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Shosuke Watanabe* Saburo Otsuki† Yoshio Nakashima‡

Kazuyasu Edamatsu** Katsusuke Mitsunobu††

*Okayama University,

†Okayama University,

‡Okayama University,

**Okayama University,

††Okayama University,

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Shosuke Watanabe, Saburo Otsuki, Yoshio Nakashima, Kazuyasu Edamatsu, and Katsusuke Mitsunobu

Abstract

The rate of transport of blood glutamic acid into the brain and the rate of metabolic conversion of the amino acid in the brain were derived by the use of the brain perfution method in vivo and in situ with [D.HC] ·Lglutamic acid. The net uptake of glutamic acid by the brain was observed. Most of the radioactivity released from the brain into the cerebral venous blood was found to consist of glutamine. Small but significant amounts of output as radioactive GSH and CO2 were also found. Glutamic acid transport and its rate of metabolism were lowered in the glucose. free condition. The size of the compartment of the small glutamic acid pool, which was related closely to the blood glutamic acid, and that of the large glutamic acid pool, which was related closely to the blood glucose, were calculated and compared with each other.

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GLUTAMIC ACID METABOLISM IN PERFUSED CAT BRAIN STUDIED WITH ^{14}C-LABELLED GLUTAMIC ACID

Shosuke WATANABE, Saburo OTSUKI, Yoshio NAKASHIMA, Kazuyasu EDAMATSU and Katsusuke MITSUNOBU

Department of Neuro-Psychiatry, Okayama University Medical School, Okayama, Japan (Director: Prof. S. Otsuki)

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Following the intravenous or intracisternal administration of glutamic acid, the rapid exchange of the amino acid between blood and brain and the compartmentation of glutamic acid metabolism in brain have already been shown (LAJTHA, BERL and WAELSCH 1959, BERL, LAJTHA and WAELSCH, 1961). These findings led to the present study on the quantitative aspect of the fate of blood glutamic acid after entering the brain and upon its subsequent metabolism in the brain. Utilizing the brain perfusion method, [U-^{14}C]-L-glutamic acid was added to the perfusion blood and kept at a constant specific activity throughout the experiments. The rate of incorporation of radioactivity into free glutamate, aspartate and glutamine of the brain was measured. And also, the amount of radioactive glutamine, GSH and respiratory CO_2 released into the cerebral venous blood was measured. The brain perfusion experiments were performed with the blood containing either glucose in the normal concentration or no glucose, so that the effect of hypoglycemia on the glutamic acid metabolism of the brain could be studied.

MATERIALS AND METHODS

Young adult cats were used for the experiments. Details of the technique of perfusion of the brain *in vivo* and *is situ* have already been described (GEIGER and MAGNES 1947, OKUMURA, IKEDA and WATANABE 1962). The roller type pump was used for the artificial brain perfusion. The blood once perfused through the brain was discarded with the exception of the venous blood samples for analysis. Continuous records of the electroencephalogram (EEG) and cerebral blood flow (measured with electromagnetic flow meter) were taken during the experiments.

In each experiment about 5 μc of radioactive L-glutamic acid and 500 μmoles of unlabelled-L-glutamic acid were added to 1000 ml of perfusion blood.

The perfusion blood consisted of washed bovine red blood cells suspended in a Krebs-Ringer solution containing 6 per cent dextran (average molecular weight 40,000) and 0.2 per cent bovine serum albumin. For the standard perfusion, the

essential amino acids, vitamins, and sodium salt of CMP were added to the perfusion blood (Table 1).

TABLE 1. COMPOSITION OF STANDARD PERFUSION BLOOD

Bovine erythrocytes	40 %
10 % Dextran in Krebs-Ringer solution	60 %

The following substances are added to 100 ml of the mixture.

Bovine serum albumin	200 mg
CMP - 2 Na	8 mg
Glucose	100 mg
Glutamic acid	7.5 mg

Amino acid mixture (mg)

Arg 2.71	Gly 1.80	His 1.29	Ileu 1.98
Leu 3.0	Lys 5.76	Met 2.04	Phe 2.88
Thr 2.1	Try 0.9	Val 1.92	

Vitamin mixture (mg)

A 100 i.u.	B_1 0.1	B_2 0.22	B_6 0.04
C 1.0	B_{12} 4×10^{-5}	D 10 i.u.	
Nicotinamide 0.4		D-Pantothenol 0.1	

The hypoglycemic experiment was performed by replacement with perfusion blood containing no glucose in the course of the standard perfusion.

The oxygen consumption and CO_2 output were determined by Natelson's method from the values of simultaneous samples of arterial and venous blood. For the analysis of amino acids the blood samples were deproteinized with TCA at final concentration of 5 per cent. Other procedures were the same as those used for the analysis of brain samples.

Brain cortex samples were excised during the perfusion and frozen immediately in dry ice and acetone. Five per cent TCA extracts of brain homogenates were passed through Dowex 2-X8 Cl^- column to remove TCA (LAJTHA and TOTH, 1962). To isolate glutamic and aspartic acid a column of Amberlite CG 4B Type 2 acetate form (0.9×15 cm) was used in conjunction with gradient elution by increasing acetic acid concentrations (KURAHASI, 1964). After elution of aspartic acid, GSH was eluted with 2 N HCl, hydrolyzed for 2 hr at 100°C, and rechromatographed on the Amberlite column as glutamic acid. The purity of the GSH fraction was checked by ascending paper chromatography with 75 per cent phenol and n-butanol-acetic acid-water (4 : 1 : 1) as solvents. GSH was determined only on blood samples. The break through fraction of the first Amberlite column was hydrolyzed in 2 N HCl for 2 hr at 100°C and glutamine was isolated on the Amberlite column as glutamic acid. Amino acid concentrations were determined with ninhydrin according to ROSEN (1957).

The radioactivity of samples was measured with a liquid scintillation counter (Packard Instrument Co.) using 0·3 ml of 1 M hyamine in methanol and 15 ml of toluene containing 4 g 2, 5-diphenyloxazole (PPO) and 100 mg 1, 4-bis-2 (4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) in 1 l. The counting efficiencies of

the samples were determined by external standardization. Radioactivity was expressed as disintegrations per minute (dpm).

The radioactivity of the respiratory CO_2 was determined by absorbing the CO_2 with a small piece of filter paper moistened with 1M hyamine. This is a slight modification of the method reported by GEIGER *et al.* (1960). For the calculation of radioactivity of the total TCA extract and glutamic acid in brain, the values were corrected for the radioactivity contained in an average of 1 per cent of residual blood (GOMBOS *et at.* 1962).

L-[U-^{14}C] Glutamic acid (66 mc/mole) was obtained from Daiichi Pure Chemicals. Twelve per cent dextran dissolved in Krebs-Ringer solution was obtained from Daigo Nutritional Co.

RESULTS

Standard perfusion with glucose, glutamic acid, and essential amino acids.

The surgical operation to separate the brain circulation from systemic circulation was performed under nembutal anesthesia. The influence of nembutal anesthesia wore off in the course of perfusion. After 15 to 20 minutes from the start of perfusion with the "blood" of standard composition, fast activity of 13 to 50 cycles per second with an amplitude of 40 to 50 μvolts became dominant in the EEG and continued thereafter. ^{14}C-Glutamic acid was added during the first 15 minutes of perfusion. The radioactivity of brain TCA extracts averaged about 50 per cent of that of arterial blood (Table 2). The specific activity of glutamic acid showed no significant difference between arterial and venous blood. The concentrations of the free amino acids in the brain were in the range of normal

TABLE 2. RADIOACTIVITY OF TCA EXTRACTS OF BLOOD AND BRAIN

No	Min	Blood dpm/ml	Brain dpm/g	Brain/Blood
Standard perfusion				
1	15	16000	1600	0.10
2	34	4800	1960	0.41
3	37	5000	2890	0.58
4	57	5400	3300	0.61
5	60	8000	3665	0.46
6	95	4832	2582	0.54
			Average	0.52* ± 0.074
Glucoes-free perfusion				
7	35	7300	1250	0.17
8	36	7200	2115	0.29
9	44	6000	1590	0.26
			Average	0.24 ± 0.051

* The value of Experiment 1 was excluded. Average values are given as ± S. D.

TABLE 3. FREE AMINO ACID CONCENTRATION OF THE PERFUSED BRAIN

No	Min	Glutamic acid	Glutamine	Aspartic acid
			μmole/g brain	
Standard Perfusion				
1	15	8.04	5.13	2.30
2	34	7.87	4.72	1.11
3	37	9.53	7.90	0.93
4	57	7.43	5.22	0.92
5	57	8.18	6.75	1.46
6	60	7.33	4.28	1.23
7	95	7.62	5.40	1.03
Average		8.00±0.69	5.63±1.67	1.28±0.45
Glucose-free Perfusion				
8	35	6.58	2.76	1.76
9	36	8.58	5.15	2.09
10	44	7.22	5.33	1.27
Average		7.46±0.83	4.41±1.17	1.71±0.34

values reported by TALLAN *et al.* (1954) (Table 3). In the free amino acids of the brain, glutamine exhibited the highest specific adivity, which was about 2.2 times higher than that of glutamic acid (Table 4). The specific activity of brain glutamic acid reached an average value of 0.56 per cent of that of blood glutamic acid at 15 minutes after the addition of ^{14}C-glutamic acid to the perfusion blood. This value was maintained until the 95-minute perfusion (Table 4). The specific activity of brain aspartic acid was the lowest among the amino acids determined (Table 4).

The cerebral venous blood taken after 10 minutes perfusion with ^{14}C-glutamic acid, contained radioactive glutamine, GSH and CO_2 (Fig. 1). The levels of radioactivity of these substances increased in the course of perfusion. Among them the radioactivity of glutamine rose most rapidly and reached the plateau after 20 to 30 minutes. The specific activity of blood glutamine was very much higher than that of brain glutamine. The specific activity of CO_2 was higher than the radioactivity per carbon atom of brain glutamic acid in some cases (Table 5).

Considering the time course of the rise of specific activity of brain glutamic acid and that of glutamine in cerebral venous blood during the perfusion (in which the specific activity of blood glutamic acid was kept constant), the amount of glutamic acid entering from the blood to brain was calculated (Table 6). Only glutamic acid and glutamine both in the brain and in the arterial and venous blood were considered on the calculation. In addition the calculation was based on the assumption that the

TABLE 4. SPECIFIC ACTIVITY OF METABOLITES DURING [U-^{14}C] GLUTAMIC ACID PERFUSION

dpm/μmole

No	Min	Blood			Brain		
		Glu	Glu (NH_2)	CO_2	Glu	Glu (NH_2)	Asp
Standard Perfusion							
1	15	26000	1220	11.3	187	653	4.8
2	34	9970	—	21.7	51	115	—
3	37	10800	—	13.6	65	246	30
4	57	20100	—	—	92	196	33
5	57	15000	3360	41.7	107	141	—
6	60	25000	4320	21.2	139	256	46
7	95	16700	—	46.8	84	196	—
Glucose-free Perfusion							
8	35	A*: 24200 V : 18500	1500	18.4	101	242	20
9	36	A : 17300 V : 16600	1420	61.5	74	79	0
10	44	A : 28100	0	12.2	89	302	11

* A : The specific activity of glutamic acid in arterial blood.
V : That in venous blood.

TABLE 5. RELATIVE SPECIFIC ACTIVITY OF METABOLITES DURING [U-^{14}C] GLUTAMIC ACID PERFUSION

No	Min	Blood		Brain		
		Glu (NH_2)	CO_2	Glu	Glu (NH_2)	Asp
Standard perfusion						
1	15	4.7	0.22	0.67	2.18	0.01
2	34	—	1.09	0.51	1.15	—
3	37	—	0.63	0.61	2.27	0.28
4	57	—	—	0.46	0.97	0.16
5	57	22.4	1.39	0.71	0.94	—
6	60	17.3	0.42	0.55	1.02	0.23
7	95	—	1.40	0.50	1.17	—
Average *		19.9±2.55	0.99±0.40	0.56±0.083	1.25±0.46	0.22±0.049
Glucose-free perfusion						
8	35	6.2	0.38	0.42	1.00	0.08
9	36	8.2	1.78	0.43	0.46	0
10	44	0	0.22	0.32	1.07	0.04
Average		4.8±3.49	0.79±0.70	0.39±0.049	0.84±0.27	0.04±0.032

* The values of Experiment 1 were excluded.

$$\text{Relative specific activity} = \frac{\text{dpm per carbon atoms of metabolites}}{\text{dpm per carbon atoms of blood glutamic acid}} \times 100$$

TABLE 6. SIZE OF METABOLIC COMPARTMENT OF BRAIN GLUTAMIC ACID

	A	B	C	D
	μmole/g	dpm/μmole	dpm/g/hr	μmole/g/hr
Brain Glu	8.0	110	1760	0.09
Glu (NH_2)	5.0	250	2500	0.13
Blood Glu (NH_2)	0.03	4000	7200	0.36
		Total	11,460	0.58

B : The values were calculated from the data in Table 4 by adjusting the specific activity of blood glutamic acid of each experiment to 20,000 dpm/μmole.
C : A×B×2 : see text.
D : C divided by 20,000 dpm/μmole.

radioactivity of brain glutamic acid and glutamine and that of glutamine in venous blood increased linearly from the beginning of ^{14}C-glutamic acid perfusion to 30 minutes perfusion, and maintained these values thereafter. If the specific activity of blood glutamic acid was adjusted to 20,000 dpm/μmole in each experiment, the average values of specific activities of brain glutamic acid, brain glutamine and blood glutamine are 110, 250 and 4,000 dpm/μmole, respectively. The rate of increase of radioactivity in brain glutamic acid, brain glutamine and blood glutamine becomes 1,760, 2,500 and 7,200 dpm/g brain/hr, i.e. 0.09, 0.13 and 0.36 μmole/g brain/hr, respectively. In the perfusion experiments the cerebral blood flow rate was 1 ml per g brain per minute. The value of blood glutamine was calculated on this basis. Thus, the total radioactivity entered into the brain was 11,460 dpm/g brain/hr, i.e. 0.58 μmole/g brain/hr.

Perfusion with glutamic acid in the absence of glucose

When the perfusion "blood" was replaced by the "blood" which contained no glucose, the fast activity in the EEG rapidly disappeared and there was a concomitant decrease of cerebral blood flow. The cerebral oxygen consumption dropped to 1.18 μmole/g brain/minutes, one-half of the average value obtained in the standard perfusion. There was no marked change of the concentration of the free amino acids in the brain (Table 3).

During glucose-free perfusion, the radioactivity of brain TCA extract was about 24 per cent of that of arterial blood (Table 2). The specific activity of glutamic acid in the venous blood was somewhat lower than that in the arterial blood (Table 4). The specific activities of brain glutamic acid, glutamine, and aspartic acid were generally lower than those during the standard perfusion (Table 4, 5). The output of radioactive glutamine and GSH decreased, but the level of radioactive CO_2 was similar to that in the standard perfusion (Fig. 1). The specific activity of

glutamine in the venous blood was also lower than that of the standard perfusion (Tables 4, 5).

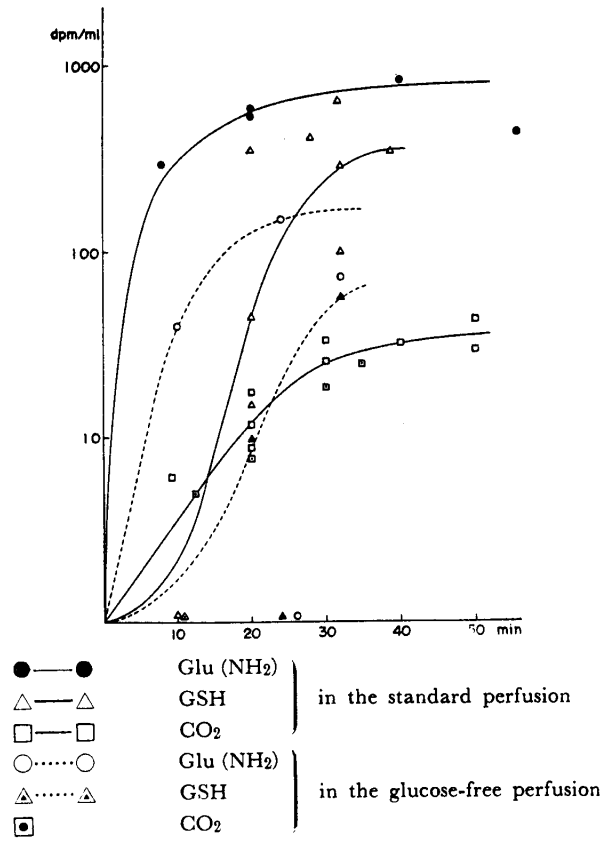

Fig. 1 Time Course of the Rise of Radioactive Metabolites in the Cerebral Venous Blood During the Brain Perfusion with [U-^{14}C] Glutamic Acid.

DISCUSSION

There was no significant difference in the specific activity of glutamic acid between arterial and venous blood in the standard brain perfusion. The specific activity of glutamic acid in cerebral venous blood would have decreased if there had been significant amounts of glutamic acid leaving the brain. This finding shows that there is a net uptake of blood glutamic acid by the brain without accompanying output of the amino acid from the brain. Or, if there were an "exchange" (the term generally used) of the amino acid between blood and brain, the rate of output is within an experimental error.

In the glucose-free perfusion, the lower specific activity of glutamic acid in cerebral venous blood indicated that the latter was significantly diluted by the brain glutamic acid which entered the blood. The influx of the amino acid into brain decreased markedly in this condition. These observations show clearly that transport of glutamic acid and maintenance of high concentration in the tissue depend on the energy consuming processes.

The specific activity of brain glutamine exceeded that of brain glutamic acid. This is confirmative evidence of the metabolic compartmentalization of brain glutamic acid. The specific activity of glutamine in venous blood was much higher than that in the brain. The newly formed glutamine derived from the glutamic acid which entered into the brain returned rapidly into the circulating blood, before the dilution occurred with the total glutamine pool of the brain. The specific activity of respiratory CO_2 was higher than that expected from the specific activity of brain glutamic acid in some cases. Similar results have been reported with ^{14}C-aspartic acid perfusion by Gombos, GEIGER and OTSUKI (1963). These authors explained the result on the basis of the possibility of randomization of radioactive carbon in each carbon atom of the utilized aspartate. In the present study, we could not rule out that possibility, but the result quite probably shows that the respiratory CO_2 is also produced in a metabolic compartment and is released into the blood.

Since the specific activity of blood glutamic acid was kept constant throughout the experiments, the rate of influx of glutamic acid into the brain, the rate of metabolic conversion to glutamine, and the rate of efflux of the glutamine can be simply calculated. The rate of influx of blood glutamic acid into the brain was about 0.6 μmole/g brain/hr as shown in Table 6. This value falls within the range previously reported by LAJTHA *et al.* (1959). Of the transported glutamic acid, only 15 per cent remained as glutamic acid in the brain, and 85 per cent was converted to glutamine. Three parts of the formed glutamine remained in the brain and seven parts entered into the venous blood. Thus, over one-half of the transported glutamic acid returned to the blood as glutamine.

It is a well known fact that the carbon source of brain glutamic acid is glucose carbon (BARKULIS *et al.*, 1960, VRBA *et al.* 1962, CREMER, 1964, GAITONDE *et al.*, 1965). In our findings (OTSUKI *et al.*, 1968) on the brain perfusion with [U-^{14}C] glucose, 80 per cent of carbon atoms of brain glutamic acid are derived from the carbon atoms of blood glucose within the hour after the beginning of the perfusion, when EEG shows predominantly fast activity. This value is much higher than that reported by GEIGER

(1962). It is assumed that the discrepancy between our results and those of GEIGER may be explained by the fact that the functional level of the brain during perfusion is different in each experiment. From our results, 70 per cent of free glutamic acid pool (8 μmole per gram brain), i. e. 5.6 μmole/g brain/hr is the influx rate from the blood glucose. This calculation is based on the assumption that the specific activity of brain glutamic acid increased linearly during the perfusion. The glutamic acid pool which comes from the blood glucose can be called a large glutamic acid pool and that which comes from the blood glutamic acid, a small glutamic acid pool. The size of the large glutamic acid pool is 5.6 μmole/g brain/hr, and that of the small glutamic acid pool is 0.5 μmole/g brain/hr as previously mentioned. The ratio of the large pool to the small pool thus becomes 9 to 1. This value is close to the value calculated by GARFINKEL (1962).

SUMMARY

The rate of transport of blood glutamic acid into the brain and the rate of metabolic conversion of the amino acid in the brain were derived by the use of the brain perfution method *in vivo* and *in situ* with [U-^{14}C]-L-glutamic acid. The net uptake of glutamic acid by the brain was observed.

Most of the radioactivity released from the brain into the cerebral venous blood was found to consist of glutamine. Small but significant amounts of output as radioactive GSH and CO_2 were also found. Glutamic acid transport and its rate of metabolism were lowered in the glucose-free condition.

The size of the compartment of the small glutamic acid pool, which was related closely to the blood glutamic acid, and that of the large glutamic acid pool, which was related closely to the blood glucose, were calculated and compared with each other.

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