The Metabolism of γ -Aminobutyrate and Glucose in Potassium Ion-Stimulated Brain Tissue *in vitro*

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1. The metabolism of γ -aminobutyrate (GABA) was investigated in cerebral-cortex slices incubated in glucose-saline medium with [$1\text{-}^{14}\text{C}$]GABA and [$\text{U-}^{14}\text{C}$]glucose as labelled substrates. 2. A rapid release of GABA from the tissue, amounting to 25–30% of the total, was observed on addition of 66m-equiv. of K^+ /l to the medium; the liberation of other amino acids was relatively small. The effect was apparently specific for K^+ ; GABA was not released on addition of equivalent amounts of Na^+ or on increasing the respiration rate with 10mM-ammonium chloride. The results show that GABA behaves like the transmitter compounds (acetylcholine, catecholamines) on K^+ stimulation, and therefore now satisfies certain of the criteria required for a transmitter in mammalian brain. 3. The release of GABA from the tissue on addition of K^+ was followed by a slow re-uptake. The rate of uptake of GABA in a medium containing 5.9m-equiv. of K^+ /l was more than four times that in a medium containing 66m-equiv. of K^+ /l. 4. The concentration of GABA in brain tissue incubated for 1h in a medium containing 66m-equiv. of K^+ /l was about 50% higher than that observed under normal conditions. 5. There was evidence that exogenous [^{14}C]GABA mixed with the endogenous pool(s), since the proportion of the total GABA released on K^+ stimulation was the same, and the specific radioactivity of the liberated GABA was close to that remaining in the tissue, whether the GABA was labelled by [$1\text{-}^{14}\text{C}$]GABA from the medium or generated in the tissue from [^{14}C]glucose. 6. On the basis of these findings and the observations outlined in the preceding papers it was possible to calculate the kinetic constants of GABA metabolism by computer simulation of the results. K^+ stimulation led to a 2.5-fold increase in the flux through the tricarboxylic acid cycle, whereas the flux in the GABA bypath was little affected; as a result the flux through the GABA bypath, which under normal conditions was 8% of that through the tricarboxylic acid cycle, decreased to 3–5%. 7. The metabolism of glutamine was greatly affected by K^+ -stimulation. The ratio of the concentration of glutamine in the slices to that in the medium, which under normal conditions was the smallest among the amino acids investigated, increased from about 17 to 63 in 1h. This effect was attributable partly to an uptake of glutamine from the medium ($1.8\mu\text{mol/h per g}$) and partly to a net increase in the total amount of glutamine ($2.6\mu\text{mol/h per g}$). At 1h after the addition of K^+ the net gain of glutamine could be accounted for by the decrease of glutamate. 8. Metabolic compartmentation was evident when brain-cortex slices were incubated in glucose-saline medium and the labelled substrate was [^{14}C]GABA, since the specific radioactivity of glutamine exceeded that of glutamate. On addition of K^+ the signs of metabolic compartmentation promptly disappeared: this effect was apparently associated with an increase in the permeability of the compartments containing labelled metabolites derived from [^{14}C]GABA. The change in the permeability, however, did not affect all the compartments; when the labelled substrate was [^{14}C]glucose the equilibration of labelled amino acids between tissue and medium was similar under normal conditions and in the presence of high concentrations of K^+ . 9. The metabolism of [^{14}C]glucose was followed by measuring oxygen uptake, respiratory $^{14}\text{CO}_2$, and incorporation of ^{14}C into amino acids. The results showed that K^+ stimulation increased the flux of glucose carbon, both in the glycolytic pathway and in the tricarboxylic acid cycle.

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In the preceding paper in which the metabolism of [$1\text{-}^{14}\text{C}$]GABA* was studied in slices of cerebral cortex incubated in glucose-saline medium, the flux through the GABA bypath was found to be about 8% of that through the tricarboxylic acid cycle (Balázs, Machiyama, Hammond, Julian & Richter, 1970). The rate of respiration of brain tissue *in vitro* is only about half of that *in vivo*, but the oxygen uptake approaches that *in vivo* when isolated brain tissue is exposed to high concentrations of K^+ or to electrical pulses (Quastel & Quastel, 1961; McIlwain, 1966). In the present experiments an attempt was therefore made to measure the turnover rate of GABA in K^+ -stimulated slices of cerebral cortex.

There is evidence that GABA and possibly glutamate are concerned in determining the excitability of neuronal membranes under normal conditions *in vivo* (Curtis & Watkins, 1965; Krnjević & Schwartz, 1967). Increased K^+ concentrations, like electrical pulses, cause a depolarization of the neuronal membrane similar to that produced by physiological stimulation (Hillman & McIlwain, 1961; McIlwain, 1966). It is known that transmitter substances such as acetylcholine and catecholamines are released from nervous tissues under these conditions (Mann, Tennenbaum & Quastel, 1939; Richter & Crossland, 1949; Mitchell, 1963; Baldessarini & Kopin, 1966) and it has been reported that K^+ stimulation results in a release of GABA from brain-cortex slices (Machiyama, Balázs & Richter, 1967; Katz, Chase & Kopin, 1969; Srinivasan, Neal & Mitchell, 1969). These observations were extended in the present work by studying the release of amino acids from brain tissue stimulated by raised concentrations of K^+ ions. Since it has been shown that the metabolism of [$1\text{-}^{14}\text{C}$]GABA in brain tissue involves metabolic compartmentation (Balázs, Machiyama & Richter, 1967; Baxter, 1968; Balázs *et al.* 1970), the effect of K^+ stimulation on the metabolic compartmentation was also studied. Part of this work has been reported in preliminary form (Machiyama, 1965; Machiyama *et al.* 1967; Balázs *et al.* 1967).

EXPERIMENTAL

Experimental animals. Adult male guinea pigs (375–425 g) were used; the animals were obtained from the same colony as in the preceding paper (Balázs *et al.* 1970).

Preparation of slices of cerebral cortex and conditions of incubation. The methods used were described by Balázs *et al.* (1970). Briefly, cerebral-cortex slices (200 mg fresh wt.) were prepared in a humid chamber; they were incubated in Warburg vessels (0.2 ml of 2 M-KOH with filter paper in centre well) in O_2 at 37.5°C in glucose-saline medium (0.127 M-NaCl, 4.7 mM-KCl, 2.5 mM- CaCl_2 , 1.2 mM-

MgSO_4 , 1.2 mM- KH_2PO_4 , 30 mM-tris-HCl buffer, pH 7.4, and 10 mM-glucose). At 10 min after the beginning of the incubation the radioactive substance, 0.5 μCi of [$1\text{-}^{14}\text{C}$]GABA (3.72 mCi/mmol) or [$1\text{-}^{14}\text{C}$]glucose (76.3 mCi/mmol) was tipped in from the side arm. KCl (180 μmol) was added to the medium either at 20 min after the addition of [$1\text{-}^{14}\text{C}$]GABA or together with [$1\text{-}^{14}\text{C}$]glucose. The final incubation volume was 3.0 ml.

Analysis of the acid-soluble fractions. Incubation was continued for the times indicated in the Results section. Slices and medium were separated and treated separately with HClO_4 (Balázs *et al.* 1970). The fractionation of the acid-soluble fraction and the separation and determination of the amino acids were done by the methods of Gaitonde (1965) as described by Balázs *et al.* (1970). Radioactivity was determined with a Nuclear-Chicago gas-flow counter.

RESULTS

In agreement with previous observations (Dickens & Greville, 1935; Ashford & Dixon, 1935) the increase in concentration of K^+ in the medium from 5.9 to 66 m-equiv./l led to considerable stimulation of the respiration rate of cerebral-cortex slices (by 117% under the present conditions). The respiration rate decreased gradually with time and it was 16% less in the second than in the first half-hour period of incubation.

Metabolism of [$1\text{-}^{14}\text{C}$]GABA in K^+ -stimulated slices. The fate of [$1\text{-}^{14}\text{C}$]GABA in brain-cortex slices incubated in glucose-saline medium, containing 66 m-equiv. of K^+ ions/l is given in Fig. 1; for comparison the results obtained at a normal K^+ concentration (5.9 m-equiv. of K^+ /l) are also shown. During the whole incubation time most of the ^{14}C was combined in amino acids under both conditions (Figs. 1a and 1b). In the K^+ -stimulated tissue the amount of [$1\text{-}^{14}\text{C}$]GABA metabolized (Fig. 1d) including conversion into neutral compounds plus carboxylic acids (Fig. 1c) and lost from the system probably as $^{14}\text{CO}_2$ (Fig. 1e), was decreased in comparison with the control slices, in spite of the increased rate of respiration under these conditions.

Release of GABA. It was previously reported that brain-cortex slices prelabelled with [^{14}C]GABA release [^{14}C]GABA on stimulation by addition of K^+ (Machiyama *et al.* 1967) (Figs. 1a, 1b and 2). The further investigation of this effect is given in the present paper. A maximal release of GABA was observed at about 5 min after the addition of potassium chloride and it amounted to 0.85 $\mu\text{mol/g}$ of tissue, or 26% of the total GABA present. Further investigation showed that the magnitude of the release is not related to the GABA taken up by the tissue during the period of preincubation with 0.14 μmol of [^{14}C]GABA. In a series of experiments in which the slices contained only endogenous GABA (Tables 1 and 2) the proportion of GABA released on addition of K^+ was the same as in the

* Abbreviation: GABA, γ -aminobutyrate.

Fig. 1. Effect of K^+ stimulation on the metabolism of $[1-{}^{14}\text{C}]\text{GABA}$. Guinea-pig cerebral-cortex slices (200 mg fresh wt.) were incubated in glucose-saline medium (K^+ concentration 5.9 m-equiv./l) in O_2 at 37.5°C . After 10 min $0.5 \mu\text{Ci}$ of $[1-{}^{14}\text{C}]\text{GABA}$ ($0.135 \mu\text{mol}$) was added and the incubation was continued for 20 min. At that time (zero time on the graphs) a KCl solution was added to the medium to give a final concn. of 66 m-equiv. of K^+ /l. (a) Radioactivity observed in the acid-soluble fraction; (b) ${}^{14}\text{C}$ combined in amino acids; (c) ${}^{14}\text{C}$ in neutral compounds and carboxylic acids, in slice ($\circ$) and medium ($\bullet$). (d) $[{}^{14}\text{C}]\text{GABA}$ metabolized = $[{}^{14}\text{C}]\text{GABA}$ added (1.08×10^5 c.p.m.) - $[{}^{14}\text{C}]\text{GABA}$ recovered at times indicated ($\circ$). (e) $[{}^{14}\text{C}]\text{GABA}$ lost from the system = $[{}^{14}\text{C}]\text{GABA}$ utilized - $[{}^{14}\text{C}]$ recovered in the acid-soluble fraction - $[{}^{14}\text{C}]\text{GABA}$ ($\circ$). Since incorporation of ${}^{14}\text{C}$ into acid-insoluble metabolites was very low under these conditions the $[{}^{14}\text{C}]\text{GABA}$ lost from the system is a measure of the formation of respiratory ${}^{14}\text{CO}_2$. For comparison the values reported by Balázs *et al.* (1970) in a control series containing 5.9 m-equiv. of K^+ /l are also reproduced: ----, slice; ---, medium.

previous experiments ($0.65 \mu\text{mol/g}$ or 26% of the total).

The amounts of other amino acids in the slices changed very little during the period of maximal release of GABA; the amount of glutamate and aspartate in the medium increased by only about 3–5% of the amount present in the slices (Figs. 3 and Table 1). The effect was apparently specific for K^+ ; GABA was not released from the slices when instead of potassium chloride an equimolar amount of sodium chloride was added to the medium (Table 1). Increasing the respiration of brain-cortex slices by adding 10 mM-ammonium chloride (Weil-Malherbe, 1938) did not lead to a release of GABA from the tissue (Table 1).

Uptake of GABA. The rapid release of GABA from the tissue on addition of K^+ was followed by a slow uptake. Under normal conditions 95% of GABA added to the medium was taken up within the first 10 min of incubation (about $0.13 \mu\text{mol}$), but in presence of 66 m-equiv. of K^+ /l the net uptake was only about $0.03 \mu\text{mol}$ in 10 min.

Content and specific radioactivity of GABA in slices. The amount of GABA in slices incubated under normal conditions decreased with time and approached the steady-state value *in vivo* of about $1.6 \mu\text{mol/g}$ (Balázs *et al.* 1970). In the K^+ -stimulated slices, on the other hand, the content of GABA increased after the initial release. The concentration reached about $3 \mu\text{mol/g}$ at 60 min (Fig. 2b).

Fig. 2. Time-courses of the change in radioactivity (a) and the amount of GABA (b) in K^+ -stimulated brain slices (200mg fresh wt.) (○) and in the medium (3ml) (●). The lines passing through the experimental points for the radioactivity and amount of GABA in the medium are the forcing functions used in the computer simulation of the system. The corresponding best solutions for the radioactivity and amount of GABA in the slice over the whole experimental period are shown as the upper curves of (a) and (b) respectively. The dashed line in (b) is the best fit over the first 30 min, when the glutamate concentration in the slice was constant.

In spite of the marked difference in the amount of GABA in the tissue, the time-course of the change in specific radioactivity of GABA was similar under both conditions (Fig. 4). The specific radioactivity of GABA in the K^+ -stimulated slices was

very close to that in the medium, indicating an exchange of GABA between tissue and medium (Fig. 7 and Table 2).

Effect of K^+ -stimulation on the metabolism of amino acids other than GABA. Under normal conditions the first amino acid to be labelled when $[^{14}\text{C}]\text{GABA}$ was metabolized was aspartate. The peak of the specific radioactivity of aspartate was reached between 20 and 30 min after the addition of $[1\text{-}^{14}\text{C}]\text{GABA}$ to the medium (Fig. 4). After the addition of potassium chloride the specific radioactivity of aspartate fell sharply; at 0.5 min it had decreased already by about 20% and at 5 min the specific radioactivity reached a value that was observed in the control only after 45 min. The evaluation of the results (see the Discussion section) indicated that these findings are attributable to the increase in flux of metabolites through the tricarboxylic acid cycle proper under the experimental conditions.

The decrease in the specific radioactivity of glutamate was slow compared with that of aspartate. The amounts of aspartate and glutamate in the control slices were about 2 and $10 \mu\text{mol/g}$ respectively, and showed relatively little change for up to 80–100 min of incubation (Balázs *et al.* 1970). In the K^+ -stimulated slices the contents of these amino acids decreased earlier than in the controls, and at 1 h they were 7.2 and $1.2 \mu\text{mol/g}$ for glutamate and aspartate respectively (see Fig. 8b). However, the retention of these amino acids was apparently unaffected and the amounts in the medium after the small initial release were comparable with those observed under normal conditions.

The specific radioactivity of alanine reflected the decrease in that of aspartate after the addition of potassium chloride (Fig. 4). The amount of alanine in the system was greatly affected by K^+ stimulation; during 1 h incubation the content of alanine ($\mu\text{mol/g}$) in the tissue increased from about 1.4 to 2.7 and the concentration doubled in the medium (from about 50 to $110 \mu\text{M}$). In the same period in the controls there was no change in the total amount of alanine. The distribution of alanine between slice and medium was, however, not affected by K^+ stimulation.

No ^{14}C was detected in serine and glycine, and both in amount and in distribution between slice and medium, the findings were similar to those obtained under normal conditions. As in the control experiments, the incorporation of ^{14}C into GSH was negligible.

Effect of K^+ stimulation on the concentration and distribution of glutamine. The concentration of glutamine in the control slices was relatively constant during the incubation period (Fig. 5). On the other hand, the concentration of glutamine increased progressively in the K^+ -stimulated slices, and at 1 h after the addition of potassium chloride

Fig. 3. Time-course of incorporation of ¹⁴C in amino acids from [1-¹⁴C]GABA in (a) K⁺-stimulated brain-cortex slices (200mg fresh wt.) (O, Δ, ∇, □) and (b) incubation medium (3ml) (●, ▲, ▼, ■). Experimental details were given in Fig. 1. O and ●, Aspartate; □ and ■, alanine; Δ and ▲, glutamate; ∇ and ▼, glutamine.

Table 1. *Specific release of GABA from K⁺-stimulated brain-cortex slices*

Guinea-pig brain-cortex slices (200mg wet wt.) were incubated in O₂ at 37.5°C in Warburg flasks containing 2.9 ml of the glucose-saline medium. At 10 min after the beginning of the incubation 180 μmol of KCl or NaCl, or 30 μmol of NH₄Cl was added from the side arm (the final volume was 3.0 ml). The slices and medium were separated 10 min after the additions and the amino acids were determined. The values are expressed as μmol/200 mg of slices and μmol/3 ml of incubation medium respectively. n.m., amount of the amino acid too small for accurate determination with the method used.

Additions (μmol/ml of incubation medium)	... None		KCl (60)		NaCl (60)		NH ₄ Cl (10)	
	Slice	Medium	Slice	Medium	Slice	Medium	Slice	Medium
GABA	0.46	n.m.	0.39	0.13	0.53	n.m.	0.46	n.m.
Glutamate	2.07	0.10	2.00	0.15	2.01	0.13	2.09	0.11
Aspartate	0.47	0.07	0.39	0.08	0.46	n.m.	0.35	n.m.
Serine	0.25	0.22	0.22	0.14	0.27	0.17	0.22	0.11
Glycine	0.32	0.22	0.34	0.18	0.33	0.14	0.33	0.14
Alanine	0.20	0.15	0.30	0.25	0.30	0.16	0.27	0.14
Glutamine	0.65	0.55	0.75	0.60	0.74	0.43	0.74	0.57

it was more than doubled. At the same time the glutamine content of the medium decreased. An increase in glutamine accumulation in K⁺-stimulated brain-cortex slices has been reported by Tsukada, Nagata, Hirano & Matsutani (1963). The amount by which the glutamine content of the tissue increased (0.88 μmol/200mg of tissue in 1 h) was more than the amount taken up from the medium (0.36 μmol). The net increase was linear in the first 30 min after the addition of potassium chloride (12 nmol/min) when the content of glutamate in slice and medium was relatively constant.

On further incubation, however, the amount of glutamate decreased; thus the net gain of glutamine in 1 h (0.52 μmol) could be accounted for by the decrease in the total amount of glutamate in the system (0.48 μmol).

Whereas brain cortex slices incubated for 1 h in a medium containing 66 m-equiv. of K⁺/l accumulated glutamine, the addition of potassium chloride produced an immediate small release of glutamine (Fig. 5). The magnitude of this release was greater than that for the other amino acids except GABA (about 11% in the first 5 min).

Fig. 4. Time-course of the specific radioactivities of amino acids in brain-cortex slices that had been incubated in glucose-saline medium containing $[1-^{14}\text{C}]\text{GABA}$ for 20 min before KCl was added (final concentrations of K^+ , 66 m-equiv./l; zero time on graph). Experimental details were given in Fig. 1. $\circ$, Aspartate; $\blacksquare$, alanine; $\triangle$, glutamate; ∇ , glutamine; $\square$, GABA. For comparison the specific radioactivities of the amino acids reported in a control set of experiments (5.9 m-equiv. of K^+ /l) are shown by the dashed lines (---) (Balázs *et al.* 1970).

Metabolic compartmentation of glutamate. Compartmentation of glutamate was observed when $[1-^{14}\text{C}]\text{GABA}$ was oxidized by brain-cortex slices under normal conditions (Balázs *et al.* 1970); the specific radioactivity of glutamine was higher than that of glutamate even before the peak of specific radioactivity of glutamate had been reached (Figs. 4 and 6). When potassium chloride was added to the medium the specific radioactivity of glutamine decreased sharply (Fig. 4), and the glutamine/glutamate specific radioactivity ratio fell below 1 (Fig. 6). The value of this ratio increased later and at 40 min after the addition of potassium chloride the two amino acids approached equilibrium.

Metabolism of $[\text{U}-^{14}\text{C}]\text{glucose}$ in K^+ -stimulated slices. In the K^+ -stimulated tissue the ratio of the rate of formation of respiratory $^{14}\text{CO}_2$ (calculated for 10 min intervals) to that under normal conditions was 2.2–2.6, and this increase was even greater than that observed in respiration rate (2–2.3). The production of $^{14}\text{CO}_2$ increased with time; the carbon dioxide/glucose specific-radioactivity ratio was 0.2 at 0–5 min and 0.47 at 30–40 min. These findings are similar to those reported by Cremer (1967); they show that the equilibration of intermediates formed from $[^{14}\text{C}]\text{glucose}$ with the metabolite pools in the tissue, although accelerated by K^+ -stimulation, was not yet completed during the experimental period (40 min).

The enhanced rate of glucose metabolism in the K^+ -stimulated slices was reflected in the rate of

Fig. 5. Effect of 66 m-equiv. of K^+ /l in the incubation medium on the content and distribution of glutamine. Experimental details were given in Fig. 1. The time-course of the change of the glutamine content in 200 mg of guinea-pig cerebral-cortex slices is given in (a) ($\circ$) and that in the medium (3 ml) in (b) ($\bullet$). The values for normal conditions are taken from Balázs *et al.* (1970); glutamine content in slice ($\triangle$) and in medium ($\blacktriangle$).

Fig. 6. Effect of K⁺-stimulation on the glutamine/glutamate specific radioactivity ratio in brain-cortex slices. Conditions for the experiments in which [1-¹⁴C]GABA was the labelled substrate were described in Fig. 1. The concentrations of K⁺ used were 66m-equiv./l (○) and 5.9m-equiv./l (●). The conditions for the experiments in which [U-¹⁴C]glucose was the labelled substrate were described in Table 2. The concentrations of K⁺ used were 66m-equiv./l (△) and 5.9m-equiv./l (▲). The arrow indicates the time of addition of 180 μmol of KCl. The results for the controls were taken from Balázs *et al.* (1970).

incorporation of ¹⁴C into amino acids (Tables 2 and 3; and Table 4 of Balázs *et al.* 1970). The specific radioactivity of alanine was twice the control values at 10min after the addition of potassium chloride, indicating an increased rate of glycolysis. The increase in the rate of incorporation of ¹⁴C into amino acids associated with the tricarboxylic acid cycle was even greater; the specific radioactivities of these amino acids were 4–8 times higher than the control values in the initial period of incubation (Table 3). The results also indicated differences in the rates of reactions associated with the cycle. Under normal conditions aspartate did not reach equilibrium with glutamate even at 40min after the addition of [U-¹⁴C]glucose, but in the presence of excess of potassium chloride the equilibrium was already attained at 5min. These observations suggest that the turnover rate of the tricarboxylic acid cycle proper was greatly increased by potassium chloride. On the other hand, the GABA/glutamate specific-radioactivity ratio was less than 1 during the whole incubation period after the addition of potassium chloride, and the plot of this ratio against time was similar to that obtained under normal conditions. One possible explanation of these results is that the flux through the GABA bypath was not stimulated by potassium chloride to the same extent as that through the tricarboxylic acid

Table 2. Incorporation of ¹⁴C into amino acids of brain-cortex slices incubated in [U-¹⁴C]glucose-saline medium containing 66m-equiv. of K⁺/l

Time of incubation (min)	Alanine			Glutamate			Aspartate			GABA			Glutamine			Serine			Glycine		
	μmol		S.A. in	μmol		S.A. in	μmol		S.A. in	μmol		S.A. in	μmol		S.A. in	μmol		S.A. in	μmol		S.A.
	slice	medium		slice	medium		slice	medium		slice	medium		slice	medium		slice	medium		slice	medium	
5	0.24	1439	485	1.98	384	142	0.40	395	91	0.36	188	142	0.65	158	42	0.21	87	56	0.30		
10	0.28	1918	883	2.00	636	277	0.32	705	179	0.39	439	272	0.75	413	97	0.22	135	100	0.34		
20	0.36	2588	1610	2.01	1054	378	0.39	1066	220	0.42	765	637	0.98	755	217	0.22	230	155	0.33		
40	0.44	3438	2825	1.77	1781	470	0.25	1692	273	0.59	1280	1255	1.40	1524	656	0.29	353	288	0.41		

Brain-cortex slices of guinea pig (200mg fresh wt.) were preincubated in glucose-saline medium for 10min, then 5μCi of [¹⁴C]glucose (1.08 × 10⁶ c.p.m.) and 180μmol of KCl were added (final vol. 3.0ml); gas phase O₂, temp. 37.5°C. The specific radioactivity (S.A.) is expressed as c.p.m./μg-atom of C; the S.A. of glucose in the medium was 6200. The contents of the amino acids in the slices are expressed in μmol/200mg of fresh tissue.

* Values of radioactivity were too low for accurate determination.

Table 3. Incorporation of ^{14}C into amino acids in brain-cortex slices incubated in $[\text{U-}^{14}\text{C}]\text{glucose-saline}$ medium containing K^+ at normal and elevated concentrations

The values for the specific radioactivities of amino acids in the slices incubated in a medium containing 66 m-equiv. of K^+ /l were taken from Table 2 and those for the normal conditions (5.9 m-equiv. of K^+ /l) from Table 4 of Balázs *et al.* 1970. Abbreviation: RSA, amino acid/glutamate specific-radioactivity ratio. The column 'Ratio' refers to the sp. radio-activity of the amino acid obtained after incubation in a medium containing 66 m-equiv. of K^+ /l relative to that with a medium containing 5.9 m-equiv. of K^+ /l.

Time of incubation (min)	Concn. of K^+ (m-equiv./l)	Alanine		Aspartate		GABA		Glutamine		Serine		Glutamate Ratio
		RSA	Ratio	RSA	Ratio	RSA	Ratio	RSA	Ratio	RSA	Ratio	
5	...	5.9	66	5.9	66	5.9	66	5.9	66	5.9	66	—
10	6.4	3.7	—	—	1.0	—	0.49	—	0.41	—	0.23	—
20	4.6	3.0	2.0	0.58	1.1	0.56	0.69	0.56	0.65	1.4	0.21	4.2
40	3.1	2.5	1.5	0.64	1.0	0.62	0.73	0.53	0.72	0.92	0.22	2.9
		3.1	1.9	0.75	0.95	0.77	0.72	0.47	0.86	0.73	0.20	2.5

cycle. The glutamine/glutamate specific-radioactivity ratio was low under normal conditions, but it increased progressively in the K^+ -stimulated tissue (Fig. 6). This effect may be related to the net increase in the amount of glutamine due to increased synthesis from glutamate under these conditions.

Some of the glucose carbon was incorporated into serine. Under normal conditions the specific radioactivity of serine was initially the highest among the amino acids other than alanine. This indicated that glycolytic intermediates are also involved in the formation of serine in brain tissue (Meister, 1965). The incorporation of ^{14}C into serine was markedly decreased in the K^+ -stimulated slices.

Equilibration of labelled amino acids between tissue and medium. It has been observed that the amino acids quickly attain a characteristic distribution between tissue and medium (see Balázs *et al.* 1970). The oxidation of substrates takes place mainly in the tissue, so that when labelled substrates such as glucose or GABA were utilized, the specific radioactivities of the derived amino acids were initially higher in the slice than in the medium. The specific radioactivities of the amino acids in the medium changed with time, apparently following those in the tissue, although the concentrations of the amino acids in the medium remained fairly

Fig. 7. Ratios of specific radioactivities of amino acids in cerebral-cortex slices to those in the incubation medium. Brain-cortex slices were incubated in glucose-saline medium and the labelled substrate was $[\text{U-}^{14}\text{C}]\text{GABA}$. For experimental details: with 66 m-equiv. of K^+ /l see Fig. 1; with 5.9 m-equiv. of K^+ /l see Fig. 4 of Balázs *et al.* (1970). Alanine, ● and ○; glutamate, ▲ and △; glutamine, ▼ and ▽; GABA, ■ and □, 66 m-equiv. of K^+ /l; ●, ▲, ▼, 5.9 m-equiv. of K^+ /l.

constant. These observations indicate an exchange of amino acids between tissue and medium. However, it took a considerable time for the specific radioactivities of the different amino acids in the medium to approach the values reached in the tissue (Fig. 7). When [1-¹⁴C]GABA was the labelled substrate and the K⁺ concentration of the medium was increased to 66m-equiv./l there was a sudden change in the rate of equilibration of the amino acids between tissue and medium (Fig. 7). Thus at 0.5 min after the addition of potassium chloride the specific radioactivities of glutamine and alanine in the medium reached values relative to those in the slice that were obtained in the controls only after 50–60 min of incubation.

The effect of K⁺ stimulation on the equilibration of amino acids between slice and medium was markedly different when the labelled substrate was [¹⁴C]glucose. Under these conditions the time-dependent change of the slice/medium specific-radioactivity ratio of the amino acids was similar to that observed in the controls (compare Table 2 with Fig. 4b of Balázs *et al.* 1970): even the apparently anomalous behaviour of glutamate in showing an increase in the ratio with time remained unchanged.

DISCUSSION

Release of GABA from K⁺-stimulated brain tissue. There is evidence that GABA may act as an inhibitory transmitter at certain synapses in the mammalian central nervous system (Curtis & Watkins, 1965; Krnjević & Schwartz, 1967) and it has been shown that GABA and the related enzyme systems in the brain are concentrated in structures rich in neurones (Roberts & Kuriyama, 1968). The subcellular distribution of glutamate decarboxylase indicates that GABA is probably formed predominantly at the nerve terminals, since this enzyme is relatively enriched in the subcellular fractions containing nerve endings. However, the utilization of GABA in the nerve endings is probably rather limited, since GABA aminotransferase is localized mainly in the non-synaptosomal mitochondria (Salganicoff & De Robertis, 1965; Van Kempen, Van den Berg, Van der Helm & Veldstra, 1965; Balázs, Dahl & Harwood, 1966; Roberts & Kuriyama, 1968). In spite of the spatial separation of the enzymes concerned in the formation and utilization of GABA, a significant turnover of GABA can be demonstrated in brain tissue. It follows that GABA apparently passes from the site of formation in the nerve terminals to the site of utilization in the adjacent cells (Salganicoff & De Robertis, 1965; Balázs *et al.* 1966; Roberts & Kuriyama, 1968): its proposed physiological action on the postsynaptic membrane would be exerted during this translocation. The termination of the inhibitory action of

GABA may be mediated by the efficient uptake mechanism operating specifically in nervous tissues (Elliott, 1965; Blasberg & Lajtha, 1965; Iversen & Neal, 1968; Balázs *et al.* 1970).

The main missing link in characterizing GABA as a synaptic transmitter has been a convincing demonstration of its release from nervous tissues as a result of stimulation (Roberts & Kuriyama, 1968). A report that GABA is liberated from the surface of the cortex under certain conditions of activation of the brain (Jasper, Khan & Elliott, 1965) has been discussed by other investigators (Iversen & Neal, 1968) and the meaning of these results is not yet clear (Van Harreveld & Kooiman, 1965; Crowshaw, Jessup & Ramwell, 1967). The observation of Machiyama *et al.* (1967) that GABA is released from brain slices as a result of stimulation by K⁺ has been extended in the present studies. When the extracellular concentration of K⁺ was increased to a value that is known to depolarize neuronal membranes (Hillman & McIlwain, 1961) about one-third of the GABA content of the cerebral-cortical tissue was released. Other amino acids were relatively little affected, and the release was apparently specific for K⁺. These observations suggest that GABA is not homogeneously distributed in the cerebral cortex. It is known that transmitter compounds such as acetylcholine and catecholamines are liberated from nervous tissues on K⁺ stimulation both *in vivo* and *in vitro*. They are also concentrated in the fraction of brain homogenates containing the nerve-ending particles (Whittaker, 1965; De Robertis, 1967). A similar enrichment of GABA in the synaptosomal fraction is not yet generally accepted (Mangan & Whittaker, 1966; see however Iversen & Snyder, 1968), but a Na⁺-dependent binding of GABA to subcellular particles including synaptosomes and synaptic vesicles has been observed (Elliott, 1965; Roberts & Kuriyama, 1968). The finding that GABA behaves like other known transmitters under conditions of K⁺ stimulation suggests that the nerve terminals, which are common sites for all transmitter compounds, are affected by high concentrations of K⁺. Clearly in the present experiments the conditions of stimulation were unphysiological. However, the demonstration that GABA is released as a result of stimulation gives further support for the view that GABA serves as a transmitter in the mammalian brain.

Compartmentation of GABA and its metabolism. Evidence was obtained in the present study that the [¹⁴C]GABA taken up by the tissue from the medium mixed with endogenous GABA, since the proportion of the total GABA released on K⁺ stimulation was the same whether or not exogenous GABA was contained in the slices (25–30%; Tables 1 and 2, and Fig. 2). This conclusion was

further supported by the observation that the specific radioactivity of the GABA released on stimulation was similar to that in the tissue, whether the tissue was prelabelled by exogenous [^{14}C]GABA or the labelled GABA was generated in the tissue from [^{14}C]glucose (Table 2 and Fig. 7). However, the specific release of a fixed proportion of GABA indicated that GABA is compartmented in brain slices.

It was previously observed that the utilization of [$1\text{-}^{14}\text{C}$]GABA through the tricarboxylic acid cycle gives rise to intermediates that do not mix freely with other pools in the tissue, since the specific radioactivity of glutamine was higher than that of glutamate (Balázs *et al.* 1970). On the other hand [$\text{U-}^{14}\text{C}$]glucose apparently entered all the compartments. When slices oxidizing [^{14}C]GABA in glucose-saline medium were stimulated by K^+ , the signs of metabolic compartmentation promptly disappeared (Fig. 6). A similar effect of high K^+ concentrations on the metabolic compartmentation of glutamate in brain slices oxidizing [$\text{U-}^{14}\text{C}$]aspartate has been described by Berl, Nicklas & Clarke (1968). They interpreted the results as indicating a relative decrease in the flow of ^{14}C from aspartate into the 'small' pool of glutamate that is associated with the synthesis of glutamine of high specific radioactivity. It cannot be concluded from the present results whether K^+ stimulation leads to a true or an apparent disappearance of the metabolic compartmentation. The evidence that K^+ stimulation increased permeability of the metabolic compartment(s) containing labelled intermediates derived from [^{14}C]GABA supports the first alternative (Fig. 7). On the other hand, the present experiments showed that the metabolism of GABA was considerably affected by K^+ stimulation; the flux through the GABA bypath was decreased relative to that through the tricarboxylic acid cycle (see below) and there was a biphasic change in the concentration of GABA in the tissue during the incubation period. It is therefore possible that the relative rates of flow of labelled metabolites from [^{14}C]GABA in the different compartments changed in comparison with the controls. An additional factor that may influence the glutamine/glutamate specific-radioactivity ratio is the effect of K^+ stimulation on the metabolism of glutamine (Fig. 5). A net increase in the amount of glutamine (about 50 nmol/min per g) probably related to an increase in ammonia production took place immediately after the addition of potassium chloride to the medium. The distribution of glutamine between tissue and medium was also markedly affected by the high concentration of potassium salts in the medium. Glutamine was taken up by the tissue at a relatively high rate (approx. 30 nmol/min per g), and the slice/medium glutamine concentration ratio

($\mu\text{mol/ml}$ of tissue-water divided by $\mu\text{mol/ml}$ of medium) increased from about 17 to 63 during 60 min incubation. Therefore both the relative rates of glutamine formation in the different metabolic compartments and the relative pool sizes may be affected by K^+ stimulation.

Stimulation of glucose metabolism in isolated brain tissue by high concentrations of potassium salts. It is known that K^+ stimulation results in an increase in the rates of oxygen uptake, glucose utilization and lactate formation in isolated brain tissue (see Quastel & Quastel, 1961; McIlwain, 1966; Rolleston & Newsholme, 1967). These effects are probably related to an increase in the concentration of phosphate acceptors in the tissue (McIlwain, 1952; Ács, Balázs & Straub, 1953; Rolleston & Newsholme 1967), which may result from the stimulated activity of processes associated with the maintenance of the normal ionic gradients between tissue and extracellular fluid (McIlwain, 1952). The stimulation of the rate of glycolysis was indicated in the present experiments by the enhanced incorporation of ^{14}C from [^{14}C]glucose into alanine (see Kini & Quastel, 1959). The high specific radioactivity of the amino acids associated with the tricarboxylic acid cycle relative to the values observed under normal conditions gave evidence of the increased flux in the cycle. The present observations agree with the qualitative findings of Kini & Quastel (1959) relating to the conversion of glucose carbon into these amino acids. However, they have also indicated that information based on the measurement of radioactivity alone is not sufficient to allow valid conclusions to be drawn as to the rate of synthesis of a particular metabolite. Both the amount and the distribution of the amino acids between tissue and medium may change, as has been shown to occur with glutamine and GABA, and these factors will influence the interpretation of the results.

Computer simulation of the reaction kinetics of GABA. A mathematical model based on the simplified system represented in Scheme 1 was implemented on an analogue computer. The dynamic response of the model under appropriate conditions was then adjusted to correspond to the performance of the real system (experimental results presented in Figs. 1-4) and certain constants were thus evaluated.

A value for C_2 (GABA-utilization rate constant) was obtained by using equations for the radioactivities (R) of GABA in the brain-cortex slice and in the medium. From the notation of Scheme 1, and noting that no ^{14}C is passed from glutamate during the formation of GABA, these equations are:

$$\begin{aligned}\dot{R}_2 &= -C_2 R_2 - (K_3 R_2 / S_2) \\ \text{and } \dot{R}_3 &= K_3 R_2 / S_2\end{aligned}$$

Scheme 1. Scheme of reactions used for computer simulation. The radioactivities and quantities of substrates are represented by R and S respectively; the numerical suffixes used relate the symbols to the specific intermediates. Reaction rate constants (per min) and fluxes (in $\mu\text{mol/min per g}$) are represented by the symbols C and K respectively and are given the same suffix as the precursor metabolite. The reversible flux of GABA exchange is taken as positive in the direction slice to medium and is represented by K_3 . The computed values of the kinetic constants are indicated, with alternative solutions in parentheses (see the text); the values obtained under normal conditions of incubation are given in **bold type** (Hammond *et al.* 1970).

Substitution between the equations and integration gives:

$$R_2 = (C_2 \int R_2 dt) - R_3 \quad (1)$$

In this equation, R_3 (the radioactivity of the medium) may be treated as a known arbitrary function given by the experimental results. The function as implemented on the computer is shown in Fig. 2(a) together with the solution for R_2 that, by adjustment of the value of C_2 , gave best agreement with the experimental results for GABA radioactivity in the slice. The value of C_2 so determined was 0.014/min, which may be compared with the value 0.019/min previously determined as the value of the GABA-utilization rate constant under normal conditions (Hammond, Julian, Machiyama & Balázs, 1970).

The equations for the quantities of GABA as a function of time, derived on the basis of Scheme 1 are:

$$\dot{S}_2 = C_1 S_1 - C_2 S_2 - K_3$$

and $\dot{S}_3 = K_3$

The quantity of glutamate in the slice (S_1) remained substantially constant for the first 30 min after the addition of potassium chloride to the medium. The flux of GABA formation ($C_1 S_1$) was therefore implemented as a constant on the analogue compu-

ter. Nominating K_1 as this constant, substitution between the above equations and integration gives:

$$\dot{S}_2 = K_1 t - (C_2 \int S_2 dt) - S_3 \quad (2)$$

The S_3 function (quantity of GABA in the medium) was implemented on the analogue computer as a second known function and the value of C_2 was taken as 0.014/min. When the solutions of eqn. (2) for S_2 , corresponding with various values of K_1 , were compared with the experimental results the value for K_1 that gave best agreement over the first 30 min was $0.0285 \mu\text{mol/min per g}$. Over the full experimental time of 60 min (which includes the period during which the quantity of glutamate decreased) the value of K_1 that gave best agreement with the results was $0.0305 \mu\text{mol/min per g}$. The solutions corresponding to these values, together with the S_2 function used in the simulation, are shown in Fig. 2(b). A reasonable fit to the S results was also obtained when it was assumed that the utilization rate constant of GABA was comparable with that observed under normal conditions ($C_2 = 0.019/\text{min}$). The value for K_1 under these conditions was $0.0535 \mu\text{mol/min per g}$ (see Machiyama *et al.* 1967). The flux of GABA formation under normal conditions has been estimated as $0.0315 \mu\text{mol/min per g}$ by Hammond *et al.* (1970).

Computer simulation of the reaction kinetics of aspartate. When the equations for the radioactivity combined in oxaloacetate and aspartate respectively are defined, allowance must be made for the fact that no ^{14}C enters the oxaloacetate pool from α oxoglutarate despite labelling of the latter during metabolism of $[1-^{14}\text{C}]\text{GABA}$. On the basis of Scheme 1 the equations are as follows:

$$\dot{R}_4 = C_2 R_2 - C_4 R_4 + C_5 R_5 - C_4^* R_4$$

$$\dot{R}_5 = C_4 R_4 - C_5 R_5$$

In the computer implementation of these further equations the function $C_2 R_2$ is available from the simulation of GABA kinetics described above. Of the remaining constants, the values of C_4 and C_5 have been evaluated for normal conditions as 63/min and 0.12/min respectively (Hammond *et al.* 1970). A simulation of the K^+ -stimulated metabolism was effected by assuming that C_4 and C_5 were the same as determined for the control and by adjusting C_4^* to give the best agreement with the values for the radioactivity combined in aspartate (R_5) (Fig. 8a). The value of C_4^* was under these conditions 447/min.

The equations for the quantities of oxaloacetate and aspartate are seen from Scheme 1 to be:

$$\dot{S}_4 = C_2 S_2 - C_4 S_4 + C_5 S_5 - C_4^* S_4 + C_6 S_6$$

$$\text{and } \dot{S}_5 = C_4 S_4 - C_5 S_5$$

If the contribution of the tricarboxylic acid cycle to oxaloacetate formation ($C_6 S_6$) is assumed to be a constant (K_6), and if C_4 , C_5 and C_4^* are allocated their previous values of 63, 0.12 and 447/min respectively, computer solutions for S_5 may be obtained. The solution that is in best agreement with the experimental values for aspartate content is shown in Fig. 8(b) and corresponds to a K_6 value of $0.95 \mu\text{mol/min per g}$. This value, in conjunction with a derived value for the oxaloacetate-utilization constant of 447/min implies an oxaloacetate content of 2.2 nmol. Since it is by no means certain that the values of C_4 and C_5 remain unchanged in the presence of 66 mequiv. of K^+/l , the effect of halving the value of C_4^* to correspond to an increased oxaloacetate content (about 4.4 nmol) was investigated. The new values that gave best agreement to the aspartate radioactivity results were 38 and 0.13/min for C_4 and C_5 respectively, but the value of K_6 that agreed best with the time-course of aspartate quantity remained substantially unchanged at $0.92 \mu\text{mol/min per g}$. This value may be compared with the value of $0.37 \mu\text{mol/min per g}$, which by computer simulation has been shown to be compatible with experimental findings under normal conditions (Hammond *et al.* 1970).

The results indicate therefore that the flux in the tricarboxylic acid cycle proper was more than

Fig. 8. Computer simulation of the time-course of the change in the radioactivity (a) and the amount of aspartate (b) in K^+ -stimulated brain tissue (labelled substrate, $[1-^{14}\text{C}]\text{GABA}$). Experimental details were as described in Figs. 1-4.

doubled by K^+ stimulation, but the flux through the GABA pathway was little affected. The findings mean therefore that the contribution of the GABA bypath to total glucose oxidation, which was only about 8% under normal conditions, is proportionately even less in the K^+ -stimulated tissue (3-5%).

In obtaining the above system constants no account has been taken of the functional compartmentation of the metabolites. However, examination of the control series of experiments has shown that errors arising from this simplification of approach are likely to be small (Hammond *et al.* 1970). Further evidence that compartmentation need not be invoked in the above analysis arises in the observations that the specific radioactivity of the GABA released by K^+ stimulation was near to that of the GABA remaining in the tissue. The same results were observed whether labelled GABA was generated in the tissue from $[^{14}\text{C}]\text{glucose}$ or $[^{14}\text{C}]\text{GABA}$ was introduced into the tissue through uptake from the medium; further, the results were the same whether potassium chloride was added to the medium at the same time as the labelled

substrate (experiments with [¹⁴C]glucose as the substrate) or 20 min later (experiments with [¹⁴C]-GABA). These results suggest either that the rate constants of GABA metabolism are similar in the different compartments or that according to the scheme in the preceding paper (Scheme 2 of Balázs *et al.* 1970) the formation of GABA takes place predominantly in one of the metabolic compartments (II) and is then transferred to and oxidized mainly in another compartment (I), where the rate of GABA production is relatively small.

Effect of high K⁺ concentrations on brain tissue in vitro. K⁺ stimulation is associated with a considerable decrease in the concentration of compounds containing high-energy phosphate bonds, which may lead to secondary effects. It is known that tissue slices swell under these conditions and it has been suggested that the swelling is intracellular (Pappius & Elliot, 1956). Investigations with the electron microscope have shown that, at least in frog brain, the swelling affects preferentially the glial cells: there is little or no swelling of the neurones, although the synaptic vesicles gradually disappear (Zadunaisky, Wald & De Robertis, 1963). Changes in the permeability of the different metabolic compartments could therefore be related to a differential effect of K⁺ on neurones, nerve terminals and glial cells (Fig. 7 and Table 2). The marked increase in the overall content of glutamine and GABA may also be related to structural alterations produced by a high concentration of K⁺ in the incubation medium.

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