino acid analog. The production of energy via respiration was possibly supplemented by production of ATP from glycolysis, and this resulted in an increased rate of incorporation of glycine.

At this point, it is of interest to note that the initial rate of glycine entry into the ascites cells in the experiment reported in Chart 1 was 43.6 μmoles/hour/ml packed cells when measured over the first 7 minutes after the addition of glycine. The rate of glycine incorporation into the alcohol-insoluble fraction was constant over the first 60 minutes and was approximately equal to 0.35 μmoles/hour/ml packed cells. The addition of 10 mM glucose increased the rate to approximately 0.5 μmoles glycine incorporated/hour/ml packed cells. Thus, the rate of glycine incorporation is only about 1 per cent of the initial rate of glycine uptake.

The effect of methionine analogs.—In view of the marked effect of methionine on glycine retention and the report of Tenenhouse and Quastel (17) that S-ethyl cysteine inhibits the concentration of

### Table 1

<table>
<thead>
<tr>
<th>Interval (MIN.)</th>
<th>Further Additions</th>
<th>Flask 1</th>
<th>Flask 2</th>
<th>Flask 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–30</td>
<td>Nil</td>
<td>0.176</td>
<td>0.190</td>
<td>0.154</td>
</tr>
<tr>
<td>30–60</td>
<td>5 mM L-leucine</td>
<td>0.185</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 mM DL-valine</td>
<td></td>
<td>0.164</td>
<td></td>
</tr>
<tr>
<td>60–90</td>
<td>10 mM Glucose</td>
<td>0.245</td>
<td>0.239</td>
<td>0.259</td>
</tr>
</tbody>
</table>

The results quoted in this table were from the same experiment as that quoted in Chart 1. The amino acids were added after 30 min. and the glucose 30 min. later.
glycine by ascites cells, it was decided to examine the effect of several methionine analogs on glycine retention by the cells. Compounds having the general structure R-CH₂-CH(NH₂)-COOH were examined, where R was CH₃ (methionine), CH₃-CH₂-S-CH₂-(ethionine), CH₃-CH₂-S-(S-ethyl cysteine), CH₃-CH₂-O-CH₂-(O-ethyl homoserine), CH₃-O-CH₂-(O-methyl homoserine), and HOCH₂-(homoserine). When added in amounts sufficient to yield a final concentration of 5 mM, these compounds decreased the concentration of glycine in the ascites cells by more than 50 per cent. Typical results obtained in one experiment with methionine, O-ethyl homoserine (the oxygen analog of ethionine), and S-ethyl cysteine are shown in Table 2. It should be noted that in no case was any significant inhibitory effect on the incorporation of glycine into the insoluble fraction observed. The results obtained with the other amino acids (methionine, homoserine, and its O-methyl derivative) were similar to those described above.

The effects of ethionine, S-ethyl cysteine, O-ethyl homoserine, and glucose on the uptake of glycine-1-C¹⁴ by Ehrlich ascites cells during incubation aerobically at 37°C.

<table>
<thead>
<tr>
<th>TIME (MIN.)</th>
<th>FURTHER ADDITIONS</th>
<th>umoles GLYCINE/ML PACKED CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Flask 1</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>4.9</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>6.6</td>
</tr>
<tr>
<td>45</td>
<td>5 mM DL-ethionine*</td>
<td>3.7</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>45</td>
<td>5 mM DL-S-ethyl*</td>
<td>2.9</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>75</td>
<td>5 mM DL-O-ethyl*</td>
<td>3.5</td>
</tr>
<tr>
<td>90</td>
<td>10 mM Glucose†</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* Amino acids added after 30 min.
† Glucose added after 60 min.

The time of addition of methionine. —The present investigations have been directed toward a study of the attainment of a new lower steady-state level of methionine employed (3.67 nM) the expected concentration factor (uMoles glycine/ml packed cells divided by uMoles glycine/ml medium) was 1.42 and the observed value was 2.16 compared with a concentration factor of 4.5 in the absence of methionine. Several attempts were made to reduce the concentration factor to unity by addition of excess methionine, but in no case could it be reduced to very much below a value of two. The present results, however, do indicate that at the lower concentrations of methionine it seems to compete in some way with glycine for the transporting system and that it has a high affinity for this system.

At no concentration did methionine seem to have any effect on the rate of glycine incorporation into the alcohol-insoluble fraction.

The effect of methionine at various concentrations. —In all the foregoing experiments the glycine concentration was 2 mM and that of all of the other amino acids was 5 mM. The effects of several concentrations of methionine on the extent of glycine retention by the ascites cells was also investigated. The results obtained in such an experiment are shown in Chart 2. It is apparent that, at a concentration in the medium of 0.55 mM (4.95 uMoles added to 9 ml.), methionine causes an efflux of approximately 25 per cent of the glycine originally present in the cell. If there is competition between glycine and methionine for a limited number of sites involved in the transport of methionine, then the reciprocal of the new equilibrium level inside the cell should bear a linear relationship to the concentration of the methionine (compare the fact that the reciprocal of the velocity of an enzyme-catalyzed reaction obeys a similar law with reference to the concentration of a competitive inhibitor). The results shown in Chart 2 are also plotted by this method, and it is apparent that at the lower concentrations such a relation-
of glycine by ascites cells which had previously been caused to accumulate glycine. It was of importance to determine whether this level was the same as that reached when the glycine and methionine were added simultaneously. The results shown in Chart 3 indicate that the position of the equilibrium attained is the same whether it is approached by an influx of glycine (through simultaneous addition of glycine and methionine) or by an efflux of glycine (through pre-packing with glycine followed by addition of methionine). It would appear, therefore, that, once it has entered the cells, the glycine is maintained in a state of dynamic equilibrium with that remaining in the external medium.

Let \( a = \frac{\text{total counts/min added}}{\text{ml medium}} \); 
\( \mu_0 = \frac{\text{\# moles glycine initially present}}{\text{ml medium}} \); 
\( \mu = \frac{\text{\# moles nonradioactive glycine added}}{\text{ml medium}} \); 
\( M = \text{maximum concentration of glycine within the cells} \); 
\( m_0 = \text{initial concentration of glycine within the cells} \); 
\( m = \text{concentration of glycine inside the cells after addition of cold glycine to the medium} \); 
\( K = \text{characteristic constant} \).

Initial concentration of glycine in the medium
\[
\text{Initial concentration} = \frac{\mu_0}{1000} M
\]

Final concentration
\[
\frac{\mu_0 + \mu}{1000} M
\]

The effect of glycine.—The studies of Heinz (10, 11) have shown conclusively that radioactive glycine may leave ascites cells very rapidly as the result of an exchange reaction with nonradioactive glycine in the medium. On addition of extra nonradioactive glycine to the medium, there must be a net passage of glycine into the cells, since increase in the external glycine concentration leads to an increase in the internal glycine concentration. On the other hand, if there occurs an exchange reaction or a continued leakage of previously concentrated glycine, then the radioactivity, as glycine, in the cells must decrease despite the net passage of glycine in the opposite direction. The results shown in Chart 4a indicate that an efflux of radioactive glycine does occur and that, in agreement with the results of Heinz and Mariani (10), the rate is much greater than the rate of entry of glycine into the cells and is of the same order as the rate of exchange diffusion (11).

The extent of the decrease in radioactivity of the glycine within the cells may be calculated as follows:

\[
M = \frac{1}{m_0} + \frac{1000K}{\mu_0}
\]

and
\[
M = \frac{1}{m} + \frac{1000K}{\mu + \mu_0}
\]
On division,

\[ m = \frac{1 + \frac{1000K}{\mu + \mu_0}}{1 + \frac{1000K}{\mu_0}}. \]

Initial specific activity

\[ = \frac{a}{\mu_0} \text{ counts/min/\(\mu\)mole}; \]

Final specific activity

\[ = \frac{a}{\mu + \mu_0} \text{ counts/min/\(\mu\)mole}. \]

Thus

\[ \frac{\text{Initial radioactivity in cell}}{\text{Final radioactivity in cell}} = \frac{\frac{m_0a}{\mu_0}}{\frac{m_0}{\mu_0}} = \frac{m_0(\mu + \mu_0)}{m_0} \]

\[ = \frac{\mu + \mu_0}{\mu_0} \left( 1 + \frac{1000K}{\mu + \mu_0} / 1 + \frac{1000K}{\mu_0} \right), \]

Initial radioactivity

\[ = \frac{\mu + \mu_0 + 1000K}{\mu_0 + 1000K}, \]

Final radioactivity

\[ = 1 + \frac{\mu}{\mu_0 + 1000K}. \]

Since the initial radioactivity, \(\mu_0\), and \(K\) are constant, it follows that the reciprocal of the final amount of radioactivity in the cell is proportional to the number of \(\mu\)moles extra glycine added, and, in accordance with this prediction, when the results were plotted by this method, the straight-line relationship shown in Chart 4 was obtained. The present results emphasize again that the rates of influx and efflux of glycine are in agreement with the concept of a true dynamic equilibrium between the glycine of the medium and that of the cell.

The effects of other amino acids.—The effects of several other amino acids on the retention of glycine were also investigated, and the results obtained are presented in Table 3. The presence of threonine or alanine, like that of methionine, leads to a decrease in the amount of glycine within the ascites cells, but these amino acids have no effect on the rate of glycine incorporation. The inhibitions of glycine uptake and incorporation observed on addition of serine are probably due, in some part, to glycine-serine interconversion. It is, however, of interest to note that the introduction of a \(\beta\)-phenyl group into the alanine or serine molecules removes completely the inhibitory effects of these amino acids on glycine retention. Most of these amino acids have been studied by Christensen et al. (8) and by Riggs et al. (15), but it should be noted that in the present experiments the observation by the former workers that the presence of the basic amino acids causes an increase in glycine uptake could not be confirmed. In confirmation of the results of Rabinovitz et al. (13), a stimulation of glycine incorporation was always observed on addition of glutamine. In some instances the presence of glutamate led to a similar though much lower stimulation. A further increase in the rate of glycine incorporation was always observed when glutamate or glutamine were added in the presence of glucose.

The effects of metabolic inhibitors on glycine retention and glycine incorporation.—It is now well established (2, 10, 14) that, in the presence of substances which bring about a depletion of the cellular reserve of ATP, there is a failure of Ehrlich ascites cells to concentrate glycine and to incorporate it into proteins. The inhibitory effects of
dinitrophenol (DNP) and of cyanide, as well as the reversal of these effects on addition of glucose, are all explicable in terms of ATP production. In view of the great difference in the relative rates of uptake of glycine and its incorporation into protein, described above, it was of interest to determine the effect of these metabolic inhibitors on the retention of glycine by ascites cells and its incorporation into the alcohol-insoluble fraction. The results presented in Chart 5b show clearly that on addition of 0.1 mM DNP there was an immediate and complete inhibition of glycine incorporation into the alcohol-insoluble fraction of the ascites cells. The effect of 0.1 mM DNP on the uptake of glycine was not immediately apparent, but within the next 10 min. the onset of an extensive efflux of glycine from the cells was observed (Chart 5a). If the DNP was added at a time when the glycine concentration inside the cells was still increasing at a rapid rate, it had no apparent effect on this influx for a period of up to 10 min. (cf. the addition of DNP after 7 min. in Chart 5a). On addition of the DNP at a time when the cells and the glycine-containing medium had come into equilibrium with each other, there was still a lag period of 5–10 min., but after this period there was again a rapid loss of glycine (cf. the addition of DNP after 30 min. in Chart 5a). Subsequent addition of glycine, which yields ATP by glycolysis, a process which is insensitive to the presence of either DNP or cyanide, led to a reversal of the efflux, and glycine once more began to accumulate in the cells. Simultaneously there was an immediate restoration of the incorporation of glycine into the alcohol-insoluble fraction. The rate of incorporation of glycine into the insoluble fraction after addition of glucose in presence of DNP was considerably less than the rate of incorporation prior to the addition of DNP and less than the rate of incorporation in the control (Chart 5b). This effect may be the result of the rapid drop in pH in presence of glucose and DNP or to an irreversible effect of DNP on the incorporating system. The results obtained when cyanide was employed were similar to those described above which were obtained on addition of DNP.

Glycine retention by S-37 ascites cells.—The results obtained with the Ehrlich ascites cells have shown wide differences in the capacities of various amino acids to influence the retention of glycine by these cells. It was of interest to determine whether similar relationships would hold when other tumor cells were employed in this form. An ascitic form of Sarcoma 37 was developed in DBA mice as described under "Materials and Methods," and the resultant cell preparation was employed for comparative studies. In a typical experiment the effects of 5 mM glutamate, glycine, or methionine were investigated and, subsequently, the effects of 10 mM glucose. It is apparent from the results shown in Table 4 that the effects observed were exactly analogous to those observed with Ehrlich ascites cells. Thus, addition of glycine led to a de-

<table>
<thead>
<tr>
<th>TIME (MIN.)</th>
<th>FURTHER ADDITIONS</th>
<th>µMOLES GLYCINE/ML PACKED CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>5 mM glycine*</td>
<td>Flask 1: 5.5, Flask 2: 6.0, Flask 3: 7.8</td>
</tr>
<tr>
<td>30</td>
<td>5 mM DL-methionine*</td>
<td>Flask 1: 6.7, Flask 2: 6.6, Flask 3: 8.9</td>
</tr>
<tr>
<td>45</td>
<td>5 mM L-glutamate*</td>
<td>Flask 1: 6.1, Flask 2: 4.1, Flask 3: 7.5</td>
</tr>
<tr>
<td>60</td>
<td>10 mM glucose†</td>
<td>Flask 1: 5.8, Flask 2: 3.6, Flask 3: 7.8</td>
</tr>
</tbody>
</table>

* Amino acids added after 30 min.
† Glucose added after 60 min.
crease in the amount of radioactivity inside the cell, methionine caused a rapid and extensive efflux of glycine but did not influence glycine incorporation, and glutamate had no effect on the retention of glycine but, in the presence of glucose, stimulated glycine incorporation into the alcohol-insoluble fraction.

The site of action of the inhibitory amino acids.—In the present experiments the passage of radioactivity out of ascites cells has been interpreted in terms of efflux of glycine (except in the experiments in which nonradioactive glycine was added). The efflux resulting from the addition of the amino acids or metabolic inhibitors may result from an increased rate of efflux due to leakage or to various types of exchange reactions and may also be the result of an inhibition of the influx of the radioactive glycine. Since the inhibitory effect of methionine was immediately apparent when it was added at the same time as the radioactive glycine, i.e., when the only possible direction of flow of the radioactive glycine could be into the cells, it seemed most likely that methionine had an inhibitory effect on the uptake of glycine. To determine whether or not methionine also had an effect on the process of efflux of glycine, cells were allowed to come into equilibrium with a medium containing 3.2 mM glycine-1-\(^{14}\)C by incubation for 30 min. This higher concentration of glycine was employed to increase the amount of radioactivity retained by the cells. They were then separated from the medium by centrifugation, washed once by resuspension in 5 ml. ice-cold calcium-free Ringer solution, and finally made up to a volume of 2 ml. in the same solution. This ice-cold suspension of cells was then added to 10 ml. fresh incubation medium at 37° C. containing no glycine but with the additions noted in Chart 6. Samples were taken at intervals, and the radioactive glycine present in the cells and in the medium was estimated as before. The results obtained (Chart 6) indicate that there was an extensive loss of glycine from the cells within 1 minute and a further loss which extended over a period of approximately 30 min. The presence of 5 mM methionine or of 0.1 mM DNP seemed to have little effect on the rate or extent of either of these two losses of glycine. The initial rapid loss was not due to leakage of the glycine during washing, since the total recovery of glycine from the cells and the medium closely approximated that present in the cells prior to washing. The over-all efflux of glycine was apparently uninfluenced by the energy level of the cell, since DNP was without effect on it, as previously indicated by Heinz (9).

**DISCUSSION**

In the present work an attempt has been made to determine some of the conditions controlling the rate of efflux of glycine from Ehrlich ascites carcinoma cells and to investigate the mechanisms involved in this efflux. The three factors, suggested by Heinz and Mariani (10), to participate in the establishment of an elevated steady-state concentration of glycine within ascites cells should be considered in this connection. There can be no doubt of the existence of an efflux or leakage of glycine from the cells which is independent of the availability of energy. The present results (Chart 6) would indicate that this process is potentially an extremely rapid one. In agreement with this conclusion is the observation (Chart 4) that a new steady state was rapidly attained when nonradioactive glycine was added. This rapid reaction, which is of the same order as the exchange reactions discussed by Heinz (10, 11), coupled with the process of active transport, would therefore constitute a system capable of maintaining glycine in the medium and in the cell in a dynamic state of equilibrium. The question of the contribution of exchange reactions, including exchange-diffusion (11), naturally arises. It has been shown conclusively by Heinz and Walsh (11) that the presence of "preloaded" amino acids may greatly stimulate the rate of entry of radioactive glycine, sarcosine, and alanine. From the definition of "exchange dif-
fusion” (11) it would be expected that addition of an exchangeable amino acid to cells “preloaded” with another amino acid should also facilitate the exit of the “preloaded” amino acid. This possibility was explored, but the results presented in Chart 6 indicate that the presence of methionine has little or no effect on the rate of exit of “preloaded” radioactive glycine from the ascites cells. It can only be concluded that under the present experimental conditions the process of “exchange diffusion” does not contribute to the efflux of glycine on addition of methionine.

It is of interest to note that, of the amino acids examined, those which carry a complete net positive or negative charge at neutral pH were without effect on glycine retention. Thus, both glutamic acid and the basic amino acids did not influence the glycine concentration. Of the neutral amino acids, those which are structurally related to methionine, such as the corresponding oxygen analog or the corresponding ethyl compounds, were found to be the most effective inhibitors. On the other hand, substitution of the carbon chains of serine or alanine with a phenyl group completely removed the inhibitory effects observed with these two amino acids. These results point out that specific sites must be involved in the over-all process of glycine transport. The demonstration by Tenenhouse and Quastel (17) that the inhibition of glycine accumulation by S-ethyl cysteine obeys the same law as that obeyed in classical competitive inhibition points to the same conclusion. In the present work, addition of methionine at low concentrations has led to results which are of the same mathematical form. However, it should be pointed out that such results would also obtain if specificity were involved only in a process of selective adsorption preceding active transport of glycine or other amino acids into the cell.

The question of the nature of this process of active transport then arises. Of this process little is known except that it eventually requires ATP and is markedly decreased when the supply of ATP is reduced. In this connection, it is of interest to note (Chart 5) that, on addition of 0.1 mM DNP, glycine incorporation into the alcohol-insoluble fraction ceased within 1–2 minutes but glycine continued to be concentrated by the cell for as long as 10 minutes. The incorporation of glycine, in which ATP is known to participate directly, is therefore much more sensitive to change in the ATP concentration than is the transport of glycine. Such a situation would result if ATP did not participate directly in transport of the amino acid but rather served to maintain a carrier system or to preserve the ascites cells in a condition conducive to amino acid transport. Such a conclusion would be supported by the fact (16) that 0.1 mM DNP causes practically complete loss of ATP from ascites cells within 5 minutes. It may be supposed that the primary function of ATP in this system is to maintain the carrier agent in an effective form. On the disappearance of ATP from the system the carrier agent may be converted to an inactive form, and the completion of this process might well take several minutes. During this period the agent could continue to cause glycine accumulation, but eventually, when all the agent had been converted to an inactive form, active transport would cease, glycine would continue to leak out, and ultimately a new lower level of equilibrium would be attained. In this connection, it is of interest to note that Riggs, Walker, and Christensen (15) have shown that potassium efflux accompanies glycine influx. If a concentration of potassium ions is the reaction which directly requires ATP, then the uptake of glycine could actually be an exchange with potassium, and the lag, observed on addition of DNP, the time required for depletion of potassium ions.

**SUMMARY**

1. When Ehrlich ascites carcinoma cells were incubated with glycine-1-C\(^{14}\), the amino acid was concentrated within the cells and an elevated steady-state level was approached in approximately 30 minutes. Addition of alanine, serine, or amino acids structurally related to methionine led to a rapid and extensive loss of the previously concentrated glycine from the cells.

2. The final equilibrium level of glycine within the cell in the presence of methionine was the same whether it was approached via an influx of glycine (inhibitors added initially) or via an efflux of glycine (inhibitors added when a steady state had been established in their absence).

3. The initial rate of glycine-1-C\(^{14}\) uptake into the cells was approximately 100 times as great as the rate of glycine incorporation into the alcohol-insoluble fraction of the cells.

4. The decrease in radioactivity of the alcohol-soluble fraction of the ascites cells on addition to the medium of nonradioactive glycine or of methionine may be correlated with the amount of amino acid added. Methionine appeared to compete with glycine for entry into the cell.

5. Studies of the rate of efflux of glycine-1-C\(^{14}\) from ascites cells led to the conclusion that, under the present experimental conditions, the only mechanism involved in the net efflux of glycine...
was passive diffusion. The inhibitory effects observed were due to primary effects on the process of uptake.

ACKNOWLEDGMENTS

It is a pleasure to thank Prof. J. H. Quastel, F.R.S., for his continued interest in this work. It is also a pleasure to thank the National Cancer Institute of Canada for their financial support of a program of work, of which this represents a part.

Thanks are due to Dr. L. Berlinguet for a supply of O-methyl- and O-ethyl homoserine.

REFERENCES


The Influence of Amino Acids and Antimetabolites on Glycine Retention by Ehrlich Ascites Carcinoma Cells

R. M. Johnstone and P. G. Scholefield