The Uptake and Efflux of Glycine from Rat Cerebral-Cortex Slices

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(Received 26 May 1971)

The kinetics of the influx and efflux of radioactive L-glycine was studied in slices of rat cerebral cortex. The influx showed saturation kinetics and was inhibited by L-alanine. Influx was dependent on the presence of Na⁺ ions and a metabolizable substrate. The efflux of glycine was accelerated by alanine. It was concluded that carrier-mediated facilitated diffusion was the mechanism of glycine uptake by, and efflux from, cerebral slices.

Van Slyke & Meyer (1912) showed that there is a higher concentration of amino acids in the tissues than in the plasma. In mammalian brain, glutamic acid, glutamine, γ -aminobutyric acid and aspartic acid account for 60% of the free L-amino acid nitrogen (Tallan, Moore & Stein, 1954, 1958), and other amino acids are found in concentrations approximately equal to those of plasma. The kinetics of their uptake is best studied in cerebral slices, to avoid the complications of the blood-brain barrier. Cerebral slices concentrate glutamic acid (Stern, Eggleston, Hems & Krebs, 1949) and glycine (Varnadis & Quastel, 1961). Several membrane carriers for particular neutral, basic and heterocyclic amino acids have been postulated (Blasberg & Lajtha, 1966), and a correlation has been shown between the degree of concentration of glycine and the concentration of ATP in the tissue (Abadom & Scholefield, 1962). We have also shown a parallelism between the accumulation of various amino acids and the activity in the tissue of ATP plus creatine phosphate, which suggests that amino acid uptake is metabolically dependent (Barbosa, Joanny & Corriol, 1968).

In the present paper we have examined in cerebral slices the influx of glycine and its inhibition by alanine, the efflux of glycine and its acceleration by alanine, and the dependence of uptake of glycine on metabolism and the presence of Na⁺ ions.

METHODS

Wistar rats, weighing 200-250 g, were killed by neck fracture and the brains removed rapidly. Two slices, 0.35 mm thick, each weighing 60 mg, were cut from each hemi-cortex with a 'bow-cutter' (McIlwain & Rodnight, 1962), and incubated in Krebs-Ringer bicarbonate-glucose saline solution as described by Joanny & Hillman (1963).

¹⁴C-labelled L-glycine, at a final concentration of 0.5 μ Ci/5 ml, was dissolved in the incubation medium, to which was added non-radioactive glycine (at least 1 mM). All the amino acids were L-isomers, and all labelled amino acids were obtained from Calbiochemical Corp., Los Angeles, Calif., U.S.A.

The water content of tissue incubated under the different conditions was determined in at least six slices, similarly treated and evaporated to dryness overnight at 105°C in an oven; the difference between the incubated and dried weight of a slice was taken as the tissue water. The uptake of glycine by tissue was measured by the tissue water/incubation medium radioactivity ratio. Adherent fluid was removed by touching tissue to glass, after the tissue had been incubated, and it was then homogenized immediately in 2 ml of ice-cold trichloroacetic acid (10%, w/v). After centrifugation for 10 min at 3000g, a 0.5 ml sample of the supernatant was added to 7 ml of liquid-scintillation fluid, kept in the dark at 4°C (Bray, 1960). Incubation fluid (0.1 ml) was similarly treated with 10% trichloroacetic acid to correct for self-absorption, and a blank of 0.5 ml of trichloroacetic acid alone was also used. The radioactivity of each sample was counted for 10 min by using a Beckman 7066 scintillation counter, with an external standard to correct for quenching.

Determination of the influx and efflux of glycine. The cerebral slices were preincubated for 30 min at 37°C in a Krebs-Ringer solution without glycine, and then were transferred to a similar solution containing glycine in the concentration selected. The mean concentration in the tissue water was expressed as a ratio to that in the incubating medium. Efflux was measured after the tissue had been preincubated for 30 min in a medium containing 1 mM-glycine, and then transferred to a Krebs-Ringer medium without this amino acid. In all experiments, each value is a mean of results from at least six slices unless otherwise indicated.

The nucleotides were adsorbed on to activated carbon (Crane & Lipmann, 1963) and the ATP was eluted with 1 M-HCl at 100°C; ATP was measured by phosphorylation of glucose in the presence of hexokinase, and by causing the glucose 6-phosphate to react with NAD⁺. The NADH formed is proportional to the ATP added

(Kornberg, 1955). Calibration was carried out by addition of ATP to the column and running it through the procedure. Creatine phosphate was measured after barium precipitation by the method of Le Page (1946), modified by Woodman & McIlwain (1961).

RESULTS

Tissue accumulation of glycine. After 30 min incubation in the presence of 0.5 mM-glycine, the tissue water had accumulated this amino acid to a mean concentration 23-fold that of the medium; as the glycine concentration in the medium was increased the ratio was much less, until at 5 mM there was not significantly more glycine in the tissue water than in the medium (Fig. 1). During incubation in 1 mM-glycine the rate of accumulation was more rapid initially, and the curve had almost reached a steady concentration by 60 min, although glycine was then present in a ratio of 11 relative to the medium (Fig. 2, top curve). Sved (1958) found that equilibrium was not reached completely for 3 h, but in view of our previous finding of considerable changes in tissue metabolism by that time (Corriol, Joanny & Millet, 1964), incubation of tissues in this series of experiments was allowed to continue for only 60–100 min.

Dependence on energy metabolism. The dependence of uptake on the substrate in the medium is shown in Table 1. Glucose (10 mM), or pyruvate, lactate, oxaloacetate or α -oxoglutarate (all 20 mM), each permitted the glycine to accumulate in the tissue water in a concentration 8–11-fold that in the medium after 60 min incubation. Fumarate (20 mM) permitted a concentration in the tissue fluid of only fivefold, and succinate (20 mM) only 3.9-fold, that of the medium after 60 min incubation. In the presence of 10 mM-glucose, 1 mM-sodium azide gave glycine ratios of 6.3, and 0.2 mM-2,4-dinitrophenol gave ratios of 5.3. Incubation in the absence of substrate permitted tissue concentration of 3.9, whereas anaerobic incubation in the presence of glucose (10 mM), or in its absence, did not permit any significant tissue accumulation of glycine with respect to the medium after 60 min.

In the presence of glucose (10 mM), or pyruvate, lactate, oxaloacetate or α -oxoglutarate (all 20 mM), neither ATP nor creatine phosphate concentrations in the tissue were significantly different between substrates; succinate and fumarate permitted tissue concentration of these organic phosphates only about half those of the other substrates, but this was nearly double their concentration in the absence of added substrate (Table 2).

Cerebral slices were preincubated in Krebs–Ringer glucose saline for 60 min, and then 0.2 mM-2,4-dinitrophenol was added; the concentration

Fig. 1. Ratio of glycine in the tissue water of rat cerebral slices to that in the incubating medium after 60 min incubation in Krebs–Ringer bicarbonate–glucose saline containing 0–5 mM-glycine. For measurement of tissue water see the Methods section. The points are the mean values for six slices each.

Fig. 2. Uptake of radioactive glycine during 60 min incubation from a medium containing 1 mM-glycine in the absence (○) and presence (●) of 1 mM-alanine. The tissue had previously been preincubated for 30 min in Krebs–Ringer bicarbonate without glycine. The bars are the standard deviations of the mean values from at least four slices. Note that the uptake of glycine is inhibited by the presence of alanine.

Table 1. *Effect of (a) anaerobiosis and inhibitors, and (b) different substrates, on the glycine ratio after 60 min incubation*

In this and other tables, glycine ratios are the concentration of glycine in the tissue water relative to 1 mM concentration in the incubating medium (see the Methods section); the mean and standard deviations are given for the number of slices indicated in parentheses.

	Glycine ratio
(a) Effect of anaerobiosis and inhibitors	
Incubation conditions	
Glucose, 10 mM; O ₂ + CO ₂ (95:5)	9.25 ± 0.15 (6)
Glucose, 10 mM; N ₂ + CO ₂ (95:5)	1.13 ± 0.15 (6)
No glucose; N ₂ + CO ₂ (95:5)	1.12 ± 0.2 (6)
No glucose; N ₂ + CO ₂ (95:5)	3.86 ± 0.2 (6)
Glucose, 10 mM; 2,4-dinitrophenol (0.2 mM); aerobic	5.28 ± 0.32 (6)
Glucose, 10 mM; NaN ₃ (1 mM); aerobic	6.26 ± 0.6 (6)
Glucose, 10 mM; NaCN (1 mM); aerobic	7.0 ± 0.45 (6)
(b) Effect of added substrate	
Substrate (all 20 mM)	
Pyruvate	8.39 ± 0.82 (6)
Lactate	11.46 ± 0.85 (6)
Oxaloacetate	9.78 ± 0.9 (6)
α-Oxoglutarate	9.26 ± 0.74 (6)
Succinate	3.91 ± 0.43 (8)
Fumarate	5.11 ± 0.36 (8)

of glycine in the tissue water fell from 9.6 ± 0.5 ($n = 4$) to 4.8 ± 0.3 ($n = 4$) after a further 40 min. After 60 min aerobic incubation, followed by 40 min incubation in an atmosphere of N₂ + CO₂ (95:5), the glycine concentration fell from 9.8 ± 0.6 ($n = 4$) to 3.7 ± 0.2 ($n = 6$).

Dependence on cations in the incubation medium. Cerebral slices incubated for 60 min in Krebs–Ringer bicarbonate–glucose saline with 1 mM-glycine contained nearly 10-fold the concentration of glycine in their tissue water. This was decreased to a mean of 5.3-fold when Na⁺ replaced the K⁺ in the incubating medium; it was decreased to a mean between 1.4- and 1.72-fold by replacement of the Na⁺ by choline, tris or Li⁺ salts. The tissue-water content was not significantly different in these latter media (Table 3).

The glycine in the tissue water was examined after 60 min preincubation in Krebs–Ringer glucose solution, and after further incubation for 5 and 40 min in similar solutions in which the Na⁺ had been substituted by tris or Li⁺ plus tris. It can be seen that in the two last-named media the concentration ratio of glycine in the tissue water relative to the medium had decreased, by 0.8–1.85, to about 8 after 5 min. However, after 40 min incubation in the Na⁺-free media the concentration ratio was only about 2 (Table 4).

Table 2. *Effect of different substrates on the ATP and creatine phosphate concentrations of cerebral slices incubated in oxygenated Krebs–Ringer bicarbonate saline for 60 min*

See Table 1 for details.

Substrate	Concn. of ATP (μmol/ml of incubated tissue)	Concn. of creatine phosphate (μmol/ml of incubated tissue)
None added	0.36 ± 0.04 (8)	0.42 ± 0.1 (8)
Glucose (10 mM)	1.18 ± 0.06 (8)	1.80 ± 0.15 (10)
Pyruvate (20 mM)	1.31 ± 0.05 (8)	2.2 ± 0.25 (10)
Lactate (20 mM)	0.90 ± 0.10 (8)	1.68 ± 0.2 (10)
α-Oxoglutarate (20 mM)	1.12 ± 0.12 (8)	1.58 ± 0.15 (8)
Succinate (20 mM)	0.42 ± 0.03 (8)	0.51 ± 0.1 (12)
Pyruvate (20 mM)	0.40 ± 0.03 (8)	0.46 ± 0.1 (12)

Table 3. *Effect of incubation in K⁺- and Na⁺-free media on the glycine ratio*

For experimental details see the text and Table 1.

Modification of Krebs–Ringer medium	Glycine ratio	Tissue-water content (%)
None	9.87 ± 0.4 (6)	64.3 ± 2.6 (4)
K ⁺ -free, Na ⁺ -substituted	5.30 ± 0.2 (8)	65.0 ± 0.3 (4)
Na ⁺ -free, choline-substituted	1.4 ± 0.1 (8)	69.0 ± 0.36 (4)
Na ⁺ -free, tris-substituted	1.68 ± 0.1 (8)	70.5 ± 0.35 (8)
Na ⁺ -free, LiCl (125 mM), choline bicarbonate (25 mM)	1.72 ± 0.1 (4)	70.8 ± 0.4 (4)

Fig. 3. Lineweaver-Burk plots of the uptake of glycine from media containing 0–5 mM-glycine; the initial rates were measured from graphs of the form of Fig. 2 (upper curve). ○, From experiments with Na⁺-free media; ●, from experiments with normal media.

Fig. 4. Efflux of glycine into glycine-free Krebs-Ringer bicarbonate-glucose saline from cerebral slices pre-incubated for 30 min in 1 mM-glycine solution. ○, Alanine absent; ●, 10 mM-alanine present. Note that the alanine accelerates the efflux of glycine.

Fig. 5. Tissue was preincubated for 30 min in a medium containing 1 mM-glycine, and then transferred either to a glycine-free, normal Na⁺ ion medium (○) or to a glycine-free, Na⁺-free medium (●).

Kinetics of the influx and efflux of glycine. After 60 min preincubation of the slices in normal Krebs-Ringer glucose saline, radioactive glycine was added; its rate of penetration into tissue water was measured for the initial 3 min in the presence of glycine concentrations of 0.5–5 mM, and Lineweaver-Burk plots were constructed (Blasberg & Lajtha, 1966; Joanny, Corriol & Hillman, 1969). Extrapolation of these curves in normal Krebs-Ringer solution gave an apparent K_m of 4.5 mM, and an apparent V_{max} of 2.5 $\mu\text{mol/min per ml}$ of tissue water (Fig. 3). The complete substitution of Na⁺ by choline induced a substantial decrease in the rate of penetration, the apparent K_m being 10.5 mM, whereas V_{max} was unchanged.

The efflux of glycine was followed after 30 min preincubation of the tissue in the normal medium containing 1 mM-glycine and subsequent transfer of the tissue to a glycine-free medium. The mean rate of efflux of glycine (0.134 $\mu\text{mol/min per ml}$ of tissue water) during the initial 5 min was considerably less than the rate of efflux (0.47 $\mu\text{mol/min per ml}$ of tissue water) into a medium containing 1 mM-glycine. The efflux of glycine was followed in the presence of 10 mM-alanine, and was found to rise to a rate of 0.3 $\mu\text{mol/min per ml}$ of tissue water after 40 min incubation, and to continue rising thereafter (Fig. 4). Incubation of the slices in choline-sub-

Table 4. *Effect of incubation of cerebral slices in Na⁺-free media on the glycine ratio*

For experimental details see the text and Table 1.

Modification of Krebs-Ringer medium Time of incubation (min) ...	Glycine ratio		
	0	5	40
None	9.7±0.65 (4)	8.4±0.6 (4)	6.2±0.3 (4)
Na ⁺ -free, tris-substituted	9.6±0.6 (4)	7.8±0.5 (6)	1.96±0.3 (6)
Na ⁺ -free, LiCl (125 mM), tris (25 mM)	9.95±0.6 (4)	8.1±0.4 (4)	2.1±0.35 (4)

stituted Na⁺-free Krebs-Ringer solution also increased the mean rate of efflux to 0.3 μ mol/min per ml of tissue water (Fig. 5). After 5 min incubation in tris-substituted or Li⁺-substituted Krebs-Ringer solution, the tissue-water concentrations of glycine were not significantly different from those in the normal Krebs-Ringer solution, but after 40 min in these Na⁺-free media they were only double that of the medium, and one-third of that of slices incubated in the normal Krebs-Ringer solution (Table 4). After 40 min the concentrations were not significantly different from those in the choline-substituted medium (Table 3).

DISCUSSION

It has been shown that cerebral slices accumulate glycine from the incubation medium (Fig. 1) and that this accumulation is much greater with the relatively low medium concentrations equivalent to those found *in vivo* (Tews, Carter, Roa & Stone, 1963). It is dependent on substrate provided; most of the citric acid-cycle intermediates permitted about the same concentrations, except for fumarate and succinate, which were much less effective; these two substrates have also been shown not to support high concentrations of creatine phosphate (Woodman & McIlwain, 1961) and it was concluded that they probably cannot cross the cell membrane (Joanny, Hillman & Corriol, 1966). The correlation shown here between ATP plus creatine phosphate concentrations (Table 2) and the effectiveness of substrates in accumulating glycine confirms previous findings with glycine (Abadom & Scholefield, 1962), and with glutamate, arginine, histidine, proline and tryptophan (Barbosa *et al.* 1968).

The diminution of glycine concentration by cyanide, azide, dinitrophenol and hypoxic incubation (Table 1) is further evidence of dependence on energy supply to the tissue, and parallels previous observations with glycine (Mase, Takahashi & Okata, 1962), as well as with γ -aminobutyric acid and lysine (Cherayil, Kandera & Lajtha, 1967). This lowering of amino acid concentrations seems to have resulted from both a decrease in influx and an

acceleration of efflux from the tissues (Tsukada, Nagata, Hirano & Matsutani, 1963).

The ability of the tissue to concentrate glycine was dependent on the presence of K⁺ and Na⁺, whose absence did not change the tissue-water content significantly (Tables 3 and 4). The ability to maintain glycine concentrations was much depressed after 40 min in the absence of these cations, and this has been shown with several other amino acids (Barbosa *et al.* 1968). This was partly due to an increased efflux (Fig. 5). However, Lineweaver-Burk plots of the uptake showed a decreased affinity of the tissue for glycine in the absence of Na⁺ (Fig. 3). Smith (1967) examined the kinetics of the uptake of neutral amino acids into cerebral slices and found a discontinuity in the curves, which he interpreted as being due to two mechanisms, one that could be saturated and a second that could not be saturated and was extracellular.

A simple hypothesis to relate the dependence of the 'glycine pump', on the one hand, to 'high-energy phosphates' in the tissue and, on the other hand, the presence of Na⁺ in the medium, is as follows: the Na⁺ diffuses into the cells and activates the adenosine triphosphatases to break down intracellular ATP, which releases energy for the 'glycine pump'. If the 'high-energy phosphates' are already depleted because the tissue cannot utilize substrate, or if little Na⁺ is available to diffuse in from the extracellular space, the rate of glycine pumping will be diminished.

Relatively few studies have been published on the kinetics of amino acid uptake in cerebral tissue. However, in lymphatic glands or lens tissue, the lowering of Na⁺ concentration from 150 to 50 mM increased the apparent K_m considerably without affecting the apparent V_{max} of the uptake of L-aminobutyric acid (Kipnis & Parrish, 1965; Cotlier & Beaty, 1967; Thier, Blair, Fox & Segal, 1967). However, in Ehrlich ascites-tumour cells, lowering the medium Na⁺ concentration affected the apparent K_m as well as the apparent V_{max} with respect to glycine (Wheeler, Inui, Hollenberg, Eavenson & Christensen, 1965), and in calvarium cells of rat foetus, with respect to glycine, proline

and hydroxyproline (Finerman & Rosenberg, 1966).

As the medium content of glycine was increased, the ability of the tissue to concentrate glycine decreased (Fig. 1), which is compatible with a carrier mechanism. Further, the influx of glycine was inhibited by alanine, whereas its efflux was accelerated by the latter amino acid (Figs. 2 and 4), which indicates counter-acceleration with a mobile carrier. Lineweaver-Burk plots showed saturation kinetics, but only in the presence of Na^+ in the medium (Figs. 3 and 5; Tables 3 and 4). These are three criteria for carrier-mediated facilitated diffusion (Wilbrandt & Rosenberg, 1961) and the process has been shown to be energy-dependent (Tables 1 and 2).

This work was carried out during the tenure by E.B. of a J. March Foundation studentship. The technical help of Mr Maurice Riol is gratefully acknowledged. The work was carried out with the support of I.N.S.E.R.M. (France) and the Handicapped Children's Aid Committee (England), to both of whom we express our thanks.

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