Mechanism of Conversion of Aspartate into Glutamate in Cerebral-Cortex Slices

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1. When slices of guinea-pig cerebral-cortex slices are incubated with [U-¹⁴C]-aspartate and non-radioactive glucose as substrates, the specific radioactivities of the citric acid-cycle intermediates are lower than that of the glutamate isolated from the same vessels. 2. Glutamate was significantly labelled when [1-¹⁴C]-aspartate and glucose were present in the incubation medium. These results would not be expected on the basis of simple conversion of aspartate into glutamate through the citric acid cycle, since the C-1 position of oxaloacetate is decarboxylated in the conversion of isocitrate into α -oxoglutarate. 3. It appears that aspartate is converted into glutamate by citric acid-cycle mechanisms; however, the carbon 'skeleton' is not immediately condensed with acetyl-CoA to form citrate but first follows the cycle in a reverse direction to fumarate or succinate and then proceeds in the forward direction. 4. The conversion of aspartate into glutamate appears to be compartmentalized.

The interconversion of aspartate and glutamate has been considered to proceed by transamination reactions involving the citric acid cycle (Krebs, 1935; Krebs & Bellamy, 1960; Borst, 1962). This interpretation was based on investigations utilizing homogenates and mitochondrial preparations of various organs. Cohen, Simon, Berry & Chain (1962) presented some results that suggested the possibility of a non-citric acid-cycle conversion of glutamate into aspartate. Haslam & Krebs (1963) confirmed some of their findings but could not duplicate all of the work, and these latter workers considered that all the conversion could be accounted for by citric acid-cycle mechanisms. We considered that, as long as the possibility of a non-citric acid-cycle pathway had been raised for the conversion of glutamate into aspartate, the mechanism of conversion of aspartate into glutamate should be investigated thoroughly.

MATERIALS

Reagents. Inorganic salts and organic solvents were Fisher Scientific Co. (Pittsburgh, Pa., U.S.A.) certified reagent grade or equivalent.

Chloroform was washed three times with an equal volume of water and distilled over K₂CO₃. Ether was washed with acid FeSO₄ (100 ml. of 2M-FeSO₄ in 0.5N-HCl/l. of ether)

and distilled over NaOH pellets. Butanol was distilled over NaOH pellets.

Substrates. These were purchased from the following sources. Non-radioactive D-glucose and L-aspartic acid were A grade from Calbiochem (Los Angeles, Calif., U.S.A.). L-[U-¹⁴C]Aspartic acid and D-[U-¹⁴C]glucose were from Nuclear-Chicago Corp. (Des Plaines, Ill., U.S.A.); DL-[3-¹⁴C]aspartic acid and DL-[4-¹⁴C]aspartic acid were from Niche Inc. (Bethesda, Md., U.S.A.); DL-[1-¹⁴C]aspartic acid was from Chem Tracer Corp. (Cambridge, Mass., U.S.A.).

Specific activity of the radioactive chemicals at the time of purchase ranged from 2 to 21 mc/m-mole. All radioactive amino acids were purified as follows.

The amino acid was dissolved in 2.0 ml. of water and extracted three times with an equal volume of ether. The aqueous material was made acidic by the addition of 0.2 ml. of 5N-HCl, added to a Dowex 50 (X8; 200-400 mesh) column (1.0 cm. $\times$ 15.0 cm.) that had been equilibrated with 2N-HCl and the column washed with water until the effluent gave a negative test with acidic AgNO₃. A 0.5 column volume of water was passed through the column and the amino acid eluted with 2N-NH₃. The NH₃ was removed under vacuum and the residual radioactive amino acid was used in the experiments. No radioactive amino acid was used 6 weeks after purification without repurification.

Celite. Celite 535 (Johns-Manville and Co. Inc., New York, N.Y., U.S.A.) was prepared for use by mixing 500 g. with 5 l. of N-NaOH for 2 hr. The mixture was allowed to settle and the aqueous phase discarded. The precipitate was then rinsed with five 3 l. volumes of water. The final precipitate was suspended in 5 l. of N-HCl, mixed for 2 hr., allowed to settle and the aqueous phase discarded. The

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precipitate was rinsed with ten 3l. volumes of deionized water, allowing for settling and decanting after each rinse. The precipitate was checked with pH paper to ensure neutrality. After the acid wash and water rinses, the Celite 535 was dried in an oven overnight at 100°. The dry Celite was suspended in ether poured into a large glass column and washed with 3l. of ether. The Celite was then spread in a thin layer on aluminium foil and air-dried overnight. It was then heated in an oven at 100° for 5 hr. and stored in a sealed glass bottle until used.

METHODS

Tissue preparations and identification of products

Incubation medium for slices. Each incubation vessel contained 2.80 ml. of the following saline medium: 98 mM-NaCl-27 mM-KCl-1.2 mM-MgSO₄-0.4 mM-KH₂PO₄-17.5 mM-Na₂HPO₄, adjusted to pH 7.4. Each vessel also contained 0.20 ml. of 0.10 M-[¹⁴C]aspartate (2.5 μC/vessel), as indicated in the Tables. Where indicated 0.02 ml. of 1.0 M-glucose (non-labelled) was added as co-substrate with the aspartate.

Tissue slices. Cerebral-cortex slices were prepared according to the method of McIlwain (1951). Adult guinea pigs were stunned by a cervical blow, decapitated and the brains quickly removed. Six animals were used in each experiment. The two top cortical slices from each hemisphere were obtained by using a recessed glass plate as a guide and a large razor blade. The average weight of each slice was 125 mg. Two slices were placed in each Warburg vessel. The centre well contained a filter-paper wick saturated with 0.200 ml. of 0.25 N-NaOH. The entire medium was maintained at 0° to minimize endogenous respiration. All vessels were then placed in a Warburg water bath at 37.0° equilibrated with O₂ for 10 min. and incubated for 1.5 hr. At least two control vessels, containing all components except tissue slices, were always included.

The time elapsed from killing the animals to incubation never exceeded 25 min.

Amino acid determination. After determination of CO₂, the slices and incubation media were pooled, those with aspartate alone being kept separate from those in which aspartate and glucose were the co-substrates. The vessels were washed with 3 ml. of 95% (v/v) ethanol followed by two additional washings with 1 ml. of 95% ethanol. The slices were homogenized in the saline solution, after the addition of the ethanol washings, in a Potter-Elvehjem-type homogenizer. The homogenate was kept at 0° for 1 hr. and then centrifuged for 20 min. at 4000g. Usually 20% of the supernatant was used for the amino acid determination. The remainder was freeze-dried to a final volume of 6 ml. and placed on a Celite column for isolation of the intermediates of the citric acid cycle, as described below.

The amino acids were separated on a Dowex 50 (X8; 200-400 mesh) column by the method of Moore & Stein (1951). Fractions of volume 2 ml. were collected. A 97% recovery of standard amino acids was achieved. The amino acids were determined quantitatively by the ninhydrin method of Yemm & Cocking (1955), 0.2 ml. samples being taken from each tube.

For radioactivity measurements 0.2 ml. from each tube was mixed with 19 ml. of scintillation fluid in each vial and

counted in the manner described below for the degradation of aspartate.

In at least two experiments from each group analyses were also carried out on an Automatic Amino Acid Analyzer (Spinco Division, Beckman Instrument Co., Palo Alto, Calif., U.S.A.).

Determination of the citric acid-cycle intermediates. A 6 ml. portion of the homogenate was placed on a Celite 535 column (2 g. of Celite/ml. of supernatant) for extraction and quantitative determination of the citric acid-cycle intermediates by the method described by Swim & Utter (1957). A 94% recovery was achieved with standard citric acid-cycle intermediates. After quantitative determination, 1.0 ml. of the water layer was pipetted into vials containing 19 ml. of scintillation fluid and counted in the manner outlined below for carbon dioxide under 'Degradation of aspartate'.

Identification of amino acids and citric acid-cycle intermediates. The amino acids were identified on the basis of column elution position with pure known samples. After separation on the Dowex 50 column, the appropriate fraction was desalted and spotted on 8 in. × 8 in. Whatman no. 1 filter paper and the two-dimensional chromatograms were developed with phenol-water-NH₄OH (sp.gr. 0.88) (840:110:19, w/v/v) in the presence of HCN, and water-ethanol-lutidine-diethylamine (40:25:55:4, by vol.), by using the carefully controlled conditions of Logothetis & Bovis (1961).

After separation on the Celite column the eluate was spotted on 1.5 in. × 20 in. Whatman no. 1 filter paper and the chromatograms were developed by the method described by Varner (1957) for citric acid-cycle intermediates. These intermediates also were identified by gas-liquid chromatography.

The quantitative determination of succinate was also checked enzymically by the method of Umbreit, Burris & Stauffer (1957).

Homogenate preparation. The brain was removed and prepared as described by McIlwain (1951), except that after removal of the basal ganglia the white matter was peeled off and the remaining cerebral cortex placed in a cold tared beaker. The beaker was weighed and the tissue placed in a cold Potter-Elvehjem-type homogenizer containing 2.0 ml. of the saline medium for each Warburg vessel to be incubated. Then 2 ml. of the homogenate was added to each cold Warburg vessel (200-300 mg. of tissue/vessel) containing the following solution: 0.020 ml. of 1.0 M-D-glucose, 0.020 ml. of 1.00 M-L-[¹⁴C]aspartate (5 μC/vessel) and 0.96 ml. of the saline medium.

The incubation conditions were identical with those in experiments conducted with the slices.

Glutamate isolated for degradation studies. After incubation, the media and tissue were homogenized with enough HClO₄ to bring the concentration of HClO₄ to 1.5% (v/v). The homogenized tissue was centrifuged at 15000g for 30 min. and the supernatant decanted. The perchlorate was precipitated with 5 N-KOH, the volume reduced to 2 ml. on a rotary evaporator and the remaining solution centrifuged at 1000g for 10 min. The supernatant was brought to pH 1.5-2.0 with HCl, the solution was again centrifuged at 15000g for 30 min. and the supernatant added to a Dowex 50 column (2.5 cm. × 155 cm.) mounted on the Automatic Amino Acid Analyzer. The glutamate fraction was collected and desalted.

Desalting of glutamate. After the glutamate was isolated on the Automatic Amino Acid Analyzer, the contents of the appropriate tubes were pooled, brought below pH 2 with 6N-HCl and added to a Dowex 50 (X8) column that had been equilibrated with 2N-HCl. The citrate buffer was eluted with 3 column volumes of water and the glutamate then eluted with 3 column volumes of 2N-NH₃. The NH₃ was removed and the sample dried under vacuum. The dried sample was dissolved in 5 ml. of water and transferred to a 25 ml. flask. To this radioactive glutamate solution was added 1.5 ml. of 1.00M-monosodium glutamate solution.

Degradation of glutamate. Glutamate was degraded stepwise according to the method of Mosbach *et al.* (1951).

Degradation of aspartate. The aspartic acid was first converted into alanine by incubation with *Clostridium welchii* SR 12 according to the method of Najjar (1957). The CO₂ was trapped in the centre well of the incubation vessel, which contained NaOH, determined quantitatively by titration [Metrohm automatic titrator (Metrohm Ltd., Herisau, Switzerland), with an accuracy of ± 0.002 ml.] to pH 8.3 ± 0.02 and the radioactivity determined by scintillation counting in a Nuclear-Chicago Corp. counter with an efficiency of 55% as determined with a standard source. Alanine was isolated on the Automatic Amino Acid Analyzer, determined quantitatively by the ninhydrin method after pooling the contents of the appropriate tubes (Yemm & Cocking, 1955) and diluted 10.0-fold with unlabelled alanine. The carboxyl group was removed with chloramine-T according to the method of Dakin (1917). The resulting acetaldehyde, identified as its semicarbazone, m.p. 167–168.5° (literature m.p. 169°), and its *p*-nitrophenylhydrazine, m.p. 179–180° (literature m.p. 182°; Shriner, Fuson & Curtis, 1962), was cooled in an ice bath. To this cold solution was added 2 ml. of 10N-H₂SO₄ and 1 ml. of *m*-chromic acid. The solution was allowed to warm to room temperature with stirring and kept for 30 min. At the end of this period the acetic acid was steam-distilled and its purity and identity were established by means of gas-liquid chromatography.

The acetic acid (C-2 and C-3 of the original aspartate) was degraded in the same manner as the acetic acid from glutamate was degraded.

Synthesis of derivatives for gas-liquid chromatography

Amino acids. The *N*-acetyl-*n*-pentyl esters of the amino acids were produced according to the method of Johnson, Scott & Meister (1961).

Citric acid-cycle intermediates. The organic acid was suspended in 5 ml. of a methanol-boron trifluoride mixture and refluxed for 5 min. in a test tube. After the tube had been cooled, 5 ml. of hexane was added followed by 10 ml. of water. The tube was shaken and after the two phases had separated the hexane layer was removed. The water-methanol-boron trifluoride layer was washed twice with 2 ml. of hexane and the hexane portions were combined. The hexane solution was washed twice with 2 ml. of water and all but 1 ml. of the hexane removed by evaporation under a stream of N₂. The material was now ready for chromatography.

Fatty acids. The fatty acids (butyric acid, propionic acid and acetic acid) were injected into the column as the free acid dissolved in hexane; no production of derivatives was necessary.

Chromatographic conditions for gas-liquid chromatography

Amino acids. A Barber-Coleman model 10 gas chromatograph equipped with a U-shaped glass column and a ⁹⁰Sr argon ionization detector was employed. A column (6 ft. $\times$ 3 mm. internal diam.) packed with 1% Carbowax 1540 on 60–80-mesh Chromosorb W was used for partition, the following conditions being used: column temperature, 148°; flash heater, 250°; cell bath, 235°; inlet pressure, 25 lb./in.² of argon. Derivatives were injected with a 10 μ l. syringe, with benzene as the solvent.

Citric acid-cycle intermediates. The column (6 ft. $\times$ 3 mm. internal diam.) was packed with 16% diethylene glycol succinate on 60–80-mesh Chromosorb W, the following conditions being used for partition: column temperature, 150°; flash heater, 225°; cell bath, 235°; inlet pressure, 30 lb./in.² of argon.

Fatty acids. The column (6 ft. $\times$ 3 mm. internal diam.) was packed with 16% diethylene glycol succinate on 60–80-mesh Chromosorb W, the following conditions being used for partition: column temperature, 110°; flash heater, 150°; cell bath, 175°; inlet pressure, 25 lb./in.² of argon.

RESULTS

Reliability of degradation procedures. Table 1 lists the results of triplicate analyses of [¹⁴C]-glutamate. Both enzymic and chemical analyses gave identical results for C-1. Both reliability and reproducibility of the method are evident from the results.

Labelling of aspartate precursors. Table 2 demonstrates that the four [¹⁴C]aspartic acid samples used as substrates were labelled as presumed within acceptable limits.

Relative specific activities of amino acids. Table 3 lists the relative specific activities of glutamate isolated from tissue slices after incubation of the tissues with equimolar aspartate and glucose. When [1-¹⁴C]aspartate was used as labelled substrate, the glutamate isolated at the end of the experiment had a relative specific activity of 0.23 ± 0.05 . When [4-¹⁴C]aspartate was the source of ¹⁴C the glutamate had a relative specific activity of 0.32 ± 0.07 . These two relative specific activities are not significantly different. When [3-¹⁴C]-aspartate was used as the sole labelled substrate the isolated glutamate had a relative specific activity of 0.32 ± 0.05 . [U-¹⁴C]Aspartate yielded glutamate with a relative specific activity of 0.31 ± 0.06 . There is therefore no difference in the relative specific activities of the isolated glutamate regardless of where the exogenous aspartate substrate was originally labelled. As shown by the results of the incubation with [U-¹⁴C]glucose about one-third of the glutamate originated from glucose within the 1.5 hr. incubation period.

It is also apparent from Table 3 that the alanine

Table 1. *Labelling of [¹⁴C]glutamate used as test runs for the degradation procedure*Observed percentage distributions are given as means $\pm$ s.e.m.

Labelled compound	Distribution of label in glutamate (%)											
	C-1*		C-1†		C-2		C-3		C-4		C-5	
	Calc.	Observed	Calc.	Observed	Calc.	Observed	Calc.	Observed	Calc.	Observed	Calc.	Observed
[1- ¹⁴ C]-Glutamate	100	99.0 $\pm$ 0.2	100	99.8 $\pm$ 0.1	0	0	0	0	0	0	0	0.2 $\pm$ 0.1
[5- ¹⁴ C]-Glutamate	0	0	0	0	0	0	0	0	0	0.5 $\pm$ 0.2	100	99.5 $\pm$ 0.2
[U- ¹⁴ C]-Glutamate	20	19.5 $\pm$ 0.1	20	19.6 $\pm$ 0.5	20	20.2 $\pm$ 0.3	20	20.9 $\pm$ 0.7	20	21.2 $\pm$ 0.3	20	18.1 $\pm$ 0.8

* Enzymic determination by the method of Meister *et al.* (1951).† The α -carboxyl group of glutamate was removed with chloramine-T. The resulting succinic semialdehyde was reduced to butyric acid by a modified Wolff-Kishner reduction. The butyric acid was broken down by successive applications of the Schmidt degradation. Details are given in the text.Table 2. *Labelling of [¹⁴C]aspartate used as substrate*Aspartate was first converted into alanine by *C. welchii*. The alanine was converted into acetaldehyde by reaction with chloramine-T. The acetaldehyde was oxidized to acetic acid, which was, in turn, degraded by means of the Schmidt degradation. Observed percentage distributions are given as means $\pm$ s.e.m.

Labelled compound	Distribution of label in aspartate (%)							
	C-1		C-2		C-3		C-4	
	Calc.	Observed	Calc.	Observed	Calc.	Observed	Calc.	Observed
[1- ¹⁴ C] Aspartate	100	99.8 $\pm$ 0.2	0	0	0	0	0	0.2 $\pm$ 0.2
[3- ¹⁴ C] Aspartate	0	0	0	0.3 $\pm$ 0.1	100	99.7 $\pm$ 0.2	0	0
[4- ¹⁴ C] Aspartate	0	0.5 $\pm$ 0.2	0	0	0	0	100	99.5 $\pm$ 0.3
[U- ¹⁴ C] Aspartate	25	24.5 $\pm$ 1.0	25	26.1 $\pm$ 0.8	25	23.7 $\pm$ 1.0	25	25.7 $\pm$ 1.0

Table 3. *Relative specific activities of isolated glutamate and alanine*Cerebral-cortex slices were incubated under O₂ for 1.5 hr. with 20 μ moles of aspartate, 20 μ moles of glucose and 2.8 ml. of a saline medium of composition 98 mM-NaCl-1.2 mM-MgSO₄-27 mM-KCl-17.5 mM-Na₂HPO₄-4 mM-KH₂PO₄, pH 7.4. In each experiment 5 μ C of ¹⁴C-labelled substrate was present/vessel. CO₂ was isolated as Na₂CO₃ from the centre well containing 0.20 ml. of 0.3 N-NaOH. Details are given in the text. Relative specific activities are given as means $\pm$ s.e.m., and are defined as:

$$\frac{\text{counts/min./}\mu\text{mole of amino acid isolated}}{\text{counts/min./}\mu\text{mole of added labelled substrate}}$$

Substrates	Relative specific activity	
	Glutamate	Alanine
[U- ¹⁴ C] Aspartate + glucose	0.31 $\pm$ 0.06	
[1- ¹⁴ C] Aspartate + glucose	0.23 $\pm$ 0.05	0.12 $\pm$ 0.03
[3- ¹⁴ C] Aspartate + glucose	0.32 $\pm$ 0.05	
[4- ¹⁴ C] Aspartate + glucose	0.32 $\pm$ 0.07	0.06 $\pm$ 0.02
[U- ¹⁴ C] Glucose + aspartate	0.32 $\pm$ 0.05	

derived from [1-¹⁴C]aspartate contained twice as much label as alanine derived from [4-¹⁴C]aspartate.*Relative specific activities of citric acid-cycle intermediates.* At the end of a 1.5 hr. incubation[U-¹⁴C]aspartate contributed only 8–12% of the carbon atoms present in the citric acid-cycle intermediates, whereas [U-¹⁴C]glucose contributed 55–80% (Table 4). These findings may have

Table 4. *Relative specific activities of several isolated citric acid-cycle intermediates*

The conditions of incubation were as described in Table 3. Relative specific activities are given as means $\pm$ s.e.m., and are defined as:

		counts/min./ μ g. atom of C of isolated material
		counts/min./ μ g. atom of C of added labelled substrate
Substrates	Isolated compound	Relative specific activity of isolated compound
[U- ¹⁴ C] Aspartate + glucose	α -Oxoglutarate	0.12 $\pm$ 0.02
	Fumarate	0.08 $\pm$ 0.03
	Pyruvate (includes oxaloacetate)	0.06 $\pm$ 0.03
	Succinate	0.10 $\pm$ 0.03
[U- ¹⁴ C] Glucose + aspartate	α -Oxoglutarate	0.80 $\pm$ 0.12
	Fumarate	0.55 $\pm$ 0.06
	Succinate	0.60 $\pm$ 0.08
[U- ¹⁴ C] Aspartate	α -Oxoglutarate	0.19 $\pm$ 0.06
	Succinate	0.07 $\pm$ 0.02
	Pyruvate (includes oxaloacetate)	0.04 $\pm$ 0.01

Table 5. *Relative specific activity of glutamate derived from [U-¹⁴C] aspartate at three time points*

[U-¹⁴C] Aspartate + glucose were used as co-substrates. Experimental conditions were as described in Table 3. Relative specific activities are given as means $\pm$ s.e.m., and are defined as:

		counts/min./ μ mole of isolated glutamate
		counts/min./ μ mole of added [U- ¹⁴ C] aspartate
Incubation period (min.)	Relative specific activity of glutamate	
5	0.0	
15	0.06 $\pm$ 0.03	
90	0.30 $\pm$ 0.03	

several possible explanations: (a) aspartate may be quantitatively much less important than glucose as a precursor of citric acid-cycle intermediates; (b) aspartate may be preferentially used early in the incubation and its progeny molecules have by 1.5 hr. largely left the intermediate pool; (c) glucose may be being preferentially used for formation of intermediates (with, perhaps, aspartate to be called on later when the glucose is gone); (d) most cells (or intracellular compartments, or both) may convert glucose into these intermediates whereas only a few cells (or compartments or both) may convert aspartate into these intermediates. Possibilities (b) and (c) imply a temporal compartmentalization of metabolism, whereas (d) implies a spatial compartmentalization. Though either or both of these types of compartmentalization might be operative, Table 4 shows that the relative specific activities of the citric acid-cycle intermediates are the same at the end of 1.5 hr. regardless of whether [¹⁴C] aspartate is the sole added substrate or

[¹⁴C] aspartate + glucose are present as co-substrates. This finding would tend to rule out (c).

Table 5 shows that the relative specific activity of glutamate is lower at 5 and 15 min. than it is at 90 min. This would appear to rule out (b). On the basis of these last two findings it would appear that temporal compartmentalization is not a pre-dominating factor. This brings spatial compartmentalization into serious consideration (Berl, Takagaki, Clarke & Waelsch, 1962a,b).

Degradation study. The results of the degradation studies of glutamate listed in Table 6, with two assumptions, are consistent with the second postulate mentioned in the introduction of this paper, i.e. with citric acid-cycle involvement in the conversion of the carbon 'skeleton' of aspartate into glutamate. The assumptions that must be made are: (1) that most of the carbon atoms of aspartate entering the citric acid cycle and becoming available for the condensation reaction must reach some point where they can be randomized, i.e. at fumarate or succinate; (2) that there are two pools of oxaloacetate within the compartment where aspartate is metabolized (only one of these pools is available for the condensation reaction but both are sources of pyruvate). The first assumption is required to explain the facts (Table 6) that [^{3-¹⁴C]} aspartate gives approximately equivalent labelling at C-2 and C-3 of glutamate, and that the glutamate isolated after incubation with [^{1-¹⁴C]} aspartate contains label. The second assumption is necessary to explain the observation that [^{3-¹⁴C]} aspartate yields unequal labelling at C-4 and C-5 of glutamate. If assumption (1) alone were operative one would expect approximately equal labelling at C-4 and C-5 of glutamate as well as at C-2 and C-3.

Comparison of the relative specific activities of compounds isolated from slices and homogenate. The

Table 6. *Labelling of glutamate obtained after incubation of slices with [¹⁴C]aspartate and glucose*

Glutamate was isolated on the Automatic Amino Acid Analyzer after incubation of the slices with glucose and [¹⁴C]aspartate labelled in the position indicated. Details of the degradation technique are given in Table 1. Percentage distributions are given as means $\pm$ s.e.m.

Substrates	Distribution of label in glutamate (%)				
	C-1	C-2	C-3	C-4	C-5
[1- ¹⁴ C] Aspartate + glucose	91.5 $\pm$ 1.1	0	0	0	8.5 $\pm$ 1.1
[3- ¹⁴ C] Aspartate + glucose	8.3 $\pm$ 0.9	43.7 $\pm$ 1.1	36.0 $\pm$ 1.1	8.3 $\pm$ 0.8	3.7 $\pm$ 0.8
[4- ¹⁴ C] Aspartate + glucose	94.0 $\pm$ 1.1	0	0.8 $\pm$ 0.8	0	5.2 $\pm$ 1.0
[U- ¹⁴ C] Aspartate + glucose	26.2 $\pm$ 2.0	27.3 $\pm$ 1.8	25.9 $\pm$ 1.5	11.5 $\pm$ 1.0	9.0 $\pm$ 0.8

Table 7. *Relative specific activities of several compounds derived from [U-¹⁴C]aspartate*

Homogenates were incubated under O₂ for 1.5 hr. in a total of 3 ml. containing 20 μ moles of [U-¹⁴C]aspartate (1.5 μ C) and 20 μ moles of glucose in a saline medium of composition 98mM-NaCl-1.2mM-MgSO₄-27mM-KCl-17.5mM-Na₂HPO₄-4mM-KH₂PO₄, pH 7.4. CO₂ was trapped as Na₂CO₃ in the centre well on filter paper saturated with 0.20 ml. of 0.3 N-NaOH. Three replicate determinations were made in the homogenate study. Details are given in the text. Relative specific activities are given as means $\pm$ s.e.m., and are defined as:

Compound	Relative specific activity of compound	
	$\frac{\text{counts/min.}/\mu\text{mole of isolated compound}}{\text{counts/min.}/\mu\text{mole of added [U-}^{14}\text{C]aspartate}}$	
	Homogenate	Slice
Glutamate	0.10 $\pm$ 0.03	0.31 $\pm$ 0.06
α -Oxoglutarate	0.14 $\pm$ 0.04	0.12 $\pm$ 0.02
Fumarate	0.16 $\pm$ 0.04	0.08 $\pm$ 0.03
Succinate	0.14 $\pm$ 0.04	0.10 $\pm$ 0.03

relative specific activity of the glutamate isolated from the homogenate is approximately equal to the relative specific activity of the α -oxoglutarate isolated from the homogenate. This finding is different from that found in the slice, as shown in Table 7. It was this observation in relation to slice metabolism that necessitated consideration of spatial compartmentalization.

DISCUSSION

For the simple reaction sequence:

a labelled compound A should yield an intermediate B with a specific activity at least as high as that of its product C.

If B has a specific activity lower than that of C one would be sceptical of B's being an intermediate

in the reaction sequence. If, however, B were also produced in another reaction, e.g.:

and compound B in the second reaction were not allowed to mix with the B in the first reaction, but on isolation of the final products all of B were isolated as a single pool, one might find that B has a specific activity lower than that of C. This could occur since the non-radioactive B in the second reaction would dilute out the specific activity of the radioactive B in the first reaction.

Aspartate is generally believed to be converted into glutamate by reactions involving the citric acid cycle (Krebs & Lowenstein, 1960). If this assumption is correct, one would expect to find that, starting with [¹⁴C]aspartate, the specific activity of the citric acid-cycle intermediates would be higher than the specific activity of glutamate. On incubation of cerebral-cortex slices with labelled aspartate this was not found to be the case. However, it was found that when tissue slices were incubated with [¹⁴C]aspartate and glucose as added co-substrate the specific activities of the isolated citric acid-cycle intermediates were about one-half to one-fifth that of the glutamate. This can be explained by one of two hypotheses: (1) most of the glutamate could be derived from aspartate by an unknown pathway not involving the citric acid cycle; (2) certain metabolites, such as glucose, are metabolized by all cells or compartments, or both, whereas other compounds, such as aspartate (or amino acids in general), may be metabolized only by certain cells or compartments, or both.

If the first postulate were true, it would be unlikely for the labelling pattern in glutamate to be consistent with citric acid-cycle conversion of uniquely-labelled [¹⁴C]aspartate into glutamate.

If the second postulate were true, the labelling pattern of glutamate derived from [¹⁴C]aspartate should be consistent with citric acid-cycle inter-conversion. In addition, when [U-¹⁴C]glucose + aspartate were used as co-substrates, one should

Position of label isolated in glutamate	Position of label in aspartate ...	No randomization			With randomization at fumarate		
		1- ¹⁴ C	4- ¹⁴ C	3- ¹⁴ C	1- ¹⁴ C	4- ¹⁴ C	3- ¹⁴ C
1 CO ₂ H		0	0	0	0	0	0
2 CH-NH ₂		0	0	0	0	0	0
3 CH ₂		0	0	0	0	0	0
4 CH ₂		0	0	100	0	0	50
5 CO ₂ H		0	0	0	0	0	50

Scheme 1. Mechanism for entry of aspartate into the citric acid cycle and subsequent conversion into glutamate. Two pools of oxaloacetate are shown.

C-1 of pyruvate (originally the C-1 of oxaloacetate) being released as carbon dioxide in this reaction. The carbonyl carbon of the acetate moiety of acetyl-CoA originates at C-2 of oxaloacetate and the methyl carbon at C-3 (Krebs & Lowenstein, 1960).

Scheme 1 shows that, if $[4\text{-}^{14}\text{C}]$ aspartate were incubated with tissue, the label should appear only

at C-1 of glutamate, assuming that conversion occurs via the citric acid cycle. Almost all the label is found at C-1 (Table 6).

A small percentage of the label appears at C-5 of glutamate. No known mechanism can account for this observation and it may be that another, unknown, pathway, of minor significance and not involving the citric acid cycle, may be present.

If [$1\text{-}^{14}\text{C}$]aspartate were converted into oxaloacetate and condensed with acetyl-CoA to form citrate, the subsequent reactions would produce a glutamate molecule that contained no ^{14}C (see Table 8). However, Table 6 shows that ^{14}C is found at both C-1 and C-5 of glutamate in approximately the same ratio as that found when [$4\text{-}^{14}\text{C}$]aspartate was used as substrate.

Table 3 shows that glutamate derived from [$1\text{-}^{14}\text{C}$]aspartate has almost the same specific activity as that derived from [$4\text{-}^{14}\text{C}$]aspartate. Assuming that the carbon 'skeleton' of aspartate is converted into glutamate via the citric acid cycle, the only way that these observations can be accounted for is by assuming a reversal of the citric acid cycle to fumarate, thus randomizing the carbon 'skeleton', and that after this the carbon 'skeleton' proceeds in the forward direction.

The ratio of isotope found at C-2 and C-3 of glutamate derived from [$3\text{-}^{14}\text{C}$]aspartate is consistent with the randomization theory outlined above. If randomization did not occur, there should be little labelling of glutamate at C-3: specifically, less than half as much as at C-4. This is so because C-3 of aspartate becomes C-2 of α -oxoglutarate. Subsequently C-2 of α -oxoglutarate becomes either C-1 or C-4 of succinate. This step would then preclude any labelling at C-3 of glutamate from C-3 of oxaloacetate (Scheme 1). The only source of label at C-3 of α -oxoglutarate, and thus of glutamate, from [$3\text{-}^{14}\text{C}$]aspartate would be from C-4 of α -oxoglutarate after the α -oxoglutarate has undergone another turn of the cycle.

Since the label at C-4 and C-5 of glutamate is derived from aspartate via oxaloacetate and pyruvate one would expect the same distribution ratio at C-4 and C-5 that one finds at C-2 and C-3 (the oxaloacetate that condenses with acetyl-CoA is believed to originate from the same pool that gives rise to pyruvate). However, there is approximately twice as much label at C-4 as there is at C-5.

The approximately equal label distribution at C-2 and C-3 and unequal label distribution at C-4 and C-5 of glutamate from [$3\text{-}^{14}\text{C}$]aspartate could be explained on the basis of two pools of oxaloacetate, such as those shown in Scheme 1. [$3\text{-}^{14}\text{C}$]Aspartate would be converted into oxaloacetate, and this compound could then follow pathway (1) and the label would be in the methyl group of pyruvate and through subsequent reactions appear at C-4 of glutamate.

The molecules from the first pool of oxaloacetate can also follow pathway (2) (Scheme 1) and enter the second pool of oxaloacetate (within the citric acid cycle). If the combined turnover number of fumarase and malate dehydrogenase is about 10–20 times as great as the turnover number of the condensing enzyme, the labelling pattern of the second

pool of oxaloacetate will be symmetrical. These symmetrically labelled molecules could now follow pathway (3). As a result of the combination of pathways (1) and (3), label would appear at C-4 and C-5 of glutamate in the unequal label-distribution pattern that is observed. The outline of the pathways listed in Scheme 1 can account for all the findings of the degradation studies except for the small amount of label that appears at C-5 of glutamate from [$1\text{-}^{14}\text{C}$] and [$4\text{-}^{14}\text{C}$]aspartate.

The relative specific activities of alanine from [$1\text{-}^{14}\text{C}$] and [$4\text{-}^{14}\text{C}$]aspartate (Table 3) have the same relationship to each other as that found at C-4 and C-5 of glutamate. This relationship would be predicted from the theory presented.

If compartmentalization of the type postulated exists, one would predict that, when [$\text{U-}^{14}\text{C}$]glucose was used as the source of ^{14}C , one would find that the citric acid-cycle intermediates would be labelled more highly than the glutamate. This would be expected since it was postulated that glucose was utilized in most if not all compartments, whereas aspartate was metabolized by only a few compartments. When [$\text{U-}^{14}\text{C}$]glucose and aspartate were used as co-substrates with slices, it was found that the citric acid-cycle intermediates were labelled 2–3 times as much as the glutamate. This finding is consistent with the compartmentalization theory (Tables 3 and 4). Waelsch, Berl, Rossi, Clarke & Purpura (1964) have presented results for carbon dioxide fixation that are consistent with these findings.

It is indicated from the present findings that the carbon 'skeleton' of aspartate is converted into glutamate mainly by reactions involving the citric acid cycle and that these reactions are compartmentalized.

A question that remains at this point concerns the type of compartmentalization that exists in the tissue; i.e. are the compartments maintained as a result of (1) membrane permeability or as a result of (2) enzyme localization.

If the first possibility were operative, one would expect to find that, if a homogenate were used in place of slices, the specific activity of the citric acid-cycle intermediates would be at least as high as the specific activity of the glutamate, assuming that [^{14}C]aspartate was used as the added radioactive substrate.

If the second possibility (enzyme localization) were the main factor contributing to the maintenance of compartmentalization one would expect, on use of a homogenate, very little difference in the interrelationship between glutamate and citric acid-cycle intermediates over that found with slices.

Table 7 clearly shows that, whereas in the slice the relative specific activity of glutamate is higher

than the relative specific activity of the citric acid-cycle intermediates, the homogenate shows a reversal of these findings. These facts are consistent with the first hypothesis; i.e. the compartments are maintained as a primary result of membrane permeability.

Neither the theory of compartmentalization nor the theory of selective permeability are new. It has been known for some time that the cell is not a homogeneous solution with all biochemical reactions occurring uniformly through the cell, but that various reactions occur in discrete locations within well-defined physical boundaries. It has also been known for some time that cell walls are impermeable, or at least almost impermeable, to the transport of certain materials, and that this selective permeability is not uniform for all cells. For example, bilirubin enters the cells of the basal ganglia quite easily but cannot be detected in the cerebral cortical cells (Becker & Vogen, 1948). It is this latter type of compartmentalization that is believed to be operative in the studies that are outlined above. It is not meant, necessarily, that the cell itself is the unit of operation, but rather that some type of membrane or barrier is present that is selective in allowing the entrance of aspartate, and that this membrane or barrier is destroyed on homogenization.

Though the possibility of two pools of oxaloacetate and the theory of compartmentalization maintained by membrane or barrier selective permeability is convenient for the explanation of most of the present findings, it does not explain all of the facts.

The most apparent inconsistency in the theory presented resides in the observation that the relative specific activities of glutamate derived from [1-¹⁴C]- and [4-¹⁴C]-aspartate are 0.23 and 0.32 respectively instead of the 0.15 that would be predicted in both cases. To what extent, if any, these findings require the theory to be modified remains to be seen.

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