Compartmentation of Glutamate Metabolism in Brain

EVIDENCE FOR THE EXISTENCE OF TWO DIFFERENT TRICARBOXYLIC ACID CYCLES IN BRAIN

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1. ^{14}C from $[1-^{14}\text{C}]$ glucose injected intraperitoneally into mice is incorporated into glutamate, aspartate and glutamine in the brain to a much greater extent than ^{14}C from $[2-^{14}\text{C}]$ glucose. This difference for $[1-^{14}\text{C}]$ glucose and $[2-^{14}\text{C}]$ glucose increases with time. The amount of ^{14}C in C-1 of glutamate increases steadily with time with both precursors. It is suggested that a large part of the glutamate and aspartate pools in brain are in close contact with intermediates of a fast-turning tricarboxylic acid cycle. 2. ^{14}C from $[1-^{14}\text{C}]$ acetate and $[2-^{14}\text{C}]$ acetate is incorporated to a much larger extent into glutamine than into glutamate. An examination of the time-course of ^{14}C incorporated into glutamine and glutamate reveals that glutamine is not formed from the glutamate pool, labelled extensively by glucose, but from a small glutamate pool. This small glutamate pool is not derived from an intermediate of a fast-turning tricarboxylic acid cycle. 3. It is proposed that two different tricarboxylic acid cycles exist in brain.

After intracerebral injection of labelled glutamate the specific radioactivity of glutamine in the brain is found to be higher than that of glutamate for at least 30 min. (Berl, Lajtha & Waelsch, 1961). Intravenous injection of radioactive bicarbonate (Waelsch, Berl, Rossi, Clarke & Purpura, 1964) and leucine (Roberts & Morelos, 1965; Berl & Frigyesi, 1968; Patel & Balázs, 1969) leads to similar results. When $[\text{U}-^{14}\text{C}]$ glucose is used as a precursor, however, the specific radioactivity of glutamine is never observed to be higher than that of glutamate (Cremer, 1964; Gaitonde, Dahl & Elliot, 1965; C. J. Van den Berg & P. Mela, unpublished work). The pathway leading from leucine to glutamate-glutamine probably involves acetyl-CoA (see Meister, 1965), whereas glucose carbon atoms must pass through acetyl-CoA before entering the tricarboxylic acid cycle. Taken together, all the above results suggest that at least two metabolically different acetyl-CoA pools and two segregated, but presumably identical, pathways from acetyl-CoA and oxaloacetate to glutamate-glutamine occur in brain.

Since multiple turns of the tricarboxylic acid cycle lead to the preferential elimination of C-1 of acetyl-CoA as carbon dioxide, in comparison with

C-2 (Weinman, Strisower & Chaikoff, 1957), it is apparent that metabolites derived directly from tricarboxylic acid-cycle intermediates should be more highly labelled by $[2-^{14}\text{C}]$ acetyl-CoA than by $[1-^{14}\text{C}]$ acetyl-CoA. The ratio of ^{14}C incorporation from $[2-^{14}\text{C}]$ acetyl-CoA to ^{14}C incorporation from $[1-^{14}\text{C}]$ acetyl-CoA in a metabolite of acetyl-CoA is therefore dependent on the contribution of cycling in the tricarboxylic acid cycle to the labelling of this metabolite.

^{14}C from $[1-^{14}\text{C}]$ acetate is incorporated to a larger extent into glutamine than into glutamate (O'Neal & Koeppe, 1966). A comparison of the time-course of incorporation of ^{14}C from specifically labelled glucose with that from acetate should therefore give some information of the relation between the two glutamate pools and the tricarboxylic acid cycle. In this paper we report data on the incorporation of ^{14}C from $[2-^{14}\text{C}]$ glucose, $[1-^{14}\text{C}]$ glucose, $[1-^{14}\text{C}]$ acetate and $[2-^{14}\text{C}]$ acetate into glutamate, aspartate and glutamine in the mouse brain *in vivo*. The results obtained strongly support the hypothesis that two different tricarboxylic acid cycles exist in brain. This hypothesis has been suggested by Garfinkel (1966) on the basis of a computer analysis of literature data and by O'Neal & Koeppe (1966), who reported that acetate and some related compounds were preferentially incorporated into glutamine in brain. A preliminary note has been published (Van den Berg, Mela & Waelsch, 1966).

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MATERIALS AND METHODS

[1-¹⁴C]Glucose and [2-¹⁴C]glucose (specific radioactivity of each compound 3mc/m-mole) and sodium [1-¹⁴C]acetate and sodium [2-¹⁴C]acetate (specific radioactivity of each compound 2mc/m-mole) were obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.). Some experiments were done with [1-¹⁴C]glucose and [2-¹⁴C]glucose obtained from Nuclear-Chicago Corp. (Des Plaines, Ill., U.S.A.) and gave equivalent results.

Mice (Swiss Webster Albino, males, 24–26g. body wt.) were injected intraperitoneally with the labelled compounds. Fed animals were given 10 μ C of glucose or 15 μ C of acetate, and a group of starved animals (18hr. without food, resulting in a weight decrease from 25 to 21g.) received 5 μ C of glucose. The labelled substances were all dissolved in 0.2ml. of 0.9% NaCl. No carrier was added.

The animals were killed by immersion in liquid N₂. The brains were then removed, pulverized in solid CO₂ and extracted with 0.25M-trichloroacetic acid (1.5ml./300mg. of brain). Care was taken to keep the brain samples at solid-CO₂ temperature until the trichloroacetic acid was added. The samples were kept overnight in a refrigerator; 8ml. of water was added to each and the tubes were centrifuged. The precipitate was then washed twice with 8ml. of water. The combined extracts were neutralized with NaOH and fractionated on Dowex 1 (X4; acetate form; 200–400 mesh) (AG1-X4; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) as described by Cheng & Waelsh (1963) and Cheng & Mela (1966). The neutral fraction was hydrolysed with 2M-HCl (1hr. at 100°) and the glutamate formed from glutamine was isolated on Dowex 1. The neutral fraction obtained in this step was passed through a column of Dowex 50 (X4; 200–400 mesh) (AG 50W-X4; Bio-Rad Laboratories) to isolate glucose. The Dowex 50 was converted into the pyridinium form by washing first with 2M-HCl, then with 2M-pyridine and finally with 0.2M-pyridine-formate buffer, pH2.90. The neutral fraction from the Dowex 1 procedure was adjusted to pH2.90 with HCl and then applied to a column of Dowex 50. The first fraction eluted with the pyridine-formate buffer, pH2.90, contains glucose. The radiochemical purity of the fractions from several repre-

sentative experiments was confirmed by one-dimensional paper chromatography in three different solvent systems. The radiochemical purity of the isolated amino acids was greater than 95%, that of the glucose greater than 90%. The recovery of standard radioactive substances carried through the whole procedure was greater than 95%. Samples were counted at infinite thinness in a Nuclear-Chicago end-window counter, with an efficiency of about 30%. Glutamate and aspartate were determined by the method of Cheng & Mela (1966) and glucose by the method of Lowry, Passonneau, Hasselberger & Schulz (1964).

The results obtained were multiplied by appropriate factors, so that they correspond to a uniform injection of 10 μ C of glucose and 15 μ C of acetate in an animal of 25g. body wt. All values are expressed as c.p.m./ μ mole.

The amount of ¹⁴C in the C-1 of glutamate (and the glutamate obtained from glutamine) was determined with glutamate decarboxylase (from *Clostridium welchii*, Worthington Biochemical Corp., Freehold, N.J., U.S.A.). The enzyme was dissolved in a 0.2M-sodium acetate buffer, pH5. Enough enzyme was added to decarboxylate 49–50% of synthetic DL-[1-¹⁴C]glutamate (obtained from New England Nuclear Corp.). After an incubation of 1hr. at 37° the reaction was stopped with trichloroacetic acid and the amount of ¹⁴C remaining in the solution measured in a Packard liquid-scintillation counter, together with the appropriate controls.

In a number of experiments we measured the concentration of lactate in the brain samples by the method of Lowry *et al.* (1964). The lactate concentration was found to be 1.50 μ moles/g. wet wt. (s.d. 0.28; *n* 8) and the glucose concentration 2.01 μ moles/g. wet wt. (s.d. 0.42; *n* 11). These results indicate that post-mortem artifacts were minimal (Lowry *et al.* 1964).

RESULTS

Experiments with [1-¹⁴C]- and [2-¹⁴C]-glucose

Specific radioactivities of glucose, glutamate and aspartate. The rate of uptake of radioactive glucose in the brain is fast (Table 1). The specific radio-

Table 1. *Specific radioactivity of glucose and the specific radioactivity of glucose relative to the specific radioactivity of glutamate in the brain at various time-intervals after the injection of [1-¹⁴C]glucose and [2-¹⁴C]glucose*

Fed animals were injected with 10 μ C and starved animals with 5 μ C of the labelled compound. The specific radioactivity is expressed as c.p.m./ μ mole for an injected dose of 10 μ C of the labelled compound in an animal of 25g. body wt. Results for the fed animals are the means $\pm$ s.d. of four experiments.

Time (min.)	10 ⁻³ $\times$ Specific radioactivity of glucose (c.p.m./ μ mole)		Specific radioactivity of glucose/specific radioactivity of glutamate	
	[1- ¹⁴ C]Glucose	[2- ¹⁴ C]Glucose	[1- ¹⁴ C]Glucose	[2- ¹⁴ C]Glucose
Fed animals				
5	19.1 $\pm$ 3.3	17.5 $\pm$ 0.8	9.5	6.9
15	15.9 $\pm$ 5.0	29.0 $\pm$ 5.8	1.3	2.4
30	10.1 $\pm$ 3.1	23.3 $\pm$ 13.9	0.6	1.4
Starved animals				
5	—	42.4	—	9.6
15	49.7, 31.9	47.8, 48.9, 61.5	1.8	3.6
30	35.9, 27.2	37.8, 18.0, 17.3	1.0	1.5
45	19.3	14.7, 16.2	0.6	1.3

Table 2. *Specific radioactivity of glutamate and specific radioactivity of aspartate and glutamine relative to the specific radioactivity of glutamate in the brain after the injection of [1-¹⁴C]glucose and [2-¹⁴C]glucose*

Values for glutamate and aspartate in parentheses were obtained after the subtraction of the amount of ¹⁴C in C-1 of glutamate, as given in Table 5. The animals were starved overnight. Results are expressed as means $\pm$ s.d. of the numbers of experiments indicated, for an injected dose of 10 μ C of the labelled compound.

Time (min.)	Specific radioactivity of glutamate (c.p.m./ μ mole)	Relative specific radioactivity of aspartate	Relative specific radioactivity of glutamine	No. of expts.
Precursor: [1- ¹⁴ C]glucose				
5	2500; 9300	0.52, 0.59	0.37, 0.26	
15	20400 $\pm$ 4600	0.88 $\pm$ 0.04 (0.92)	0.50 $\pm$ 0.08	4
30	28700 $\pm$ 4200	0.92 $\pm$ 0.05 (1.01)	0.62 $\pm$ 0.04	4
45	25900	0.94	0.82	
Precursor: [2- ¹⁴ C]glucose				
5	4400	0.57	0.37	
15	14800 $\pm$ 3800 (12600)	0.85 $\pm$ 0.04 (1.01)	0.63 $\pm$ 0.16	3
30	15300 $\pm$ 4700 (11100)	0.80 $\pm$ 0.03 (1.09)	0.88 $\pm$ 0.19	3
45	12000, 12300	0.63, 0.66	0.83, 0.70	

activity of glucose in the brain of fed animals at 15 and 30 min. after the injection of [2-¹⁴C]glucose is higher than that in the brain of animals injected with [1-¹⁴C]glucose (Table 1). This difference between [2-¹⁴C]- and [1-¹⁴C]-glucose in the degree of labelling of glucose in the brain decreased when starved animals were used (Table 1).

The incorporation of radioactivity into glutamate from both precursors is rapid and extensive (Table 2). The ratio of the specific radioactivity of glucose to the specific radioactivity of glutamate decreases with the length of the experimental period with both precursors in the fed and starved animals (Table 1). This ratio decreases faster when [1-¹⁴C]glucose was the precursor than when [2-¹⁴C]glucose was the precursor. This faster decrease indicates that more radioactivity from [1-¹⁴C]glucose than from [2-¹⁴C]glucose accumulates in glutamate.

The results of the experiments with both precursors can be compared directly by calculating the ratio of the specific radioactivity of the amino acid after [1-¹⁴C]glucose to the specific radioactivity of the same amino acid after [2-¹⁴C]glucose ([1-¹⁴C]glucose/[2-¹⁴C]glucose labelling ratio). This ratio increases continuously with time for glutamate and aspartate in the starved animals (Table 3). The [1-¹⁴C]glucose/[2-¹⁴C]glucose labelling ratio for glutamate and aspartate increases only slightly in the fed animals. The much larger specific radioactivity of the glucose-precursor pool in the brain of animals injected with [2-¹⁴C]glucose as compared with the animals injected with [1-¹⁴C]glucose might explain the observation that the [1-¹⁴C]glucose/[2-¹⁴C]glucose labelling ratio for glutamate and aspartate does not increase very much with time in the fed animals. As the specific radioactivity of glucose in the brain of animals injected with [2-¹⁴C]-

glucose is approximately twice that in animals injected with [1-¹⁴C]glucose, the [1-¹⁴C]glucose/[2-¹⁴C]glucose labelling ratio for glutamate in the fed animals should be about 0.5 at 15 and 30 min., when equal amounts of [1-¹⁴C]glucose and [2-¹⁴C]glucose are incorporated into glutamate. The observed values of the [1-¹⁴C]glucose/[2-¹⁴C]glucose labelling ratio are 0.91 and 1.03 (Table 3).

Despite the considerable variation of the results and the differences in the extent of labelling of the glucose precursor pool in brain we may conclude that more radioactivity from [1-¹⁴C]glucose than from [2-¹⁴C]glucose accumulates in glutamate and aspartate in the brain. The increase in the [1-¹⁴C]glucose/[2-¹⁴C]glucose labelling ratio for glutamate and aspartate with time is therefore very likely a result of turning of the tricarboxylic acid cycle within the brain and not of processes occurring outside the brain.

Comparison of the specific-radioactivity curves for glucose and glutamate. The maximal specific radioactivity of glucose in the [2-¹⁴C]glucose experiments occurs at about 15 min. (Table 1). The carboxyl group of acetyl-CoA is the precursor of C-5 of α -oxoglutarate (and glutamate). The oxaloacetate formed from α -oxoglutarate is labelled in both carboxyl groups. After condensation of this oxaloacetate with acetyl-CoA, one carboxyl group is lost in the conversion of isocitrate into α -oxoglutarate; the other carboxyl group becomes C-1 of α -oxoglutarate (or glutamate). Thus: glucose C-2 $\rightarrow$ acetyl-CoA C-1 $\rightarrow$ α -oxoglutarate C-5 $\rightarrow$ oxaloacetate C-1 and C-4 $\rightarrow$ carbon dioxide and α -oxoglutarate C-1 $\rightarrow$ carbon dioxide.

The sequence of reactions for acetyl-CoA C-2 is very complicated and it is impossible to analyse these results properly in terms of precursor-

Table 3. $[1-^{14}\text{C}]\text{Glucose}/[2-^{14}\text{C}]\text{glucose}$ labelling ratio and $[2-^{14}\text{C}]\text{acetate}/[1-^{14}\text{C}]\text{acetate}$ labelling ratio of glutamate, aspartate and glutamine

Labelling ratios were calculated from the average specific radioactivities of the amino acids with each of the precursors. Values for glutamate in parentheses were obtained after subtraction of the amount of ^{14}C in C-1 of glutamate, as given in Table 5. Fed and starved animals were used for the glucose experiments and fed animals only for the acetate experiments.

Time (min.)	No. of expts.		$[1-^{14}\text{C}]\text{Glucose}/[2-^{14}\text{C}]\text{glucose}$ labelling ratio		
	$[1-^{14}\text{C}]\text{Glucose}$	$[2-^{14}\text{C}]\text{Glucose}$	Glutamate	Aspartate	Glutamine
Fed animals					
5	4	4	0.79	0.90	0.97
15	4	4	0.91	1.00	0.91
30	4	4	1.03	1.37	1.13
Starved animals					
5	2	1	1.34	1.27	1.25
15	4	3	1.38 (1.54)	1.41	1.15
30	4	3	1.87 (2.32)	2.16	1.35
45	1	2	2.14	3.12	2.29
Time (min.)			$[2-^{14}\text{C}]\text{Acetate}/[1-^{14}\text{C}]\text{acetate}$ labelling ratio		
	$[2-^{14}\text{C}]\text{Acetate}$	$[1-^{14}\text{C}]\text{Acetate}$			
5	3	3	1.53 (1.70)	1.46	1.11 (1.19)
10	1	1	1.71	1.94	1.29
15	3	3	1.95 (2.69)	2.27	0.90 (1.04)
20	1	1	2.56	4.37	1.23
25	1	1	3.15	5.41	1.36
30	3	3	3.47 (4.85)	4.30	1.66 (1.97)

product relations. A valid comparison of glucose with glutamate can thus only be made between the specific radioactivity of glucose and the specific radioactivity of C-5 of glutamate in the $[2-^{14}\text{C}]\text{glucose}$ experiments. Subtraction of the amount of ^{14}C in C-1 of glutamate gives a reasonable estimate of the specific radioactivity of C-5 of glutamate. The amount of ^{14}C in C-2, C-3 and C-4 of glutamate in the brains of rats injected with $[2-^{14}\text{C}]\text{glucose}$ is 8–15% (O'Neal & Koeppe, 1966). This amount is not large enough to affect appreciably the analysis of the results. The maximum specific radioactivity of C-5 of glutamate occurs at about 15 min. (Table 2). The glucose and glutamate maxima are thus very close. The maximal specific radioactivity of C-5 of glutamate is much lower than the maximal specific radioactivity of glucose. It should be realised that the specific radioactivity of acetyl-CoA will be half the specific radioactivity of glucose from which this acetyl-CoA is formed, as two molecules of acetyl-CoA are formed from one molecule of glucose. In the starved animals the maximal specific radioactivity of acetyl-CoA entering the tricarboxylic acid cycle is therefore about 26 000 c.p.m./ μmole (Table 1), which is about twice the maximal specific radioactivity of C-5 of glutamate (Table 2). It follows directly from these results that more than half of the glutamate pool is labelled by glucose. This is a minimal estimate, as glucose is not the immediate precursor of glutamate. The maximal specific radioactivity of glutamate in the $[1-^{14}\text{C}]\text{glucose}$

glucose experiments is even higher than the calculated maximal specific radioactivity of the acetyl-CoA, showing in a dramatic way that ^{14}C accumulates in glutamate as a consequence of the turning of the tricarboxylic acid cycle and that a proper analysis of these data is very complicated.

Percentage of ^{14}C in C-1 of glutamate and glutamine. There is always a larger amount of ^{14}C in C-1 of glutamate isolated from the brain of animals injected with $[2-^{14}\text{C}]\text{glucose}$ than in that from the brain of animals injected with $[1-^{14}\text{C}]\text{glucose}$ (Table 5). The rate of glucose utilization in the brain of adult mice is 0.76 $\mu\text{mole/g. wet wt./min.}$ (Lowry *et al.* 1964). The amount of acetyl-CoA entering the tricarboxylic acid cycle is twice this amount or 1.52 $\mu\text{moles/g. wet wt./min.}$ The combined concentration of tricarboxylic acid-cycle intermediates in mouse brain is about 1.7 $\mu\text{moles/g. wet wt.}$ (Goldberg, Passonneau & Lowry, 1966). The amount of tricarboxylic acid-cycle intermediates relative to the rate of acetyl-CoA entering the cycle is therefore not very large. One would therefore expect almost equal fractions of an intermediate in the first, second, third and following turns of the cycle after the entrance of acetyl-CoA.

In the first turn of the cycle after the entrance of $[1-^{14}\text{C}]\text{acetyl-CoA}$ all the radioactivity present in $\alpha\text{-oxoglutarate}$ (glutamate) is located in C-5 of $\alpha\text{-oxoglutarate}$. In the second turn 50% of the ^{14}C is lost and 50% is present in C-1 of $\alpha\text{-oxoglutarate}$ (glutamate). Of the radioactivity present in

α -oxoglutarate 33.3% should therefore be in C-1 of α -oxoglutarate when $[2-^{14}\text{C}]\text{glucose}$ is used as a precursor. When intermediates of the tricarboxylic acid cycle are in rapid equilibrium with compounds related to these intermediates, the pool of intermediates present (pool of carbon compounds through which acetyl-CoA carbon atoms have to move before conversion into carbon dioxide) is much larger. This slows down the rate of movement of carbon atoms, entering as acetyl-CoA, from the first to the second cycle, resulting in a time-dependent increase in the amount of ^{14}C in C-1 of α -oxoglutarate. Dilution of intermediates of the cycle by compounds related to these intermediates will therefore result in a time-dependent increase of the amount of radioactivity in C-1 of α -oxoglutarate. The time-course of the increase in amount of radioactivity is, of course, dependent on the time-course of the specific radioactivity of the precursor entering the cycle and the rate of equilibrium between cycle intermediates and the compounds related to them. If equilibrium is fast, then it is clear that the value of 33.3% of the radioactivity in C-1 of α -oxoglutarate will be reached when the specific radioactivity of the precursor entering the cycle (acetyl-CoA) starts to decrease. The value of 33.3% will be lower when there is carbon dioxide fixation at the pyruvate level in the brain. Carbon dioxide fixation has been shown to occur in the brain by Berl, Takagaki, Clarké & Waelsch (1962*a,b*). O'Neal & Koeppe (1966) found indeed 8–15% ^{14}C in C-2, C-3 and C-4 of glutamate isolated from the brains of rats injected with $[2-^{14}\text{C}]\text{glucose}$. The theoretical value of 33.3% of ^{14}C in C-1 of α -oxoglutarate or glutamate will therefore be actually a little lower, say about 30%.

We have no information on the time-course of the specific radioactivity of acetyl-CoA, but the time-course of the specific radioactivity of glucose is probably almost the same. In a number of experi-

ments (results not shown) we found that the specific radioactivity of lactate in the brain was always about 50% of the specific radioactivity of glucose, indicating that the assumption with regard to acetyl-CoA is valid. The specific radioactivity of glucose in the $[2-^{14}\text{C}]\text{glucose}$ experiments starts to decrease between 15 and 30 min. (Table 1). The amount of radioactivity in C-1 of glutamate is about 30% at 30 min. after the injection of $[2-^{14}\text{C}]\text{glucose}$ (Table 5). The results reported as analysed in this section strongly suggest therefore that glutamate and other compounds related to tricarboxylic acid-cycle intermediates are in a very rapid equilibrium with intermediates of the cycle. A rapid equilibrium of glutamate and α -oxoglutarate *in vitro* has been found by Balázs & Haslam (1965).

Comparison of results for glutamate and aspartate. The rate of incorporation of ^{14}C into aspartate is somewhat lower than the corresponding rate for glutamate (Fig. 1 and Table 2). The rapid equilibrium of labelled α -oxoglutarate and glutamate mentioned before offers itself as a reasonable explanation. Dilution by other compounds related to tricarboxylic acid-cycle intermediates might conceivably occur as well.

The relative specific radioactivity of aspartate is about 0.9 for both precursors at 15 and 30 min. It is better to compare the specific radioactivity of aspartate with the specific radioactivity of C-2, C-3, C-4 and C-5 of glutamate. The amount of ^{14}C in C-2, C-3, C-4 and C-5 of glutamate is a reasonable estimate of the amount of ^{14}C in C-5, as it has been shown that the amount of ^{14}C in C-2, C-3 and C-4 is 8–15% (O'Neal & Koeppe, 1966). The specific radioactivity of aspartate, relative to the specific radioactivity of C-2, C-3, C-4 and C-5 of glutamate is about 1.0 (Table 2). In the experiments with $[2-^{14}\text{C}]\text{glucose}$ it is also possible to compare the specific radioactivity of aspartate with the specific radioactivity of the C-1, C-2 and C-3 of glutamate,

Fig. 1. Time-course of the specific radioactivities of (a) aspartate (○, ●), (b) glutamate (□, ■) and glutamine (△, ▲), in the brain of starved mice, injected intraperitoneally with $[2-^{14}\text{C}]\text{glucose}$ (■, ●, ▲) or $[1-^{14}\text{C}]\text{glucose}$ (□, ○, △). Vertical bars indicate the standard deviations.

multiplied by 2, because one carboxyl group of oxaloacetate (or aspartate) is lost in the conversion of isocitrate into α -oxoglutarate (or glutamate). The specific radioactivity of aspartate is about three times the calculated specific radioactivity of the C-1, C-2 and C-3 of glutamate at 15 min. This value is, however, decreased to 1.4 at 30 min. Complete isotopic equilibrium between glutamate and aspartate and between aspartate and glutamate would, if the complete glutamate and aspartate pools are involved, lead to values of about 1.

The $[1-^{14}\text{C}]\text{glucose}/[2-^{14}\text{C}]\text{glucose}$ labelling ratio for aspartate increases faster with time than the same ratio for glutamate (Table 3). But again we should compare C-2, C-3, C-4 and C-5 of glutamate with aspartate. The ratio for C-2, C-3, C-4 and C-5 of glutamate increases now a little faster than the ratio for aspartate (Table 3).

Glutamine. The specific radioactivity of glutamine increases with time, but more slowly than the specific radioactivity of glutamate (Fig. 1 and Table 2). The $[1-^{14}\text{C}]\text{glucose}/[2-^{14}\text{C}]\text{glucose}$ labelling ratio does not increase very much between 5 and 30 min. (Table 3). This latter finding suggests that isotopic equilibrium between glutamate and glutamine is less rapidly established than between glutamate and α -oxoglutarate.

Experiments with $[1-^{14}\text{C}]\text{acetate}$ and $[2-^{14}\text{C}]\text{acetate}$

Rate of labelling of glutamate, aspartate and glutamine. $[1-^{14}\text{C}]\text{Acetate}$ corresponds to $[2-^{14}\text{C}]\text{glucose}$ and $[2-^{14}\text{C}]\text{acetate}$ corresponds to $[1-^{14}\text{C}]\text{glucose}$ with respect to the labelling of the carboxyl

group and methyl group respectively of acetyl-CoA. The specific radioactivity of glutamate is highest at between 5 and 15 min. after the introduction of $[1-^{14}\text{C}]\text{acetate}$ into the animal (Fig. 2 and Table 4). The maximum is reached later in the $[2-^{14}\text{C}]\text{acetate}$ experiments. Aspartate follows glutamate closely. Glutamine is exceptional, a maximum being reached at about 5 min. with both precursors. The specific-radioactivity curves of glutamine for both precursors are almost identical. This is not so with glutamate and aspartate. A striking difference between the glucose and the acetate experiments is that the specific radioactivity of glutamine is much higher than the specific radioactivity of either glutamate or aspartate, with acetate as a precursor, whereas the specific radioactivity of glutamine is always lower than the specific radioactivity of glutamate and aspartate with glucose as a precursor. Acetate is therefore a specific precursor of glutamine and of the glutamate pool labelled by bicarbonate, leucine and other compounds.

The rapid incorporation of ^{14}C from $[^{14}\text{C}]\text{acetate}$ is not surprising in view of the fact that the acetate pool in brain is probably very small. Busch & Baltrush (1954) found, a few minutes after the injection of labelled acetate, almost no radioactivity in the volatile acid fraction from the brain; most of the radioactivity was already in non-volatile acid compounds, mostly glutamate and aspartate. We also found ^{14}C in glucose in the brain, but the specific radioactivity of glucose in the first 15 min. was much lower than the specific radioactivity of the amino acids (results not shown). At 30 min.,

Fig. 2. Time-course of the specific radioactivities of (a) aspartate (○, ●), (b) glutamate (□, ■) and glutamine (△, ▲), in the brain of mice, injected intraperitoneally with $[1-^{14}\text{C}]\text{acetate}$ (▲, ●, ■) or $[2-^{14}\text{C}]\text{acetate}$ (○, △, □).

Table 4. *Specific radioactivity of glutamate and the relative specific radioactivity of aspartate and glutamine in the brain of mice at various time-intervals after the intraperitoneal injection of 15 μ C of [1- 14 C]acetate and [2- 14 C]acetate*

Values in parentheses for the relative specific radioactivity of aspartate were obtained after the subtraction of the amount of 14 C in C-1 of glutamate, as given in Table 5. Results are expressed as means of the numbers of experiments indicated.

Time (min.)	Specific radioactivity of glutamate (c.p.m./ μ mole)	Relative specific radioactivity of aspartate	Relative specific radioactivity of glutamine	No. of expts.
Precursor: [1- 14 C]acetate				
5	1779	0.46 (0.64)	4.81	3
10	2310	0.57	3.00	1
15	1946	0.59 (0.89)	3.27	3
20	1721	0.45	2.85	1
25	1283	0.40	2.92	1
30	1339	0.47 (0.89)	2.45	3
Precursor: [2- 14 C]acetate				
5	2726	0.44 (0.46)	3.55	3
10	3960	0.64	2.25	1
15	3800	0.70 (0.75)	1.46	3
20	4398	0.78	1.37	1
25	4039	0.68	1.26	1
30	4647	0.70 (0.79)	1.17	3

however, the specific radioactivity of glucose, especially in the experiments with [2- 14 C]acetate, is of the same order of magnitude as the specific radioactivity of the amino acids. Although we have not proved that acetate enters the brain as such, we may safely assume that most of the 14 C enters the brain in the first 15 min., in the form of acetate, and that acetate is converted into acetyl-CoA in the brain. Acetyl-CoA synthetase is present in the brain (Schuberth, 1965).

Comparison of results for [1- 14 C]acetate and [2- 14 C]acetate. The ratio of the specific radioactivity of each of the amino acids after [2- 14 C]acetate to the specific activity after [1- 14 C]acetate is given in Table 3. This [2- 14 C]acetate/[1- 14 C]acetate labelling ratio increases rapidly for glutamate and aspartate, whereas it stays constant for almost 20 min. for glutamine. Radioactivity from [1- 14 C]acetate and [2- 14 C]acetate is thus incorporated into glutamine in about the same ratio, or the glutamate precursor pool of glutamine is not formed from an intermediate of a rapidly turning tricarboxylic acid cycle. The glutamate labelled by acetate is certainly derived from an intermediate of a rapidly turning cycle. This follows directly from the increase of the [2- 14 C]acetate/[1- 14 C]acetate labelling ratio for glutamate. The immediate precursor of glutamine is supposed to be glutamate, but the glutamate that is labelled by acetate does not seem to be the precursor of glutamine. It is thus probable that the glutamate precursor pool of glutamine is very small.

Percentage of 14 C in C-1 of glutamate and glutamine. If it is true that glutamine is derived from an

intermediate of a slowly turning cycle, then we should expect a small amount of 14 C in C-1 of glutamine. As is shown in Table 5, the percentage of 14 C in the C-1 of glutamine is indeed much lower than the corresponding value for glutamate. However, it is clear that there is an appreciable incorporation of 14 C into C-1 of glutamine. Some glutamine is probably derived from the glutamate labelled after acetate. This explains partly the incorporation of 14 C into C-1 of glutamine.

Comparison of results for glutamate and aspartate. The rates of labelling of glutamate and aspartate are almost the same (Fig. 2 and Table 4). The [2- 14 C]acetate/[1- 14 C]acetate labelling ratio for aspartate increases with time somewhat faster than the ratio for glutamate (Table 3). As with glucose this situation is reversed when the [2- 14 C]acetate/[1- 14 C]acetate labelling ratio for aspartate is compared with this ratio for C-2, C-3, C-4 and C-5 of glutamate (Table 3). The relative specific radioactivity of aspartate remains always lower than 1.0 (Table 4), even when the specific radioactivity of aspartate is compared with the specific radioactivity of C-2, C-3, C-4 and C-5 of glutamate (Table 4). Aspartate or oxaloacetate derives carbon atoms from tricarboxylic acid-cycle intermediates, but also directly from pyruvate. In unpublished experiments we found an appreciable incorporation of 14 C from [3,4- 14 C₂]glucose into aspartate (approximately 10% of the incorporation of 14 C from [2- 14 C]glucose into aspartate). There is, of course, no direct pathway from acetate to aspartate. This then might explain the difference in the relative

Table 5. *Percentage of the amount of ^{14}C present in C-1 of glutamate and glutamine isolated from the brain of animals injected with $[1\text{-}^{14}\text{C}]$ - and $[2\text{-}^{14}\text{C}]$ -glucose or $[2\text{-}^{14}\text{C}]$ - and $[1\text{-}^{14}\text{C}]$ -acetate*

Results are expressed as means of the numbers of experiments indicated in parentheses.

Time (min.)	$[1\text{-}^{14}\text{C}]\text{Glucose}$				$[2\text{-}^{14}\text{C}]\text{Glucose}$			
	^{14}C in C-1 of glutamate (%)		^{14}C in C-1 of glutamine (%)		^{14}C in C-1 of glutamate (%)		^{14}C in C-1 of glutamine (%)	
	Fed	Starved	Fed	Starved	Fed	Starved	Fed	Starved
5	—	—	—	—	2.0 (1)	9.4 (1)	7.2 (1)	2.6 (1)
15	3.7 (1)	5.0 (2)	6.1 (1)	5.7 (2)	18.2 (1)	14.9 (3)	18.6 (2)	15.0 (3)
30	10.3 (2)	10.0 (2)	8.3 (2)	10.3 (2)	24.6 (2)	27.3 (3)	20.6 (2)	19.5 (3)
45	—	—	—	—	—	33.3 (1)	—	25.5 (1)

	$[2\text{-}^{14}\text{C}]\text{Acetate}$		$[1\text{-}^{14}\text{C}]\text{Acetate}$	
	^{14}C in C-1 of glutamate (%)		^{14}C in C-1 of glutamine (%)	
5	4.6 (1)	1.9 (1)	14.1 (1)	7.6 (2)
15	7.9 (1)	4.1 (1)	33.1 (2)	18.7 (2)
30	13.7 (1)	14.6 (1)	38.3 (3)	27.9 (2)

specific radioactivity for aspartate in the glucose and the acetate experiments.

Conversion of glutamine into glutamate. In the $[1\text{-}^{14}\text{C}]\text{acetate}$ experiments the specific radioactivity of glutamate is always lower than the specific radioactivity of glutamine, even in the decreasing part of the curve. When one compares the total amount of ^{14}C present in both amino acids, a different picture emerges: the total amount of ^{14}C in glutamate is higher than the total amount of ^{14}C in glutamine between 15 and 30 min. after the injection of labelled acetate. It is thus possible that glutamine is a precursor of glutamate. The total contribution of glutamine to glutamate is not very large; this follows from the low specific radioactivity of glutamate. The larger amount of ^{14}C in C-1 of glutamate than in C-1 of glutamine is easily explained by the effect on the large glutamate pool of turning of the tricarboxylic acid cycle. The relatively high labelling of aspartate is consistent with this explanation. $[^{14}\text{C}]\text{Glutamine}$ that is injected directly into the brain is in fact converted into $[^{14}\text{C}]\text{glutamate}$ at an appreciable rate (Berl *et al.* 1961).

DISCUSSION

Two glutamate pools: two-cycle model. Two glutamate pools (a large and a small one) exist in brain. The results obtained with $[1\text{-}^{14}\text{C}]\text{glucose}$ and $[2\text{-}^{14}\text{C}]\text{glucose}$ further suggest that the large glutamate pool is in close contact with α -oxoglutarate from a rapidly turning tricarboxylic acid cycle. The acetate results on the other hand

suggest that the small pool has a different relation to the tricarboxylic acid cycle.

We may conclude that glucose and acetate are not the precursors of the same homogeneous acetyl-CoA pool in brain. The assumption is made, of course, that all the glucose and acetate carbon atoms pass through acetyl-CoA before being incorporated into glutamate, aspartate and glutamine. Compartmentation of acetyl-CoA with respect to the tricarboxylic acid cycle and the synthesis of acetylcholine has been postulated by Cheng, Nakamura & Waelsch (1967).

Leucine (Roberts & Morelos, 1965; Berl & Frigyesi, 1968), butyrate (O'Neal & Koeppe, 1966) and acetate (O'Neal & Koeppe, 1966) are effective precursors of the small glutamate pool and therefore of glutamine. These compounds are precursors of acetyl-CoA. Bicarbonate (Waelsch *et al.* 1964), aspartate (Berl *et al.* 1961) and propionate (O'Neal, Koeppe & Williams, 1966; C. J. Van den Berg & P. Mela, unpublished work) are also effective precursors of the small glutamate pool. These substances are all precursors of C_4 compounds and thus of oxaloacetate. The relative specific radioactivity of glutamine is about 2–4 with all these precursors, suggesting that they are precursors of the same small glutamate pool.

These results suggest that there are in brain two segregated, but presumably identical, pathways from acetyl-CoA and oxaloacetate to α -oxoglutarate and glutamate. We might therefore conclude that two different tricarboxylic acid cycles occur in the brain.

Berl *et al.* (1962a) have shown that the α -amino

group of glutamine incorporated more ^{15}N from [^{15}N]ammonium acetate than does the α -amino group of glutamate. This implicates a role for glutamate dehydrogenase in the formation of the small glutamate pool. The intracellular localization of glutamate dehydrogenase, as studied with sucrose density gradients of mitochondrial preparations from brain, is not identical with the localization of succinate dehydrogenase (Salganicoff & De Robertis, 1965; Neidle, Van den Berg & Grynbaum, 1969). Acetyl-CoA synthetase has almost the same intracellular localization as glutamate dehydrogenase (Neidle *et al.* 1969). It seems thus that the two tricarboxylic acid cycles are localized in physically distinct particles. Baxter (1968) found that γ -aminobutyrate is also a specific precursor of the small glutamate pool. γ -Aminobutyrate transaminase is not localized in the particles where most of the cytochrome oxidase is present (Van Kempen, Van den Berg, Van der Helm & Veldstra, 1965) but has almost the same intracellular localization as glutamate dehydrogenase (Salganicoff & De Robertis, 1965). This argues again for the hypothesis that two different tricarboxylic acid cycles are present in the brain.

Are both glutamate pools present in the same cell?

Different types of cells, neurones and glial cells, are present in the brain. The two tricarboxylic acid cycles might well occur in different cells. Glutamate dehydrogenase (Lowry, Roberts & Chang, 1956) and γ -aminobutyrate transaminase-succinate semialdehyde dehydrogenase (Van Gelder, 1965*a,b*) are present both in neurones and glial cells. If we take these enzymes as criteria for the presence of the small pool, then we can conclude that the small pool is not present in one cell type only. This follows also from the finding that both populations of mitochondria are present in cerebral cortex and in corpus callosum of the rat (Neidle *et al.* 1969). The argument is weak, however, since the presence of an enzyme at a certain site does not prove that it is operating, nor is there any proof that all the glutamate dehydrogenase has a function in the formation of the small glutamate pool.

Large glutamate and aspartate pools. A large fraction of the glutamate present in the brain is labelled by glucose, and most of the carbon atoms from glucose pass through the glutamate pool before being converted into carbon dioxide. The same is true for aspartate. The rapid equilibrium between glutamate, aspartate and tricarboxylic acid-cycle intermediates, and the time-course of labelling of the amino acids, all suggest that a large fraction of both amino acids are almost intermediates of the tricarboxylic acid cycle. The concentration of glutamate, aspartate, α -oxoglutarate and oxaloacetate are approximately those demanded for the equilibrium of glutamate-aspartate transaminase

(Goldberg *et al.* 1966). The α -amino group of glutamate is rapidly incorporated into aspartate, although complete equilibrium was not attained (Berl *et al.* 1962*a*). Rapid isotopic equilibrium between glutamate and α -oxoglutarate *in vitro* was found by Balázs & Haslam (1965). Lindsay & Bachelard (1966) found *in vivo* a higher specific radioactivity of α -oxoglutarate than glutamate in the first 5–10 min. after the injection of [$\text{U-}^{14}\text{C}$]glucose into rats. Gaitonde (1965), however, found a lower specific radioactivity of α -oxoglutarate than of glutamate. The specific radioactivity of α -oxoglutarate is always lower than the specific radioactivity of glutamate in experiments with labelled bicarbonate (Waelsch *et al.* 1964). The reported data for α -oxoglutarate are thus contradictory. In some experiments we isolated succinate; its specific radioactivity, from the succinate concentration as determined by Goldberg *et al.* (1966), was always of the same order of magnitude as the specific radioactivity of glutamate and aspartate.

The incorporation of ^{14}C from [$\text{U-}^{14}\text{C}$]glucose into glutamate and aspartate is decreased to almost the same extent by pentobarbitone sodium (Bachelard & Lindsay, 1966), Pentothal (C. J. Van den Berg, Lj. Kržalić, P. Mela & H. Waelsch, unpublished work) and γ -hydroxybutyrate (Godin, Mark & Mandel, 1968). The slowing down of the flow of carbon atoms through the tricarboxylic acid cycle thus affects the labelling of glutamate and aspartate in about the same way. This is what one would expect if a large fraction of glutamate and aspartate pools are in rapid equilibrium with tricarboxylic acid-cycle intermediates.

General remarks. The brain uses mainly glucose as its source of energy, most of which is used for the maintenance of its functional activity (McIlwain, 1966). The metabolic pathways leading to the production of energy are probably under direct control of this 'functional activity'. It would therefore not be surprising if the metabolism of compounds not directly involved in energy production were separated from the reaction pathways involved in energy production. Metabolic pathways used for biosynthetic reactions or for the removal of waste material would then be separated from those pathways that should be critically and highly controlled by the need of energy for the functional activity of the brain.

Compartments certainly exist in other organs as well. Liver mitochondria are probably heterogeneous (Swick, Stange, Nance & Thomson, 1967). Incorporation of radioactive bicarbonate into amino acids in liver suggests compartmentation of amino acid metabolism (Berl *et al.* 1962*a,b*). Fritz (1967) proposed a compartmentation of acetyl-CoA metabolism in liver; he assumes that these compartments are located in different spaces within

the mitochondrion. In brain it seems that metabolically different acetyl-CoA pools are located in different particles. Fritz (1967) postulated these compartments to account for peculiarities observed with the metabolism of fatty acids. Butyrate and acetate are lower fatty acids, and are also precursors of the small glutamate pool in brain.

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