

Locations of Amino Acids in Brain Slices from the Rat TETRODOTOXIN-SENSITIVE RELEASE OF AMINO ACIDS

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1. Amino acids, particularly glutamate, γ -aminobutyrate, aspartate and glycine, were released from rat brain slices on incubation with protoveratrine (especially in a Ca^{2+} -deficient medium) or with ouabain or in the absence of glucose. Release was partially or wholly suppressed by tetrodotoxin. 2. Tetrodotoxin did not affect the release of glutamine under various incubation conditions, nor did protoveratrine accelerate it. 3. Protoveratrine caused an increased rate of formation of glutamine in incubated brain slices. 4. Increased K^+ in the incubation medium caused release of γ -aminobutyrate, the process being partly suppressed by tetrodotoxin. 5. Incubation of brain slices in a glucose-free medium led to increased production of aspartate and to diminished tissue contents of glutamates, glutamine and glycine. 6. Use of tetrodotoxin to suppress the release of amino acids from neurons in slices caused by the joint action of protoveratrine and ouabain (the latter being added to diminish reuptake of amino acids), it was shown that the major pools of glutamate, aspartate, glycine, serine and probably γ -aminobutyrate are in the neurons. 7. The major pool of glutamine lies not in the neurons but in the glia. 8. The tricarboxylic cycle inhibitors, fluoroacetate and malonate, exerted different effects on amino acid contents in, and on amino acid release from, brain slices incubated in the presence of protoveratrine. Fluoroacetate (3mM) diminished the content of glutamine, increased that of glutamate and γ -aminobutyrate and did not affect respiration. Malonate (2mM) diminished aspartate and γ -aminobutyrate content, suppressed respiration and did not affect glutamine content. It is suggested that malonate acts mainly on the neurons, and that fluoroacetate acts mainly on the glia, at the concentrations quoted. 9. Glutamine was more effective than glutamate as a precursor of γ -aminobutyrate. 10. It is suggested that glutamate released from neurons is partly taken up by glia and converted there into glutamine. This is returned to the neurons where it is hydrolysed and converted into glutamate and γ -aminobutyrate.

Tetrodotoxin, a neurological poison of great potency, is well known to abolish at low concentrations (e.g. 0.3 μM) the generation of action potentials in a variety of excitable tissues and the associated influx of Na^+ into such tissues (Kao, 1966; Narahashi *et al.*, 1964; Nakamura *et al.*, 1965). Whereas it has no effect on the rate of respiration of rat brain-cortex slices incubated in a physiological glucose medium, tetrodotoxin blocks the stimulation of respiration that occurs either on application of electrical impulses or in the presence of protoveratrine or when Ca^{2+} is omitted from the medium (Chan & Quastel, 1967, 1970; Okamoto & Quastel, 1970*b*). McIlwain (1967) similarly found that tetrodotoxin inhibits the respiratory response induced by electrical stimulation of incubated brain-cortex slices from the guinea pig and that the K^+ content of the electrically stimulated slices is enhanced by the presence of tetrodotoxin. The increased influx of Na^+ that occurs when electrical pulses are applied to incubated rat brain-cortex

slices or when protoveratrine (10 μM) is present in the incubation medium is completely blocked by tetrodotoxin at small concentrations (Chan & Quastel, 1970; Okamoto & Quastel, 1970*b*). It was concluded that tetrodotoxin causes its potent metabolic effects in the brain by blocking the influx of Na^+ that occurs on electrical stimulation or in the presence of protoveratrine.

Tetrodotoxin also suppresses the increased uptake of Na^+ that occurs in rat brain-cortex slices under a variety of incubation conditions, e.g. the absence of glucose from the incubation medium, or the presence of ouabain (Okamoto & Quastel, 1970*b*), or at the onset of anoxia (Shankar & Quastel, 1972).

We now find that under some incubation conditions there are increased rates of release of certain amino acids, e.g. glutamate, aspartate, γ -aminobutyrate and glycine from brain slices and that these increased rates are wholly, or partially, blocked by small concentrations of tetrodotoxin. A brief preliminary report

of these results has been made (Benjamin & Quastel, 1971).

Much investigation has shown the existence of at least two separate pools in brain tissue that differ in their contents of amino acids and in their rates of amino acid uptake. Segregation of glutamate and γ -aminobutyrate and other amino acids into 'small' or 'large' compartments occurs as demonstrated by the results of studies of amino acid metabolism both *in vitro* and *in vivo* (Berl *et al.*, 1961; Waelsch *et al.*, 1964; Roberts & Morelos, 1965; O'Neal & Koeppe, 1966; Garfinkel, 1966; Machiyama *et al.*, 1967; Berl *et al.*, 1968; Margolis *et al.*, 1965; Van den Berg *et al.*, 1969; Berl & Frigyesi, 1969; Nicklas *et al.*, 1969; Clarke *et al.*, 1970; Machiyama *et al.*, 1970; Berl & Clarke, 1970; Van den Berg, 1970). The 'small' compartment seems to be associated with a relatively small glutamate pool but possesses the major amount of glutamine present in the brain tissue. The 'large' compartment appears to contain the major amount of glutamate. Indirect evidence indicates that the 'large' compartment may consist of neuronal structures (Balázs *et al.*, 1970; Machiyama *et al.*, 1970; Cocks *et al.*, 1970; Patel & Balázs, 1971), whereas the 'small' pool, which is associated with high glutamine content, is thought to consist of glial tissue (Tower, 1960; Margolis *et al.*, 1968; Balázs *et al.*, 1970). Studies of the subcellular distribution of glutamate indicate its presence in the neurons (Kuhar & Snyder, 1970).

As it is known that glia are not electrically excitable cells and do not generate action potentials (Kuffler & Nicholls, 1966; Kuffler, 1967), it has been concluded that the effects of tetrodotoxin are confined to the neurons.

We have studied certain incubation conditions that are most effective in releasing amino acids from incubated rat brain-cortex slices and we have estimated how much of this release is abolished by the presence of tetrodotoxin. The amino acid that is retained in the brain tissue by the addition only of tetrodotoxin is considered to be located in the neurons. Its amount represents a minimum value of the amino acid content of the neurons in the incubated brain-cortex slices. Experiments, carried out on these lines, may give, therefore, measurements of minimum values of the contents of a variety of amino acids in the neurons.

The results of these experiments are described below.

Experimental

Materials

Animals. Adult rats (usually male) of the Wistar strain, weighing 150–200g, were used and were obtained from the Vivarium, Department of Zoology,

or from the Animal Unit, Faculty of Medicine, University of British Columbia, B.C., Canada. All the animals had free access to food and water.

Chemicals. All common laboratory chemicals were of reagent grade and were used without further purification. Tetrodotoxin was obtained from Calbiochem., Los Angeles, Calif., U.S.A., protoveratrine from K & K Laboratories, Plainview, N.Y., U.S.A., ouabain and EGTA [ethanedioxybis(ethylamine)-tetra-acetate] from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Methods

Preparation of brain-cortex slices. Rats were killed by stunning, the brains were removed and cerebral-cortex slices were prepared with a Stadie-Riggs tissue slicer. The slices were not more than 0.4mm thick. One dorsal and one lateral slice ('first slices') weighing approx. 90mg wet wt. from the same brain were used.

Incubation procedure. The slices were weighed quickly, after removal of adherent fluid by touching with filter paper, and were suspended in a precooled incubation medium contained in a vessel of the conventional Warburg manometric apparatus. The incubation medium was a Krebs-Ringer phosphate solution of the following composition: NaCl, 128mM; KCl, 5mM; CaCl₂, 2.8mM; MgSO₄, 1.3mM; Na₂HPO₄, 10mM (adjusted to pH7.4 with HCl). In a Ca²⁺-deficient medium, CaCl₂ was omitted from the medium. Glucose, when added, was 10mM. Additions were made to the medium, as noted in the Tables below, but the final volume in the manometric vessel was kept at 3ml. Incubations were carried out at 37°C in O₂ usually for 1h.

Amino acid analyses. 1. Tissue. At the end of the incubation period the tissue was removed from the incubation medium, then it was quickly dipped into chilled Krebs-Ringer phosphate-glucose medium (to remove the adherent incubation medium) and was homogenized at once in 3ml of cold 5% (w/v) trichloroacetic acid. After centrifuging, the sediment was washed with 2ml of 5% trichloroacetic acid and the washings were added to the supernatant from the first centrifugation. The final supernatant was extracted three times with equal volumes of diethyl ether to remove the trichloroacetic acid and the remaining diethyl ether in the supernatant was removed by evaporation. A portion of the diethyl ether-free solution was used for amino acid analysis.

2. Medium. A sample (2ml) of the incubation medium was centrifuged in the presence of 0.1ml of 100% (w/v) trichloroacetic acid. The precipitate was washed with 2ml of 5% trichloroacetic acid and the washings were added to the supernatant. This was then extracted with diethyl ether to remove trichloroacetic acid, the diethyl ether was removed by evaporation and a portion of the solution was used for

amino acid analysis. The samples prepared as above from either the tissue or the incubation medium were analysed for amino acids on appropriate columns of a Beckman 120B amino acid analyser. To obtain a true value of glutamine (which co-elutes with serine), a portion of the solution, obtained from either tissue or incubation medium, was heated with 10% (w/v) trichloroacetic acid for 75 min at 75°C. The solution was then extracted with diethyl ether to remove trichloroacetic acid, the diethyl ether was removed and the amino acids were measured. The glutamine was removed in this manner and the observed value for serine was subtracted from the total value of glutamine and serine previously obtained, thus giving a measure of glutamine present in the sample.

Amino acid concentration ratios (tissue/medium). Ratios of the concentrations of amino acids in the tissue ($\mu\text{mol/g}$ initial fresh wt.) to those in the medium at the termination of the experiment ($\mu\text{mol/ml}$) are referred to in the text. They were calculated from the values of amino acid concentrations in the tissues given in the Tables below and from the amounts of amino acids released from the tissue into the total volume (3 ml) of the incubation medium. The values obtained were of a similar order to those recorded by Balázs *et al.* (1970).

Reproducibility of results. Each experiment was done at least four times. Results given below are the means of the values obtained together with the S.D.

Results

Effects of protoveratrine, ouabain and tetrodotoxin on amino acid content in, and release from, rat brain-cortex slices incubated in glucose-saline media

Values of the contents of amino acids in rat brain-cortex slices, freshly prepared and immediately before incubation, were found to approximate to those already recorded in the literature (e.g. Carver, 1965; Roberts & Morelos, 1965; Flock *et al.*, 1966; Himwich & Agrawal, 1969; Piccoli *et al.*, 1971).

Incubation in a physiological glucose-saline medium in O_2 for 1 h caused relatively small changes in the total (i.e. tissue+medium) quantities of amino acids from those found in the fresh tissue. There was an increase in glutamine content from 4.40 to 6.37 $\mu\text{mol/g}$ and a fall of glutamate content from 11.83 to 9.97 $\mu\text{mol/g}$ (Table 1). There were small increases in the contents of aspartate, γ -aminobutyrate, glycine and alanine of 0.52, 0.61, 0.60 and 0.58 $\mu\text{mol/g}$ respectively.

Addition of protoveratrine (5 μM), which is known to affect the kinetics of glucose metabolism in incubated brain slices (Wollenberger, 1955*a,b*; Kini & Quastel, 1960) and to generate action potentials in nervous tissue (Shanes, 1958; Ulbricht, 1969), caused

significant decreases in the contents of glutamate and aspartate in the tissue, with no corresponding increases in the contents in the medium (Table 1), and very small changes in the quantities of the other amino acids investigated. The changes due to protoveratrine were abolished by tetrodotoxin (2 μM), which by itself caused no significant changes in the amino acid contents of the tissue and medium (Table 1).

The changes caused by protoveratrine in the contents of glutamate and aspartate in the tissue could not be caused by diminished rates of conversion of glucose into glutamate and aspartate, as the yields of labelled glutamate and aspartate from labelled glucose by incubated rat brain-cortex slices are enhanced by the presence of protoveratrine (5 μM) (Kini & Quastel, 1960).

Experiments were done with ouabain, because it blocks the active transport of amino acids into brain slices (Gonda & Quastel, 1962*a*; Nakazawa & Quastel, 1968). Incubation in the presence of ouabain caused large decreases in the tissue contents of all the amino acids investigated and corresponding increases in the amino acid concentrations in the incubation medium (Table 1). The total (tissue+medium) contents of glutamate (11.29 $\mu\text{mol/g}$) or of aspartate (3.88 $\mu\text{mol/g}$) in the presence of ouabain were not markedly changed from the values (9.97 and 3.88 $\mu\text{mol/g}$ respectively) obtained in the absence of ouabain. However, the total (tissue+medium) content of glutamine was diminished (from 6.37 to 3.12 $\mu\text{mol/g}$) and that of γ -aminobutyrate was increased from 2.59 to 4.32 $\mu\text{mol/g}$.

Addition of tetrodotoxin (2 μM) substantially decreased the effect of ouabain on the loss of glutamate and aspartate, but had only minor effects on the loss of the other amino acids (Table 1). These results suggest that specific tetrodotoxin-sensitive changes in ionic balance caused by ouabain result in the release of glutamate and aspartate from the brain slices incubated in a physiological glucose-saline medium. The release becomes evident under these incubation conditions because the uptake processes are blocked by ouabain.

Effects of protoveratrine and tetrodotoxin on amino acid content in, and release from, rat brain-cortex slices incubated in a glucose-free medium

Experiments were carried out to observe the effects of lack of glucose on the amino acid contents of brain tissue and on their release from the tissue. The contents of all amino acids in the tissue, except aspartate, were markedly decreased from normal (Tables 1 and 2) and the contents of all amino acids in the incubation medium, except glutamine, were increased, particularly γ -aminobutyrate, aspartate and glycine. The decrease in the tissue concentration of glutamate

Table 1. *Effects of tetrodotoxin, protoveratrine and ouabain on amino acid content in, and release from, incubated rat brain-cortex slices*

Rat brain-cortex slices were incubated in 3 ml of Krebs-Ringer phosphate medium containing 10 mM-glucose in O₂ at 37°C for 1 h in the presence or absence of mixtures of tetrodotoxin (2 μM), protoveratrine (5 μM) and ouabain (0.1 mM) as shown below. Values for amino acids in tissue (Tissue) are expressed as μmol/g initial wet wt. and those in the medium (Medium) as μmol/5 ml per g wet wt.

Amino acid	Addition ...		None		Tetrodotoxin		Protoveratrine		Ouabain		Ouabain + tetrodotoxin		Protoveratrine + ouabain		Protoveratrine + tetrodotoxin	
	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium
Glutamate	9.20 ± 0.14	0.77 ± 0.14	8.82 ± 0.01	0.87 ± 0.02	6.00 ± 0.83	0.76 ± 0.06	4.95 ± 0.26	6.34 ± 0.40	6.94 ± 0.36	2.18 ± 0.18	2.52 ± 0.11	8.33 ± 0.48	7.14 ± 0.12	2.42 ± 0.16	2.42 ± 0.16	2.42 ± 0.16
Glutamine	3.45 ± 0.30	2.92 ± 0.28	2.88 ± 0.11	2.62 ± 0.13	4.10 ± 0.39	2.83 ± 0.66	0.71 ± 0.05	2.41 ± 0.12	0.53 ± 0.04	1.65 ± 0.08	0.74 ± 0.04	2.56 ± 0.41	0.53 ± 0.03	2.07 ± 0.25	0.53 ± 0.03	2.07 ± 0.25
γ-Aminobutyrate	2.56 ± 0.20	0.03 ± 0.02	2.58 ± 0.11	0.04 ± 0.03	2.18 ± 0.39	0.10 ± 0.04	1.39 ± 0.06	2.93 ± 0.35	1.71 ± 0.06	2.42 ± 0.26	0.63 ± 0.02	3.23 ± 0.15	1.47 ± 0.13	2.18 ± 0.25	1.47 ± 0.13	2.18 ± 0.25
Aspartate	3.45 ± 0.23	0.43 ± 0.05	3.64 ± 0.20	0.38 ± 0.06	2.15 ± 0.12	0.42 ± 0.01	1.75 ± 0.32	2.13 ± 0.05	3.32 ± 0.02	0.95 ± 0.26	1.20 ± 0.07	2.92 ± 0.18	3.28 ± 0.19	1.09 ± 0.02	3.28 ± 0.19	1.09 ± 0.02
Glycine	1.03 ± 0.15	0.36 ± 0.06	0.88 ± 0.03	0.27 ± 0.02	1.01 ± 0.06	0.36 ± 0.01	0.59 ± 0.03	1.12 ± 0.05	0.82 ± 0.02	0.84 ± 0.26	0.25 ± 0.07	1.22 ± 0.18	0.66 ± 0.81	0.81 ± 0.02	0.66 ± 0.81	0.81 ± 0.02
Alanine	0.75 ± 0.05	0.06 ± 0.04	0.82 ± 0.03	0.38 ± 0.02	0.85 ± 0.06	0.47 ± 0.08	0.42 ± 0.03	1.50 ± 0.08	0.45 ± 0.16	1.15 ± 0.16	0.26 ± 0.01	1.59 ± 0.04	0.46 ± 0.03	1.28 ± 0.05	0.46 ± 0.03	1.28 ± 0.05
Taurine	0.05 ± 4.10	0.04 ± 1.89	0.02 ± 3.92	0.02 ± 2.05	0.06 ± 3.08	0.09 ± 2.55	0.02 ± 1.82	0.06 ± 4.25	0.03 ± 3.31	0.03 ± 2.06	0.01 ± 1.76	0.14 ± 4.49	0.05 ± 2.07	0.19 ± 3.89	0.05 ± 2.07	0.19 ± 3.89
Serine	0.35 ± 1.12	0.08 ± 0.82	0.01 ± 0.96	0.01 ± 0.67	0.34 ± 1.10	0.15 ± 0.75	0.25 ± 0.64	0.09 ± 1.74	0.13 ± 1.43	0.13 ± 0.90	0.13 ± 0.28	0.71 ± 1.76	0.50 ± 0.72	0.35 ± 1.33	0.50 ± 0.72	1.33 ± 1.33
Threonine	0.13 ± 0.44	0.05 ± 0.28	0.12 ± 0.48	0.09 ± 0.26	0.07 ± 0.42	0.30 ± 0.30	0.03 ± 0.20	0.13 ± 0.64	0.12 ± 0.62	0.04 ± 0.28	0.01 ± 0.10	0.15 ± 0.66	0.02 ± 0.21	0.06 ± 0.60	0.02 ± 0.21	0.60 ± 0.60
Total	26.10 ± 0.06	7.94 ± 0.05	24.98 ± 0.03	7.54 ± 0.02	20.89 ± 0.02	8.54 ± 0.06	12.47 ± 0.11	23.06 ± 0.11	17.01 ± 0.01	14.55 ± 0.06	7.74 ± 0.01	26.76 ± 0.01	16.54 ± 0.01	15.67 ± 0.01	16.54 ± 0.01	15.67 ± 0.01

Table 2. *Effects of tetrodotoxin (2 μ M) and protoveratrine (5 μ M) on amino acid content in, and release from, rat brain-cortex slices incubated in glucose-free media*

For experimental details see the text. Amounts of amino acids in rat brain-cortex slices (Tissue), μ mol/g initial wet wt., or in the 3 ml of incubation medium (Medium), μ mol/g initial wet wt., after incubation in O₂ for 1 h at 37°C in Krebs-Ringer phosphate medium in the absence of glucose are shown.

Addition ... Amino acid	None		Tetrodotoxin		Protoveratrine		Protoveratrine + tetrodotoxin	
	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium
Glutamate	2.03	0.96	3.14	0.47	1.12	2.32	2.57	0.53
	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm
	0.33	0.12	0.34	0.11	0.10	0.30	0.01	0.03
Glutamine	0.51	1.74	0.48	1.33	1.17	2.61	0.34	1.79
	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm
	0.20	0.10	0.20	0.22	0.10	0.18	0.03	0.04
γ -Aminobutyrate	1.38	0.41	2.04	0.35	0.65	0.93	1.20	0.42
	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm
	0.22	0.08	0.05	0.04	0.14	0.07	0.02	0.02
Aspartate	7.79	1.98	8.60	0.78	5.68	4.31	8.43	1.21
	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm
	0.27	0.13	0.04	0.06	0.42	0.32	0.01	0.07
Glycine	0.62	0.70	0.91	0.52	0.36	1.02	0.82	0.47
	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm
	0.12	0.10	0.11	0.12	0.10	0.10	0.02	0.01
Alanine	0.17	0.52	0.20	0.36	0.07	0.72	0.08	0.33
	\pm	\pm	\pm	\pm	\pm	\pm	\pm	\pm
	0.04	0.10	0.03	0.09	0.02	0.03	0.01	0.02

was to be expected, as this amino acid forms a major fuel of the brain in the absence of substrates from the incubation medium. Moreover, as glutamate oxidation is accompanied by marked production of aspartate, the increase in the concentration of aspartate in tissue was also to be expected. With every amino acid investigated, the ratio of the concentration in tissue to that in the medium was greatly decreased from the normal value by incubation of the tissue in the glucose-free medium. This result was perhaps partly caused by depletion of tissue ATP in the absence of glucose, with consequent suppression of active uptake of the amino acids.

The possibility that disturbance of the ionic balance at the cell membrane plays a significant role in the release of amino acids from the brain tissue incubated in the glucose-free medium was shown by the effects of the addition of tetrodotoxin (2 μ M) to the incubation medium. Tetrodotoxin caused significant retention in the tissue of glutamate, γ -aminobutyrate, aspartate and glycine (Table 2) and a decreased concentration of amino acids, particularly glutamate and aspartate, in the incubation medium (Table 2). The amino acid concentration ratio (tissue to medium) was significantly increased, for glutamate, γ -amino-

butyrate, aspartate and glycine, when tetrodotoxin was present.

Addition of protoveratrine (5 μ M) to the glucose-free incubation medium caused an even more pronounced decrease in the concentration of glutamate, γ -aminobutyrate and glycine in the tissue (Table 2). Aspartate in the tissue was also decreased, but glutamine in the tissue was enhanced. These changes in concentrations of amino acids in the tissue caused by protoveratrine were accompanied by notable increases in the concentration of amino acids, particularly glutamate, γ -aminobutyrate and aspartate, in the incubation medium (Table 2). These effects of protoveratrine were either abolished, or greatly decreased, by tetrodotoxin (Table 2). The amino acid concentration ratio (tissue to medium) found in the presence of protoveratrine was enhanced by the addition of tetrodotoxin from 14 to 143 for glutamate, from 21 to 86 for γ -aminobutyrate, from 39 to 209 for aspartate, from 10 to 52 for glycine; it decreased, however, from 13 to 5 for glutamine (Table 2). A noteworthy effect of the presence of protoveratrine was the considerable increase in the total content of glutamine. A value of 2.25 μ mol/g (0.51 in tissue+1.74 in medium) found in the absence of

Table 3. *Effects of tetrodotoxin (2 μM), protoveratrine (5 μM) and sodium L-glutamate (2.5 mM) on amino acid content in, and release from, rat brain-cortex slices incubated in Ca²⁺-deficient media*

For experimental details see the text. Amounts of amino acids in rat brain-cortex slices (Tissue), μmol/g initial wet wt., or in the 3 ml of incubation medium (Medium), μmol/g initial wet wt., after incubation in O₂ for 1 h at 37°C in Ca²⁺-deficient media are shown.

Addition ... Amino acid	None		Tetrodotoxin		Protoveratrine		Protoveratrine + tetrodotoxin		Sodium L-glutamate		Sodium L-glutamate + tetrodotoxin	
	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium
Glutamate	9.75 ±	1.17 ±	10.87 ±	0.72 ±	7.07 ±	2.63 ±	9.37 ±	0.60 ±	20.17 ±	(1.73) ±	22.61 ±	(1.73) ±
	0.34 ±	0.11 ±	0.31 ±	0.04 ±	0.68 ±	0.30 ±	0.09 ±	0.04 ±	0.09 ±	0.06(mM) ±	1.39 ±	0.05(mM) ±
Glutamine	1.96 ±	2.41 ±	2.00 ±	2.46 ±	2.53 ±	3.09 ±	1.50 ±	2.21 ±	3.81 ±	3.39 ±	3.82 ±	3.69 ±
	0.02 ±	0.14 ±	0.02 ±	0.19 ±	0.27 ±	0.34 ±	0.20 ±	0.04 ±	0.20 ±	0.18 ±	0.21 ±	0.11 ±
γ-Aminobutyrate	2.30 ±	0.05 ±	2.44 ±	0.03 ±	1.55 ±	0.55 ±	2.56 ±	0.08 ±	3.21 ±	0.26 ±	3.85 ±	0.16 ±
	0.05 ±	0.03 ±	0.10 ±	0.02 ±	0.06 ±	0.05 ±	0.48 ±	0.04 ±	0.34 ±	0.04 ±	0.02 ±	0.02 ±
Aspartate	2.95 ±	0.45 ±	3.21 ±	0.31 ±	2.43 ±	0.71 ±	3.70 ±	0.49 ±	5.69 ±	2.73 ±	6.30 ±	2.73 ±
	0.19 ±	0.05 ±	0.28 ±	0.01 ±	0.35 ±	0.04 ±	0.48 ±	0.13 ±	0.07 ±	0.03 ±	0.30 ±	0.05 ±
Glycine	0.88 ±	0.44 ±	0.76 ±	0.34 ±	0.65 ±	0.60 ±	0.91 ±	0.36 ±	0.81 ±	0.38 ±	0.99 ±	0.26 ±
	0.17 ±	0.10 ±	0.12 ±	0.03 ±	0.11 ±	0.10 ±	0.02 ±	0.10 ±	0.03 ±	0.07 ±	0.08 ±	0.02 ±
Alanine	0.73 ±	0.70 ±	0.68 ±	0.80 ±	0.55 ±	0.76 ±	0.85 ±	0.83 ±	0.98 ±	1.34 ±	1.06 ±	0.50 ±
	0.23 ±	0.21 ±	0.27 ±	0.06 ±	0.10 ±	0.14 ±	0.02 ±	0.21 ±	0.04 ±	0.50 ±	0.12 ±	0.22 ±

protoveratrine rose to $3.78 \mu\text{mol/g}$ (1.17 in tissue +2.61 in medium) in the presence of the drug (Table 2). Such a marked increase did not occur when tetrodotoxin was present, nor was it so clearly evident when glucose was present in the incubation medium (Table 1).

Effects of protoveratrine and tetrodotoxin on amino acid content in, and release from, rat brain-cortex slices incubated in Ca^{2+} -deficient media

Absence of Ca^{2+} from the glucose-containing incubation medium caused relatively small changes from the normal in the amino acid contents of the tissue slices after aerobic incubation for 1 h (Table 3), though there seemed to be a significant decrease in the content of glutamine in the tissue. A series of experiments was done with EGTA added to the Ca^{2+} -deficient incubation medium to remove as many Ca^{2+} ions as possible from the medium. The results showed no significant changes of amino acid contents in either the tissue or the incubation medium from those found in the absence of EGTA.

Addition of protoveratrine ($5 \mu\text{M}$) to the Ca^{2+} -deficient medium caused changes in the contents of amino acids in the tissue similar to those found in the normal incubation medium (Tables 1 and 3), but it caused substantial increases in the content of glutamate, γ -aminobutyrate and aspartate in the medium (Tables 1 and 3). These effects of protoveratrine were abolished by the addition of tetrodotoxin (Table 3). The amino acid concentration ratios (tissue to medium) in the presence of protoveratrine in the Ca^{2+} -deficient media increased on addition of tetrodotoxin from 80 to 468 for glutamate, from 86 to 950 for γ -aminobutyrate, from 102 to 226 for aspartate, from 32 to 76 for glycine (Table 3). No significant changes caused by tetrodotoxin occurred with glutamine or alanine.

The effects of protoveratrine in promoting tetrodotoxin-sensitive losses of amino acid from slices were considerably greater in the Ca^{2+} -deficient medium than in the normal incubation medium, the amino acids most markedly affected being glutamate, γ -aminobutyrate, aspartate and glycine.

The total quantity of glutamine found in the tissue and medium, at the end of the incubation period of 1 h, was greater in the presence of protoveratrine than in its absence (Table 3). The value of $4.37 \mu\text{mol/g}$ was increased in the presence of protoveratrine to $5.62 \mu\text{mol/g}$. It was the only amino acid, among those investigated, that showed an increase in the total value on incubation in the presence of protoveratrine. The increase was abolished by the addition of tetrodotoxin.

Experiments were done to observe whether the addition of sodium L-glutamate (2.5mM) to the Ca^{2+} -deficient medium would result in increased losses of

amino acids from the incubated brain slices. Table 3 shows that there were significantly increased amounts of glutamine, γ -aminobutyrate and aspartate both in the tissue and the medium after incubation for 1 h, but that these amounts were not markedly affected by the presence of tetrodotoxin.

Effects of protoveratrine and tetrodotoxin on amino acid content in, and release from, rat brain-cortex slices incubated in glucose-free Ca^{2+} -deficient media

Absence of both glucose and Ca^{2+} from the incubation medium increased the loss of amino acids, particularly glutamate, γ -aminobutyrate and aspartate, from slices incubated aerobically for 1 h (Table 4); the loss was greater than that found in an incubation medium devoid only of glucose (Table 2) or of Ca^{2+} (Table 3). The rates of release of the amino acids were diminished by the presence of tetrodotoxin (Table 4). Amino acid concentration ratios (tissue to medium) increased, on addition of tetrodotoxin (Table 4), from 23 to 117 for glutamate, from 23 to 168 for γ -aminobutyrate, from 49 to 210 for aspartate and from 12 to 34 for glycine.

Addition of protoveratrine to the glucose-free Ca^{2+} -deficient medium increased the loss of all amino acids, except glutamine and alanine (Table 4), and the loss was diminished by tetrodotoxin. Amino acid concentration ratios (tissue to medium) found with protoveratrine present increased on addition of tetrodotoxin (Table 4) from 17 to 56 for glutamate, from 6 to 114 for γ -aminobutyrate, from 41 to 114 for aspartate and from 9 to 26 for glycine. No significant changes occurred with glutamine or alanine.

Addition of sodium L-glutamate to the glucose-free Ca^{2+} -deficient medium caused an increase in the tissue contents of amino acids, particularly glutamate, glutamine, γ -aminobutyrate and aspartate, after an incubation period of 1 h and also in the contents of these amino acids, particularly aspartate, in the incubation medium (Table 4). The presence of tetrodotoxin caused some retention of the amino acids (except for glutamine) in the tissue and decreased the contents of these amino acids in the incubation medium.

Effects of increased K^+ and of tetrodotoxin on amino acid content in, and release from, incubated rat brain-cortex slices

Incubation of slices in Krebs-Ringer phosphate medium containing 10mM -glucose, in which K^+ was increased by 45mequiv/l caused a significant increase in the content of γ -aminobutyric acid in the tissue and an increased content of γ -aminobutyrate in the incubation medium (Tables 5 and 1). There was some retention of glutamine in the tissue slices. Less definite changes occurred with the other amino acids

Table 4. Effects of tetrodotoxin (2 μM), protoveratrine (5 μM) and sodium L-glutamate (2.5 mM) on amino acid content in, and release from, rat brain-cortex slices incubated in glucose-free Ca²⁺-deficient media

For experimental details see the text. Amounts of amino acids in rat brain-cortex slices (Tissue), μmol/g initial wet wt., or in the 3 ml of incubation medium (Medium), μmol/g initial wet wt., after incubation in O₂ for 1 h with Ca²⁺ omitted and in the absence of glucose are shown.

Addition	...	None		Tetrodotoxin		Protoveratrine		Protoveratrine + tetrodotoxin		Sodium L-glutamate		Sodium L-glutamate + tetrodotoxin	
		Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium
Glutamate		1.62	2.08	3.01	0.77	1.48	2.68	2.39	1.29	5.14	2.15	9.33	1.96
		±	±	±	±	±	±	±	±	±	±	±	±
Glutamine		0.12	0.19	0.38	0.12	0.07	0.42	0.08	0.19	0.02	0.02mm	0.60	0.07mm
		0.22	1.24	0.17	0.75	0.37	0.97	0.27	0.80	0.75	3.01	0.75	2.41
γ-Aminobutyrate		±	±	±	±	±	±	±	±	±	±	±	±
		0.06	0.19	0.03	0.14	0.02	0.01	0.07	0.10	0.09	0.18	0.07	0.26
Aspartate		0.80	1.07	1.73	0.31	0.29	1.39	1.45	0.38	1.37	1.25	2.52	0.70
		±	±	±	±	±	±	±	±	±	±	±	±
Glycine		0.17	0.08	0.29	0.05	0.03	0.01	0.01	0.06	0.30	0.13	0.23	0.17
		6.39	3.92	8.04	1.15	6.63	4.81	7.67	2.02	9.65	12.07	12.05	6.02
Alanine		±	±	±	±	±	±	±	±	±	±	±	±
		0.42	0.18	0.06	0.10	0.37	0.06	0.37	0.16	0.20	0.22	0.20	0.51
	0.33	0.80	0.62	0.54	0.27	0.93	0.57	0.50	0.57	0.74	0.82	0.37	
	±	±	±	±	±	±	±	±	±	±	±	±	±
	0.02	0.03	0.13	0.04	0.02	0.05	0.03	0.02	0.03	0.07	0.02	0.02	0.08
	0.07	0.60	0.21	0.45	0.02	0.47	0.51	0.05	0.51	0.12	0.49	0.22	0.84
	±	±	±	±	±	±	±	±	±	±	±	±	±
	0.05	0.06	0.04	0.07	0.01	0.01	0.04	0.01	0.01	0.03	0.01	0.03	0.29

Table 5. *Effects of increased K⁺ and of tetrodotoxin (2 μM) on amino acid content in, and release from, rat brain-cortex slices incubated in various media*
 For experimental details see the text. Amounts of amino acids in rat brain-cortex slices (Tissue), μmol/g initial wet wt., or in the 3 ml of incubation medium (Medium), μmol/g initial wet wt., after incubation in O₂ for 1 h in the presence or absence of glucose or Ca²⁺ are shown.

Amino acid	KCl (45mm)		KCl (45mm) + tetrodotoxin		KCl (45mm); Ca ²⁺ absent		KCl (45mm); tetrodotoxin; Ca ²⁺ absent		KCl (45mm); glucose absent		KCl (45mm) + tetrodotoxin; glucose absent	
	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium
Glutaminate	8.38 ±	0.88 ±	8.31 ±	0.60 ±	9.45 ±	1.20 ±	9.37 ±	1.24 ±	1.29 ±	2.63 ±	1.27 ±	2.52 ±
Glutamine	4.93 ±	0.08 ±	0.13 ±	0.02 ±	0.19 ±	0.13 ±	0.17 ±	0.09 ±	0.27 ±	0.21 ±	0.16 ±	0.04 ±
γ-Aminobutyrate	0.05 ±	0.07 ±	0.18 ±	0.05 ±	0.09 ±	0.07 ±	0.10 ±	0.18 ±	0.09 ±	0.09 ±	0.08 ±	0.04 ±
Aspartate	3.98 ±	0.63 ±	3.80 ±	0.32 ±	2.93 ±	0.34 ±	2.74 ±	0.21 ±	0.72 ±	0.74 ±	0.76 ±	0.53 ±
	0.04 ±	0.02 ±	0.40 ±	0.01 ±	0.34 ±	0.04 ±	0.03 ±	0.01 ±	0.36 ±	0.05 ±	0.28 ±	0.12 ±
	2.31 ±	0.43 ±	2.12 ±	0.41 ±	2.33 ±	0.57 ±	2.42 ±	0.59 ±	7.08 ±	6.07 ±	7.78 ±	5.33 ±
	0.13 ±	0.01 ±	0.16 ±	0.02 ±	0.08 ±	0.06 ±	0.23 ±	0.02 ±	0.32 ±	0.93 ±	0.06 ±	0.50 ±
Glycine	1.03 ±	0.67 ±	1.00 ±	0.47 ±	0.67 ±	0.54 ±	0.71 ±	0.59 ±	0.48 ±	1.16 ±	0.49 ±	1.10 ±
	0.05 ±	0.08 ±	0.04 ±	0.01 ±	0.03 ±	0.11 ±	0.05 ±	0.06 ±	0.07 ±	0.06 ±	0.07 ±	0.07 ±
Alanine	1.13 ±	0.77 ±	1.13 ±	0.67 ±	0.57 ±	1.04 ±	0.48 ±	1.04 ±	0.18 ±	1.00 ±	0.17 ±	0.91 ±
	0.06 ±	0.03 ±	0.02 ±	0.02 ±	0.04 ±	0.01 ±	0.04 ±	0.02 ±	0.04 ±	0.02 ±	0.04 ±	0.05 ±

examined. The increased content of γ -aminobutyrate in the high K^+ medium was decreased to some extent by tetrodotoxin (Table 5).

In a Ca^{2+} -deficient medium increased K^+ concentrations did not cause any significant changes in the glutamate content of the tissue or medium, in contrast with the effects of protoveratrine (Tables 5 and 3). It would appear, therefore, that the changes in amino acid fluxes in brain slices incubated with increased K^+ may differ markedly from those caused by the presence of protoveratrine.

When glucose was absent from the incubation medium, increased K^+ promoted increased concentrations of glutamate, γ -aminobutyrate, aspartate, glycine and alanine in the incubation medium but no change in glutamine (Tables 2 and 5). Accompanying changes took place in the contents of amino acids in the tissue (Tables 2 and 5) and the total (tissue + medium) content of aspartate was considerably increased. The addition of tetrodotoxin caused no significant changes in the contents of amino acids both in the tissue and medium, except for a small decrease in the content of γ -aminobutyrate in the medium.

Effects of the combined presence of protoveratrine and ouabain on amino acid release

It was found, after a series of experiments utilizing various incubation conditions, that the optimum medium for the release of amino acids from slices incubated under aerobic conditions at $37^\circ C$ for 1 h, consisted of Krebs-Ringer phosphate medium containing 10 mM-glucose together with protoveratrine ($5 \mu M$) and ouabain (0.1 mM). The presence of protoveratrine caused membrane ionic changes with resultant loss of amino acids from the tissue (Tables 2 and 3), whereas that of ouabain blocked the re-uptake of the released amino acids against a concentration gradient (Gonda & Quastel, 1962a; Quastel, 1965). Table 1 shows the contents of amino acids in the tissue and in the medium at the end of the incubation period.

It will be observed that the presence of protoveratrine and ouabain caused a loss from the brain tissue of $26.10 - 7.74 = 18.36 \mu mol/g$ of the total amino acids investigated, i.e. a loss of 70%. At the same time, the incubation medium (3 ml) gained amino acids to the extent of $26.76 - 7.94 = 18.82 \mu mol/g$, a value approximately equal to that lost by the tissue. The losses from the tissue of the individual amino acids were as follows: glutamate (73%), glutamine (78%), γ -aminobutyrate (75%), aspartate (65%), glycine (74%), alanine (65%), taurine (57%), serine (75%), threonine (77%). Thus all the amino acids in the tissue suffered substantial losses, taurine being rather less affected than the others.

The total (tissue+medium) quantity of amino

acids was not significantly affected by the combined action of protoveratrine and ouabain. However, the total (tissue+medium) value for glutamine decreased by 48%, whereas that for γ -aminobutyrate increased by 49%; that for alanine increased by 55%, and that for glutamate increased only slightly (9%) (Table 1).

Effects of tetrodotoxin

Tetrodotoxin ($2 \mu M$) had little or no effect, within the range of experimental error, on the contents of amino acids both in the tissue and in the incubation medium under the normal incubation conditions. However, it did cause large changes in the presence of protoveratrine and ouabain.

Results given in Table 1 show that, on the addition of tetrodotoxin, the total quantity of amino acids in the tissue was $24.98 \mu mol/g$ and this was decreased by the combined presence of protoveratrine and ouabain to $16.54 \mu mol/g$, i.e. a loss of $8.44 \mu mol/g$, equivalent to 34% of the content in the tissue. The gain in amino acids in the medium was $15.67 - 7.54 = 8.13 \mu mol/g$, approximately equal to the loss from the tissue. Therefore tetrodotoxin decreased a loss of 70% of amino acids from the tissue, caused by protoveratrine and ouabain, to 34%. This suggests that at least 36% of the tissue content of amino acids is located in the neurons. This is a minimum value, as it is unlikely that the tetrodotoxin causes a complete block of the neuronal efflux of amino acids, or that the block is equally effective with all amino acids or with all conditions that generate membrane ionic changes.

Alterations in the contents of individual amino acids

Glutamate. Table 1 shows that the content of glutamate in the tissue was decreased by protoveratrine and ouabain in the presence of tetrodotoxin from 8.82 to $7.14 \mu mol/g$, i.e. a loss of $1.68 \mu mol/g$ or 19% of the content of glutamate in the tissue. As the loss amounted to 73% in the absence of tetrodotoxin, it follows that the difference, i.e. $73 - 19 = 54\%$, represents the percentage of the glutamate in the tissue located in the neurons. Evidently more than half the glutamate in the brain-cortex slices is present in the neurons. Considerations of the glutamate contents in the incubation medium (Table 1) suggest the same conclusion. The increase of the concentration of glutamate in the medium, caused by the presence of protoveratrine and ouabain, amounted to $8.33 - 0.77 = 7.56 \mu mol/g$, whereas with added tetrodotoxin the increase amounted to $2.42 - 0.87 = 1.55 \mu mol/g$. The difference, $6.01 \mu mol/g$, represented the amount retained by the neurons, which therefore constituted $6.01/9.20 \times 100 = 65\%$ of the glutamate present in the tissue at the end of the incubation period.

Aspartate. The content of aspartate in the tissue

was decreased by protoveratrine and ouabain, in the presence of tetrodotoxin, from 3.64 to 3.28 $\mu\text{mol/g}$, i.e. a loss of 0.36 $\mu\text{mol/g}$ or of 10% of the content of aspartate in the tissue (Table 1). As the loss amounted to 65% in the absence of tetrodotoxin it appears that the difference, i.e. 65–10 = 55%, represents the percentage of the aspartate in the tissue, located in the neurons. Increase of the concentration of aspartate in the medium in the presence of protoveratrine and ouabain amounted to 2.49 $\mu\text{mol/g}$ (Table 1), whereas with tetrodotoxin added the increase amounted to 0.71 $\mu\text{mol/g}$. The difference, 1.78 $\mu\text{mol/g}$, represents the amount retained by the neurons and therefore constitutes $1.78/3.45 \times 100 = 51\%$ of the amount of aspartate normally present in the tissue under the given experimental condition.

It seems therefore that, as with glutamate, the neurons are the site of more than half the aspartate present in the brain.

γ -Aminobutyrate. Calculations similar to those given above, from the values recorded in Table 1, indicate that at least 32% of the γ -aminobutyrate in the tissue is located in the neurons. The actual amount must be considerably greater than this, because the effects of tetrodotoxin on γ -aminobutyrate efflux caused by protoveratrine under various incubation conditions (Tables 2, 3 and 4) indicated an almost complete block of the release of γ -aminobutyrate.

Glycine and serine. Calculations similar to those described above from the results in Table 1 show that at least 50% of the content of either glycine or serine in the tissue is in the neurons.

Taurine, alanine and threonine. Similar calculations show that at least 21% of the content of alanine in the tissue, 10% of the content of taurine in the tissue and 6% of the content of threonine in the tissue are located in the neurons. It is not possible to decide, from these values, whether the neurons are the locations of the major pools of these amino acids.

Glutamine. Table 1 shows that the percentage decrease in glutamine in the tissue in the presence of protoveratrine and ouabain was $(3.45 - 0.74)/3.45 \times 100 = 78\%$ and the percentage decrease, with tetrodotoxin added, was $(2.88 - 0.53)/2.88 \times 100 = 81\%$. Thus the presence of tetrodotoxin had no diminishing effect on the proportion of glutamine released from the brain tissue in the presence of protoveratrine and ouabain. It appears therefore that glutamine is not retained in the brain tissue by tetrodotoxin under conditions where the release of glutamate and other amino acids is greatly affected.

Moreover the presence of protoveratrine (5 μM) had no effect on the content of glutamine in the tissue in the presence of ouabain, whereas it caused significant decreases in the tissue contents of glutamate, γ -aminobutyrate, aspartate, glycine and alanine (Table 1).

Effects of sodium malonate and sodium fluoroacetate on cerebral amino acid content and release

It has been suggested (Clarke *et al.*, 1970) that the suppression of cerebral glutamine synthesis by low concentrations of fluoroacetate (Lahiri & Quastel, 1963) is caused by the localization of its effect in a special compartment where glutamine synthesis takes place. The fluoroacetate is considered to block the operation of the tricarboxylic acid cycle in this compartment thereby decreasing the amount of ATP available for glutamine synthesis.

Experiments were therefore done to observe the effects of two tricarboxylic acid-cycle inhibitors, sodium malonate and sodium fluoroacetate, on both the respiration and on the contents of amino acids of brain slices incubated in a medium containing protoveratrine. It is well known that protoveratrine causes stimulation of brain respiration. The stimulated respiration is abolished by tetrodotoxin (Okamoto & Quastel, 1970b) and is considered, therefore, to be associated with the neurons.

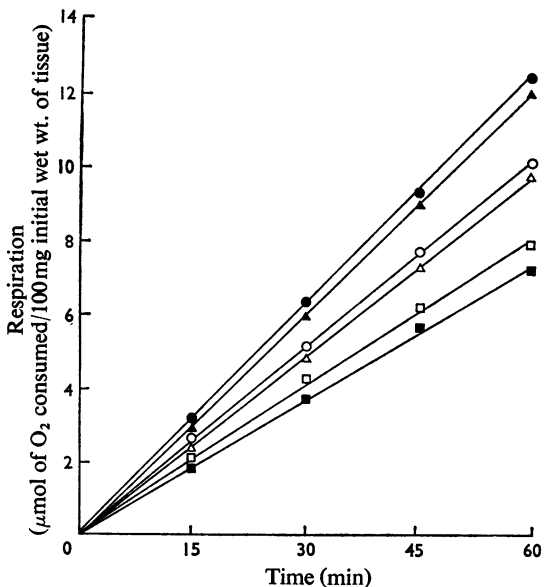


Fig. 1. Respiration of rat brain-cortex slices incubated in O_2 at 37°C in Krebs-Ringer phosphate medium containing 10 mM-glucose in the presence or absence of protoveratrine (5 μM), sodium fluoroacetate (3 mM) or sodium malonate (2 mM).

For experimental details see the text. ○, No addition; □, with malonate; △, with fluoroacetate; ●, protoveratrine added; ■, protoveratrine + malonate added; ▲, protoveratrine + fluoroacetate added.

Results given in Fig. 1 show that the protoveratrine-stimulated respiration was blocked by malonate (2mM), but not by fluoroacetate (3mM). This result is consistent with the conclusion that fluoroacetate, in contrast with malonate, at the concentrations investigated does not suppress the operation of the tricarboxylic acid cycle in the neurons.

Malonate (2mM) and fluoroacetate (3mM) caused different effects on the amounts of amino acids in, and on their release from, brain slices incubated in the presence of protoveratrine (Table 6).

The major effect of fluoroacetate (3mM) was to bring about a diminution in the total (tissue + medium) content of glutamine, amounting to 2.45 μ mol/g, accompanied by an increase in that of glutamate amounting to 2.45 μ mol/g. There was an increase in the content of γ -aminobutyrate amounting to 1.71 μ mol/g and a slight decrease in the value for aspartate. There were minor changes in the contents of the other amino acids. These results with fluoroacetate resemble those obtained in studies of the amounts of labelled amino acids formed from labelled glucose (Lahiri & Quastel, 1963). Fluoroacetate caused a significant increase in the content of glutamate in the tissue, but not in the medium, and a significant decrease in the content of glutamine in the tissue, but not in the medium. The increase in γ -aminobutyrate was mostly confined to the tissue.

The major effect of malonate was to diminish the total (tissue + medium) amount of aspartate by 1.59 μ mol/g and to increase that of γ -aminobutyrate by 1.92 μ mol/g. There were no statistically significant effects on the total amounts of glutamine or other amino acids (Table 6). Malonate caused significant increases in the contents of glutamate and glutamine in the medium with accompanying decreases in the contents of these amino acids in the tissue. It caused a large decrease in the value of aspartate in the tissue with a relatively small change in the content of aspartate in the medium. A large increase occurred in γ -aminobutyrate in the tissue with a small, but significant, change in the content of γ -aminobutyrate in the medium. The content of alanine in the tissue was also significantly decreased by malonate. Changes in the amounts of the other amino acids investigated were not statistically significant.

Effects of glutamine and sodium L-glutamate on cerebral amino acid content and release

Experiments were done to observe the effects of the addition of L-glutamine (2.5mM) to the incubation medium on the amino acid contents of rat brain-cortex slices. Krebs-Ringer phosphate solution containing 10mM-glucose, but deficient in Ca^{2+} , was used as the incubation medium, because, with this medium, the addition of L-glutamate was found to promote the release of γ -aminobutyrate and aspartate.

Table 6. *Effects of sodium malonate or sodium fluoroacetate on amino acid content in, and release from, incubated rat brain-cortex slices in the presence of protoveratrine*

Addition ...	Protoveratrine			Protoveratrine+malonate			Protoveratrine+fluoroacetate		
	Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total
Glutamate	7.04 \pm 0.06	0.78 \pm 0.06	7.82	6.07 \pm 0.37	1.22 \pm 0.01	7.29	9.31 \pm 0.69	0.96 \pm 0.10	10.27
Glutamine	3.56 \pm 0.07	2.73 \pm 0.25	6.29	2.77 \pm 0.54	3.88 \pm 0.09	6.65	0.91 \pm 0.17	2.93 \pm 0.59	3.84
γ -Aminobutyrate	2.92 \pm 0.37	0.06 \pm 0.03	2.98	4.65 \pm 0.01	0.25 \pm 0.03	4.90	4.58 \pm 0.47	0.11 \pm 0.02	4.69
Aspartate	1.98 \pm 0.10	0.51 \pm 0.01	2.49	0.53 \pm 0.15	0.37 \pm 0.05	0.90	1.39 \pm 0.04	0.53 \pm 0.08	1.92
Glycine	0.89 \pm 0.07	0.30 \pm 0.03	1.19	0.79 \pm 0.07	0.53 \pm 0.07	1.32	0.98 \pm 0.07	0.48 \pm 0.03	1.46
Alanine	1.07 \pm 0.01	0.59 \pm 0.06	1.66	0.66 \pm 0.04	0.79 \pm 0.01	1.45	1.04 \pm 0.01	0.86 \pm 0.07	1.90
Taurine	3.61 \pm 0.28	2.40 \pm 0.10	6.01	2.98 \pm 0.32	3.17 \pm 0.05	6.15	3.52 \pm 0.08	3.10 \pm 0.12	6.62
Serine	0.97 \pm 0.08	0.70 \pm 0.01	1.67	0.86 \pm 0.07	0.99 \pm 0.13	1.85	1.07 \pm 0.08	0.95 \pm 0.05	2.02
Threonine	0.51 \pm 0.02	0.25 \pm 0.02	0.76	0.37 \pm 0.06	0.38 \pm 0.02	0.75	0.35 \pm 0.03	0.37 \pm 0.03	0.72
Total	22.55	8.32	30.87	19.68	11.58	31.26	23.15	10.29	33.44

Rat brain-cortex slices were incubated in 3ml of Krebs-Ringer phosphate medium containing 10mM-glucose in O_2 at 37°C for 1 h in the presence of protoveratrine (5 μ M) with or without sodium malonate (2mM) or sodium fluoroacetate (3mM). Values in tissue for amino acids (Tissue) are expressed as μ mol/g initial wet wt. and those in the medium (Medium) as μ mol/3 ml per g wet wt.

Table 7. Effects of L-glutamate and L-glutamine on amino acid content in, and release from, rat brain-cortex slices

Rat brain-cortex slices were incubated in 3 ml of Krebs-Ringer phosphate medium containing 10 mM-glucose, but in the absence of Ca²⁺, at 37°C for 1 h with or without L-glutamate (2.5 mM) or L-glutamine (2.5 mM). Values in tissue of amino acids (Tissue) are expressed as μmol/g initial wet wt. and those in the medium (Medium) as μmol/3 ml/per g initial wet wt.

Addition ...	None			L-Glutamate			L-Glutamine		
	Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total
Amino acid									
Glutamate	9.75 ± 0.34	1.17 ± 0.11	10.92	20.17 ± 0.09	(1.73 ± 0.06 mM)	7.20	10.10 ± 0.08	2.27 ± 0.28	12.37
Glutamine	1.96 ± 0.02	2.41 ± 0.14	4.37	3.81 ± 0.20	3.39 ± 0.18	7.20	12.22 ± 0.09	(1.62 ± 0.06 mM)	4.07
γ-Aminobutyrate	2.30 ± 0.05	0.05 ± 0.03	2.35	3.21 ± 0.34	0.26 ± 0.04	3.47	4.04 ± 0.06	0.03 ± 0.01	4.07
Aspartate	2.95 ± 0.19	0.45 ± 0.05	3.40	5.69 ± 0.07	2.73 ± 0.03	8.42	2.96 ± 0.23	0.63 ± 0.01	3.59
Ammonia	1.47 ± 0.09	5.69 ± 0.63	7.16	1.48 ± 0.45	4.88 ± 0.36	6.36	1.51 ± 0.12	9.93 ± 0.49	11.44

Results are shown in Table 7, which also gives comparable results obtained with sodium L-glutamate (2.5 mM). The addition of L-glutamine caused an increased value of the content of γ-aminobutyrate in the tissue, but no increased release of γ-aminobutyrate, and an increased quantity of ammonia and some increase in the concentration of glutamate in the medium. There was no increased formation of aspartate. Addition of glutamate (2.5 mM), however, caused a smaller increase of γ-aminobutyrate in the tissue, but an increased release of γ-aminobutyrate, increased formation and release of both aspartate and glutamine, and a somewhat diminished formation of ammonia. Thus the addition of glutamine and of L-glutamate to the incubation medium caused significantly different effects both in amino acid content and amino acid effluxes in rat brain-cortex slices.

Discussion

The results show that incubation with protoveratrine (particularly in a medium deficient in Ca²⁺), or with ouabain, or in the absence of glucose causes an increased release of a number of amino acids from rat brain-cortex slices into the medium and that the amino acid concentration ratios between tissue and medium are decreased. The amino acids most markedly affected are glutamate, γ-aminobutyrate, aspartate and glycine. Moreover, the processes involved in the increased release of these amino acids or in bringing about decreased concentration ratios have tetrodotoxin-sensitive components. As sensitivity to tetrodotoxin according to electrophysiological evidence (Kao, 1966) is associated with the blocking of the Na⁺ channels concerned in excitation, it seems possible that the release of amino acids from the tissue induced by these conditions is in some way associated with concomitant changes in membrane ionic balance. This result has obvious physiological significance, as all the amino acids in question are considered to possess transmitter properties in the central nervous system (Curtis & Johnston, 1970). It may be noted (Jones & McIlwain, 1971) that electrical stimulation of guinea pig brain slices also causes increased release of amino acids into the incubation medium.

As the effects of protoveratrine are presumably confined to the electrically excitable cells of the nervous system, i.e. the neurons, it is probable that the tetrodotoxin-sensitive release of amino acids is also confined to the neurons. It is likely, therefore, that the amino acids glutamate, γ-aminobutyrate, aspartate and glycine released by the action of protoveratrine emanate from the neurons.

It has been noted (Gottesfeld & Elliott, 1971) that rat brain-cortex slices loaded with γ-aminobutyrate, by previous incubation in the presence of this substance, lose this amino acid on incubation in a γ-aminobutyrate-free medium at a rate that is

accelerated by protoveratrine, and that the protoveratrine effect is abolished by tetrodotoxin. Presumably this phenomenon is analogous with that recorded above. Results by Bowery & Brown (1971) support the conclusion that there is neuronal uptake of γ -aminobutyrate.

The tetrodotoxin-sensitive release of glycine (Tables 2, 3, 4), though small, is significant. Electrical stimulation seems not to affect the rate of glycine uptake (Nakazawa & Quastel, 1968), nor does it affect the release of glycine from brain-cortex slices that have been allowed to take up glycine (Mitchell *et al.*, 1969). However, these results may only reflect the fact that the rate of uptake of glycine keeps pace with its release. Processes of efflux and influx obviously control the actual amounts of amino acids in the tissue under various incubation conditions (Cherayil *et al.*, 1967).

Although ouabain or protoveratrine causes tetrodotoxin-sensitive amino acid release in incubated rat brain slices, the responses of these two drugs towards tetrodotoxin differ. Tetrodotoxin causes only a partial suppression of the effect of ouabain in stimulating amino acid release, or of Na^+ uptake (Okamoto & Quastel, 1970*b*), into brain slices. It causes, however, a complete abolition of the activity of protoveratrine with either of these processes. This phenomenon may be explained by the conclusion that protoveratrine, like electrical stimuli, acts only on the neurons, whereas ouabain affects both neurons and glia by suppression of the Na^+ -stimulated adenosine triphosphatase and its dependent amino acid uptake mechanism. If an amino acid, therefore, is concentrated for the most part in the glia, it will respond to ouabain but its efflux may have no tetrodotoxin-sensitive component.

The results given above show that, in contrast with the changes in glutamate, γ -aminobutyrate, aspartate and glycine concentration ratios (tissue to medium), that of glutamine is little affected by the various incubation conditions. Even with ouabain, which suppresses the synthesis of glutamine from glucose (Gonda & Quastel, 1962*b*) and causes a greatly diminished concentration ratio between tissue and medium after 1 h incubation, the addition of tetrodotoxin has no significant effect on this concentration ratio (Table 1). Such results suggest that the main pool of glutamine in normal brain tissue is not in the neurons but in the glia, a conclusion in accord with previous observations and suggestions (Tower, 1960; Margolis *et al.*, 1968).

The action of tetrodotoxin in increasing the content of glutamate in the tissue of brain slices incubated in a glucose-free medium containing 2.5 mM-sodium L-glutamate (Table 4), indicates that some of the exogenous glutamate taken up by the tissue enters the neurons.

Incubation of the slices in a glucose-free medium

has marked effects on the contents of the amino acids both in the tissue and in the incubation medium. There are considerable increases in the contents of aspartate in the tissue and medium and decreases in the contents of glutamate, γ -aminobutyrate, glutamine, and glycine in the tissue (Table 2). Similar results have been obtained in the brains of rats suffering from insulin hypoglycaemia (De Ropp & Snedeker, 1961; Dawson, 1953; Cravioto *et al.*, 1951) and in rat brains perfused with a glucose-free saline medium (Mukherji *et al.*, 1971). The results, therefore, obtained with incubated slices reflect, at least qualitatively, the results obtained with rat brain *in vivo*, so far as brain amino acids are concerned. Interestingly, the concentrations of glutamate and aspartate in brain-cortex slices from both rat and guinea pig for incubation periods of 1 h are near to the values *in vivo* (Balázs *et al.*, 1970).

A notable effect of protoveratrine, when added to a glucose-free (Table 2) or a Ca^{2+} -free medium (Table 3), is to cause a significant increase in the total (tissue + medium) quantity of glutamine, whereas the total quantities of the other amino acids investigated are not significantly changed. This effect is abolished by the presence of tetrodotoxin. A possible explanation for this result is that release of glutamate from neurons by protoveratrine results in an increased concentration of glutamate in the intracellular space, with consequent increased uptake of glutamate by the glia and, therefore, an increased amount of conversion, in the glia, of glutamate into glutamine. Thus uptake of glutamate by glia may represent a mechanism for the decrease in the extracellular concentration of glutamate, released from neurons, during functional activity. This suggests that the movement of glutamate from neuron to glia resembles that apparently occurring with the movement of K^+ under the same conditions (Okamoto & Quastel, 1970*b*).

Results obtained with high K^+ in the incubation medium (Table 5) indicate relatively small changes in the contents of glutamate and aspartate in rat brain-cortex slices incubated in a glucose-saline medium, but they show an increased release of γ -aminobutyrate, which is only partially suppressed by the addition of tetrodotoxin. It has been reported (Srinivasan *et al.*, 1969; Machiyama *et al.*, 1967, 1970) that the effect of increased K^+ is to accelerate the release of γ -aminobutyrate from incubated brain tissue. Our results are in accordance with this finding.

When the concentration of K^+ , however, is increased in a glucose-free medium considerable decreases in the concentration ratios (tissue to medium) of glutamate, aspartate, γ -aminobutyrate and glycine (Tables 2 and 5) occur; the increased rates of release of the amino acids are little affected by tetrodotoxin (Table 5). The effect of tetrodotoxin

may be obscured because of the large depolarization due to high concentrations of K^+ (Kuffler, 1967; Gibson & McIlwain, 1965; Hodgkin & Huxley, 1952). A possible explanation for the apparent K^+ stimulation of amino acid release is that there is a decrease in the concentration of ATP in the tissue (Okamoto & Quastel, 1970a), resulting in a further suppression of the amino acid-uptake processes occurring in a glucose-free incubation medium.

Experiments done to identify the possible locations of glutamate and glutamine and other amino acids in incubated rat brain-cortex slices led to the conclusion that the major pools of glutamate, aspartate, glycine, serine and probably γ -aminobutyrate are in the neurons. The major pool of glutamine, however, is not in the neurons and therefore must be in the glia. These results have been obtained by using tetrodotoxin to suppress the increased neuronal release of amino acids caused by the joint action of protoveratrine and ouabain. The results found with glutamate and aspartate definitely indicate that over 50% of the contents of these amino acids in the tissue are located in the neurons. The inference about the location of glutamine depends on the observation that tetrodotoxin has no retarding effect on the release of this amino acid under circumstances where it has significant retarding effects on the release of glutamate, aspartate, glycine and γ -aminobutyrate.

This conclusion about the glial location of the major pool of glutamine is supported by results obtained by using the tricarboxylic acid cycle inhibitors, fluoroacetate (3 mM) and malonate (2 mM). It was found that whereas malonate blocked the increased brain-tissue respiration caused by protoveratrine, fluoroacetate had no such effect. The lack of action of fluoroacetate, at a concentration that suppresses cerebral glutamine synthesis, is consistent with the conclusion that it acts, at 3 mM, on the operation of the tricarboxylic acid cycle in a compartment other than that in the neurons, i.e. in the glia. This conclusion is in accord with the findings of Clarke *et al.* (1970), who concluded that the fluoroacetate effect is confined to the 'small' pool in which glutamine synthesis takes place. Such a conclusion is further supported by the observation that malonate and fluoroacetate exert different effects on the contents of amino acids of rat brain-cortex slices when respiration has been stimulated by the presence of protoveratrine. Thus whereas fluoroacetate suppresses glutamine formation at the expense of glutamate, whose content in the tissue increases, malonate has no such action on these amino acids, but it suppresses the formation of aspartate. The malonate effect is explicable, as it will block the cerebral conversion of succinate into aspartate (Gonda & Quastel, 1962b). Its lack of action on glutamine biosynthesis, at the relatively low concentration necessary to suppress the protoveratrine-stimulated

respiration, is consistent with the conclusion that its main effect is on the stimulated neurons, where it will have no significant effect on glutamine formation in the tissue, if the major site of the latter operation is in the glia.

Both inhibitors cause an increase in the quantities of γ -aminobutyrate. Possibly malonate may cause this by diversion of glutamate from oxidative breakdown through the tricarboxylic acid cycle towards the γ -aminobutyrate pathway, and fluoroacetate may cause this by increasing the neuronal concentration of glutamate.

Neuron-glia interrelations

The results recorded above lead to the conclusion that the neuron is the site of the major pool of glutamate in brain-cortex slices, and that during excitation glutamate is released from the neurons. Part of the glutamate is taken up by the glia and there converted into glutamine, as the glia seem to be the site of the major pool of glutamine. Glutamine, however, is also released from the tissue by extracellular glutamate. The extracellular glutamine is taken up by the neurons. This is shown by the results in Table 7, which indicate that glutamine causes an increased formation of γ -aminobutyrate, and is even more active in this respect than glutamate at a similar concentration. Glutamic acid decarboxylase, whose activity is responsible for the formation of γ -aminobutyrate, seems to be confined to the neurons (Neal & Iversen, 1969; Balázs *et al.*, 1966; Salganicoff & de Robertis, 1965), so that an increase of γ -aminobutyrate in the tissue on incubation with glutamine leads to the inference that glutamine penetrates the neurons. Presumably the neuronal entry of glutamine is followed by hydrolysis, with release of ammonia (Table 7) and decarboxylation of glutamate. The fact that glutamine appears to be a more effective precursor of γ -aminobutyrate than glutamate (Table 7) in incubated rat brain-cortex slices indicates that extraneuronal hydrolysis of glutamine is not essential for γ -aminobutyrate formation from glutamine and that hydrolysis of glutamine may take place inside the neuron rather than outside it.

The facts therefore indicate a cycle of events in which neurons and glia are coupled to allow part of the glutamate, released from the neurons during excitation, to be withdrawn by the glia from the extraneuronal space and to be returned to the neurons eventually in the form of glutamine.

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