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The Metabolism of Glutamate in Homogenates and Slices of Brain Cortex

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Glutamate is a highly active metabolite in the cerebral cortex. It can be converted into α-oxoglutarate by transamination or dehydrogenation; it can be decarboxylated to γ-aminobutyrate, and its carbon skeleton can be synthesized from glucose and intermediates of glucose metabolism (see Waelsch & Lajtha, 1961; Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari, 1955; Kini & Quastel, 1959; Chain, Cohen & Pocchiari, 1962; Sellinger, Catazaro, Chain & Pocchiari, 1962; Krebs & Bellamy,

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1960). The extent to which the various reactions occur depends partly on environmental circumstances; for example, low concentrations of glucose favour the removal of glutamate and high concentrations prevent it (Waelsch, 1949; Cravioto, Massieu & Izquierdo, 1951; Takagaki, Hirano & Tsukada, 1957). Information on the factors determining the rates of the various metabolic pathways is still scanty; the present experiments, employing a combination of isotopic and quantitative chemical methods, are aimed at supplying such information.

EXPERIMENTAL

Materials. DL-[1-^{14}C]Glutamic acid and α-oxo[5-^{14}C]-glutarate were obtained from The Radiochemical Centre, Amersham, Bucks. When L-[1-^{14}C]glutamate was required a solution of sodium DL-[1-^{14}C]glutamate was mixed with sufficient unlabelled L-glutamate to reduce the proportion of D-glutamate to a chemically negligible value (5% or less). The 50% of the radioactivity remaining in the D-[1-^{14}C]-glutamate did not interfere, as shown by the fact that no $^{14}CO_2$ was liberated from D-[1-^{14}C]glutamate by homogenates or slices of cerebral cortex. L-[5-^{14}C]Glutamate was prepared from unlabelled glutamate and α-oxo[5-^{14}C]-glutarate by an isotope-exchange reaction catalysed by aspartate aminotransferase (Nisonoff, Barnes, Enns & Schuching, 1954; Jenkins & Sizer, 1959). A mixture of 15 μmoles of α-oxo[5-^{14}C]glutarate (60 μc) and 75 μmoles of L-glutamate, dissolved in 2·5 ml. of water, plus 0·5 ml. of 0·1 M-phosphate-buffer, pH 8·0, was incubated for 3 hr. at room temperature with 0·1 ml. of aspartate aminotransferase (2 mg./ml.; C. F. Boehringer und Soehne, Mannheim, Germany). L-[5-^{14}C]Glutamate was separated from the reaction mixture by the method of Haslam & Krebs (1963) and the material obtained was neutralized and dissolved in 0·375 ml. of water plus 4·625 ml. of 0·2 M-sodium glutamate to give a 0·2 M solution with a specific activity of approx. 50 μc/m-mole. This solution was stored at −18°.

D-[1-^{14}C]Glutamate was prepared from DL-[1-^{14}C]-glutamate by incubation of the latter with a suspension of *Clostridium welchii* as in the manometric estimation of glutamate (Krebs, 1948). When no further CO_2 was evolved the vessel contents were deproteinized with perchloric acid and, after precipitation of potassium perchlorate with 5 N-KOH, D-[1-^{14}C]glutamic acid was isolated by paper chromatography with the butan-1-ol–propionic acid–water solvent of Benson *et al.* (1950). After elution from the paper with water and freeze-drying, the D-[1-^{14}C]glutamic acid obtained was dissolved in 0·2 M-sodium D-glutamate to give a final specific activity of about 20 μc/m-mole. The final product yielded no $^{14}CO_2$ on addition to a suspension of *Clostridium welchii.*

Sodium pyruvate was prepared from technical pyruvic acid as described by Bartley & Davies (1954). Sodium parapyruvate was obtained by hydrolysis with 0·5 M-Na_2CO_3 solution of the αγ-lactone of parapyruvic acid (γ-hydroxy-γ-methyl-α-oxoglutaric acid) prepared as described by de Jong (1901). All other materials were analytical reagent grade (British Drug Houses Ltd.).

Tissue preparations. Homogenates of rat-brain cerebral cortex and slices of adult guinea-pig cerebral cortex were used. The animals were killed by a sharp blow to the neck followed by decapitation.

For the preparation of homogenates the brains were removed and immersed in ice-cooled 0·154 M-KCl for 2 min. The weighed grey matter was homogenized in a stainless-steel Potter–Elvehjem homogenizer with 2–3 vol. of cold (0°) incubation medium containing (per 100 ml.) 52 ml. of 0·154 M-KCl, 10 ml. of 0·1 M-potassium phosphate buffer, pH 7·4, 8·3 ml. of 0·2 M-$MgCl_2$, 8·0 ml. of M-glycylglycine buffer (pH adjusted to 7·4 with NaOH) and water to 100 ml. This is a modification of the medium of Case & McIlwain (1951), incorporating a higher concentration of phosphate. After homogenization further medium was added to the homogenate to give a final volume of 7·5 ml./g. of brain tissue. The homogenates were incubated at 30° in conical Warburg vessels with O_2 in the gas phase. Each vessel contained 3·0 ml. of homogenate. This was added to 1·0 ml. of an ice-cooled solution comprising 0·2 ml. of 0·02 M-AMP and 0·02 M-ATP (both as sodium salts), 0·2 ml. of 0·154 M-$KHCO_3$, substrates (usually as 0·2 M solutions of the sodium salts) and 0·154 M-KCl to volume, prepared before the killing of the animal. The final tissue concentration was 10% (w/v). The side arm contained 0·5 ml. of 2 N-HCl and the centre well 0·2 ml. of CO_2-free 2 N-NaOH. The vessel contents were kept at 0° until the zero time of the incubation, which was continued for 20–40 min. and was terminated by tipping the 2 N-HCl from the side arms. When the respiratory $^{14}CO_2$ was to be collected, shaking was continued after addition of acid to the main compartments until no further change in gas pressure occurred (approx. 1 hr.). The acidified tissue suspensions were stored at −18° until analysed. Rat-liver homogenates used for comparison with brain homogenate were prepared and incubated as described by Haslam & Krebs (1963).

Slices of guinea-pig cerebral cortex were incubated aerobically in Warburg vessels at 37°, the suspending medium being a phosphate saline (Krebs, 1933) from which calcium was omitted. Slices (about 200 mg. fresh wt.) were blotted with filter paper, weighed on a torsion balance and placed in the incubation medium in Warburg vessels with two side arms. One side arm contained substrates (usually as 0·2 M-solutions of the sodium salts) and the other 0·5 ml. of 2 N-HCl. Carbon dioxide-free 2 N-NaOH was placed in the centre wells. The gas spaces of the vessels were filled with O_2 and the vessels were shaken at 37° for 5 min., before the substrates (if any) were added from a side arm to give a final volume of 4·0 ml. (including the slices). The initial concentration of added substrates was 2·5 mM. Incubation was then continued for 90–120 min. and was terminated by tipping 2 N-HCl from the second side arm. Further details were as described for brain homogenates, except that before storage the slices plus acidified incubation medium were homogenized in a glass Potter–Elvehjem homogenizer. The slices and incubation medium were not analysed separately.

Chemical analyses. Glutamate plus glutamine and aspartate were estimated manometrically by the method of Krebs & Bellamy (1960). No attempt was made to differentiate in the analyses between glutamate and glutamine, so that measurements of glutamate removal may include endogenous glutamine and refer to glutamate metabolized by reactions other than glutamine synthesis. Alanine was estimated by the ninhydrin reaction as described by Krebs & Bellamy (1960), after chromatographic separation by the two-dimensional solvent system described below. Pyruvate

and α-oxoglutarate were estimated by the method of Holzer & Holldorf (1957). Citrate was determined as described by Taylor (1953).

Chromatography. The products of [1-^{14}C]- and [5-^{14}C]-glutamate metabolism were separated by two-dimensional chromatography and the radioactive areas were located by radioautography. Aspartate was identified by its colour reaction with ninhydrin and when radioactive by co-chromatography with authentic material. The chromatograms were developed, in a descending direction, successively with phenol–formic acid–water (500:13:167, w/v/v) as described by Kornberg (1958) and with butan-1-ol–propionic acid–water (47:22:31, by vol.; Benson *et al.* 1950). The details of the methods used were as described by Large, Peel & Quayle (1961).

Radioactive tracer techniques. The general techniques used and the corrections applied have been described by Sakami (1955) and Haslam & Krebs (1963). The radioactivity of ^{14}C-labelled materials was estimated by conversion of the labelled C atoms into $^{14}CO_2$, which was absorbed in 2N-NaOH, plated as $Ba^{14}CO_3$ on filter-paper disks and so counted with a mica end-window Geiger–Müller tube. The specific activity of added and residual L-[1-^{14}C]glutamate was measured by decarboxylation with *Clostridium welchii* in a manometer vessel (Krebs, 1948), and plating and counting of the $^{14}CO_2$ as $Ba^{14}CO_3$. Similarly, D-[1-^{14}C]-glutamate was decarboxylated with chloramine T (Gibson & Wiseman, 1951) and the specific activity of [4-^{14}C]-aspartate formed from labelled glutamate was estimated by decarboxylation with *Nocardia globerula* (Crawford, 1958; Krebs & Bellamy, 1960). [5-^{14}C]Glutamate was converted into $^{14}CO_2$ by combustion as described by Van Slyke & Folch (1940).

RESULTS

Fate of [1-^{14}C]*glutamate as sole added substrate in homogenates of rat cerebral cortex.* In brain homogenates, as in brain mitochondria (Krebs & Bellamy, 1960), added glutamate was converted almost quantitatively (80–90%) into aspartate (Table 1). The amounts of $^{14}CO_2$ and aspartate formed from [1-^{14}C]glutamate were equal. On chromatography of the non-volatile products no radioactivity was detectable in the aspartate, and the specific activity of C-4 of aspartate was less than 1% of that of C-1 of glutamate. Thus a γ-decarboxylation of glutamate, as proposed by Cohen, Simon, Berry & Chain (1962), did not occur. The average specific activity of the glutamate removed in vessel 2 (Table 1) relative to the original [1-^{14}C]-glutamate can be calculated from the amount of glutamate removed and the amounts of $^{14}CO_2$ and α-oxoglutarate formed; it was $15{\cdot}1/(17{\cdot}1–0{\cdot}3) = 0{\cdot}90$. This dilution would be expected if the exogenous glutamate mixed freely with the endogenous glutamate (assumed from the analyses of Schwerin, Bessman & Waelsch, 1950, to constitute 70–75% of the measured glutamate plus glutamine).

Endogenous glutamate (and possibly glutamine) was also converted into aspartate in the homogenate (Table 1, vessel 1). The formation of aspartate and carbon dioxide from glutamate requires 1½ mol.prop. of oxygen and therefore accounted for 35% of the uptake of oxygen. In another experiment where the period of incubation was shorter (20 min.) the formation of aspartate from endogenous glutamate accounted for 68% of the consumption of oxygen. The parellelism between the disappearance of glutamate and the decline of endogenous respiration suggests that glutamate was one of the principal endogenous respiratory substrates in the homogenates, especially during the early period of incubation.

Effects of inhibitors on glutamate metabolism in homogenates of rat cerebral cortex. The conversion of endogenous glutamate into aspartate was almost completely inhibited by 10 mM-malonate (see also Borst, 1962) but only slightly decreased by 2 mM-2-fluorocitrate (Table 2). This indicates that succinate is an intermediate in the conversion of glutamate into aspartate. There was no net removal of added [1-^{14}C]glutamate in the presence of 10 mM-malonate, and no significant aspartate

Table 1. *Fate of* [1-^{14}C]*glutamate in homogenates of rat cerebral cortex*

Cortex homogenate was incubated for 30 min. with [1-^{14}C]glutamate (specific activity 16·0 μc/m-mole). The additions to the homogenate included (final concn.) 1 mM-AMP and 1 mM-ATP. Results refer to 4·0 ml. of homogenate, which initially contained 6·3 μmoles of glutamate plus glutamine. Tissue dry wt. was 71 mg./vessel.

Vessel no.	1	2
Substrate added	None	[1-^{14}C]Glutamate (40·8 μmoles)
Metabolic changes (μmoles)		
O_2	−18·1	−34·6
$^{14}CO_2$	—	+15·1
Glutamate plus glutamine	−4·5	−17·1
Aspartate	+4·2	+15·1
α-Oxoglutarate	+0·1	+0·3

Table 2. *Effects of malonate and 2-fluorocitrate on the fate of endogenous glutamate in homogenates of rat cerebral cortex*

Results refer to 4 ml. of homogenate incubated for 30 min. in Warburg vessels. Tissue dry wt. was 80 mg./vessel. AMP and ATP were added to the homogenate (final concn. of each 1 mM). The endogenous glutamate plus glutamine was initially 5·9 μmoles/vessel.

Vessel no.	1	2	3
Additions	None	Malonate (10 mM)	2-Fluorocitrate (2 mM)
Metabolic changes (μmoles)			
O_2	−21·6	−11·1	−13·4
Glutamate plus glutamine	−3·7	−0·3	−2·6
Aspartate	+3·6	+0·1	+2·8

formation (Table 3). The small increase in the glutamate content of the homogenate during the incubation was probably a result of proteolysis (Bellamy, 1962). Formation of $^{14}CO_2$ was diminished by 86%, which suggests that in the absence of malonate at least this percentage of glutamate removal was due to transamination with oxaloacetate. This conclusion was confirmed by the finding that fumarate abolished the inhibition of glutamate removal and aspartate formation by malonate (Table 3, vessel 3). Fumarate did not completely restore the formation of $^{14}CO_2$ but all the [1-^{14}C]glutamate removed could be accounted for as $^{14}CO_2$ plus α-oxoglutarate. Fumarate raised the yield of the latter.

Further information on the mechanisms of glutamate removal was obtained from the effects of parapyruvate, an inhibitor of α-oxo-glutarate oxidation (Montgomery & Webb, 1956), on the metabolism of added [1-^{14}C]glutamate. Like malonate, parapyruvate almost completely inhibited the formation of aspartate (92%) and $^{14}CO_2$ (91%) from [1-^{14}C]glutamate (Table 3, vessel 4). However, the glutamate removal was inhibited by only 44% and the disappearance of glutamate was associated with the formation of an equivalent amount of α-oxoglutarate. Thus in the presence of parapyruvate a removal of glutamate and a formation of α-oxoglutarate occurred without transamination with oxaloacetate. This removal of glutamate was probably due to a transamination between glutamate and parapyruvate, as has been reported to occur in extracts of *Clostridium propionicum* (Goldfine, 1960). Parapyruvate was found to react slowly with glutamate to yield α-oxoglutarate in the presence of purified aspartate aminotransferase, as shown by the oxidation of $NADH_2$ on addition of glutamate dehydrogenase and ammonium sulphate (R. Balázs & R. J. Haslam, unpublished work).

Addition of fumarate to the system inhibited by parapyruvate (Table 3, vessel 5) restored glutamate removal and aspartate formation to values approaching those found in the absence of parapyruvate, but the restoration was less effective than that occurring on addition of fumarate to the malonate-inhibited system. The formation of $^{14}CO_2$ from [1-^{14}C]glutamate remained about 90% inhibited by parapyruvate after addition of fumarate. The effects of parapyruvate on [1-^{14}C]-glutamate metabolism suggest that in brain homogenate at least 90% was metabolized by a pathway involving α-oxoglutarate dehydrogenase and confirm that the removal of glutamate was almost entirely dependent on its initial transamination. The effects of malonate and parapyruvate on $^{14}CO_2$ formation imply that under the test conditions a direct decarboxylation of glutamate to γ-aminobutyrate could not account for more than 10% of glutamate metabolism. This conclusion was confirmed by the finding that the anaerobic formation of $^{14}CO_2$ from [1-^{14}C]glutamate under the same conditions was about 8% of that formed aerobically.

Fate of [1-^{14}C]*glutamate in the presence of pyruvate or glucose in homogenates of rat cerebral cortex.* The addition of 10 mM-pyruvate inhibited the net removal of [1-^{14}C]glutamate by 61%, $^{14}CO_2$ formation by 36% and aspartate formation by over 80% (Table 4, vessel 2). The $^{14}CO_2$ formation was thus over 50% greater than the net glutamate (plus glutamine) removal. These observations can be explained by the formation of unlabelled glutamate from acetyl-CoA derived from the pyruvate and oxaloacetate derived from the [1-^{14}C]glutamate.

Table 3. *Effects of malonate and parapyruvate on the fate of* [1-^{14}C]*glutamate in homogenates of rat cerebral cortex*

Results refer to 4 ml. of homogenate incubated for 20 min. in Warburg vessels. Tissue dry wt. was 78 mg./vessel. AMP and ATP were added (final concn. of each 1 mM). Specific activity of the [1-^{14}C]glutamate was 8·0 μc/m-mole. Each vessel contained initially 5·7 μmoles of endogenous glutamate plus glutamine and 1·8 μmoles of endogenous aspartate.

Vessel no.	1	2	3	4	5
Additions	[1-^{14}C]Glutamate (19·5 μmoles)	[1-^{14}C]-Glutamate (19·5 μmoles) and malonate (40 μmoles)	[1-^{14}C]Glutamate (19·5 μmoles), malonate (40 μmoles) and fumarate (18·0 μmoles)	[1-^{14}C]-Glutamate (19·5 μmoles) and parapyruvate (40 μmoles)	[1-^{14}C]Glutamate (19·5 μmoles), parapyruvate (40 μmoles) and fumarate (18·0 μmoles)
Metabolic changes (μmoles)					
O_2	−29·1	−9·4	−19·1	−6·0	−13·0
$^{14}CO_2$	+10·0	+1·40	+7·12	+0·85	+1·13
Glutamate plus glutamine	−12·0	+4·4	−11·6	−6·7	−10·3
Aspartate	+9·8	+0·3	+11·0	+0·9	+7·3
α-Oxoglutarate	+0·3	+0·1	+2·9	+8·5	+14·2
Percentage inhibition of $^{14}CO_2$ formation by additions other than [1-^{14}C]glutamate	—	86	29	91	89

This is supported by the finding that addition of pyruvate caused a decrease of the specific activity of the [1-^{14}C]glutamate during incubation (Table 5, last row). The formation of the unlabelled glutamate may be due to a rapid isotope exchange between [1-^{14}C]glutamate and unlabelled α-oxoglutarate formed through the tricarboxylic acid cycle (see Discussion). In the presence of pyruvate net transamination between glutamate and oxaloacetate was decreased, though exchange transamination between glutamate and α-oxoglutarate occurred. The reduction in $^{14}CO_2$ formation arose partly from dilution of the α-oxoglutarate and glutamate pools by unlabelled material. The size of this effect can be roughly calculated on the assumption that the specific activity of the glutamate pool decreased linearly. On this basis the mean relative specific activity of the [1-^{14}C]glutamate in the presence of pyruvate was 0·73 (from data in Table 5), which would result in the liberation of 2·3 μmoles of $^{14}CO_2$ less than in the absence of pyruvate. As the actual decrease in the formation of $^{14}CO_2$ caused by pyruvate was 6·8 μmoles, it follows that, in addition to reducing glutamate removal, pyruvate reduced the oxidative decarboxylation of α-oxoglutarate by about 24%. Some of the labelled material was retained in the glutamate pool and the rest accumulated as α-oxoglutarate, the concentration of which rose by a factor of 10–20 on addition of pyruvate.

The effect of added glucose (10 mM) on [1-^{14}C]-

Table 4. *Effects of pyruvate and glucose on the metabolism of* [1-^{14}C]*glutamate in homogenates of rat cerebral cortex*

Conditions of incubation were as in Table 1, except that the period of incubation was 40 min. Tissue dry wt., 84mg./vessel. Each vessel contained initially 6·5 μmoles of glutamate + glutamine and 0·1 μmole of α-oxoglutarate.

Vessel no.	1	2	3
Substrates added	[1-^{14}C]Glutamate (40·8 μmoles)	[1-^{14}C]Glutamate (40·8 μmoles) and pyruvate (37·1 μmoles)	[1-^{14}C]Glutamate (40·8 μmoles) and glucose (40 μmoles)
Metabolic changes (μmoles)			
O_2	−43·2	−45·9	−38·2
$^{14}CO_2$	+18·9	+12·1	+14·4
Glutamate plus glutamine	−19·9	− 7·7	−14·7
Aspartate	+18·0	+ 2·4	+13·0
Pyruvate	0·0	−24·0	0·0
α-Oxoglutarate	+ 0·2	+ 3·9	+ 0·2
Citrate plus *cis*-aconitate plus isocitrate	+ 0·1	+ 1·6	—
100 × ($^{14}CO_2$ formation)/glutamate removal	95	157	98
Percentage inhibition of glutamate removal by unlabelled substrate	—	61	26
Percentage inhibition of $^{14}CO_2$ formation by unlabelled substrate	—	36	24

Table 5. *Fate of added* [1-^{14}C]*glutamate and relative specific activity of residual glutamate in vessels* 1, 2 *and* 3 *of Table* 4

Residual [1-^{14}C]glutamate (plus [1-^{14}C]glutamine) was determined by estimation of the $^{14}CO_2$ liberated by *Clostridium welchii*, and is expressed as μmoles of labelled glutamate of the original specific activity. The relative specific activity of the residual glutamate plus glutamine is expressed as a fraction of the specific activity of the added [1-^{14}C]glutamate.

Vessel no.	1	2	3
Substrates added	[1-^{14}C]Glutamate (40·8 μmoles)	[1-^{14}C]Glutamate (40·8 μmoles) and pyruvate	[1-^{14}C]Glutamate (40·8 μmoles) and glucose
Fate of added [1-^{14}C]glutamate:			
Residual [1-^{14}C]glutamate plus [1-^{14}C]glutamine (μmoles)	22·6	25·4	26·7
α-Oxoglutarate formed (μmoles)	0·2	3·9	0·2
$^{14}CO_2$ formed (μmoles)	18·9	12·1	14·4
Sum	41·7	41·4	41·3
Rel.sp.activity of residual glutamate plus glutamine:			
Residual [1-^{14}C]glutamate plus [1-^{14}C]glutamine (μmoles) (*a*)	22·6	25·4	26·7
Total residual glutamate plus glutamine (μmoles) (*b*)	27·4	39·6	32·6
Rel.sp.activity (*a*)/(*b*)	0·83	0·64	0·82

glutamate metabolism (Table 4, vessel 3) was much smaller than that of pyruvate and there was no disparity between $^{14}CO_2$ formation and glutamate removal. Glutamate removal was inhibited by 26%, $^{14}CO_2$ formation by 24% and aspartate formation by 28%. Glucose had no effect on the relative specific activity of the residual [1-^{14}C]-glutamate (Table 5). These results and the absence of any appreciable α-oxoglutarate accumulation are in contrast with the effects of addition of pyruvate and suggest that in the homogenates fewer glucose carbon atoms than pyruvate carbon atoms entered the tricarboxylic acid cycle, probably because of the low rate of glycolysis in homogenates.

As shown in Table 5, the whole of the [1-^{14}C]-glutamate added to vessels 1, 2 and 3 of Table 4 can be accounted for by the α-oxoglutarate and $^{14}CO_2$ formed and the radioactivity in the residual glutamate. This confirms the reliability of the techniques used, as the combined errors in accounting for [1-^{14}C]glutamate were in each vessel less than 2·5%.

Fate of [5-^{14}C]*glutamate in homogenates of rat-brain.* [5-^{14}C]Glutamate yielded very little $^{14}CO_2$ in brain homogenate when added as the sole substrate (Table 6, vessel 1); the $^{14}CO_2$ formed was equivalent to less than 10% of the glutamate removed. This finding, as well as the slight excess of glutamate removal over aspartate formation, can be accounted for by the assumption that some (19% in this experiment) of the oxaloacetate formed from glutamate passed through the tricarboxylic acid cycle as far as α-oxoglutarate, so losing half its ^{14}C in the conversion of isocitrate into α-oxoglutarate. Alternatively some oxaloacetate may have released $^{14}CO_2$ by undergoing decarboxylation to pyruvate.

Table 6. *Effect of pyruvate on the fate of* [5-^{14}C]-*glutamate in homogenates of rat cerebral cortex*

Results refer to 4 ml. of homogenate, incubated for 40 min. in Warburg vessels. Tissue dry wt. was 84 mg./vessel. AMP and ATP were added (final concn. of each 1 mM). The specific activity of the [5-^{14}C]glutamate was 50 μc/m-mole. Each vessel contained initially 6·5 μmoles of endogenous glutamate plus glutamine.

Vessel no.	1	2
Substrates added	[5-^{14}C]Glutamate (32·0 μmoles)	[5-^{14}C]Glutamate (32·0 μmoles) and pyruvate (37·1 μmoles)
Metabolic changes (μmoles)		
O_2	−38·7	−41·1
$^{14}CO_2$	+ 1·36	+ 3·81
Glutamate plus glutamine	−15·6	− 6·2
Aspartate	+12·6	+ 2·6
Pyruvate	0·0	−22·7
α-Oxoglutarate	+ 0·1	+ 2·2
Alanine	+ 0·1	+ 3·0

The aspartate formed was radioactive and the specific activity of C-4 of aspartate relative to C-5 of [5-^{14}C]glutamate was 0·46. This is consistent with the oxidation of glutamate through the tricarboxylic acid cycle, which would lead to an equal distribution of the ^{14}C between the two carboxyl groups of aspartate, so giving a theoretical relative specific activity of 0·5 for C-1 and C-4.

When both pyruvate and [5-^{14}C]glutamate were added (Table 6, vessel 2) the $^{14}CO_2$ yield was almost trebled, compared with that from [5-^{14}C]glutamate alone, and was equivalent to more than 60% of the glutamate removed. Both glutamate removal and aspartate formation were diminished, the latter more than the former, and the difference between aspartate formation and glutamate removal was almost equal to the $^{14}CO_2$ formation. This indicates that the glutamate not converted into aspartate was oxidized further through the tricarboxylic acid cycle, so losing all its ^{14}C in the decarboxylations of isocitrate and α-oxoglutarate. Thus the addition of pyruvate, although decreasing the total amount of glutamate used, increased both the proportion and amount of glutamate oxidized beyond oxaloacetate. Most (90%) of the amino nitrogen of the glutamate removed in the presence of pyruvate was accounted for by the formation of alanine and aspartate (Table 6, vessel 2).

Fate of [1-^{14}C]- *and* [5-^{14}C]-*glutamate in slices of guinea-pig cerebral cortex.* As some of the findings on rat-brain homogenate are in conflict with the results obtained by Cohen *et al.* (1962) with slices of guinea-pig cortex (see the Discussion section), additional experiments were carried out with this material. Except for quantitative differences, the results obtained resembled very closely those obtained with homogenates (Table 7). Added glutamate (2·5 mM) formed aspartate but the yield was lower, 60% compared with 80–90% in homogenates. The $^{14}CO_2$ formation from [1-^{14}C]glutamate was almost equal to the glutamate removed (by reactions other than the synthesis of glutamine), and the relative specific activity of C-4 of the aspartate was less than 0·01.

When no substate was added the endogenous glutamate disappeared but was not converted into aspartate (Table 7). The complete oxidation of the endogenous glutamate (or glutamine) requiring 4½ moles of oxygen for each mole could account for up to about 50% of the endogenous respiration. It follows that other readily oxidizable endogenous substrates were also present, as Takagaki *et al.* (1957) have found.

When [5-^{14}C]glutamate was added to slices the aspartate formed was labelled and the relative specific activity of C-4 of aspartate was found to be 0·32 (expected value 0·50). The difference between the glutamate removed and aspartate formed was equal

to the amount of $^{14}CO_2$ formed from [5-^{14}C]glutamate (Table 7). This $^{14}CO_2$ constituted a much higher percentage of the glutamate removed in slices (35%) than in homogenates (10%). These findings do not necessarily signify that more glutamate is oxidized beyond oxaloacetate in the slices, as in this case the $^{14}CO_2$ formation from [1-^{14}C]-glutamate would be expected to be higher than the glutamate removal even in the absence of glucose owing to the synthesis of unlabelled glutamate. The considerable formation of $^{14}CO_2$ from [5-^{14}C]glutamate and the low specific activity of C-4 of aspartate suggest that ^{14}C may have been lost at the oxaloacetate level by an exchange with unlabelled carbon catalysed by a carbon dioxide-fixing enzyme.

Effects of glucose and pyruvate on the fate of glutamate in slices of guinea-pig cerebral cortex. The addition of glucose decreased the removal of glutamate (through reactions other than glutamine formation) by more than 50% and the formation of aspartate by 80%, but it did not significantly diminish the $^{14}CO_2$ formation from [1-^{14}C]glutamate (Table 7). In this respect glucose acted similarly to pyruvate in the homogenates, where the inhibition of net glutamate removal was also much greater than that of $^{14}CO_2$ formation. This effect can again be attributed to the re-formation of unlabelled glutamate from the added substrates. It follows from these findings that the specific activity of the residual [1-^{14}C]glutamate (plus glutamine) must have decreased in the presence of glucose, which is contrary to the claims of Sellinger *et al.* (1962). The mean $^{14}CO_2$ formation from [5-^{14}C]glutamate was not significantly increased by glucose, and remained approximately equal to the difference between glutamate removal and aspartate formation (Table 7).

Pyruvate inhibited glutamate removal, aspartate formation and $^{14}CO_2$ formation from [1-^{14}C]glutamate, all by about 20% (Table 7). The decrease in glutamate removal was highly significant ($P < 0.001$) whereas the effects on aspartate formation ($0.2 > P > 0.1$) and $^{14}CO_2$ formation were not. The $^{14}CO_2$ formation from [1-^{14}C]-glutamate was approximately equal to the amount of glutamate removed in the presence of pyruvate. This result, as well as the smallness of the effects of pyruvate on the removal of glutamate and the formation of aspartate, are in contrast with the larger effects of pyruvate in homogenates and of glucose in slices. The formation of $^{14}CO_2$ from [5-^{14}C]glutamate was slightly increased by pyruvate but the significance of this effect is not certain ($0.1 > P > 0.05$).

Effect of pyruvate on the fate of [1-^{14}C]*glutamate in homogenates of rat liver.* The effects of pyruvate on glutamate metabolism in liver homogenates differ quantitatively from those in brain homogenates. When glutamate (10 mM) was the sole added substrate the yield of aspartate in liver homogenates was lower than in brain (69%) and the deficit was accounted for by the formation of alanine (28%) and α-oxoglutarate (3%) (Table 8, vessel 2). Thus less aspartate and more alanine was formed than in homogenates of cerebral cortex under the same conditions. The lower yield of aspartate in liver can be explained by the decarboxylation to pyruvate of some of the oxaloacetate formed from glutamate, and transamination between pyruvate and glutamate.

The $^{14}CO_2$ liberated from [1-^{14}C]glutamate (Table 8, vessel 2) was equivalent to 83% of the glutamate removed. Thus, in liver as in brain, the removal of [1-^{14}C]glutamate was accompanied by the release of $^{14}CO_2$, which is consistent with the oxidation of glutamate through the tricarboxylic acid cycle.

The addition of pyruvate substantially altered the pattern of glutamate metabolism in liver homogenate (Table 8, vessel 3). Glutamate removal was increased by some 60%, aspartate formation was diminished by 90% and the accumulation of both alanine and α-oxoglutarate was increased severalfold. Almost the whole of the amino nitrogen of the glutamate removed was recovered as alanine, which

Table 7. *Fate of glutamate in the presence and absence of glucose and pyruvate in slices of guinea-pig cerebral cortex*

Results are expressed as μmoles of glutamate removed or of metabolite formed/g. of tissue (wet wt.)/hr. Mean values are given ±S.E.M. and number of observations are given in parentheses. All added substrates were initially 2·5 mM. Specific activities of the [1-^{14}C]glutamate and [5-^{14}C]glutamate used were 50 μC/m-mole. Further experimental details are given in the Experimental section.

	Metabolic changes (μmoles/g./hr.)				
Additions	Oxygen	Glutamate plus glutamine	Aspartate	$^{14}CO_2$ from [1-^{14}C]glutamate	$^{14}CO_2$ from [5-^{14}C]glutamate
None	−30·2 ± 3·6 (5)	− 3·0 ± 1·3 (5)	−0·3 ± 0·7 (5)	—	—
Glutamate	−46·1 ± 4·4 (7)	−14·4 ± 0·3 (7)	+8·7 ± 0·6 (6)	+13·8 ± 0·5 (4)	+5·0 ± 0·7 (3)
Glutamate and glucose	−65·1 ± 8·7 (5)	− 7·1 ± 0·5 (5)	+1·7 ± 0·6 (5)	+13·3 ± 1·2 (2)	+6·4 ± 0·8 (3)
Glutamate and pyruvate	−66·9 ± 10·5 (3)	−11·2 ± 0·6 (3)	+6·8 ± 1·1 (3)	+11·5 (1)	+8·1 ± 1·0 (2)

Table 8. *Effects of pyruvate on the fate of* [1-^{14}C]*glutamate in homogenates of rat liver*

Results refer to 4 ml. of homogenate incubated for 30 min. in Warburg vessels. Tissue dry wt. was 115 mg./vessel. Specific activity of the [1-^{14}C]glutamate used was 10 μc/m-mole. Each vessel contained 3·2 μmoles of endogenous glutamate plus glutamine.

Vessel no.	1	2	3
Substrates added	None	[1-^{14}C]Glutamate (36·6 μmoles)	[1-^{14}C]Glutamate (36·6 μmoles) and unlabelled pyruvate (40·0 μmoles)
Metabolic changes (μmoles)			
O_2	−20·0	−23·2	−21·6
$^{14}CO_2$	—	+12·6	+13·4
Glutamate plus glutamine	−0·5	−15·2	−24·3
Aspartate	+1·6	+10·5	+1·0
Alanine	−0·4	+4·3	+24·2
Pyruvate	−0·1	+0·0	−29·7
α-Oxoglutarate	0·0	+0·4	+8·7

Table 9. *Aminotransferase activities of homogenates of rat liver and cerebral cortex*

The method of Delbrück, Zebe & Bücher (1959) was used. Results refer to 37°.

	Activity (μmoles/g. fresh wt./min.)	
Tissue	Aspartate aminotransferase	Alanine aminotransferase
Liver	310	37
Cerebral cortex	93	2

shows that pyruvate had largely replaced oxaloacetate as the α-oxo acid initiating glutamate oxidation by transamination, and that transamination rather than dehydrogenation was the main primary reaction of glutamate under the test conditions.

The differences in the effects of pyruvate in liver and brain, including the stimulation of glutamate removal by pyruvate in liver and inhibition in brain, can be attributed to the much higher alanine-aminotransferase activity in liver. Data on the aminotransferases of liver and cerebral cortex are given in Table 9. The activity of the alanine aminotransferase of liver was about 12% of that of the aspartate aminotransferase in liver and about 2% in cerebral cortex (see also Cohen & Hekhuis, 1941; Matzelt, Oriol-Bosch & Voigt, 1962; Fellenberg, Eppenberger, Richterich & Aebi, 1962).

DISCUSSION

Pathways of glutamate metabolism in cerebral cortex. The observations are all consistent with the occurrence of the following reactions, already well established by previous work: (1) glutamate + oxaloacetate ⇌ α-oxoglutarate + aspartate; (2) glutamate + pyruvate ⇌ α-oxoglutarate + alanine; (3) glutamate → γ-aminobutyrate + carbon dioxide; (4) glutamate − 2H → α-oxoglutarate + ammonia; (5) α-oxoglutarate → oxaloacetate; (6) γ-aminobutyrate + α-oxoglutarate → succinic semialdehyde + glutamate; (7) succinic semialdehyde → oxaloacetate; (8) oxaloacetate → pyruvate + carbon dioxide; (9) pyruvate → acetyl-CoA + carbon dioxide; (10) oxaloacetate + acetyl-CoA → citrate; (11) citrate → α-oxoglutarate.

This list is not a comprehensive survey of the reactions involving glutamate in cerebral cortex, but is limited to those which are relevant to the subsequent discussion. The experimental findings do not support the occurrence of reactions resulting in the conversion of glutamate into aspartate via γ-decarboxylation as proposed by Cohen *et al.* (1962).

Homogenates of cerebral cortex. Since aspartate and alanine do not take part in any reactions other than (1) and (2) their yields are a measure of these reactions. The yield of γ-aminobutyrate, on the other hand, represents only the minimum extent of (3) since some γ-aminobutyrate can be removed by (6). Aspartate was formed in quantities equivalent to 80–90% of the glutamate removed when the latter was the sole substrate. Under these conditions no alanine was detectable and the amount of γ-aminobutyrate found was less than 8% of the glutamate removed. This leaves some 10% of the glutamate unaccounted for, which has probably been removed by the glutamate-dehydrogenase reaction (4). These results, in combination with the effects of malonate, show that glutamate was mainly oxidized through transamination with oxaloacetate, followed by the conversion of the α-oxoglutarate formed into oxaloacetate (reaction 5), as previously shown for brain mitochondria (Krebs & Bellamy, 1960) and for many other tissues (Krebs, 1950; Müller & Leuthardt, 1950; Krebs & Bellamy, 1960; Borst & Slater, 1960; Jones & Gutfreund, 1961; Borst, 1962).

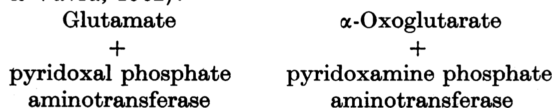
The fact that $^{14}CO_2$ was formed from [5-^{14}C]-glutamate shows that some glutamate was metabolized beyond the stage of oxaloacetate, probably because the latter condensed with acetyl-CoA according to (10). One turn of the tricarboxylic acid cycle (reactions 11 and 5) would then cause the complete release of ^{14}C as $^{14}CO_2$. Alternatively C-5 of glutamate could be set free as $^{14}CO_2$ by reactions (8) and (9). The formation of $^{14}CO_2$ from [5-^{14}C]-glutamate accounted for about 10% of the glutamate removed (the same value as the amount not accounted for by the recovery of the amino acids). This confirms the earlier conclusion that about 10% of the glutamate removed was metabolized by reaction (4). The data on the formation of $^{14}CO_2$ from [1-^{14}C]glutamate in the presence of malonate or parapyruvate, or under anaerobic conditions, show that reaction (3) accounted for not more than 10% of the glutamate removed, a figure which corresponds to the contribution of the γ-aminobutyrate pathway (see Roberts, 1956) to the oxidative metabolism of cat-brain homogenate as estimated by McKhann, Albers, Sokoloff, Mickelsen & Tower (1960).

Slices of cerebral cortex. In slices about 40% of the glutamate removed was not recovered as amino nitrogen and the fraction of the ^{14}C of the [5-^{14}C]-glutamate removed which appeared as $^{14}CO_2$ was also about 40%. However, the glutamate concentration in the slice experiments was lower than in the homogenate experiments, so that the results do not permit the conclusion that the relative proportions of the reactions (1) and (4) differ in slices and homogenates. McKhann *et al.* (1960) have calculated from isotope data that over 40% of the oxidative metabolism of glutamate may pass through the γ-aminobutyrate pathway in slices of cat cerebral cortex. The present experiments provide no evidence on whether the conversion of glutamate into aspartate in slices involved the reactions (1) and (5) or reactions (3), (6), (7) and (1). The latter sequence converts glutamate into aspartate via γ-aminobutyrate. That aspartate may be a product of the oxidation of γ-aminobutyrate has been shown by Tsukada, Hirano, Nagata & Matsutani (1960).

Effects of glucose and pyruvate on the metabolism of glutamate. Although added and endogenous glutamate are under some conditions readily oxidized by brain preparations *in vitro*, the concentration of glutamate *in vivo* is normally maintained at about 10 mM (Krebs, Eggleston & Hems, 1949; Schwerin *et al.* 1950). Thus *in vivo* the oxidation of glucose either prevents the oxidation of glutamate or causes glutamate to be resynthesized as rapidly as it is utilized. The present experiments confirm the previous observations (Stern, Eggleston, Hems & Krebs, 1949; Waelsch, 1949; Takagaki *et al.* 1957) that glucose inhibits the removal of glutamate from brain slices and they also show that pyruvate has the same effect in brain homogenate. The almost complete suppression of aspartate formation by these substrates indicates that the reaction of glutamate which is inhibited is its transamination with oxaloacetate. The reason for the inhibition requires further investigation. The occurrence of a net transamination depends in the first instance upon the concentration of the reactants in relation to the equilibrium position. It is possible that the addition of glucose or pyruvate so affects the concentrations of α-oxoglutarate and oxaloacetate as to make them unfavourable for a net transamination between glutamate and oxaloacetate. In fact the addition of pyruvate increased the concentration of α-oxoglutarate in brain homogenate more than tenfold (Table 4). This concept is also supported by the observations made on liver homogenates, where on addition of pyruvate the transamination between glutamate and pyruvate supplanted that between glutamate and oxaloacetate, although the activity of the aspartate aminotransferase is very high (see also Ratner & Pappas, 1949). As shown in Table 8, addition of pyruvate caused a large accumulation of α-oxoglutarate, thus probably increasing the α-oxoglutarate:oxaloacetate ratio. The fact that pyruvate did not inhibit aspartate formation in slices, whereas it did so in homogenates, cannot yet be explained.

Fall of specific activity of [^{14}C]glutamate in the presence of pyruvate or glucose. Pyruvate, when added to homogenates, and glucose, when added to slices, cause a decrease of the specific activity of added [1-^{14}C]glutamate, indicating the formation of unlabelled glutamate (Tables 5 and 7). Another aspect of this phenomenon is the liberation of $^{14}CO_2$ from [1-^{14}C]glutamate in excess of the amounts of glutamate removed (Tables 4 and 7). These observations have presumably the same basis as the incorporation of radioactivity from ^{14}C-labelled pyruvate or glucose into the amino acids of brain (described by Winzler, Moldave, Rafelson & Pearson, 1952; Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari, 1955, 1959; Busch, 1955; Kini & Quastel, 1959; Shrivastava & Quastel, 1962; Vrba, Gaitonde & Richter, 1962).

The dilution of labelled glutamate by glucose or pyruvate or the appearance of carbon atoms of glucose or pyruvate in unlabelled glutamate can be fully explained by the participation of glutamate in the following half-reaction of transamination (Nisonoff *et al.* 1954; Jenkins & Sizer, 1959; Velick & Vavra, 1962):



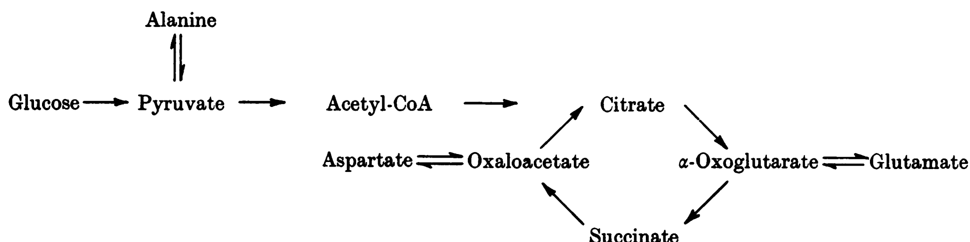
This half-reaction occurs with great speed, according to Velick & Vavra (1962) 2·7 times as fast as the overall transaminase reaction catalysed by the aspartate aminotransferase, and will cause a rapid exchange between the carbon atoms of α-oxoglutarate arising in the tricarboxylic acid cycle and of glutamate, provided that the aminotransferase is present in the same cell compartment as the enzymes of the tricarboxylic acid cycle. The ready oxidation of glutamate to aspartate through the tricarboxylic acid cycle indicates that this is the case. The above half-reaction is a side step of the cycle, consisting of the backward and forward reaction of a reversible system; it does not involve any overall metabolic change. The α-oxoglutarate on the other hand, which is present in very low concentrations, undergoes a real metabolic change by conversion into oxaloacetate and resynthesis via citrate. It is essential to make a distinction between 'isotope exchange' and the 'metabolic incorporation of isotopes'. The essential feature of metabolic incorporation is the involvement of irreversible steps so that the maintenance of the steady state necessitates different enzyme mechanisms for the formation and removal of the metabolite.

The extent to which isotope exchange is responsible for the appearance of substrate carbon atoms in amino acids, or of amino acid carbon atoms in carbon dioxide, can be roughly assessed from the known metabolic rates and enzyme capacities of the tissue. The rate of oxygen consumption of cerebral cortex is about 100 μmoles/g. wet wt./hr. at 40° (Q_{O_2} 11). As one turn of the tricarboxylic acid cycle corresponds to an uptake of approximately 3 molecules of oxygen, the rate at which the intermediates of the tricarboxylic acid cycle are formed and used is about 33 μmoles/g. wet wt./hr. The assay of aspartate-aminotransferase activity gives values of 5600 μmoles/g. wet wt./hr. (Table 9). This corresponds to a value of 15000 μmoles/g./hr. for the rate of the exchange reaction between α-oxoglutarate and glutamate (Velick & Vavra, 1962). Although this value, based on measurements in which high concentrations of α-oxoglutarate and aspartate were added to a tissue preparation, may be very much higher than the rate under physiological conditions, it is evident that the rate of the exchange reactions may be two orders of magnitude greater than the rate of formation of α-oxoglutarate and oxaloacetate in the tricarboxylic acid cycle. This implies that most molecules of the α-oxoglutarate and oxaloacetate formed in the course of respiration are likely to exchange their carbon atoms with glutamate and aspartate before they react in the tricarboxylic acid cycle.

Factors affecting the rate of appearance and disappearance of ^{14}C *in the free amino acids of tissues.* The aminotransferase activities, and hence the rates of the exchange reactions, are of the same order in the major tissues such as liver, muscle, brain or kidney. If glucose carbon atoms are nevertheless incorporated more rapidly into the amino acid pool of brain, this must be due to factors other than aminotransferase activity. Most important of all is the rate at which glucose is converted into acetyl-CoA—a prerequisite for the formation of the α-oxo acids. This is far more rapid in brain than in liver, kidney or resting muscle owing to the rather low rate of glycolysis of these tissues. Another factor is the pool size of glutamate, which is substantially greater in brain than in most other tissues of the rat (Schwerin *et al.* 1950). A third factor operating when glucose is injected into the whole animal (Busch, Fujiwara & Keer, 1960; Vrba *et al.* 1962) is the fact that the blood supply of brain is relatively larger per unit of weight than that of muscle or liver. Thus more isotope reaches the brain. Finally added glucose may enter metabolic processes more rapidly in brain than in other tissues because of its low glucose content (Gey, 1956), the average glucose concentration of brain being 8 mg./100 g. compared with 82 mg. in liver, 51 mg. in kidney and 22 mg. in muscle, and because of the inability of brain, in contrast with most other tissues, to rely on substrates other than glucose as a source of energy.

The conversion of a substrate into the intermediates of the tricarboxylic acid cycle is liable to depend on experimental conditions and these must therefore affect the appearance of substrate carbon atoms in glutamate. As the rate of glycolysis is greatly reduced by converting a tissue into an homogenate, it is not surprising that the glucose carbon atoms appear in glutamate in slices but not in homogenates.

These considerations are equally relevant to the interpretation of experiments in which the labelling of $^{14}CO_2$ is measured after the addition of ^{14}C-labelled substrates (see Sacks, 1957, 1961; Allweis & Magnes, 1958; Chain *et al.* 1962; Sellinger *et al.* 1962). Though the main principles discussed, especially the distinction between isotope exchange and metabolic incorporation, have already been clearly expressed by Albers & Jakoby (1960) (see also Weinman, Strisower & Chaikoff, 1957), they do not appear to have been fully appreciated by recent authors (see Sellinger *et al.* 1962; Chain *et al.* 1962; Vrba *et al.* 1962). Thus Chain *et al.* (1962) report that addition of unlabelled glutamate or aspartate reduces the specific activity of the $^{14}CO_2$ produced by slices of cerebral cortex respiring in the presence of uniformly labelled [^{14}C]glucose and infer that the amino acids inhibit the oxidation of glucose and are therefore preferentially oxidized. However, this observation can also be explained by the retention of radioactivity in the amino acid



Scheme 1. Exchange reactions (indicated by the arrows in two directions) in cerebral cortex.

pools after isotope exchange between labelled α-oxo acids and the corresponding amino acids. Vrba *et al.* (1962) conclude from the rapid incorporation of glucose carbon atoms into amino acids that their formation and oxidation constitutes a main pathway of glucose metabolism in brain. This conclusion is not justified as the observations can be explained by exchange transamination in combination with the special features of brain metabolism discussed above.

Even if the data on the distribution of ^{14}C between amino acids and carbon dioxide found after administration of labelled substrates can largely be accounted for on the basis of the concept of the exchange reactions illustrated in Scheme 1, it still remains open whether glutamate participates directly as an intermediate in a modified tricarboxylic acid cycle in which the reactions between α-oxoglutarate and succinate are replaced by those of the γ-aminobutyrate pathway, involving the steps: glutamate → γ-aminobutyrate → succinic semialdehyde → succinate (Roberts, 1956; Roberts, Rothstein & Baxter, 1958; Wilson, Hill & Koeppe, 1959; Albers & Brady, 1959; Salvador & Albers, 1959; Roberts, 1960; McKhann & Tower, 1961). The extent to which this pathway occurs is uncertain. It is not likely to be more than 10 %, but, even if it were the main pathway, the rapid labelling of the amino acid pools would still be the result of exchange, i.e. side reactions, rather than evidence of a special role in brain of the amino acids as intermediary metabolites.

SUMMARY

1. The fate of [1-^{14}C]- and [5-^{14}C]-glutamate has been studied in the presence and absence of pyruvate or glucose in homogenates of rat cerebral cortex and slices of guinea-pig cerebral cortex.

2. In homogenates 80–90 % of the [1-^{14}C]-glutamate removed was converted into aspartate and $^{14}CO_2$. Endogenous glutamate was also converted into aspartate and this accounted for about 50 % of the endogenous oxygen uptake.

3. Malonate and parapyruvate (γ-hydroxy-γ-methyl-α-oxoglutarate) almost completely inhibited the formation of aspartate and $^{14}CO_2$ from [1-^{14}C]-glutamate. Addition of fumarate removed the inhibitions of asparate formation and in the presense of malonate, but not of parapyruvate, largely restored $^{14}CO_2$ production. These findings indicate that in homogenates the oxidation of glutamate depends almost entirely on its initial transamination with oxaloacetate.

4. In homogenates pyruvate inhibited the net removal of [1-^{14}C]glutamate by 60 %, aspartate formation by 80 % and the production of $^{14}CO_2$ by 36 %. The formation of $^{14}CO_2$ from [1-^{14}C]glutamate was, in the presence of pyruvate, more than 50 % greater than the net glutamate removal and the specific activity of the residual glutamate decreased. The observations can be explained by a synthesis of unlabelled α-oxoglutarate through the tricarboxylic acid cycle and isotope exchange between this unlabelled α-oxoglutarate and the labelled glutamate.

5. Addition of glucose to homogenate equally decreased glutamate removal and $^{14}CO_2$ production from [1-^{14}C]glutamate (25 %).

6. The formation of $^{14}CO_2$ from [5-^{14}C]glutamate in homogenates was equivalent to about 10 % of the glutamate removed. Pyruvate trebled $^{14}CO_2$ production. The specific activity of C-4 of aspartate formed from [5-^{14}C]glutamate was 0·46 (expected 0·50) of that of C-5 of the glutamate.

7. In slices of cerebral cortex about 60 % of the glutamate removed was converted into aspartate. The formation of $^{14}CO_2$ from [1-^{14}C]glutamate was equivalent to the glutamate removed and that from [5-^{14}C]glutamate to the difference between the glutamate removed and the aspartate formed. The aspartate formed from [1-^{14}C]glutamate was unlabelled whereas that from [5-^{14}C]glutamate was radioactive. The effects of glucose on glutamate metabolism in cortex slices were similar to those of pyruvate in homogenates.

8. In rat-liver homogenate the glutamate removed can be almost entirely accounted for by the formation of aspartate (69 %) and alanine (29 %).

In contrast with homogenate of cerebral cortex addition of pyruvate stimulated glutamate removal and pyruvate replaced oxaloacetate as the α-oxo acid initiating glutamate oxidation through transamination. The differences between liver and brain can be explained by differences in alanine-aminotransferase activities.

9. The metabolic behaviour of glutamate in the preparations of cerebral cortex can be fully accounted for by the tricarboxylic acid cycle and associated reactions. The factors that are responsible for rapid incorporation of isotope from glucose into glutamate and aspartate in brain, both *in vivo* and *in vitro*, are discussed and it is emphasized that the rapid labelling of amino acids may be the result of isotope exchanges that are side reactions of the tricarboxylic acid cycle.

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Properties of Crystalline L-Aspartate 4-Carboxy-Lyase from *Achromobacter* sp.

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L-Aspartate 4-carboxy-lyase (EC 4.1.1.12) has been shown to be present in a number of micro-organisms (for review, see Braunstein, 1960). The enzyme, which is unusual in its strong stimulation by α-oxo acids, was partially purified by Nishimura, Manning & Meister (1962) and was shown to contain firmly bound pyridoxal 5′-phosphate; it has subsequently been crystallized from *Achromobacter* sp. (Wilson, 1963) and has been extensively purified from *Alcaligenes faecalis* (Meister, Nishimura & Novogradsky, 1962).

It is the main purpose of the present paper to describe some of the properties of the L-aspartate 4-carboxy-lyase of *Achromobacter* sp. and, in particular, to discuss the mode of binding of pyridoxal 5′-phosphate to the protein and to L-aspartate. A preliminary report of part of this work has been presented to The Biochemical Society (Wilson & Kornberg, 1963).

METHODS AND MATERIALS

Preparation of L-*aspartate* 4-*carboxy-lyase*. The enzyme was purified by the procedure of Wilson (1963) except that the conditions of crystallization were modified to obtain more uniform crystals. The first crop of crystals was obtained by dialysis of the carboxymethylcellulose-treated extract ('Step V') against 0·2M-sodium acetate, pH 7·0, instead of against 0·1M-buffer as previously described. It is important that the dialysis be performed without stirring of the buffer. The enzyme was recrystallized by the same procedure. The resulting crystals (Fig. 1) were regular hexagonal plates that became broken and less regular with time (the photographs shown in Fig. 1 were taken after the crystals had been stored for 3 days).

Two preparations of enzyme were used in most of the experiments described below. One had an initial specific activity of 107 units/mg. of protein and contained 1 mole of pyridoxal 5′-phosphate/120 000 g. of protein, and was used in the spectrophotometric experiments; the other had an initial specific activity of 90 units/mg. of protein and contained 1 mole of pyridoxal 5′-phosphate/60 000 g. of protein; this preparation was used in the ultracentrifugal studies (see Appendix and Fig. 2) and in the remaining experiments.

The enzyme was stable when stored in concentrated solution at pH 7 and 4°. Activity was lost very rapidly in dilute solution or at pH 5·0; it is recommended that dilution before assay be made in 0·5% bovine serum albumin.

A solution of the second preparation of enzyme in 0·5M-sodium acetate, pH 7·0, containing 8·8 mg. of protein/ml., lost less than 15% of its activity, when assayed in the presence of pyruvate, after storage for 2½ months. However, its activity assayed in the absence of pyruvate doubled after this storage. This stored enzyme, which was therefore stimulated less by pyruvate than was freshly prepared enzyme, is referred to below as 'aged' enzyme.

Assay methods. Method *A*. L-Aspartate 4-carboxy-lyase was assayed manometrically at 30°. Evolution of CO_2 was measured for 30 min. after the addition of L-aspartate, and was linear with time and with enzyme concentration. The main compartments of Warburg flasks contained, in a final volume of 1·8 ml., 200 μmoles of sodium acetate (pH 5·0), 0·5 μmole of sodium pyruvate, and enzyme (2–2000 μg. of protein). L-Aspartate (20 μmoles) was added from the side arm after equilibration for 15 min. One unit of enzyme is defined as that quantity which catalyses the evolution of 1 μmole of CO_2/min. under these conditions.

Method *B*. When lower substrate concentrations were required, the activity of the enzyme was measured by assay of the radioactivity of $^{14}CO_2$ from the decarboxylation of L-[U-^{14}C]aspartate. The complete system contained, in the main compartments of double-armed Warburg manometer cups, 200 μmoles of sodium acetate (pH 5·0), 0·5 μmole of sodium pyruvate, 0·176 μg. of purified L-aspartate 4-carboxy-lyase, and water to bring the reaction volume to 2·0 ml. after the addition of the contents of the first side arm. The centre wells contained 0·2 ml. of 2N-KOH. L-[U-^{14}C]Aspartate (0·05–0·20 μmole) was added from the