SUBCELLULAR GLUTAMATE AND GLUTAMINE POOLS IN RAT BRAIN

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Bachelor of Science

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Peru, Nebraska

1970

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 1973

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ACKNOWLEDGMENTS

The author gratefully acknowledges the guidance, assistance, and counsel provided by his major professor, Dr. R. E. Koeppe, during the course of these investigations and in the preparation of this thesis.

The author also wishes to express appreciation to Dr. I. K. Mushahwar for suggestions and assistance during the study.

To the author's wife, Sarah, goes a special thanks for her patience, encouragement, and understanding during these years of graduate studies.

The author is indebted to the Department of Biochemistry for facilities and financial support during these investigations.

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CHAPTER I

INTRODUCTION

Compartmentation of metabolic events was first conceived in an effort to account for observations which could not be explained by the simple precursor-product relationship as described by Zilversmit, Entenman, and Fishler (1). In general, two types of compartments can be defined. One is the anatomical compartment which can be described by distinct anatomical boundaries, e.g., the plasma, a whole organ such as the brain, a particular type of cell, or a subcellular structure such as mitochondria. The other type of compartment is the metabolic compartment which can be described only in biochemical terms. However, a metabolic compartment may subsequently be related to anatomical structures. By definition, metabolic compartmentation refers to the presence of more than one distinct pool of a given metabolite. These pools are necessarily not in rapid equilibrium with each other.

It should not be assumed that a metabolic compartment need be related to an anatomical structure. It is known

that the affinity of succinate dehydrogenase for succinate generated in the mitochondria from α-ketoglutarate is 100 times that for added succinate (2, 3). Further, the effective concentration of succinate is 200 times the overall steady-state concentration (4). Thus, the affinity of succinate dehydrogenase for the complex of succinate with the forming enzyme is greater than that for free succinate. This enzyme-substrate complex can be considered a separate metabolic pool.

A two-compartment system is based on the criterion that after injection of an isotopicly labeled precursor of a metabolic product, the specific activity of the product rises within minutes to values four to five times as great as that of its precursor. In a one-compartment system, under the same circumstances, the specific activity of the product would increase as the specific activity of the precursor decreased until the specific activity of the product would be equal to or slightly greater than that of the precursor.

Lajtha, Berl, and Waelsch (5, 6) showed that within five minutes after intracisternal administration of tracer amounts of ^{14}C-glutamate to rats, the ratio of the specific activity of brain glutamine to that of glutamate was approximately five while the total count in glutamate

plus glutamine remained unchanged during this period. Also, the peak specific activity of glutamine reached at five minutes was almost twice that of the highest glutamic acid value observed at 25 seconds.

Takagaki et al. (7) and Berl et al. (8), in their studies of detoxification of ammonia by brain, provided another means of studying compartmentation of the glutamate-glutamine system in this organ. Infusion of ^{15}N-ammonium acetate at a constant rate into the carotid artery of the cat resulted in an ^{15}N content of the amide group of cerebral glutamine higher than that of liver or blood glutamine indicating the glutamine was synthesized in the brain. The high atom % excess of ^{15}N found in the amide group was easily explained by the de novo net synthesis of glutamine since an increased level of glutamine in brain was observed. This increased level of brain glutamine is in agreement with the observations of Flock et al. (9) on hepatectomized animals. However, the α-amino group of brain glutamine had many times the atom % excess found in brain glutamic acid. Thus, the new glutamine must have been derived from a pool of glutamic acid not in rapid equilibrium with the total content of brain glutamate.

These studies on ammonia metabolism hinted that there may be a carbon dioxide fixation mechanism in the brain to replenish the Krebs cycle intermediates lost due to net synthesis of glutamine. It was shown that upon intracarotid infusion of ^{14}C-sodium bicarbonate, brain amino acids showed considerable activity and the specific activity of glutamine was greater than or equal to the specific activity of glutamate (10-12). Simultaneous administration of ammonium acetate and ^{14}C-bicarbonate resulted in an increase in the specific activity of glutamine relative to that of aspartic acid and glutamic acid indicating an increased CO_2 fixation into glutamine.

It was shown that increased blood glutamate levels failed to raise the brain glutamate levels (13, 14) suggesting a blood-brain barrier to the passage of glutamate into the brain. These results were confirmed by administration of tracer amounts of ^{14}C-glutamate (15) in which a relatively small amount of radioactivity entered the brain. In contrast to earlier reports (16, 17), it was shown that glutamine does not enter the brain readily (8, 18).

O'Neal and Koeppe (18, 19) studied the labeling patterns of glutamate, glutamine, and aspartate after

administration of ^{14}C-labeled acetate, butyrate, glucose, lactate, and glycerol. They confirmed the results of Cremer (20) and Gaitonde (21) by noting that when labeled glucose is administered to rats, the specific activity of brain glutamate is always greater than that of glutamine. They also reported that when glucogenic precursors were administered (such as glucose, lactate, or glycerol), the specific activity of glutamine was always less than that of glutamate and when glutamate, glutamine, or ketogenic precursors were utilized (such as acetate or butyrate), the specific activity of glutamine was always greater than that of glutamate. More recently, it was shown that upon the administration of L-phenylalanine-U-^{14}C (22) and ethanol - 1 - ^{14}C (23) to rats, the specific activity of brain glutamine was always higher than that of brain glutamate. These results were explained by assuming that there were two pools of Krebs cycle intermediates, both accessible to pyruvate but only one readily accessible to metabolites that exhibit compartmentation (18). However, Cremer (24) found that upon administration of D-β-hydroxy butyrate - 3 - ^{14}C or acetoacetate - 3 - ^{14}C, the specific activity of glutamine was less than that of glutamate. This suggested that the formation of acetyl-CoA from these compounds took place in a compartment different from

the one in which acetate is converted to acetyl-CoA but similar to the one in which acetyl-CoA is formed from glucose. A scheme similar to that of O'Neal and Koeppe was suggested by Van den Berg et al. (25-27) in which they postulated at least two distinct Krebs cycles operating independently of each other in the brain. Figure 1 represents the current concept of compartmentation in brain as described by Berl and Clarke (28). One Krebs cycle presumably consists of relatively larger pools of intermediates that interchange rapidly with a large pool of glutamic acid and a small pool of glutamine; the other cycle, composed of smaller pools of intermediates, interchanges rapidly with the small pool of glutamic acid which equilibrates with the large pool of glutamine. The cycle containing the larger pools of intermediates is most likely used for energy production, being well protected from depletion of its intermediates by the large stabilizing pool of glutamic acid. The other cycle probably serves more as a "synthetic" cycle, e.g., ammonia detoxification.

Berl and Frigyesi (29) showed that upon administration of ^{14}C-leucine, the RSA (specific activity relative to glutamic acid) of glutamine was greater than one in most parts of the brain of the cat (cortex, hippocampus,

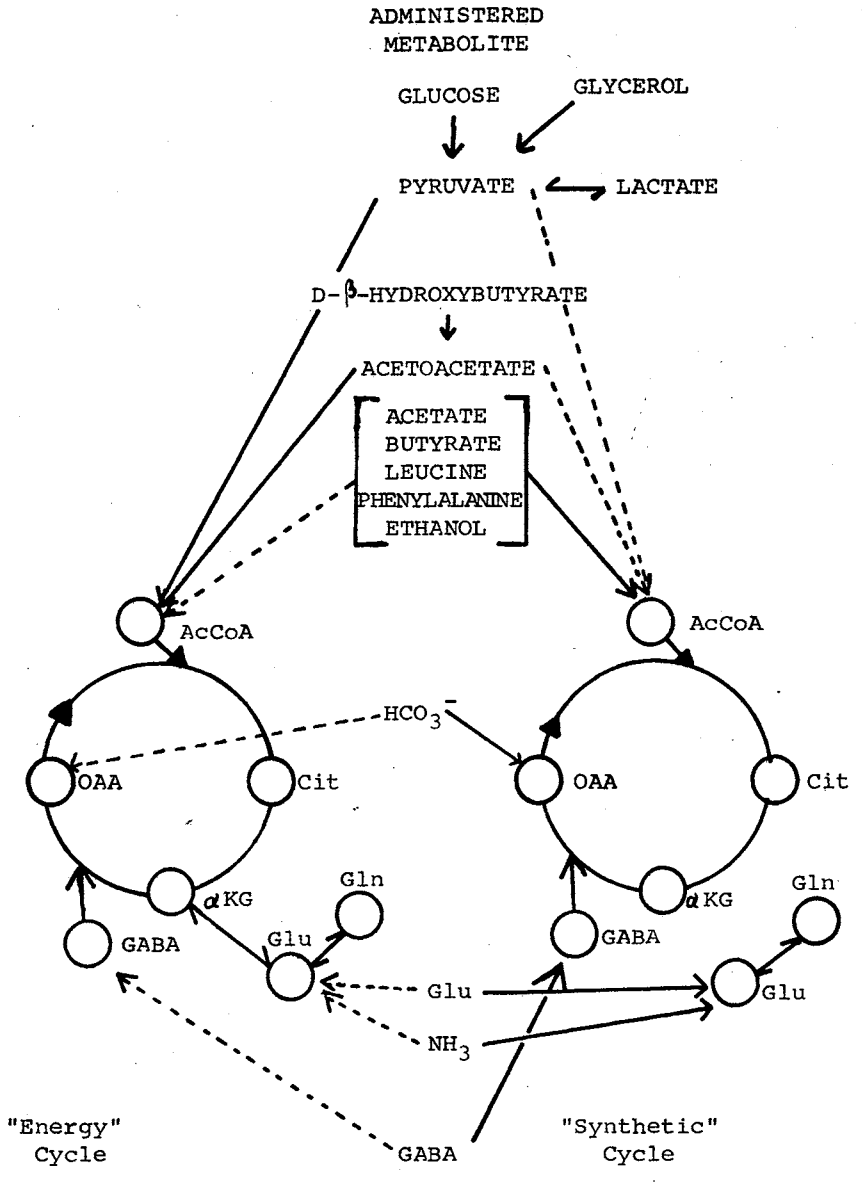

Figure 1. Schematic Description of Compartmentation of Amino Acid Metabolism in Brain. The relative sizes of the pools are not known. Solid lines represent the preferred metabolic pathways. Broken lines represent minor pathways. Cit, citrate; αKG, α-ketoglutarate; OAA, oxaloacetate; Glu, glutamic acid; Gln, glutamine; GABA, γ-aminobutyric acid

thalamus, pons, and medulla); this is consistent with the degradation pattern of leucine to acetyl-CoA. In contrast, in the cerebellum and caudate nucleus, the RSA of glutamine was less than one. Since administration of acetate-1-^{14}C resulted in a RSA of glutamine greater than one in all of the different parts of the brain of the cat, the postulate is that in the cerebellum and caudate nucleus, acetyl-CoA formation from leucine takes place in a different compartment from that in which acetyl-CoA is derived from acetate.

Berl (30) studied the maturational development of the glutamate-glutamine compartmentation system in the cerebral cortex of cats. His results show it appearing during the fourth week after birth. Similar studies (31) showed that this system developed two days after birth in the hippocampus but not until six weeks in the cerebellum, mesodiencephalon, and brainstem. There is some correlation between maturation of this compartmentation system and morphological development. Morphological development of neurons and synaptic structures proceeds rapidly during the four weeks following birth (32, 33). Elaboration of dendritic and axonal plexuses occurs in the latter part of this period but continues after four weeks. Neuronal and synaptic organization is considerably more mature

in the hippocampus at birth (34). In the cerebellum, Purkinje cells and other neural elements are not mature until after six weeks (35). The participation of glutamine synthetase in the development of the glutamate-glutamine compartmentation system was also studied (36), but only in the neocortex did the increase in enzyme activity parallel the appearance of compartmentation.

Until a few years ago, the glutamate-glutamine compartmentation system could be demonstrated only in vivo. In 1968, Berl et al. (37) reported results from work in vitro similar to those obtained in vivo. Preparation of the brain tissue slices at 0°C led to a large inhibition of incorporation of radioactivity from aspartate into glutamine but did not affect the labeling of glutamate appreciably. This inhibition could be reversed by preincubation of the slices at 37°C followed by transferal to fresh medium before addition of tracer amounts of radioactive aspartate; this increased the RSA of glutamine from a value less than one to a value greater than one. When the tissue slices were prepared at room temperature, a RSA of glutamine greater than one was obtained using labeled aspartate but preincubation and transferal to a new medium resulted in a higher RSA of glutamine. Berl et al. (37) also demonstrated carbon dioxide fixation

by brain slices using sodium bicarbonate-^{14}C. In addition, they illustrated the somewhat reciprocal effects of K^+ and Ca^{++} in the medium on the RSA of glutamine. Omission of Ca^{++} from the medium resulted in a decrease in the RSA of glutamine from a value greater than one to 0.2 using ^{14}C-labeled aspartate or glutamate as substrate. Addition of a high concentration of K^+ to the medium had a similar effect (38) but in this case, γ-aminobutyric acid-1-^{14}C was used as substrate. It was suggested that K^+ and Ca^{++} played reciprocal roles in regulating respiration in brain slices (39, 40).

Experimental methods to date have limited the consideration of compartmentation to metabolic definitions rather than to specific anatomical boundaries. It does appear, however, that at least some of the metabolic pools may be of anatomical origin. One can thus postulate a number of possibilities to account for compartmentation--at the cellular and intracellular level. Since the brain is quite heterogeneous with respect to its different cell types, one could conceive of particular cell types which are preferentially penetrated by certain compounds, e.g., glucogenic as glucose, lactate, and glycerol, while other cell types are preferentially penetrated by another class of compounds, e.g., ketogenic as acetate and butyrate.

However, it would be difficult to account for the ^{15}N-ammonia and ^{14}C-bicarbonate data on this basis. One might conceive of one cell type containing a small pool of glutamic acid which is actively converted to glutamine while another cell type, containing a large pool of glutamic acid, is slowly converted to glutamine. However, such disproportionate distribution of glutamic acid and glutamine has not been observed in the cerebral tissue (41, 42). The anatomic relationship of subcellular structures may also be important to compartmentation. Such may be the case in the relationship between the mitochondria and the endoplasmic reticulum since the latter structure has high glutamine synthetase activity (43).

The idea that subcellular structures are the major contributors to the compartmentation system is most prominent. De Robertis et al. (44) subfractionated crude mitochondrial preparations from rat cerebral cortex using discontinuous sucrose gradients. Electron micrographs of the different subfractions revealed the presence of mitochondria from different sources, e.g., those contained in the nerve endings and typical free mitochondria. These subfractions show different distributions of enzymes, including those related to the metabolism of

glutamate and glutamine (45-47). Also, Balazs et al. (48) have reported a heterogeneous enzyme distribution in mitochondrial subfractions isolated by Whittaker's discontinuous gradient method (49).

It should be realized that even though an enzyme may be present in a particular subcellular structure, it may not be operating *in vivo*. Despite this, mitochondrial heterogeneity (different populations of mitochondria) fits well with the concept of at least two Krebs cycles in brain cells operating independently of each other.

The purpose of this research was to investigate the relationship of the glutamate and glutamine compartments to subcellular structures. The concentrations of glutamate, glutamine, and in some cases aspartate in various subcellular fractions were determined. The fractions isolated were the non-particulate fraction and a number of particulate fractions which included the total particulate, the crude nuclear, the crude mitochondrial, the pure or free mitochondrial, and the microsomal fractions.

The distribution of radioactivity in the different subcellular fractions isolated after addition of glutamate-U-^{14}C or glutamine-U-^{14}C to the homogenization buffer was determined. The results showed that the glutamate and glutamine in the non-particulate fraction were not

equilibrating rapidly with the glutamate and glutamine in the particulate fractions during the fractionation procedure.

Experiments were performed in which rats were injected with ^{14}C-glucose or ^{14}C-acetate. The specific activities of glutamate and glutamine in the various brain fractions were then determined. After administration of glucose-2-^{14}C the RSA of glutamine in the non-particulate and the total particulate fractions was always less than one. Furthermore, the specific activities of glutamate and glutamine in the supernatant fraction were in essence the same as those of glutamate and glutamine in the particulate portion.

After administration of acetate-1-^{14}C, the RSA of glutamine in the non-particulate fraction was consistently greater than one while that in the total particulate fraction was equal to or less than one. The RSA of glutamine in the crude nuclear fraction was greater than one but in the crude mitochondrial fraction the RSA of glutamine was much less than one.

Although these experiments have not defined the "small" glutamate pool, the finding of striking differences in specific activities of the subcellular compartments

after giving labeled acetate is encouraging and suggests that this experimental approach may contribute to our understanding of brain metabolism.

CHAPTER II

EXPERIMENTAL

A. Materials Used

The D-glucose-2-^{14}C (SA = 3 mCi/mmole) and sodium acetate-1-^{14}C (SA = 59 mCi/mmole) were obtained from Amersham/Searle Corporation, Arlington Heights, Illinois. Both were obtained in freeze-dried form and diluted in distilled water for injection. One sample of sodium acetate-1-^{14}C (SA = 59 mCi/mmole), which was used for rats 63-66, was obtained from New England Nuclear Corporation, Boston, Mass.

The L-glutamic acid-U-^{14}C (SA = 9 mCi/mmole) and L-glutamine-U-^{14}C (SA = 9 mCi/mmole) were obtained from Calbiochem, Los Angeles, California, in freeze-dried form. The glutamine-U-^{14}C was dissolved in water and an aliquot passed over a Dowex 1- x 8 (100-200 mesh) acetate column after adjusting the pH to 7. The column was washed slowly with 50 mℓ water. Glutamic acid will remain on the column while glutamine will pass through (18). The

water effluent, containing the glutamine-U-^{14}C, was evaporated under a vacuum at 37°C and diluted to a known volume. Carrier glutamine was added to an aliquot of this stock glutamine-U-^{14}C solution and the mixture passed over another Dowex 1- x 8 acetate column after adjusting the pH to 7. 100% of the activity put on the column was recovered in the water effluent. The glutamic acid isolated from the same column (to be discussed) contained less than 0.4% of the activity added to the column. Thus, the stock glutamine-U-^{14}C solution contained less than 0.5% glutamic acid.

The glutamic acid-U-^{14}C was also dissolved in water and the pH adjusted to 7. It was purified by adding it to a Dowex 1- x 8 acetate column and isolating the glutamic acid from the column.

Glutaminase was obtained from Sigma Chemical Company, St. Louis, Missouri.

B. Animal Experiments

Albino rats obtained from the Holtzman Company were used in all of the experiments. All animals were allowed to eat ad libitum a stock laboratory diet up to the time of the experiment. The duration of the labeling experiments was always 10 minutes. The animals were

sacrificed by decapitation with a guillotine. Unless stated otherwise, the entire brain (cerebrum and cerebellum) was excised. When very small amounts of amino acid were to be assayed, the brains of three rats subjected to the same treatment were pooled.

After excision, the brain was blotted with filter paper to remove the blood and weighed. It was then homogenized in 0.25 M sucrose dissolved in 0.1 M phosphate buffer, pH 7.0, for 1½-2 minutes (15-20 strokes) with a loose teflon pestle (0.43 mm clearance). For those experiments in which only one brain was excised, 5 mℓ of buffer were used while 30 mℓ were used for the experiments in which three brains were pooled. The homogenate was centrifuged at 97,000 x g or 900 x g (depending on the experiment) at 0°C immediately. The time elapsed from decapitation to centrifugation was 4-5 minutes (except in those cases where three brains were pooled).

1 mM isoniazide was added to the homogenization buffer used for rats 47-57. Isoniazide is a potent inhibitor of many pyridoxal phosphate enzymes, including glutamic acid decarboxylase and many amino-transferases.

Glutamic acid-U-^{14}C or glutamine-U-^{14}C was added to the homogenization buffer in some experiments to test if the glutamate and/or glutamine in the non-particulate

fraction was equilibrating rapidly with the glutamate and glutamine pools in the particulate fractions. Information concerning the various brains used in these experiments is shown in Table I. Rat weights ranged from 200-400 grams unless stated otherwise.

Given in Table II is a summary of information concerning the experiments in which the animals were injected with radioactive compounds. Included are the rat weight, the brain weight, the ^{14}C-labeled compound administered, the route of injection, the amount, volume, and activity injected. For rats 47-66, only the cerebrum was excised while for all of the other animals both the cerebrum and cerebellum were excised. In many of these experiments (see Table II) 0.5 mmole of carrier sodium acetate was added to the injected sample. This increases the incorporation of radioactivity from sodium acetate-1-^{14}C into brain amino acids (Mushahwar and Koeppe, unpublished results).

C. Fractionation Procedures

For rats 1-16 and 26-46, only two fractions were obtained--a non-particulate or supernatant fraction (labeled S) and a particulate fraction (labeled Pt or

TABLE I

SUMMARY OF THE EXPERIMENTS IN WHICH GLUTAMATE-U-^{14}C OR GLUTAMINE-U-^{14}C WAS ADDED TO THE HOMOGENIZATION BUFFER SHOWING THE ACTIVITY AND THE AMOUNT ADDED

Rat No.	Compound	Activity[a]	Amount[b]
		μCi	nmoles
7	Glutamate-U-^{14}C	0.067	7.5
8	Glutamate-U-^{14}C	0.050	5.5
9	Glutamate-U-^{14}C	0.25	28
10	Glutamate-U-^{14}C	0.25	28
11	Glutamate-U-^{14}C	0.074	8.3
12	Glutamine-U-^{14}C	0.073	8.1
13	Glutamine-U-^{14}C	0.063	7.0
14	Glutamine-U-^{14}C	0.063	7.0
15	Glutamine-U-^{14}C	0.063	7.0
16	Glutamine-U-^{14}C	0.063	7.0
20	Glutamate-U-^{14}C	0.13	15
21	Glutamate-U-^{14}C	0.13	15
22	Glutamate-U-^{14}C	0.13	15
23	Glutamine-U-^{14}C	0.061	6.8
24	Glutamine-U-^{14}C	0.12	14
25	Glutamine-U-^{14}C	0.12	14
67	Glutamate-U-^{14}C	0.064	7.1
68	Glutamate-U-^{14}C	0.064	7.1
69	Glutamate-U-^{14}C	0.064	7.1

[a]Activities were determined in this lab.

[b]Determined from specific activity given by manufacturer.

TABLE II

SUMMARY OF ^{14}C-LABELING EXPERIMENTS
In Vivo

Rat No.	Rat Weight	Brain Weight	Compound Injected	Route of Injection	Amount Injected	Activity Injected	Volume Injected
	Grams	Grams			μmoles	μCi	mℓ
26	190	1.20	Glucose-2-^{14}C	I.P.[a]	3.2	9.6	0.80
27	206	1.39	Glucose-2-^{14}C	I.P.	3.2	9.6	0.80
28	193	0.68	Glucose-2-^{14}C	I.P.	3.2	9.6	0.80
29	190	1.54	Glucose-2-^{14}C	I.P.	3.1	9.4	0.85
30	140	1.40	Glucose-2-^{14}C	I.P.	3.1	9.4	0.85
31	120	1.38	Sodium Acetate-1-^{14}C	I.P.	0.39	23	0.50
32	182	1.21	Sodium Acetate-1-^{14}C	I.P.	0.39	23	0.50
33	200	1.40	Sodium Acetate-1-^{14}C	I.P.	0.39	23	0.50
34	170	1.34	Sodium Acetate-1-^{14}C	I.P.	0.45	27	1.0
35	170	1.40	Sodium Acetate-1-^{14}C	I.P.	0.45	27	1.0
36	175	1.02	Glucose-2-^{14}C	I.C.[b]	0.80	2.4	0.2
37	175	1.35	Glucose-2-^{14}C	I.C.	0.80	2.4	0.2
38	175	1.45	Glucose-2-^{14}C	I.C.	0.80	2.4	0.2
39	220	1.25	Sodium Acetate-1-^{14}C	I.C.	0.045	2.7	0.1
40	220	1.50	Sodium Acetate-1-^{14}C	I.C.	0.045	2.7	0.1
41	220	1.45	Sodium Acetate-1-^{14}C	I.C.	0.045	2.7	0.1
42	220	1.27	Sodium Acetate-1-^{14}C	I.C.	0.045	2.7	0.1
43	340	1.22	Sodium Acetate-1-^{14}C	I.C.	0.045	2.7	0.1
44	340	1.28	Sodium Acetate-1-^{14}C	I.C.	0.045	2.7	0.1
45	340	1.22	Sodium Acetate-1-^{14}C	I.C.	0.045	2.7	0.1
46	360	1.53	Sodium Acetate-1-^{14}C	I.C.	0.045	2.7	0.1
47[c]	322,319,236	3.70	Sodium Acetate-1-^{14}C	I.P.	500	27	2.0
48[c]	250,289,244	3.90	Sodium Acetate-1-^{14}C	I.P.	500	27	2.0
49[c]	264,227,247	3.30	Sodium Acetate-1-^{14}C	I.P.	500	27	2.0
50[c]	226,258,269	3.00	Sodium Acetate-1-^{14}C	I.P.	500	27	2.0
60[c]	200,200,200	3.00	Sodium Acetate-1-^{14}C	I.P.	500	27	2.0
61[c]	230,230,190	3.30	Sodium Acetate-1-^{14}C	I.P.	500	27	2.0
62[c]	150,150,150	3.10	Sodium Acetate-1-^{14}C	I.P.	500	27	2.0
63[c]	220,220,220	3.40	Sodium Acetate-1-^{14}C	I.P.	500	25	2.0
64[c]	230,230,230	3.55	Sodium Acetate-1-^{14}C	I.P.	500	25	2.0
65[c]	145,145,145	3.45	Sodium Acetate-1-^{14}C	I.P.	500	25	2.0
66[c]	145,145,145	3.60	Sodium Acetate-1-^{14}C	I.P.	500	25	2.0

[a] I.P. = Intraperitoneal

[b] I.C. = Intracisternal

[c] The brains of three rats subjected to the same treatment were pooled prior to homogenization.

total particulate). The Pt fraction was obtained by centrifuging the homogenate at 97,000 x g for 45 minutes in the Spinco Model L Preparative Ultracentrifuge, Type 50 rotor, rehomogenizing the pellet in 5 mℓ of the same buffer, and centrifuging again at 97,000 x g for 30 minutes. The two supernatants were combined to make up the S fraction.

The method of De Robertis et al. (44) was used for rats 20-25 and 47-50 to isolate a nuclear fraction (labeled P_1), a crude mitochondrial fraction (labeled P_2), a microsomal fraction (labeled P_3), and a non-particulate fraction (labeled S). The P_1 fraction was obtained by centrifuging the homogenate at 900 x g for 10 minutes, rehomogenizing the pellet in 5 mℓ of the same buffer, and centrifuging as before. The two supernates were combined and centrifuged at 10,000 x g for 15 minutes. The pellet was suspended in 5 mℓ of the homogenization buffer and centrifuged as before to get the P_2 fraction. The two supernates of the P_2 fraction were centrifuged at 54,000 x g for one hour in the Spinco Model L Preparative Ultracentrifuge, Type 30 rotor, to get the P_3 fraction. The resulting supernate was the S fraction (free of microsomes).

For rats 67-69, this same method of De Robertis was used to isolate a P_1 and a P_2 fraction but a P_3 or microsomal fraction was not isolated. Thus, the S fraction of these rats was not free of microsomes.

The method of De Robertis et al. (44) was used for rats 51-66 to obtain a relatively pure mitochondrial fraction (labeled M). The method consisted of subfractionating the P_2 or crude mitochondrial fraction in a discontinuous sucrose gradient in cellulose nitrate tubes, 1 inch x 3 inches, with the following steps: 1.4 (5 mℓ), 1.2 (7 mℓ), 1.0 (7 mℓ), and 0.8 (7 mℓ) M sucrose. The gradient was left at room temperature for two hours and in the cold for one hour prior to use. The P_2 fraction was suspended in 15 mℓ of 0.25 M sucrose buffer and 5 mℓ carefully layered on each of three gradients. They were then centrifuged for two hours at 54,000 x g in the Spinco Model L Preparative Ultracentrifuge, SW 25.1 rotor. This procedure resulted in four subfractions (A, B, C, D) layered in the gradient, and a pellet (the M fraction). This pellet (intact mitochondria) has been shown by electron microscopy to be almost free of nerve endings and synaptic vesicles as well as myelin and other subcellular structures present in the crude mitochondrial fraction (44).

For rats 51-57 and 60-66, the M fraction (pellet) in each of the three centrifuge tubes was removed by cutting out the bottom of the tube and washing it off with water into a centrifuge tube and frozen immediately. The M fractions isolated in seven experiments (21 rats) were combined and the glutamic acid isolated as described below. The amount of protein in the combined M fractions of rats 60-66 was also determined by the Biuret reaction (50).

D. Isolation and Assay of Glutamate, Aspartate, and Glutamine

The isolation of glutamate, glutamine, and aspartate was done according to the method of O'Neal and Koeppe (18). The isolated particulate fractions were suspended in 5 mℓ of 7% $HClO_4$ (51, 52), centrifuged at 10,000 x g for 10 minutes, washed once with 5 mℓ of 0.33 M $HClO_4$, and centrifuged again. The supernates were combined, neutralized with KOH, and centrifuged to remove $KClO_4$. This supernate was passed over a Dowex 1- x 8 (100-200 mesh) acetate column (1 x 22 cm). The column was first washed with 50-75 mℓ of water. Glutamine and other neutral and basic amino acids pass through the column while glutamate and aspartate remain on the column. The column was eluted with 0.5 M acetic acid at a rate of one drop every six

seconds. Approximately 3 mℓ fractions (60 drops/fraction) were collected. This procedure readily separates glutamate and aspartate from other acidic components of brain. Glutamate elutes from the column first (fractions 7-13) and aspartate follows (fractions 16-28). The glutamate and aspartate peaks were evaporated under a vacuum at 35°C and diluted to a known volumn before being assayed.

Glutamine was isolated according to the method of Mushahwar and Koeppe (23). The water effluent, containing glutamine, was evaporated under a vacuum at 35°C to near dryness and diluted to approximately 9 mℓ with 0.1 M sodium acetate buffer, pH 4.9. To this was added 0.5-1 unit of glutaminase and the solution incubated for one hour at 37°C. After incubation, the solution was neutralized and glutamic acid from glutamine was isolated as discussed above. All of the amino acids isolated were assayed by the ninhydrin method of Rosen (53) as modified by Grant (54). In all but one of the ninhydrin assays performed, the absorbance reading was at least 0.1 absorbance unit above blank (corresponding to 0.04 μmole of glutamic acid). All assays were done in either duplicate or triplicate.

The S fraction was added directly to a Dowex 1- x 8 acetate column and the amino acids isolated as described

before. Sucrose was present in the water effluent and consequently in the incubation mixture but this did not affect the enzymatic hydrolysis of glutamine to glutamic acid. The presence of sucrose did, however, make acid hydrolysis impossible since the hydrolysate turned a dark brown color (furfural polymers) and the glutamic acid could not be isolated from it.

The isolated amino acid fractions obtained from rats injected with Na-acetate-1-^{14}C were evaporated to dryness under a vacuum at 40°C several times in the presence of HCl to volatilize any labeled acetic acid. This precaution was done routinely to insure that no contamination of the glutamate peak with acetate-1-^{14}C had occurred. When Na-acetate-1-^{14}C was applied to a standard Dowex 1- x 8 acetate column the water effluent had no activity. On eluting with 0.5 M acetic acid, however, all of the activity appeared in the aspartate peak while little, if any, activity was found in the glutamic acid peak.

E. Measurement of Radioactivity

The ^{14}C-content of the isolated brain amino acids, the perchloric acid filtrates of the various particulate fractions, the non-particulate fractions, and other ^{14}C-labeled materials was measured in a Packard Tri-Carb

liquid-scintillation spectrometer with Bray's solution (55) as scintillator. The observed CPM (counts per minute) were corrected for quenching and for presentation, converted to nCi or μCi. In all but a few experiments the radioactivity counting error was 10% or less (confidence level 0.99). In some of the recovery experiments, considerably larger errors were introduced when small amounts of radioactivity were being counted. However, the error in these cases had no effect on the interpretation of the results.

CHAPTER III

RESULTS AND DISCUSSION

A. Amino Acid Distributions

A summary of the distribution of glutamate, aspartate, and glutamine in the non-particulate (S) and total particulate (Pt) fractions of rat brain is given in Table III. The combined values of the two fractions, although slightly higher, are in agreement with the reports of others (56; Mushahwar and Koeppe, unpublished results) for total brain glutamate, aspartate, and glutamine. As can be seen, the glutamate and aspartate values are fairly consistent. The glutamine values for both fractions for rat numbers 39-46, however, are obviously low. These low values are the result of incomplete hydrolysis of glutamine by glutaminase. Consequently, these values were not included in the calculation of the mean. The glutamine values for rat numbers 29, 30, and 34-38 also appear to be low, but there was no evidence of incomplete hydrolysis or incomplete recovery of glutamic acid from glutamine for these rats.

TABLE III

DISTRIBUTION OF GLUTAMATE, ASPARTATE, AND GLUTAMINE IN THE NON-PARTICULATE (S) AND TOTAL PARTICULATE (PT) FRACTIONS OF RAT BRAIN

(Values are given in μmoles per gram wet weight of tissue)

	S Fraction			Pt Fraction		
Rat No.	Glu	Asp	Gln[a]	Glu	Asp	Gln[a]
1	8.8	2.4	3.2	3.5	0.73	0.67
2	9.0	2.2		4.1	0.84	
3	7.5	2.1	3.5	4.1	0.86	0.64
4	8.7	2.6	3.7	4.4	0.98	0.88
5	7.5	2.1	3.0	4.0	0.82	0.60
6	7.7	2.5	3.7	4.1	0.94	0.63
8	8.0	2.5		5.1	0.34	
11	8.6	2.6			0.98	
15	8.7	2.5		4.3	1.1	
16	7.8	2.8		5.3	1.1	
26	8.6	2.9	3.5	4.2	1.1	0.75
27	8.6	2.3	4.0	5.6	1.0	1.1
28	8.3	4.1	3.7	3.8	0.91	0.69
29	8.5		2.2	4.1		0.39
30	8.2		2.5	4.1		0.39
31	8.5	2.5	3.2	4.6	1.2	0.94
32	8.6	2.9	3.6	4.8	1.0	1.0
33	7.9	2.2	3.7	5.1	1.1	0.89
34	9.3		2.3	3.7		0.46
35	8.3		2.6	4.8		0.63
36	8.0		2.0	4.4		0.52
37	7.2		2.3	4.3		0.36
38	7.4		2.2	4.2		0.52
39	8.1		0.50	4.7		0.60
40	8.5		0.78	3.5		0.51
41	8.8		0.84	4.1		0.44
42	8.4		0.68	4.3		0.47
43	8.5		0.96	4.0		0.34
44	8.6		1.1	3.8		0.36
45	9.1		0.70	4.3		0.36
46	8.1		0.57	3.9		0.29
Mean	8.3	2.6	3.1	4.3	0.94	0.75
±SEM[b]	±0.09	±0.12	±0.11	±0.09	±0.05	±0.05

[a]Values given for rat numbers 39-46 were not included in the calculation of the mean, see text.

[b]SEM = Standard Error of the Mean.

Glu = Glutamate; Asp = Aspartate; Gln = Glutamine.

Therefore, these values were included in the calculation of the mean.

The distribution of glutamate and glutamine in the primary subcellular fractions, e.g., S, P_1, P_2, and P_3 fractions, is shown in Table IV. The glutamate values for the S fractions were higher than those observed in previous experiments. It should be recalled that isoniazide was added to the homogenization buffer of rats 47-50. Also, the S fractions of rats 67-69 were contaminated with microsomes, causing a very slight (almost insignificant) increase in the value for glutamate in the S fraction.

B. Radioactivity Recovery Experiments

To interpret the labeling experiments <u>in vivo</u> more realistically it was necessary to show that the glutamate and glutamine pools of the particulate fractions were not interchanging rapidly with the glutamate and glutamine in the non-particulate fraction. To test for this, L-glutamate-U-^{14}C or L-glutamine-U-^{14}C were added to the homogenization buffer in separate experiments and the distribution of radioactivity in the various subfractions was determined.

The percent recoveries of radioactivity in the S and Pt fractions after addition of L-glutamate-U-^{14}C and

TABLE IV

DISTRIBUTION OF GLUTAMATE AND GLUTAMINE IN PRIMARY SUBCELLULAR FRACTIONS[a]

(Values are given in μmoles per gram wet weight of tissue)

	S Fraction		P_1 Fraction		P_2 Fraction		P_3 Fraction	
Rat No.	Glu	Gln	Glu	Gln	Glu	Gln	Glu	Gln
47	10.3	2.4	4.5	0.90	1.3	0.18	0.18	0.08
48	9.9	2.0	3.9	0.70	0.83	0.17	0.18	0.07
49	8.6	2.3	3.9	0.85	0.77	0.17	0.11	0.09
50	9.2	2.6	4.2	1.1	1.2	0.22	0.21	0.04
67	7.8		4.7		0.32			
68	7.3		4.4		0.26			
69	8.9		4.3		0.27			
Mean	8.9	2.3	4.3	0.89	0.71	0.19	0.17	0.07
±SEM[a]	±0.41	±0.13	±0.11	±0.08	±0.17	±0.01	±0.02	±0.01

[a]Data obtained by Dr. I. K. Mushahwar.

[b]SEM = Standard Error of the Mean.

Glu = Glutamate; Gln = Glutamine

L-glutamine-U-^{14}C to the homogenization buffer are shown in Tables V and VI. Although 5-10% of the radioactivity in the S fraction was recovered in the water effluent, the recovery of 80-90% in glutamic acid after addition of glutamate-U-^{14}C to the homogenization buffer (Table V) was favorable. Most important was the finding that 5% or less of the activity added was recovered in the Pt fraction.

After addition of L-glutamine-U-^{14}C to the homogenization buffer (Table VI), over 80% of the activity in the S fraction was recovered in the water effluent but 10-20% was recovered in glutamate. The recovery of 10% of the activity added in the Pt fraction, most of which was present in glutamic acid, was also unfavorable. No explanation can be given for this latter observation.

The percent recoveries of radioactivity in the primary subcellular fractions after addition of L-glutamate-U-^{14}C and L-glutamine-U-^{14}C to the homogenization buffer are shown in Tables VII and VIII. After addition of L-glutamate-U-^{14}C (Table VII), very small amounts of activity were recovered in the particulate fractions, and virtually all of the radioactivity recovered was present in glutamate. However, after addition of L-glutamine-U-^{14}C,

TABLE V

PERCENT RECOVERY OF RADIOACTIVITY IN AMINO ACIDS AFTER ADDITION OF GLUTAMATE-U-^{14}C TO BRAIN HOMOGENATE

		S Fraction[b]					P_t Fraction[b]		
Rat No.	S Fraction[a]	Glu	Asp	Gln	Water Effluent	P_t Fraction[a]	Glu	Asp	Water Effluent
7	82.4	85.6	2.6	6.6	10.5	2.6	68.0	9.1	6.3
8	76.1	89.8	1.4		5.5	5.0	78.2	1.0	10.7
9	87.5	87.7			4.8				
10	84.1	81.3			12.1				
11	73.3	90.4	0.9		6.5	4.1		2.9	13.0
Mean	80.7	87.0	1.6		7.9	3.9	73.1	4.3	10.0
±SEM[c]	±2.6	±1.6	±0.5		±1.4	±0.7		±2.5	±2.0

[a]Percent recovery based on the radioactivity added to the homogenate as 100%.

[b]Percent recovery based on the radioactivity present in the fraction prior to addition to the column.

[c]SEM = Standard Error of the Mean.

Glu = Glutamate; Asp = Aspartate; Gln = Glutamine.

TABLE VI

PERCENT RECOVERY OF RADIOACTIVITY IN AMINO ACIDS AFTER ADDITION OF GLUTAMINE-U-^{14}C TO BRAIN HOMOGENATE

		S Fraction[b]				Pt Fraction[b]			
Rat No.	S Fraction[a]	Glu	Asp	Water Effluent	Pt Fraction[a]	Glu	Asp	Gln	Water Effluent
12					7.9	58.3	3.2	4.5	30.8
13	92.3	11.4		87.4					
14	83.5	18.9		76.9					
15	79.3	15.0	0.3	84.7	10.0	75.9	3.1		26.7
16	83.0	14.0	0.5	80.8	10.2	71.9	3.7		22.5
Mean	84.5	14.8	0.4	82.5	9.4	68.7	3.3	4.5	26.7
±SEM[c]	±8.4	±1.6		±2.3	±0.7	±5.3	±0.2		±2.4

[a]Percent recovery based on the radioactivity added to the homogenate as 100%.

[b]Percent recovery based on the radioactivity present in the fraction prior to addition to the column.

[c]SEM = Standard Error of the Mean.

Glu = Glutamate; Asp = Aspartate; Gln = Glutamine.

TABLE VII

PERCENT RECOVERY OF RADIOACTIVITY IN PRIMARY SUBCELLULAR FRACTIONS AFTER ADDITION OF GLUTAMATE-U-^{14}C TO BRAIN HOMOGENATE

		S Fraction[b]			P_1 Fraction[b]			P_2 Fraction[b]		
Rat No.	S Fraction[a]	Glu	Water Effluent	P_1 Fraction[a]	Glu	Water Effluent	P_2 Fraction[a]	Glu	Water Effluent	P_3 Fraction[a]
20	86.6			3.9			0.2			0.0
21	90.0			3.7			0.2			0.0
22	84.1			2.2			0.1			0.0
67	90.1	100.0	1.9	5.2	96.0	2.3	0.0	0.0	0.0	
68	90.8	99.7	1.7	4.2	98.6	1.5	0.0	0.0	0.0	
69	91.8	100.0	1.4	3.7	99.2	0.0	0.0	0.0	0.0	
Mean	88.9	99.9	1.7	3.8	97.9	1.3	0.08	0.0	0.0	0.0
±SEM[c]	±1.2	±0.0	±0.1	±0.4	±1.0	±0.7	±0.04			

[a]Percent recovery based on the radioactivity added to the homogenate as 100%.

[b]Percent recovery based on the radioactivity present in the fraction prior to addition to the column.

[c]SEM = Standard Error of the Mean.

Glu = Glutamate.

TABLE VIII

PERCENT RECOVERY OF RADIOACTIVITY IN PRIMARY SUBCELLULAR FRACTIONS AFTER ADDITION OF GLUTAMINE-U-^{14}C TO BRAIN HOMOGENATE

Rat No.	S[a] Fraction	P_1[a] Fraction	P_2[a] Fraction	P_3[a] Fraction
23	83.2	7.0	0.9	0.0
24	83.3	10.9	0.9	0.0
25	82.6	9.3	0.8	0.0
Mean	83.0	9.1	0.9	0.0
±SEM[b]	±0.3	±1.1	±0.04	

[a]Percent recovery based on the radioactivity added to the homogenate as 100%.

[b]SEM = Standard Error of the Mean.

small but significant amounts of activity were recovered in the particulate fractions.

The enzymes responsible for the interconversion of glutamate and glutamine, namely glutamine synthetase and glutaminase, are located predominantly in the microsomal and mitochondrial fractions respectively (45). From the results given in Tables V and VII, it appears that glutamine synthetase is relatively inactive during the fractionation procedure. This was not surprising since ATP and ammonia, as well as glutamate, are necessary for glutamine synthesis. However, glutaminase is active to some extent.

Although the results indicate that the glutamate and glutamine pools of the particulate fractions are not equilibrating rapidly with the glutamate and glutamine in the S fraction, obtaining more statistical evidence for this would be justifiable. In addition, control experiments of this type with the use of inhibitors of enzymes related to the metabolism of glutamate and glutamine would be helpful. It should be noted that these data do not reflect the degree of equilibration, if any, of glutamate and/or glutamine between the different particulate fractions. Even though the results were not

as good as hoped for, especially in the case of the L-glutamine-U-^{14}C experiments (Table VI), it appeared feasible to proceed with the ^{14}C-labeling experiments *in vivo*.

C. ^{14}C-Labeling Experiments *in vivo*

The specific activities of glutamate and glutamine in the S and Pt fractions after administration of glucose-2-^{14}C are shown in Table IX. As expected, the RSA of glutamine in both subfractions was less than one. Furthermore, the specific activities of glutamate and glutamine in the S fraction were virtually the same as those of glutamate and glutamine in the Pt fraction.

It has long been known that glucose readily penetrates the brain and is rapidly metabolized, being the brain's chief energy source. Thus, it was no surprise to see that ^{14}C-glucose was labeling the "entire brain." That is, from the results shown in Table IX, it appears that neither ^{14}C-labeled glucose nor its degradation products are being metabolized in any selective part(s) of the brain. With this in mind it was apparent that experiments of this type with ^{14}C-glucose would not yield any evidence as to the location of the small glutamate-glutamine pool.

TABLE IX

SPECIFIC ACTIVITIES OF GLUTAMATE, ASPARTATE, AND GLUTAMINE IN THE S AND Pt FRACTIONS AFTER ADMINISTRATION OF GLUCOSE-2-^{14}C

(Values are given in nCi per μmole)

Rat No.	S Fraction			Pt Fraction			RSA of Gln in S	RSA of Gln in Pt
	Glu	Asp	Gln	Glu	Asp	Gln		
26	1.4	0.94	0.66	1.3	0.98	0.71	0.47	0.55
27	1.4	1.0	0.57	1.0	0.90	0.80	0.41	0.80
28	1.7	0.82	0.87	1.3	1.1	0.96	0.51	0.74
29	2.1		0.94	1.8		0.85	0.45	0.47
30	2.3		0.94	1.9		1.1	0.41	0.58
36	3.8		2.0	3.4		1.9	0.53	0.56
37	2.6		1.3	2.2		1.5	0.50	0.68
38	3.0		1.7	2.5		1.1	0.57	0.44

Glu = Glutamate; Asp = Aspartate; Gln = Glutamine.

Therefore, efforts were concentrated on labeling experiments *in vivo* using ^{14}C-acetate.

After administration of acetate-1-^{14}C, the RSA of glutamine in the S fraction was consistently greater than one while that in the Pt fraction was equal to or less than one (See Table X). These results were not entirely unexpected. The observation that the RSA of glutamine is greater than one for total brain glutamine after administration of acetate-^{14}C, coupled with the findings here that most of the glutamate and glutamine in brain is present in the soluble fraction would lead one to expect a RSA of glutamine greater than one in the S fraction. This does, however, further support the concept that acetate-^{14}C is preferentially labeling a small pool of glutamic acid. This glutamate pool and/or the glutamine pool associated with it is (are), in turn, labeling the glutamine pool in the S fraction. This necessarily implies that the so-called small glutamate-glutamine pool is present in the Pt fraction.

Thus it was thought one might localize this small pool of glutamic acid by further fractionating the total particulate fraction into three primary subcellular fractions, e.g., the crude nuclear (P_1), the crude

TABLE X

SPECIFIC ACTIVITIES OF GLUTAMATE, ASPARTATE, AND GLUTAMINE IN THE S AND Pt FRACTIONS AFTER ADMINISTRATION OF SODIUM ACETATE-1-^{14}C

(Values are given in nCi per μmole)

Rat No.	S Fraction			Pt Fraction			RSA of Gln in S	RSA of Gln in Pt
	Glu	Asp	Gln	Glu	Asp	Gln		
31	0.21	0.16	0.51	0.31	0.17	0.35	2.4	1.1
32	0.21	0.14	0.51	0.30	0.19	0.32	2.4	1.1
33	0.23	0.16	0.42	0.27	0.17	0.32	1.8	1.2
34	0.36		0.79	0.47		0.46	2.2	1.0
35	0.43		0.86	0.57		0.39	2.0	0.68
39	2.7		3.7	3.9		2.6	1.4	0.67
40	1.6		2.1	2.3		1.0	1.3	0.43
41	6.7		12.4	7.3		5.5	1.9	0.75
42	14.0		15.8	14.4		14.7	1.1	1.0
43	9.7		13.9	10.7		6.0	1.4	0.56
44	14.3		13.4	15.0		10.9	0.94	0.73
45	8.6		14.7	9.8		3.4	1.7	0.35
46	5.4		7.6	6.1		2.6	1.4	0.43

Glu = Glutamate; Asp = Aspartate; Gln = Glutamine.

mitochondrial (P_2), and the microsomal (P_3) fractions, and determining the specific activities of the glutamate and glutamine in each after administration of acetate-1-^{14}C. The results of such experiments are summarized in Tables XI and XII. The RSA of glutamine in the S and P_1 fractions was greater than one while that in the P_2 and P_3 fractions was less than one. However, in none of the fractions isolated was the specific activity of glutamate or glutamine high enough to account for the high specific activity of glutamine in the S fraction. On the other hand, the results were very favorable in that this was the first time a subfraction of brain with a RSA of glutamine less than one has been obtained after administration of acetate-1-^{14}C. Furthermore, one would expect this to be the case for the site(s) where ^{14}C-acetate is labeling glutamate. However, it should not be assumed that the glutamic acid pool that is being labeled from ^{14}C-acetate is necessarily labeling directly the glutamine pool in the same anatomical compartment. This concept is illustrated in Figure 2. It would seem at this point that more experiments of this type, but with shorter time periods, might lead to more conclusive evidence as to the localization of the small glutamate-glutamine pool(s).

TABLE XI

SPECIFIC ACTIVITIES OF GLUTAMATE AND GLUTAMINE IN THE PRIMARY SUBCELLULAR FRACTIONS AFTER ADMINISTRATION OF SODIUM ACETATE-1-^{14}C [a]

(Values are given in nCi per μmole)

Rat	S Fraction		P_1 Fraction		P_2 Fraction		P_3 Fraction	
No.	Glu	Gln	Glu	Gln	Glu	Gln	Glu	Gln
47	0.17	0.36	0.20	0.51	0.17	0.074	0.10	0.031
48	0.21	0.48	0.28	0.42	0.26	0.087	0.16	0.12
49	0.21	0.50	0.27	0.58	0.24	0.084	0.20	0.063
50	0.19	0.45	0.26	0.57	0.27	0.098	0.10	0.084

[a]Data obtained by Dr. I. K. Mushahwar.

Glu = Glutamate; Gln = Glutamine

TABLE XII

SPECIFIC ACTIVITY OF GLUTAMINE RELATIVE TO THAT OF GLUTAMATE IN PRIMARY SUBCELLULAR FRACTIONS

(Calculated from Table XI)

Rat No.	S Fraction	P_1 Fraction	P_2 Fraction	P_3 Fraction
47	2.1	2.6	0.44	0.31
48	2.3	1.5	0.33	0.75
49	2.4	2.1	0.35	0.32
50	2.4	2.2	0.36	0.84

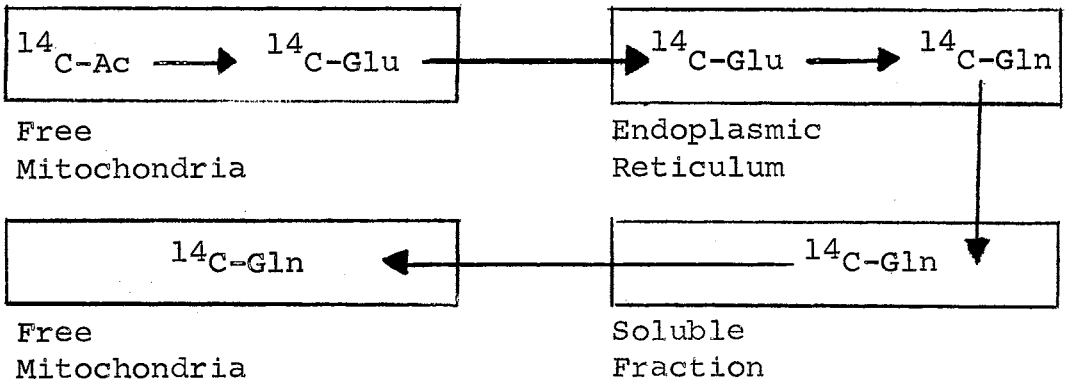

Figure 2. Schematic Description Showing Transfer of ^{14}C from Acetate to Glutamine in Brain (Ac = Acetate; Glu = Glutamate; Gln = Glutamine)

It is possible that after 10 minutes most of the ^{14}C in acetate has been transferred to glutamine in the soluble fraction via the glutamate pool that is preferentially labeled by ^{14}C-acetate. In shorter time experiments one would hope to find an increase in the SA of the "small" glutamate pool and a decrease in the SA of glutamine in the soluble fraction. At the same time, if the scheme presented in Figure 2 is correct, one would not necessarily find an increase in the SA of the glutamine pool present in the same fraction as the small glutamate pool, e.g., free mitochondria.

A correction factor to account for the glutamate-glutamine interconversions after decapitation is realistic but seemingly unnecessary. Take, for example, the interconversion of glutamate and glutamine in the S fraction. 15% of the glutamine in the S fraction appears to be converted to glutamate while possibly up to 10% of the glutamate appears to be converted to glutamine. Thus, there appears to be a net transfer of activity of at least 5% from glutamine to glutamate. Since, after acetate-1-^{14}C injection, the SA of glutamine is approximately twice that of glutamate in the S fraction, this correction would serve to increase the RSA of glutamine slightly. And since only the fact that the RSA of glutamine is greater than one is of prime importance, the correction is unnecessary.

To investigate further, the P_2 fraction was subfractionated to obtain a relatively pure mitochondrial fraction (M fraction). For rat 59 the protein recoveries in the P_2 fraction and in the three combined M fractions were determined. The results are shown in Table XIII. These recoveries are much less than those reported by De Robertis (44, 45). However, since the glutamic acid recovery was to be expressed in μmoles per mg protein in

TABLE XIII

PROTEIN RECOVERIES IN P_2 AND M FRACTIONS

	Total Protein	Protein Recovery[a]
	(mg)	(mg)
P_2 Fraction	43.1	12.7
M Fraction	1.6	0.47

[a]Values are given in mg protein per gram wet weight of brain.

the M fraction, high protein recoveries were not as important as one might expect. Furthermore, since the SA of glutamic acid in the M fraction was to be determined, purity of the M fraction was more important than a higher protein recovery and a greater chance of contamination with non-mitochondrial protein.

For rats 51-57, a total of 29 grams of rat brain (wet weight) were used and only 0.26 μmole of glutamic acid was isolated from the combined M fractions. Although the yield was less than anticipated we did proceed to inject 21 rats with sodium acetate-1-^{14}C and to determine the SA of glutamate in the combined M fractions.

Rats 60-66 (21 rats in all) were injected with sodium acetate-1-^{14}C. In these experiments a total of 23.4 grams of rat brain (wet weight) were used. 29.4 mg of protein were recovered in the combined M fractions while 0.74 µmole of glutamic acid was recovered. Expressed differently, 25.1 nmoles of glutamic acid per mg protein were recovered in the combined M fractions. The SA of the glutamic acid was 0.36 nCi/µmole. This is considerably higher than the SA of glutamic acid in the crude mitochondrial fraction observed previously in similar experiments. However, this SA did not appear to be large enough to account for the larger SA of glutamine in the S fraction observed earlier. This does not discount the possibility that this is the site where ^{14}C-acetate is labeling glutamate. As discussed before, shorter time periods may be helpful in localizing the small pool of glutamic acid which is preferentially labeled by ^{14}C-acetate.

CHAPTER IV

SUMMARY

The concentrations of glutamate, glutamine, and, in some cases, aspartate in different subcellular fractions of rat brain were determined. Two thirds or more of the total tissue content of glutamate and glutamine was present in the soluble fraction.

Glutamate-U-^{14}C or glutamine-U-^{14}C were added to the homogenization buffer in some experiments to test if either compound is metabolized significantly during the isolation procedure. The results showed that the glutamate and glutamine in the soluble fraction are not interchanging rapidly during the fractionation procedure. Furthermore, only small amounts of radioactivity were recovered in the particulate fractions. Although there were indications that a small but significant amount of glutamine was being hydrolyzed to glutamate, it appeared feasible to proceed with the ^{14}C-labeling experiments *in vivo*.

D-glucose-2-^{14}C and acetate-1-^{14}C were administered to rats and the specific activities of glutamate and

glutamine in different subcellular fractions of the brain were determined. After administration of D-glucose-2-^{14}C, the specific activity of glutamate was greater than that of glutamine in both the soluble and total particulate fractions. It did not appear that experiments of this type using D-glucose-2-^{14}C would give any insight as to the location of the so-called small glutamate-glutamine pool. Therefore, efforts were concentrated on using acetate-1-^{14}C as substrate.

After administration of acetate-1-^{14}C the specific activity of glutamate was greater than that of glutamine in the soluble, total particulate, and crude nuclear fractions, but the reverse was true in the crude mitochondrial and microsomal fractions. This was the first time that a subfraction of brain has been isolated in which the specific activity of glutamate was greater than that of glutamine, after administration of acetate-1-^{14}C.

These results give further evidence for the existence of a small glutamate pool in brain, preferentially labeled by acetate, and suggest that it may be located in one of the mitochondrial fractions.

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