Exchange Transamination and the Metabolism of Glutamate in Brain

By R. BALÁZS and R. J. HASLAM*
Medical Research Council Neuropsychiatric Research Unit, Carshalton, Surrey, and Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

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1. Experiments were performed to throw light on why the incorporation of ^{14}C from labelled carbohydrate precursors into glutamate has been found to be more marked in brain than in other tissues. 2. Rapid isotope exchange between labelled glutamate and unlabelled α-oxoglutarate was demonstrated in brain and liver mitochondrial preparations. In the presence but not in the absence of α-oxoglutarate the yield of $^{14}CO_2$ from [1-^{14}C]glutamate exceeded the net glutamate removal, and the final relative specific activities of the two substrates indicated that complete isotopic equilibration had occurred. Also, when in a brain preparation net glutamate removal was inhibited by malonate, isotope exchange between [1-^{14}C]glutamate and α-oxoglutarate and the formation of $^{14}CO_2$ were unaffected. 3. The time-course of isotope exchange between labelled glutamate and unlabelled α-oxoglutarate was followed in uncoupled brain and liver mitochondrial fractions, and the rate of exchange calculated by a computer was found to be 3–8 times more rapid than the maximal rate of utilization of the two substrates. 4. The physiological situation was imitated by the continuous infusion of small amounts of α-oxo[1-^{14}C]glutarate into brain homogenate containing added glutamate. The fraction of ^{14}C infused that was retained in the glutamate pool depended on the size of the latter, and the final relative specific activities of the two substrates indicated almost complete isotope exchange. Isotopic equilibration also occurred when α-oxoglutarate was generated from pyruvate through the tricarboxylic acid cycle in a brain mitochondrial preparation containing [1-^{14}C]glutamate. 5. The differences in the incorporation of ^{14}C from labelled glucose into the glutamate of brain and liver are discussed in terms of the rates of isotope exchange, the glutamate pool sizes and the rates of formation of labelled α-oxoglutarate in the two tissues. It is concluded that the differences between tissues in the incorporation of glucose carbon into glutamate reflect features of their metabolism largely unrelated to that of glutamate.

Work from several Laboratories has shown that ^{14}C from labelled glucose is rapidly incorporated in large amounts into the glutamate and aspartate of brain both *in vivo* and *in vitro*, whereas in liver a considerably smaller fraction was found in these amino acids (Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari, 1955; Kini & Quastel, 1959; Roberts, Flexner & Flexner, 1959; Barkulis, Geiger, Kawakita & Aguilar, 1960; Busch, Fujiwara & Keer, 1960; Smith & Moses, 1960; Vrba, Gaitonde & Richter, 1962). The high labelling of glutamate and aspartate does not necessarily indicate that these amino acids are on the main pathway of glucose oxidation in brain, but may result from rapid isotopic equilibration with intermediates of the tricarboxylic acid cycle as a result of exchange transaminations (Albers & Jakoby, 1960; Balázs & Haslam, 1963; Haslam & Krebs, 1963*b*). Thus reaction (1) occurs between α-oxo acids and the corresponding amino acids in the presence of the appropriate aminotransferases (Nisonoff, Barnes, Enns & Schuching, 1954; Jenkins & Sizer, 1959; Velick & Vavra, 1962):

α-Oxo acid		α-amino acid	
+	⇌	+	(1)
pyridoxamine phosphate aminotransferase		pyridoxal phosphate aminotransferase	

This paper is concerned primarily with the importance of exchange of ^{14}C between α-oxoglutarate and glutamate in the labelling of glutamate in brain and also with some aspects of the net metabolism of glutamate. Some of the results have already been published in a preliminary form (Balázs & Haslam, 1963).

* Present address: Department of Pharmacology, Royal College of Surgeons of England, London, W.C.2.

EXPERIMENTAL

Materials

Sodium pyruvate was prepared from pyruvic acid (British Drug Houses Ltd.) by the method of Price & Levintow (1952). The product contained no parapyruvate, as indicated by chromatography of the 2,4-dinitrophenylhydrazone according to the method of Bush & Hockaday (1960).

DL-[1-^{14}C]Glutamate was obtained from The Radiochemical Centre, Amersham, Bucks., and was mixed with unlabelled L-glutamate as described by Haslam & Krebs (1963*b*). α-Oxo[1-^{14}C]glutarate was prepared from DL-[1-^{14}C]glutamate as described by Haslam & Krebs (1963*a*).

Tissue preparations and incubations

Brain and liver mitochondrial preparations. For brain mitochondrial preparations rats were lightly narcotized with ether and decapitated. The cerebral hemispheres were homogenized (Aldridge, 1957) in 0·3 M-sucrose containing EDTA (0·5 mM) and the suspension was diluted to 10% (w/v). The mitochondrial fraction was isolated as described by Balázs, Biesold & Magyar (1963) and was suspended in a volume of the above sucrose medium equivalent to the fresh weight of tissue taken.

Rat liver mitochondrial fractions were prepared by the method of Aldridge (1957).

The basic incubation medium contained: potassium phosphate buffer, pH 7·2 (15 mM); tris–HCl buffer, pH 7·2 (20 mM); $MgCl_2$ (6 mM); EDTA (sodium salt), pH 7·2 (2 mM); sucrose introduced with the mitochondrial suspension (20–50 mM); sufficient KCl to give a final osmolarity of 0·3 with substrates and any other additions. Substrate concentrations were usually 8–10 mM; when present ADP was 2 mM and 2,4-dinitrophenol was 40 μM.

Mitochondrial suspensions were incubated in conical Warburg vessels unless otherwise mentioned. The volume of the suspension was 1·5 ml. in the absence and 2 or 3 ml. in the presence of ADP. The incubation was terminated by tipping sufficient 2 N-HCl from the side arm to give a final concentration of 0·5 N. The centre well contained 0·2 ml. of 2 N-NaOH, and when $^{14}CO_2$ was collected shaking was continued after acidification of the vessel contents until no further change in gas pressure occurred (approx. 1 hr.). Mitochondrial suspensions were incubated at 37·5°, usually for 30–60 min., with O_2 in the gas phase.

Rat-brain homogenates. To simulate the formation of α-oxoglutarate at a low concentration through the tricarboxylic acid cycle, α-oxo[1-^{14}C]glutarate was added continuously throughout incubations of brain homogenates containing added glutamate. The preparation of the homogenate and composition of the incubation medium were as described by Haslam & Krebs (1963*b*). Before incubations 3 ml. samples of homogenate were introduced into Warburg vessels containing 1 ml. of solution comprising 0·2 ml. of 0·04 M-ADP, 0·2 ml. of 0·154 M-$KHCO_3$, 0·2 ml. of 0·2 M-L-glutamate (sodium salt) and 0·154 M-KCl to volume. α-Oxo[1-^{14}C]glutarate solution of appropriate strength was infused into the main compartment of the Warburg vessels, during an incubation carried out at 30°, by means of a fine polythene tube (Portex no. 45; Portland Plastics Ltd.) inserted through the side-arm stopper and sealed into position with paraffin wax. The solution was injected at a rate of 10 μl./1 or 3 min. with an Agla micrometer syringe (Burroughs Wellcome Ltd.) driven by a synchronous electric-clock motor (Smiths). The incubation was terminated with 2N-HCl as above.

Analytical methods. Glutamate plus glutamine and aspartate were determined by bacterial decarboxylations in Warburg manometers as described by Krebs & Bellamy (1960). As glutamate and glutamine were never assayed individually the values for glutamate removal may include endogenous glutamine and refer to glutamate metabolized by pathways other than glutamine synthesis.

Pyruvate and α-oxoglutarate were determined by enzymic methods (Bergmeyer, 1962). The oxidation of NADH was measured at 340 mμ in the presence of lactate dehydrogenase and glutamate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany). Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Radioactive-tracer techniques with ^{14}C. The techniques used were as described by Haslam & Krebs (1963*a*,*b*). The initial and final specific activities of α-oxo[1-^{14}C]glutarate and [1-^{14}C]glutamate were determined by assay of $^{14}CO_2$ liberated by specific decarboxylation methods. The specific activity of α-oxo[1-^{14}C]glutarate was in some experiments also estimated by isolating and counting the 2,4-dinitrophenylhydrazone. The relative specific activities of residual substrates are defined as:

$$\frac{\text{Sp. activity of residual [1-}^{14}\text{C]substrate}}{\text{Sp. activity of added [1-}^{14}\text{C]substrate}}$$

In some experiments the Nuclear–Chicago gas-flow counter was used instead of the Geiger–Müller mica end-window tube and Panax counter.

Determination of enzymic activities. Aspartate aminotransferase (EC 2.6.1.1) was assayed in both directions. The method of Gutfreund, Ebner & Mendiola (1961) was used for determining the activity in the direction:

$$\text{Glutamate} + \text{oxaloacetate} \rightarrow \alpha\text{-oxoglutarate} + \text{aspartate}$$

The activity was measured in the reverse direction by the method of Karmen (1955) as modified by Boyd (1961).

Glutamate-dehydrogenase (EC 1.4.1.3) activity was determined by the method of Olson & Anfinsen (1952) in both directions. The medium contained ADP (1 mM) also (Mildvan & Greville, 1962).

The incubation volume was 3 ml. in each case, and the change of extinction at 340 mμ was followed with a Unicam SP.700 recording spectrophotometer. The total activity of the enzymes was measured by diluting the homogenates in Triton X-100 (final concn. 0·1%) before the assay.

RESULTS

Effects of isotope exchange on the relation between the net metabolism of [1-^{14}C]*glutamate and the yield of* $^{14}CO_2$. When [1-^{14}C]glutamate was oxidized by a brain mitochondrial fraction the $^{14}CO_2$ formed was roughly equivalent to the glutamate removed and 85% accumulated as aspartate (Table 1, vessel 1). In the presence of comparable concentrations of [1-^{14}C]glutamate and α-oxoglutarate the glutamate removal and asparate formation were both depressed by about 50%, but the formation of $^{14}CO_2$

was decreased by only 18%, so that more $^{14}CO_2$ was formed than glutamate removed. It follows that the specific activity of the residual glutamate relative to the added [1-^{14}C]glutamate must have decreased and measurement showed that its final relative specific activity was 0·45. These observations can be explained by isotope exchange between [1-^{14}C]-glutamate and α-oxoglutarate. The final specific activity of α-oxoglutarate relative to the initial value of the labelled glutamate was also close to 0·5, indicating that complete equilibration had occurred. The oxidation of α-oxoglutarate in the presence of glutamate was probably equal to the total utilization of glutamate and α-oxoglutarate, and on this basis was larger than the removal of either glutamate or α-oxoglutarate when added singly. Thus the relatively high formation of $^{14}CO_2$ is readily explained. These results indicate that ^{14}C may enter the tricarboxylic acid cycle from [1-^{14}C]glutamate independently of the net metabolism of glutamate. This was demonstrated even more conclusively by the effect of malonate on [1-^{14}C]glutamate metabolism in the presence and absence of α-oxoglutarate (Table 1). With α-oxoglutarate absent (vessel 4) malonate inhibited both [1-^{14}C]glutamate removal and $^{14}CO_2$ formation by about 80%, and the formation of aspartate was almost completely abolished, observations that are accounted for by the inhibition of the formation of oxaloacetate required for the net removal of glutamate by transamination. However, when α-oxoglutarate was added in addition to [1-^{14}C]glutamate and malonate (vessel 6) the formation of $^{14}CO_2$ was equal to that in the absence of malonate, although the glutamate removal was very small. Measurement of the final relative specific activities of the glutamate and α-oxoglutarate indicated that complete isotopic equilibration had occurred. The oxidation of α-oxo[1-^{14}C]glutarate formed by isotope exchange can thus account for the considerable formation of $^{14}CO_2$. Calculation of the relative specific activity of the α-oxoglutarate oxidized ($^{14}CO_2$ formed/α-oxoglutarate + glutamate removed) yields values approximating the relative specific activity of the residual α-oxoglutarate (Table 1, vessels 3 and 6; see also Table 3, 60 min. samples). This suggests that isotopic equilibrium between α-oxoglutarate and glutamate was established very early in the incubation. When in contrast with the experiment described in Table 1 ADP was omitted from the

Table 1. *Effects of malonate and α-oxoglutarate on* [1-^{14}C]*glutamate metabolism*

Brain mitochondrial fraction (5·2 mg. of protein) in 2·0 ml. of suspension containing ADP (2 mM) was used. The brain mitochondrial fraction included the 'fluffy layer' and thus contained appreciable adenosine-triphosphatase activity (q_{O_2} in vessel 1 = 98 μl./hr./mg. of protein). The specific activity of [1-^{14}C]glutamate was 10 μc/m-mole. Incubation was at 37·5° for 50 min.

Vessel no.	1	2	3	4	5	6
Concn. of [1-^{14}C]glutamate (mM)	9·8	—	9·8	9·8	—	9·8
Concn. of α-oxoglutarate (mM)	—	9·0	9·0	—	9·0	9·0
Concn. of malonate (mM)	—	—	—	10	10	10
Metabolic changes (μmoles):						
O_2	−19·2	−18·8	−26·2	−3·5	−10·3	−13·0
$^{14}CO_2$	+10·8	—	+8·9	+2·3	—	+8·6
Glutamate	−11·0	—	−5·1	−2·1	—	−1·1
Aspartate	+9·3	—	+4·5	+0·3	—	+0·3
α-Oxoglutarate	+0·28	−13·4	−10·1	+0·1	−14·6	−13·9
Rel. sp. activity of residual [1-^{14}C]-substrates:						
(*a*) [1-^{14}C]Glutamate (μmoles)			6·54			7·78
Glutamate (μmoles)			14·38			18·36
∴ Rel. sp. activity of [1-^{14}C]-glutamate			0·45			0·42
(*b*) α-Oxo[1-^{14}C]glutarate (μmoles)			4·26			2·09
α-Oxoglutarate (μmoles)			7·94			4·10
∴ Rel. sp. activity of α-oxo[1-^{14}C]-glutarate			0·53			0·51
Recovery of ^{14}C (as $^{14}CO_2$, [1-^{14}C]-glutamate and α-oxo[1-^{14}C]-glutarate):						
(μmoles)			19·7			18·5
(% of ^{14}C added)			100			94

incubation medium the rate of removal of glutamate was decreased to one-tenth but complete isotopic equilibration occurred as rapidly, as judged by the final relative specific activities of glutamate and α-oxoglutarate and by the calculated relative specific activity of the α-oxoglutarate oxidized.

In liver mitochondrial preparations 75% of the glutamate removed was accounted for by the formation of aspartate (Table 2, vessels 2 and 7), which therefore represents the extent of glutamate removal by transamination with oxaloacetate. The remaining 25% of the glutamate removed was probably metabolized by glutamate dehydrogenase (Jones & Gutfreund, 1961; Borst, 1962). Equimolar concentrations of α-oxoglutarate inhibited glutamate removal by about 80% (Table 2, vessels 5 and 10). As judged from the asparate formation and from the difference between the glutamate removed

Table 2. *Metabolic changes and isotopic equilibration in liver mitochondrial suspensions oxidizing [1-^{14}C]glutamate and α-oxoglutarate*

Liver mitochondrial fraction in the presence and absence of 40 μM-dinitrophenol (3·8 and 7·35 mg. of protein/vessel respectively) was used. The volume of suspension was 2·0 ml., containing ADP (2 mM), and α-oxoglutarate (9·2 mM) and [1-^{14}C]glutamate (9·8 mM) as indicated. Endogenous glutamate was 0·07 μmole/mg. of protein. The incubation temperature was 37·5°.

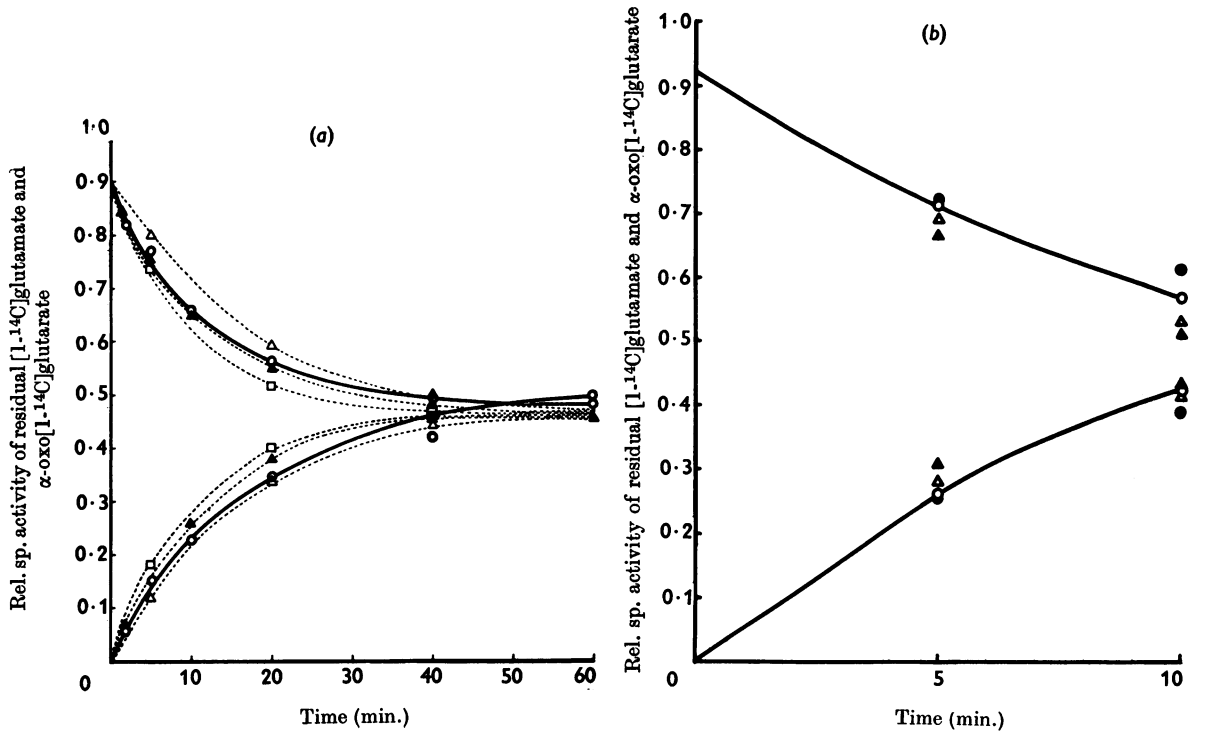
	Dinitrophenol absent					Dinitrophenol present				
Vessel no.	1	2	3	4	5	6	7	8	9	10
Substrate(s)	α-Oxo-glutarate	[1-^{14}C]-Glutamate	[1-^{14}C]Glutamate +α-oxoglutarate			α-Oxo-glutarate	[1-^{14}C]-Glutamate	[1-^{14}C]Glutamate +α-oxoglutarate		
Incubation time (min.)	60	60	5	10	60	50	50	5	10	50
Metabolic changes (μmoles):										
O_2	−9·2	−8·3	—	—	−8·6	−14·5	−13·2	—	—	−13·3
$^{14}CO_2$	—	+4·85	+0·51	+0·87	+4·24	—	+7·74	+0·64	+1·39	+6·13
Glutamate	+0·3	−6·4	−0·2	−0·5	−0·94	+0·1	−10·5	−0·6	−1·2	−2·3
Aspartate	0·0	+4·8	0·0	+0·5	+0·5	0·0	+7·3	+0·3	+1·2	+2·2
α-Oxoglutarate	−9·49	+0·31	−0·92	−1·85	−9·17	+14·6	+0·19	−0·85	−2·02	−9·82
Rel. sp. activity of residual [1-^{14}C]substrates:										
[1-^{14}C]Glutamate	—	0·93	0·55	0·49	0·48	—	—	0·71	0·58	0·48
α-Oxo[1-^{14}C]-glutarate	—	1·2	0·36	0·47	0·47	—	—	0·25	0·43	0·55
Recovery of ^{14}C (%)	—	96	95	96	94	—	—	101	101	103

Table 3. *Metabolism of [1-^{14}C]glutamate and α-oxoglutarate during isotopic equilibration in a brain mitochondrial system*

Brain mitochondrial suspension was incubated in an Erlenmeyer flask in a Dubnoff metabolic shaking incubator at 25° (Expt. A). The flask contained 6 ml. made up of the basic medium, α-oxoglutarate and [1-^{14}C]glutamate (10 μc/m-mole), ADP (2 mM) and mitochondrial protein (final concn. 3 mg./ml.). Samples (1 ml.) were withdrawn at the time-intervals indicated and were acidified and analysed. Mitochondrial suspensions were also incubated at 37·5° (Expt. B), and O_2 consumption and $^{14}CO_2$ formation were measured over 60 min. at the two temperatures in parallel Warburg incubations. In Expt. A the regression lines for the content of glutamate and α-oxoglutarate were calculated and the regression estimates at the different time-intervals were used in the subsequent computations described in the Appendix (Julian *et al.* 1965).

	Expt. A (25°)							Expt. B (37·5°)	
Incubation time (min.)	0	2	5	10	20	40	60	10	60
Metabolic changes (μmoles/ml.):									
O_2 uptake	—	—	—	—	—	—	7·20	—	11·8
$^{14}CO_2$ formed	—	—	—	—	—	—	2·81	—	4·35
Aspartate formed	—	0*	0*	0*	0·45	1·1	1·4	0·45	2·26
Concn. of substrates (μmoles/ml.):									
Total glutamate	9·84	9·60	9·60	9·30	8·95	8·70	8·22	9·14	7·29
[1-^{14}C]Glutamate	8·94	7·83	7·34	6·21	5·17	4·30	3·98	4·80	3·21
Total α-oxoglutarate	10·15	10·12	9·95	9·55	8·62	7·48	5·88	8·41	3·47
α-Oxo[1-^{14}C]glutarate	0	0·57	1·51	2·19	3·04	3·10	2·96	3·58	1·50
Rates of substrate removal (μmole/ml./min.):									
Glutamate uptake	—	0·0248†	0·0248†	0·0248†	0·0248†	0·0248†	0·0248†	0·07	0·0425
Glutamate and α-oxoglutarate	—	0·0965†	0·0965†	0·0965†	0·0965†	0·0965†	0·0965†	0·244	0·1538
Rel. sp. activity of residual [1-^{14}C]-substrates:									
[1-^{14}C]Glutamate		0·82	0·76	0·67	0·58	0·49	0·48	0·53	0·44
α-Oxo[1-^{14}C]glutarate		0·056	0·152	0·23	0·35	0·41	0·50	0·43	0·43

* Metabolic change unmeasurable. † Regression estimate.



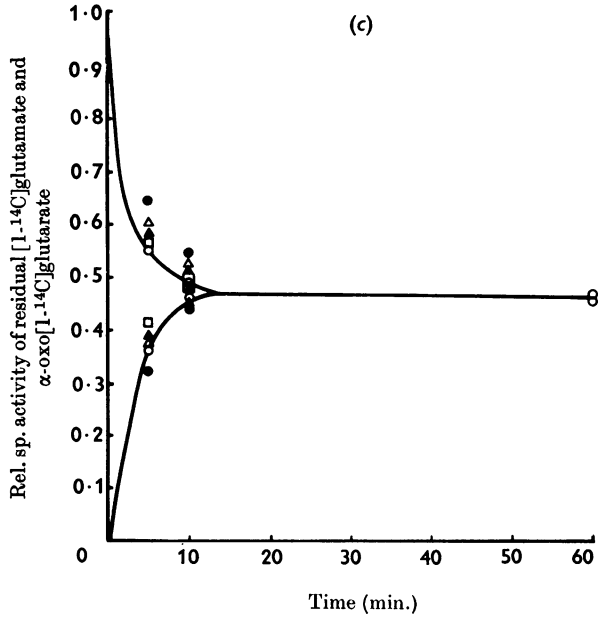


Fig. 1. Rates of exchange transamination in brain and liver mitochondrial systems. The observed values of the relative specific activities of [1-^{14}C]glutamate (upper curve) and α-oxo[1-^{14}C]glutarate (lower curve) are indicated by ○ and are connected with continuous lines. The relative specific activities calculated by the computer from different values of k (rate of exchange transamination, given in μmoles/min./sample) are indicated with different symbols (●, □, ▲, △). The computer points are connected with broken lines in Fig. 1(*a*). (*a*) Brain mitochondrial suspension (3 mg. of protein/ml.) was incubated at 25°. Experimental details are given in Table 3 (Expt. A). $k = 0{\cdot}3$ (△), 0·4 (▲) and 0·5 (□). The computed curves approximated to the observed curves when the value of k was between 0·3 and 0·4. (*b*) Uncoupled liver mitochondrial suspension (3·8 mg. of protein/vessel) was incubated at 37·5°. Experimental details are given in Table 2 (vessels 8 and 9). $k = 1{\cdot}25$ (●), 1·50 (△) and 1·75 (▲). The computed points approximated to the observed values when k was between 1·25 and 1·5. (*c*) Liver mitochondria (7·35 mg. of protein/vessel) were in the controlled state at 37·5°. Experimental details are given in Table 2 (vessels 3, 4 and 5). $k = 2{\cdot}0$ (●), 2·5 (△), 3·0 (▲) and 3·5 (□). The computed points for the values of k at 60 min. are not indicated on the Figure as they agreed to four decimal places (0·4788). The computed curves approximated to the observed curves when the value of k was between 2·5 and 3·0.

and aspartate formed, both the transamination and dehydrogenase pathways of glutamate metabolism were markedly inhibited, presumably by the effects of α-oxoglutarate on the enzymic equilibria. Despite the marked inhibition of glutamate removal by α-oxoglutarate the formation of $^{14}CO_2$ was only slightly decreased. The relative specific activities of the residual glutamate and α-oxoglutarate in vessels 5 and 10 show that complete isotopic equilibration had occurred, so accounting for the formation of $^{14}CO_2$ without an appreciable net metabolism of glutamate. In liver, as in brain preparations, the calculated relative specific activities of the α-oxoglutarate oxidized approach those of the residual α-oxoglutarate, indicating that isotopic equilibrium was achieved early in the incubation, as is also shown by the results of short-term incubations (vessels 3 and 4, and 8 and 9). Vessels 6–10 contained less tissue than vessels 1–5, but as they contained dinitrophenol their rate of oxygen consumption was higher (q_{O_2} values about 100 and 26 respectively). As a result of the lower tissue concentration, isotopic equilibration occurred rather more slowly but was nevertheless almost complete within 10 min. These results show that in liver as in brain mitochondrial preparations the transfer of ^{14}C from [1-^{14}C]glutamate to α-oxoglutarate was independent of the overall metabolic rate.

Rates of isotopic equilibration between [1-^{14}C]*glutamate and α-oxoglutarate in brain and liver mitochondrial preparations.* Brain mitochondrial preparations were incubated with approx. 10 mM-[1-^{14}C]glutamate and 10 mM-α-oxoglutarate at 25° and 37·5° (for experimental details see Table 3). The relative specific activities of the residual substrates were measured at intervals during the incubation (for 25° incubation see Fig. 1*a*), and were found to approximate gradually to each other at a rate depending on the temperature. The rates of isotope exchange between the two substrates were calculated by an Elliott 803 computer from the analytical information obtained by using the equations given in the Appendix (Julian, Balázs & Haslam, 1965). The exchange rate was also calculated for liver mitochondria from the results in Table 2, Fig. 1(*b*) and Fig. 1(*c*), and was found to be comparable with that in brain preparations at the same temperature (Table 4). Although, as judged by the q_{O_2} values, the mitochondrial preparations were in the active state, the calculated rates of isotope exchange were 3–8 times as great as the total substrate utilization. In the controlled state (e.g. Table 2, vessels 3–5, and Fig. 1*c*) the exchange rate was unaltered but the total substrate utilization was much diminished. In mitochondrial preparations glutamate and α-oxoglutarate are incompletely oxidized (e.g. compare oxygen and substrate utilization in Table 4), so that under physiological conditions the ratio of exchange transamination to α-oxoglutarate oxidation may be expected to be much greater.

Table 4. *Comparison of rates of exchange transamination with the rates of oxidation of substrates in mitochondrial systems*

The calculations are based on experiments with mitochondrial systems in the active state of respiration. The analytical data used are given in Table 2 (vessels 8, 9 and 10) for liver and in Table 3 for brain. The method of determining the rates of exchange transamination is given in the Appendix (Julian *et al.* 1965). The rates (upper and lower limits) are given in μmole/min./mg. of protein and were read off the graphs in Fig. 1(*a*) (brain, 25°) and Fig. 1(*b*) (liver, 37·5°). The rate of exchange transamination for brain mitochondrial suspensions at 37·5° was calculated from the 10 min. incubation point only (Expt. B in Table 3).

	Brain		Liver
Temp. of incubation ...	25°	37·5°	37·5°
Exchange transamination	0·1–0·13	0·27–0·4	0·33–0·39
O_2 uptake	0·04	0·065	0·07
Uptake of substrates (glutamate + α-oxoglutarate)	0·032	0·051	0·064

Continuous infusion of α-oxo[1-^{14}C]*glutarate into brain homogenates containing added glutamate.* Under physiological conditions the concentration of α-oxoglutarate is much lower than that of glutamate, and α-oxoglutarate is formed continuously through the tricarboxylic acid cycle. An experimental model of this situation was constructed by the slow infusion of α-oxo[1-^{14}C]glutarate into brain homogenate containing 5 mM- or 10 mM-unlabelled glutamate. The results in Table 5 show that when α-oxoglutarate was infused at 0·25–0·50 μmole/min. for 20 min. the final concentration of α-oxoglutarate was about 0·6–1·3 mM. The rate of oxidation of α-oxoglutarate was proportional to the rate at which it was infused and to the final concentration of α-oxoglutarate in the homogenate. This is in accordance with the high apparent K_m for the oxidation of α-oxoglutarate found by Balázs, Magyar & Richter (1962) in brain preparations. Doubling the initial concentration of glutamate from 5 to 10 mM decreased the formation of $^{14}CO_2$ from α-oxo[1-^{14}C]glutarate by about 40%, indicating that a rapid dilution of the α-oxo[1-^{14}C]glutarate pool by unlabelled glutamate had occurred. The final relative specific activity of glutamate in each vessel corresponded closely to the value that would have been obtained by complete isotopic equilibration between all the α-oxoglutarate and glutamate added. Thus complete exchange between

Table 5. *Infusion of α-oxo[1-^{14}C]glutarate into brain homogenate containing glutamate: variation of substrate amounts*

The results refer to 4 ml. of homogenate incubated for 20 min. at 30°. Each vessel contained initially 6·1 μmoles of endogenous glutamate plus glutamine. Tissue dry wt. was 78 mg./vessel.

Vessel no.	1	2	3	4
Added glutamate (μmoles/vessel)	20·0	20·0	40·0	40·0
α-Oxo[1-^{14}C]glutarate (μmoles infused/vessel)	5·0	10·0	5·0	10·0
Infusion rate (μmoles/min.)	0·25	0·50	0·25	0·50
Final concn. of α-oxoglutarate (mM)	0·56	1·15	0·60	1·35
Metabolic changes (μmoles):				
$^{14}CO_2$	+1·35	+2·51	+0·77	+1·57
α-Oxoglutarate	−2·76	−5·40	−2·60	−4·65
Glutamate	−7·0	−9·1	−12·6	−14·8
Rel. sp. activity of residual glutamate:				
[1-^{14}C]Glutamate (μmoles)	2·99	5·44	3·61	6·88
Total residual glutamate (μmoles)	19·1	17·0	33·5	31·3
∴ Rel. sp. activity of [1-^{14}C]glutamate	0·16	0·32	0·11	0·22

Table 6. *Infusion of α-oxo[1-^{14}C]glutarate into brain homogenate containing glutamate: time course*

Each vessel contained 4 ml. of homogenate incubated at 30°. Glutamate (40·3 μmoles) was added to each vessel and the endogenous glutamate plus glutamine was 5·6 μmoles. All vessels were preincubated for 5 min. to permit temperature equilibration. In one vessel the reaction was then stopped by tipping 2 N-HCl from the side arm, and the content of metabolites in this vessel served as the control. The infusion of α-oxo[1-^{14}C]glutarate (6·2 μc/m-mole) was started after 5 min. preincubation in the vessels 1–4 at a rate of 1·12 μmoles/3 min. One vessel was incubated for 12 min. without added α-oxo[1-^{14}C]glutarate. The protein content of the homogenate was 47·4 mg./vessel and the dry wt. 70 mg./vessel. The α-oxo[1-^{14}C]glutarate contained an impurity yielding $^{14}CO_2$ with ceric sulphate, and therefore the specific activity of the former was estimated by the 2,4-dinitrophenylhydrazone technique.

Vessel no.	1	2	3	4	5
α-Oxo[1-^{14}C]glutarate infused (μmoles/vessel)	1·12	2·24	3·36	4·48	0·0
Incubation time (min.)	3	6	9	12	12
Final concn. of α-oxoglutarate (mM)	0·34	0·56	0·79	0·92	0·09
Metabolic changes (μmoles):					
O_2	−2·65	−5·66	−8·97	−12·15	−12·74
$^{14}CO_2$	+0·12	+0·25	+0·46	+0·64	0·0
Glutamate	−2·69	−6·26	−8·11	−11·75	−11·33
Aspartate	+2·16	+4·34	+6·81	+6·81	+8·52
α-Oxoglutarate	−0·16	−0·40	−0·59	−1·19	+0·38
Rel. sp. activity of residual [1-^{14}C]substrates:					
[1-^{14}C]Glutamate (μmoles)	1·05	1·92	2·97	3·62	
Total glutamate (μmoles)	43·61	40·04	38·19	34·55	
∴ Rel. sp. activity of [1-^{14}C]glutamate	0·025	0·051	0·08	0·109	
Rel. sp. activity of α-oxo[1-^{14}C]glutarate	—	—	0·103	0·098	
Recovery of ^{14}C (%)	—	—	114	106	

the glutamate and α-oxo[1-^{14}C]glutarate added to vessel 1 (Table 5) would have given a relative specific activity for glutamate of 0·2, in vessel 2, 0·33 etc.

The results of an experiment in which α-oxo[1-^{14}C]-glutarate was infused for different periods of time from 3 to 12 min. (Table 6) indicate that the final specific activities of α-oxoglutarate and glutamate are approximately equal. Under the experimental conditions less than one-fifth of the ^{14}C infused was converted into $^{14}CO_2$, most being trapped in the glutamate pool. These experiments provide further evidence that the rate of isotopic exchange considerably exceeds the rates of net metabolism of α-oxoglutarate and glutamate.

Table 7. *Effects of pyruvate on the metabolism of* [1-^{14}C]*glutamate and α-oxo*[1-^{14}C]*glutarate*

The results refer to 4 ml. of mitochondrial suspension incubated at 37·5° for 50 min. The dry wt. of mitochondrial fraction was 12·4 mg./vessel. Initial substrate concentrations were approx. 10 mM. The specific activity of [1-^{14}C]glutamate was 10 μc/m-mole (39·6 μmoles added) and that of α-oxoglutarate was 6 μc/m-mole (43·7 μmoles added); 39·0 μmoles of pyruvate were added.

Vessel no.	1	2	3	4
Substrate(s) added ...	...[1-^{14}C]Glutamate	[1-^{14}C]Glutamate + pyruvate	α-Oxo[1-^{14}C]-glutarate	α-Oxo[1-^{14}C]glutarate + pyruvate
Metabolic changes (μmoles):				
O_2	−18·5	−20·8	−18·9	−22·2
$^{14}CO_2$	+12·0	+7·4	+18·1	+11·4
Glutamate	−16·4	−4·1	0·0	+1·6
α-Oxoglutarate	0·0	+1·3	−25·4	−14·2
Pyruvate	0·0	−12·4	+0·4	−11·2
Rel. sp. activity of residual [1-^{14}C]glutamate	0·96	0·79	—	—

Effects on [1-^{14}C]*glutamate metabolism of α-oxoglutarate formed through the tricarboxylic acid cycle from pyruvate.* The addition of pyruvate to a brain mitochondrial fraction containing the same concentration of [1-^{14}C]glutamate caused decreases in the formation of $^{14}CO_2$ (−38%) and in the removal of glutamate (−75%) (Table 7, vessels 1 and 2). In the presence of both substrates the yield of $^{14}CO_2$ was 80% greater than the removal of glutamate and, as expected from this observation, the specific activity of the residual [1-^{14}C]glutamate was decreased by the addition of pyruvate. These results parallel closely the effects of added unlabelled α-oxoglutarate on [1-^{14}C]glutamate metabolism (Table 1), and can be explained by the formation of α-oxoglutarate from pyruvate through the tricarboxylic acid cycle. If the whole of the pyruvate removed had equilibrated with the [1-^{14}C]glutamate present in the incubation mixture the final relative specific activity of the glutamate would have been 0·75. This is so close to the value actually obtained that almost complete equilibration must have occurred between the added [1-^{14}C]glutamate and the unlabelled α-oxoglutarate formed.

The decrease in the removal of glutamate in the presence of pyruvate results from a decrease in the transamination reaction with oxaloacetate (Haslam & Krebs, 1963*b*), probably caused by an alteration of the relative concentrations of oxaloacetate and α-oxoglutarate. Pyruvate causes an inhibition of the oxidation of α-oxoglutarate (Balázs & Richter, 1962; Haslam & Krebs, 1963*a*), as is also indicated by the decreased formation of $^{14}CO_2$ from α-oxo-[1-^{14}C]glutarate in vessel 4 of Table 7 relative to vessel 3. This may account for the increased concentration of α-oxoglutarate found in the presence of glutamate and pyruvate. Such a decrease in the oxidation of α-oxoglutarate and an increased utilization of oxaloacetate for citrate synthesis may also be expected to decrease the concentration of oxaloacetate in the presence of pyruvate.

DISCUSSION

The results presented show that in both brain and liver mitochondrial preparations exchange of ^{14}C occurs between α-oxoglutarate and glutamate several times more rapidly than the rate at which the intermediates of the tricarboxylic acid cycle are oxidized (Table 4). It is therefore to be expected that in both brain and liver α-oxoglutarate formed through the tricarboxylic acid cycle will equilibrate with any glutamate that is in the same metabolic compartment. In the present work this has been shown to occur with α-oxoglutarate derived from pyruvate in a brain mitochondrial system, so confirming results previously obtained with brain homogenates and slices (Haslam & Krebs, 1963*b*). The incorporation *in vivo* and *in vitro* of ^{14}C into glutamate from labelled glucose or pyruvate by isotope exchange must depend in the first instance on the activity of the enzyme systems catalysing the exchange, the size of the glutamate pool and the rate of formation of labelled α-oxoglutarate. If incorporation of radioactivity into glutamate under physiological conditions is by isotope exchange the differences between the tissues must be explained by differences in these three factors, which are considered individually below. These factors have been briefly discussed by Haslam & Krebs (1963*b*) and are now elaborated in greater detail in relation to the results obtained.

Rate and nature of exchange of ^{14}C. Work on purified aminotransferases has demonstrated that reaction (1) may occur between a single substrate pair participating in a transamination reaction (Nisonoff *et al.* 1954; Jenkins & Sizer, 1959; Velick & Vavra, 1962). Thus exchange between α-oxoglutarate and

Table 8. *Factors affecting the fate of* ^{14}C *from* [^{14}C]*glucose*

	Brain	Liver	References and comments
1. Aspartate 1-aminotransferase activity* (μmoles/g. wet wt./min.):			
(*a*) Aspartate + α-oxoglutarate → oxaloacetate + glutamate	97	134	25°
(*b*) Glutamate + oxaloacetate → α-oxoglutarate + aspartate	85	79	25°
2. Glutamate pool size (μmoles/g. wet wt.)	10	3	Schwerin, Bessman & Waelsch (1950)
3. Rate of formation of labelled α-oxoglutarate:			
(*a*) Glucose pool size (μmoles/g. wet wt.)	0·45	4·6	Gey (1956)
(*b*) Rate of glucose catabolism (μmoles/g. wet wt./hr.) (glucose → pyruvate)	17	7	McIlwain (1959); *in vivo* Ashmore, Cahill, Hastings & Zottu (1957); liver slices
(*c*) Alternative substrates	Insignificant	Lactate, fatty acids, amino acids	*in vivo*
(*d*) Competition between oxidation of α-oxo acids	Pyruvate preferentially oxidized	α-Oxoglutarate preferentially oxidized	Balázs & Richter (1962) Haslam & Krebs (1963*a*) Balázs, Magyar & Richter (1962)
4. Formation of labelled glutamate without exchange of ^{14}C:			
(*a*) γ-Aminobutyrate-aminotransferase activity (μmole/g. wet wt./min.)	0·49	Rate about 60% of that observed in brain	Baxter & Roberts (1961); 37° Roberts & Bregoff (1953)
(*b*) Glutamate-decarboxylase activity (μmole/g. wet wt./min.)	0·45	None	Baxter & Roberts (1961); 37°
(*c*) Glutamate-dehydrogenase activity* (μmole/g. wet wt./min.):			
(i) α-Oxoglutarate + NH_3 + NADH + H^+ → glutamate + NAD^+ + H_2O	38	218	25°
(ii) Glutamate + NAD^+ + H_2O → α-oxoglutarate + NH_3 + NADH + H^+	3·3	20·2	25°

* The total activities were measured by disrupting the structure of the subcellular particles with 0·1% Triton X-100.

glutamate is catalysed additively by all the aminotransferases. Aspartate aminotransferase in particular is very active in most tissues, including brain (see Table 8). High rates of exchange between α-oxoglutarate and glutamate are thus to be expected in most organs, and the present results confirm that this is the case in brain and liver. Under these circumstances the rate of incorporation of ^{14}C into glutamate cannot be limited by the rate of exchange transamination. Where, however, aminotransferase activities are similar to or smaller than the rates of turnover of the particular α-oxo acids, the former could limit the rate and extent of isotope incorporation into amino acids.

Effect of glutamate pool size. The present experiments utilizing the continuous-infusion technique indicate that increasing the size of the glutamate pool in brain homogenate increases the incorporation of ^{14}C into glutamate, even when the α-oxo-[1-^{14}C]glutarate pool is small and turning over rapidly. A similar effect of glutamate pool size has been demonstrated in heart homogenate and kidney slices (Busch & Baltrusch, 1955; Busch, Goldberg & Anderson, 1956) in the retention of ^{14}C from [1-^{14}C]-acetate and [2-^{14}C]pyruvate. The concentration of glutamate in rat brain (10 μmoles/g. fresh wt.) is higher than in other rat tissues measured (Schwerin, Bessman & Waelsch, 1950), so that when isotopic equilibrium is established the total ^{14}C incorporated can be greater than in other tissues. However, there are at least two distinct metabolic pools of glutamate in the brain (Berl, Lajtha & Waelsch, 1961; Cremer, 1964; Gaitonde, Dahl & Elliott, 1965). The various factors concerned in the incorporation of ^{14}C into glutamate will affect each pool separately.

Rate of formation of labelled α-oxoglutarate. In addition to the factors discussed above the amount of ^{14}C incorporated into glutamate will depend on the rate at which labelled α-oxoglutarate is formed through the tricarboxylic acid cycle. The final

specific activity of the glutamate will adjust to that of the α-oxoglutarate entering the same cell compartment. The experiments using the continuous-infusion technique illustrate both these points. Doubling of the rate of infusion of α-oxo[1-^{14}C]-glutarate almost doubled the rate of formation of labelled glutamate (Table 5), and during infusion of α-oxo[1-^{14}C]glutarate the relative specific activity of glutamate rose progressively (Table 6).

There are several factors that result in more ^{14}C from labelled glucose entering the tricarboxylic acid cycle in brain than in other tissues such as liver, with which a detailed comparison is made below (see Table 8). First, according to Gey (1956) the glucose pool of liver is at least ten times as large as that of brain, so that labelled glucose entering the liver will be diluted more than in brain before being metabolized. The very low value in brain indicates that glucose is metabolized as soon as it enters the tissue. Although adequate information on the rate of conversion of glucose into pyruvate is not available for the liver *in vivo*, it is clear from experiments *in vitro* that it is slower than in brain (Table 8), and this accords with what is known of the overall metabolic properties of the two tissues. Under many conditions glucose synthesis will be the dominant process in liver, which, unlike the brain *in vivo*, can utilize alternative substrates. Thus lactate can enter liver metabolism from the circulation and can therefore dilute labelled pyruvate formed from glucose, whereas in the brain labelled pyruvate can be diluted by only the relatively small endogenous lactate pool (about 2 μmoles/g.; McIlwain, 1959).

Busch *et al.* (1960) found that 1 min. after the injection of [1-^{14}C]glucose into rats the lactate pool in brain contained 72% of the total ^{14}C in compounds other than glucose, and that after 15 min. most of the ^{14}C has been transferred into amino acids, especially glutamate. Although in brain very little ^{14}C was retained in glucose, in the liver much of the ^{14}C remained in the glucose pool and more ^{14}C was found in lactate than in the free amino acid pools for some time after the injection. Similar results were obtained by Vrba *et al.* (1962) and Gaitonde, Marchi & Richter (1964).

Dilution of material from labelled glucose is also likely to be greater in liver than in brain at the acetyl-CoA level as a result of fatty acid oxidation. It may be concluded that, as a result of repeated dilution at the levels of glucose, pyruvate and acetyl-CoA, the specific activity of material entering the tricarboxylic acid cycle will be considerably lower in liver than in brain, so that more labelled α-oxoglutarate will be formed in the latter tissue.

Another factor to be considered is the existence of a competition between the oxidations of pyruvate and α-oxoglutarate, which occurs in both tissues (Balázs & Richter, 1962; Balázs *et al.* 1962; Haslam & Krebs, 1963*a*). In liver α-oxoglutarate is oxidized preferentially, but in brain pyruvate inhibits the oxidation of α-oxoglutarate. This will facilitate the conversion of labelled pyruvate into α-oxoglutarate in brain relative to liver and may be a factor responsible for the larger glutamate pool size in brain.

Formation of labelled glutamate without isotope exchange. To the extent to which there is in brain a bypass of the α-oxoglutarate to succinate section of the tricarboxylic acid cycle via glutamate, γ-aminobutyrate and succinic semialdehyde, there must be a true metabolic turnover of glutamate. The extent of this pathway is not established but, as the activity of γ-aminobutyrate aminotransferase is less than 1% of that of asparate aminotransferase (Table 8), the proportion of ^{14}C incorporated into glutamate by this route must be very small. Glutamate may also be synthesized by glutamate dehydrogenase, which has a fairly high potential activity in brain (though not as high as in liver; see Table 8). However, this reaction is limited by the availability of ammonia and reduced nicotinamide nucleotides, so that the net synthesis of glutamate by glutamate dehydrogenase is probably small under physiological conditions.

Significance of incorporation of ^{14}C into amino acid pools from carbohydrate precursors. The foregoing discussion has indicated that the incorporation of ^{14}C from labelled glucose into amino acids, in particular into glutamate, depends on a number of factors (Table 8). It follows that differences in this respect between the tissues have no single interpretation but require consideration of the fate of labelled glucose carbon in each tissue at each metabolic step between glucose and glutamate. The rapid and marked incorporation of ^{14}C into brain glutamate, reported both *in vivo* and *in vitro* by so many workers (e.g. Beloff-Chain *et al.* 1955; Busch *et al.* 1956, 1960; Vrba *et al.* 1962), reflects a combination of special features of brain metabolism largely unrelated to that of glutamate. What is more, as the labelling of glutamate can be accounted for by isotope exchange a role for glutamate in the pathway of glucose catabolism cannot be inferred from this evidence alone.

As a result of isotope exchange between the products of glucose metabolism and the free amino acid pools of brain, the formation of $^{14}CO_2$ from [^{14}C]glucose will not reflect the rate of glucose oxidation until isotopic equilibration has occurred. In cerebral-cortex slices this process may take about 3 hr. (Allweis, Gainer & Chaikoff, 1961). Similarly the formation of $^{14}CO_2$ from [^{14}C]glutamate does not provide a true index of glutamate oxidation when unlabelled material is simultaneously oxidized through the tricarboxylic acid cycle. Thus it has been inferred from the specific activity of respira-

tory carbon dioxide produced by rat and developing-rabbit cerebral-cortex slices incubated with [^{14}C]-glucose and unlabelled glutamate (or vice versa) that glutamate is oxidized preferentially to glucose (Chain, Cohen & Pocchiari, 1962; Swaiman, Milstein & Cohen, 1963). Measurements of the actual rate of glutamate (plus glutamine) removal in the presence and absence of glucose have shown that this is not the case (Haslam & Krebs, 1963*b*), and the present experiments show how the addition of unlabelled α-oxoglutarate to brain mitochondrial preparations may greatly increase the yield of $^{14}CO_2$ from [1-^{14}C]glutamate without causing any net loss of glutamate, when the overall transamination reaction with oxaloacetate is prevented.

The results thus illustrate the importance of combining measurements of net chemical change with information on the metabolic distribution of radioactive isotopes.

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