CONCERNING THE FORMATION IN VIVO OF SOME FREE AMINO ACIDS IN RAT BRAIN

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CHAPTER I

INTRODUCTION

The free amino acids of brain tissue have been the focal point of many biological studies. One of the reasons for this interest is that brain tissue has a relatively high content of certain of the free amino acids (1-5). Most of these are non-essential amino acids, glutamic acid, glutamine, γ - aminobutyric acid, aspartic acid and N-acetylaspartic acid making up 75 per cent of the total. Of these, N-acetylaspartic acid and γ -aminobutyric acid appear to be peculiar to nervous tissue.

The origin of these brain amino acids was not known for some time. The work of Himwich (6) had led to the general assumption that glucose was almost exclusively used by the brain as an energy source rather than for synthesis of the non-essential amino acids and other components. This assumption was based on the fact that the amount of oxygen consumed and lactic acid produced by the brain was shown to be equivalent to the amount of glucose taken up by the brain from the blood. Assuming glucose was not the main precursor of the brain amino acids, knowledge of the origin of these compounds was further confused by observations which were interpreted to indicate that the brain cell membrane, or the "blood-brain barrier," was impermeable to glutamic acid and aspartic acid (7, 8). However, this was not believed to be the case with all the amino acids because the concentration of glutamine and some of the essential amino acids in the brain increased when the blood levels of these compounds were increased (7-9). These observations suggested that free glutamic acid

and aspartic acid were formed in the brain from glutamine or some carbon source other than glucose rather than being formed in some other tissue and transported to the brain by the blood.

The availability of labeled compounds made possible experiments which have established the origin of some of these brain amino acids. Using a tracer technique in the early 1950's, Winzler and co-workers (10, 11) were able to show that glucose-U-C^{14} labeled the amino acids in brain protein. They incubated glucose-U-C^{14} with young mouse brain mince and then isolated the amino acids found in the protein hydrolysates. Their results showed radioactivity of comparable specific activity in all the amino acids isolated, with the exception of proline and threonine. When the glucose-U-C^{14} was injected intraperitoneally into baby mice the radioactivity found in the amino acids of brain protein was largely limited to aspartic acid, glutamic acid, alanine, serine and glycine.

The first direct evidence that glucose was a precursor of some of the brain amino acids was presented by Beloff-Chain et al. (12) in 1955. In their experiments, glucose-C^{14} was incubated with the brain slices for periods as long as 60 minutes. After sixty minutes, 60 per cent of the glucose which had been taken up from the medium was accounted for as lactic acid, 20 per cent as carbon dioxide and the remainder as free amino acids. The distribution in the amino acids, as per cent of total radioactivity removed from the medium, was 9.0 in glutamic acid, 1.5 in alanine, 3.0 in γ-aminobutyric acid, and 2.4 in aspartic acid. These early reports (10-12), showing the incorporation of radioactivity from labeled glucose into the amino acids of brain protein in vivo and into the free amino acids of brain in vitro, have been verified by numerous other experiments.

In 1958, Flexner et al. (13) demonstrated that radioactivity from subcutaneously injected glucose-U-C^{14} was incorporated into the free amino

acids of newborn mouse brain. The largest amount of radioactivity was incorporated into glutamic acid, the next largest amount into aspartic acid and the amount going into glutamine slightly less than that into the aspartic acid. As a result of their calculations, they concluded that glucose supplied approximately ten times as much carbon to the glutamic acid and glutamine of the cortex as did the glutamic acid of the blood. In some other work with adult mice, they observed that the rate at which carbon from glucose was incorporated into the non-essential amino acids of adult brain cortex far exceeded that of the newborn cortex (14). As with the newborn mice, glucose again supplied about ten times as much carbon to the glutamic acid of the cortex as did plasma glutamic acid.

The free amino acids of cortical samples taken from cat brain during the course of perfusion with a simplified blood containing glucose-U-C^{14} were isolated and analyzed (15, 16). The radioactivity in the amino acids rose irregularly during the first 50 minutes of perfusion; it reached a specific activity of 15 to 18 per cent of that of glucose and remained constant. Radioactivity in glutamic acid and aspartic acid showed the same irregular rise as in the total amino acids but leveled at 23 to 24 per cent of the specific activity of the glucose. The incorporation of radioactivity from the glucose into N-acetylaspartate of the cortex averaged 1 to 5 per cent of that going into aspartic acid. The pattern of entry of C^{14} into the amino acids was similar with the brain at rest, under nembutal narcosis and during convulsions induced by Metrazol. A major conclusion was that endogenous carbon sources were contributing to glycolytic and tricarboxylic acid intermediates at a rate which was almost as great as that at which they were being formed from exogenous glucose.

In some studies with tumor-bearing rats, Busch et al. (17) measured

the incorporation of radioactivity from intravenously injected glucose-1-C^{14} into the free amino acids of different tissues. In brain, 15 minutes after the injection, 75 per cent of the non-glucose radioactivity was found in the glutamic acid, aspartic acid and alanine.

In 1962, Vrba et al. (18, 19) presented data from studies with rats injected intravenously and subcutaneously with glucose-U-C^{14}. Two minutes after the intravenous administration of glucose-U-C^{14}, 41 per cent of the total brain radioactivity was in the form of α-amino acids, and this figure increased to 75 per cent after 30 minutes. Further separation of the α-amino acids indicated that the dicarboxylic α-amino acids had much higher specific activities than the other amino acids. Comparison of the specific activity of the blood and brain free amino acids was interpreted to indicate that the radioactive amino acids of the brain were formed in the brain from glucose. After subcutaneous injection of glucose-U-C^{14} the specific activity of the amino acid fraction was many times greater in the brain than in other tissue. Basic amino acids accounted for only 1 per cent, neutral amino acids for 19 per cent, and dicarboxylic amino acids for 80 per cent of the total radioactivity in the free amino acid fraction of the brain.

Jacobson (20) injected glucose-U-C^{14} intracerebrally into mice. When the amino acids were isolated, he observed incorporation of radioactivity into the aspartic acid and acetic acid moieties of N-acetylaspartic acid as well as into glutamic acid and aspartic acid.

Studies in vivo on the incorporation of radioactivity from compounds other than glucose into the amino acids of brain have also been conducted. Busch (21) injected pyruvate-2-C^{14} intravenously into tumor-bearing rats and observed the incorporation of radioactivity into the free amino acids of different tissues. The incorporation rate into brain was slower than

into any of the other tissues studied. Eight minutes after the labeled pyruvate was injected 80 per cent of the total brain radioactivity was found in the glutamic acid, aspartic acid, and alanine.

Intracerebral administration of sodium acetate-1-C^{14} to mice 1 hour before death resulted in incorporation of radioactivity into brain N-acetylaspartic acid (20). Eighty per cent of the radioactivity incorporated occurred in the acetyl position.

Potanos et al. (22) determined the distribution of radioactivity in brain glutamic acid of rats 1 hour after intracardiac injection of DL-alanine-2-C^{14}. They observed an unusually high labeling of carbon 4 of brain glutamic acid. To explain the unusual distribution of radioactivity, they invoked a serine-glycine interchange as a possible mechanism of obtaining methyl-labeled acetate from the alanine-2-C^{14} in the brain. A more plausible explanation proposed that the alanine-2-C^{14} did not penetrate the brain, but was converted to liver glycogen-1,2,5,6-C^{14} (23) and to blood glucose-1,2,5,6-C^{14} which could enter the brain and be metabolized to yield glutamic acid with considerable labeling of carbon 4 (24).

In 1962 Koeppe and Hahn (24) reported the results of studies in which rat brain glutamic acid was isolated and degraded 1 hour after pyruvate-2-C^{14}, glucose-2-C^{14}, acetate-1-C^{14} and glutamic acid-2-C^{14} had been administered intraperitoneally. Glucose was the only precursor converted in considerable amounts to brain glutamic acid by adult rats. However, in young rats (10 to 20 g) pyruvate-2-C^{14} was a fairly good precursor of brain glutamic acid. Pyruvate-2-C^{14} resulted in high labeling of carbon 4 of adult rat brain glutamic acid while glucose-2-C^{14} did not. These facts were interpreted to support further the postulate that very little pyruvate was metabolized directly by the brain, but was converted to glucose outside the brain and the label transported into the brain as glucose.

McMillan and Mortensen (25) determined the labeling patterns of free brain glutamate after the intracisternal injection of pyruvate-2-C^{14}, acetate-1-C^{14} and acetate-2-C^{14} into rats. Both labeled pyruvate and acetate, when injected directly into the brain, produced labeling patterns in brain glutamate consistent with their metabolism in the tricarboxylic acid cycle.

The incorporation of radioactivity from intravenously injected glutamic acid-U-C^{14} and glutamine-U-C^{14} into the amino acids of rat and mouse brain was studied by Lajtha, Berl and Waelsch (26). In experiments ranging from 2 to 10 minutes in duration, small amounts of radioactivity from the injected glutamic acid were incorporated into the brain glutamic acid, glutamine, γ-aminobutyric acid and glutathione. Similar amounts were incorporated into brain glutamic acid and glutamine when glutamine-U-C^{14} was used. The results were interpreted to indicate an exchange between blood and brain amino acids rather than a net uptake or uptake followed by utilization.

In addition to the origin of the brain amino acids, numerous studies have been conducted concerning the levels and rates of synthesis of these compounds under various traumatic conditions. In 1950, Dawson (27) measured the glutamine and glutamic acid content of rat brain during insulin hypoglycemia. He noted a decrease in glutamic acid when the rats were treated with insulin, but when insulin and glucose were both administered this effect of insulin was not observed.

The following year, Cravioto et al. (28) observed a similar decrease in the brain glutamic acid concentration of insulin-treated rats. Also, they observed slight decreases in the glutamine and γ-aminobutyric acid concentrations and at least a two-fold increase in aspartic acid. These latter observations were also reported by Dawson (29). In spite of the

large increase in aspartic acid content, the level of N-acetylaspartic acid in the brain of insulin-treated rats was the same as in control rats (20, 30).

Okumura et al. (31) noticed a decrease in free alanine, as well as the decrease in glutamic acid and the rise in aspartic acid, in the brain of insulin-treated rats. Insulin treatment caused decreases in the glutamic acid and γ-aminobutyric acid, increases in aspartic acid and no changes in glutamine levels of the brains of deoxypyridoxine-treated and normal rats (32).

In a study using several hypoglycemic agents, insulin was the only one which caused major changes in the levels of the amino acids of rat brain (30). The effect of insulin appeared to be due to the very low blood sugar levels it caused, because when the level of insulin was not great enough to reduce the blood sugar content to 25 mg per cent it had no effect on the brain amino acid levels.

In 1953, Flock et al. (33) observed an extremely large increase in the concentration of glutamine in the cerebrum of hepatectomized dogs, but no change was noted in the cerebrum glutamic acid content. Since this increase in glutamine was not proportional to the increase in plasma glutamine, it was thought to result from the brain detoxification of ammonia by de novo synthesis of glutamine.

In an ammonia toxicity study with rats, a 300 per cent increase in brain glutamine concentration was observed 15 minutes after a $L D_{99.9}$ dose of ammonium acetate had been injected intraperitoneally (34). The biochemical changes in the brain tissue of dogs during ammonia induced coma were reported by Clark and Eiseman (35). They noted an increase in pyruvate and glutamine concentrations, a decrease in α-ketoglutarate content and no change in the glutamic acid concentration in the brains of

dogs receiving ammonia by carotid artery infusions. They felt that this reduction of an essential citric acid cycle intermediate might interfere with energy metabolism in some way and thus be partially responsible for the induced coma.

The amino acids of cat brain were studied following N^{15}-ammonia infusion (36). Glutamine was the only cerebral component showing a considerable increase in concentration as a result of the ammonia infusion. This increase occurred without a corresponding drop in glutamic acid concentration. Experiments with C^{14}-labeled glutamic acid and the N^{15}-ammonia indicated that the additional glutamine was formed in the brain rather than being transported into the brain from the blood. For de novo synthesis of brain glutamine to occur without depletion of the citric acid cycle intermediates, the brain tissue must be able to fix carbon dioxide at a fairly rapid rate. This was demonstrated with brain tissue in vivo (37).

In addition to the increased level of brain glutamine observed following the N^{15}-ammonia infusion, it was noted that the α-amino group of cerebral glutamine contained a significantly higher concentration of isotope than that of the glutamic acid (36). This was interpreted to mean that the newly formed glutamine was derived from a small, metabolically very active compartment of glutamic acid which was not in rapid equilibrium with the total tissue glutamic acid.

The effects of thiamine deficiency on the amino acids of brain have not been studied very extensively. One study indicated that the glutamic acid content of the brain decreases somewhat in experimental thiamine deficiency (38). The work of Peters (39) and others has led to the general assumption that one of the primary defects of thiamine deficiency at the cellular level is the reduced ability to decarboxylate pyruvate. If this defect occurred in brain tissue, a noticeable effect on the level of the

free amino acids or distribution of radioactivity in the compounds from specifically labeled precursors might be expected. However, since this early work was reported, other workers have presented evidence that suggested that the lack of ability to decarboxylate pyruvate was not one of the primary defects of thiamine deficiency in the intact animal (40, 41).

Although there have been numerous studies concerning precursors, concentrations and rates and routes of synthesis of the free amino acids of brain, no comprehensive study in vivo of possible brain free amino acid precursors or of one precursor under various traumatic conditions was found in the literature. With this in mind the objectives of this study were:

1. To develop a rapid, relatively simple method to assay for and to isolate brain free glutamate, glutamine, glutathione, aspartate and N-acetylaspartate.
2. To compare the rates and routes of synthesis of these brain components from labeled glucose, glutamate, glutamine, aspartate, pyruvate, lactate, citrate, malate, butyrate, and glycerol given intravenously.
3. To investigate the effect of certain traumatic conditions (i.e., ammonium ion toxicity, hypoglycemia, thiamine deficiency) on the levels and rates and routes of synthesis of these brain components.

CHAPTER II

EXPERIMENTAL

A. Radioactive Test Compounds

The D and L isomers of lactic acid-2-C^{14} used in this study were prepared in this laboratory by Mr. N. F. Inciardi (42). The L-glutamic acid-2-C^{14} used was also prepared here by Dr. W. E. Wilson (43).

The D-glucose-2-C^{14} used in these experiments was obtained from Merck and Company Limited of Canada. This glucose was dissolved in a known volume of triple distilled water. Aliquots were spotted on Whatman No. 1 filter paper and chromatographed in a N-butanol-methyl cellosolve-water (2:1:1) solvent system (44). There was only one radioactive peak when the chromatograms were checked with a strip counter and this peak had an R_f value (0.44) similar to that of a glucose standard. Another aliquot was mixed with a known amount of unlabeled D-glucose and crystallized from an ethanol-water solution. Radioactivity determinations of the crystallized glucose were in good agreement with the manufacturer's data. A sample of glucose obtained by evaporating the ethanol-water solution to dryness after the crystals had been harvested was degraded as described by Gunsalus and Gibbs (45) to determine the distribution of the radioactivity. The results showed that 99 per cent of the total radioactivity found in carbons 1, 2 and 3 of the glucose was in carbon 2.

The L-glutamine-U-C^{14} was obtained from the California Corporation for Biochemical Research. It was diluted to a known volume with 0.9 per cent saline. One µl was placed on Whatman No. 1 filter paper and chromatographed in an 80 per cent phenol solution to which 1 per cent, by volume, of

concentrated ammonium hydroxide had been added (46). There was only one radioactive peak when this chromatogram was checked with a strip counter and this peak had an R_f value (0.60) similar to that of a known glutamine standard. Another measured sample of the labeled glutamine was mixed with 5 mmoles of L-glutamic acid. This mixture was dissolved in 3 N hydrochloric acid and autoclaved (15 lb/sq in) for 12 hours. The hydrochloric acid was removed by evaporation under reduced pressure. The glutamic acid hydrochloride was dissolved in 5 to 7 ml of hot water and treated with 25 per cent pyridine to bring the pH to 3.5 to 4.0. Two volumes of ethanol were added and the glutamic acid allowed to crystallize overnight in a refrigerator. The glutamic acid was collected by filtration, washed twice with absolute ethanol and with ether, dried in vacuo over phosphorus pentoxide, and assayed for radioactivity.

Two samples of labeled N-butyric acid, as the sodium salt, were obtained from the California Corporation for Biochemical Research. One sample was sodium butyrate-3-C^{14}, the other was sodium butyrate-1-C^{14}. These samples were dissolved in known amounts of distilled water. Aliquots of these samples were diluted with known quantities of carrier sodium butyrate and taken to dryness on a steam hot plate under a stream of filtered air. These butyrates were purified by Celite chromatography (47). The butyric acids recovered from the Celite columns were titrated to pH 8.5 with carbon dioxide-free sodium hydroxide, dried, and assayed for C^{14}.

Glycerol-2-C^{14} was obtained from California Corporation for Biochemical Research. One µl of the labeled glycerol was diluted with a known amount of carrier glycerol and the tribenzoate ester prepared (48). The melting point of the ester after the second crystallization was 74° C as compared with literature values of 75 to 76° C. The ester was assayed for radioactivity and the results indicated that the glycerol-2-C^{14}

contained C^{14} in an amount comparable to that listed on the manufacturer's label.

The sodium pyruvate-2-C^{14} used was part of the same sample Mr. N. F. Inciardi had used for preparation of the D and L lactic acid-2-C^{14}. It was obtained from Nuclear Chicago Corporation and was believed to have been diluted with distilled water to a concentration of approximately 200 μc per ml. When 1 μl was dried and assayed, it was found to contain more radioactivity than had been estimated from the manufacturer's data. However, when the 2,4-dinitrophenylhydrazone of the labeled pyruvate mixed with unlabeled potassium pyruvate was prepared and the radioactivity determined, only about one-third of the estimated radioactivity in the sample was calculated to be in pyruvate (48). Since this derivative was prepared and checked twice, there was believed to be some question about the purity of the pyruvate sample used.

A sample of DL-aspartic acid-3-C^{14} was obtained from California Corporation for Biochemical Research. An aliquot was spotted on pH 7.0 phosphate buffered filter paper and chromatographed in 76 per cent ethanol. Scanning this chromatogram with a strip counter indicated only one major radioactive peak, and this peak had an R_f value (0.20) corresponding to that of aspartic acid. A sample of the labeled aspartic acid was diluted with carrier DL-aspartic acid, crystallized from boiling water, and assayed for C^{14}.

DL-malic acid-3-C^{14} was obtained from the California Corporation for Biochemical Research. After dilution with 0.9 per cent saline, an aliquot was dried and counted. This count indicated that only about 15 per cent of the total radioactivity listed on the label by the manufacturer was present. An attempt was made to prepare the calcium salt of an aliquot of the labeled malic acid. One μl of DL-malic-3-C^{14} was diluted with 3 mmoles of carrier malic acid in about 15 ml of water, 100 mg of calcium

hydroxide was added, the mixture was heated to boiling and kept at this temperature for several minutes. After heating, the mixture was filtered and set in a refrigerator for several days. The crystals that formed were harvested by filtration, dried and the radioactivity determined. Again the results showed only about 15 per cent of the total radioactivity listed by the manufacturer as being present.

The citric acid-1,5-C^{14} was obtained from New England Nuclear Corporation. After dilution with distilled water an aliquot was dried and assayed for C^{14}. The results were in agreement with the manufacturer's data.

The test compounds and their derivatives were assayed for total radioactivity by first being oxidized to carbon dioxide by the macrocombustion method of Van Slyke et al. (49, 50). Unlabeled carrier was added to the oxidation mixture, when necessary, to obtain enough carbon dioxide for accurate measurement. The resulting radioactive carbon dioxide was measured manometrically, swept into an ionization chamber, and the quantity of radioactivity determined with a vibrating reed electrometer (51).

Data on test compounds and solutions are summarized in Table I.

B. Animal Experiments

Young albino rats (125 to 290 g) obtained from the Holtzman Rat Company were used in all experiments. With the exception of Rat 193, a female, all the animals were males. They were allowed to eat ad libitum a stock laboratory diet up to the time at which the various experimental conditions were imposed. Water was provided throughout both the pre-experimental and the experimental periods.

Rats 189, 190 and 191 were placed under mild ether anesthesia shortly before the administration of glucose into the stomach, again just before the labeled lactic acid was injected into the heart and a third time just before a blood sample was taken by heart puncture at the end of the

TABLE I

DATA ON TEST COMPOUNDS AND SOLUTIONS

Compound	Specific Activity mc/mmole	Activity μc/ml	Weight mg/ml
D and L-Lactic acid-2-C^{14}	5.5	9	0.15
D-Glucose-2-C^{14}	2.2[1]	100[2]	8.3[1]
L-Glutamic acid-2-C^{14}	0.37	53	21.0
L-Glutamine-U-C^{14}	2.1[1]	85	6.1[1]
Sodium butyrate-3-C^{14}	1.7[1]	94	6.1[1]
Sodium butyrate-1-C^{14}	2.7[1]	32	1.3[1]
Glycerol-2-C^{14}	7.0	124	1.5[1]
Sodium pyruvate-2-C^{14}	4.0[1]	65	1.8[1]
DL-Aspartic acid-3-C^{14}	6.8[1]	87	1.7[1]
DL-Malic acid-3-C^{14}	6.8[1]	10	0.2[1]
Citric acid-1,5-C^{14}	1.9[1]	160	16.0[1]

[1]Taken from data supplied by manufacturer.
[2]Sample injected into Rat 232 contained approximately 200 uc per ml.

experiment. All the other rats in these experiments, with the exception of Rat 221 which received no anesthetic, were injected subcutaneously with nembutal (60 mg/kg of body weight) 40 to 70 minutes before the labeled compounds were administered.

After fasting 48 or 72 hours, Rats 189, 190, 191, 193, 218A and 219 were given 2 g of glucose by stomach tube 30 minutes before the lactic acid-2-C^{14} was injected. The hepatic artery and portal vein of Rats 202 and 216 were ligated before the labeled lactic acid was administered. The blood sugar levels, determined by the Nelson-Somogyi Method (52), of Rats 202 and 216 were 35 and 30 mg per cent, respectively, at the time of sacrifice.

Insulin (150 units/kg body weight) dissolved in 1 ml of 0.9 per cent saline was injected intraperitoneally into Rats 209 and 214 three hours prior to the labeled glucose injection. The blood sugar levels of Rats 209 and 214 were 20 and 12 mg per cent, respectively, at the time of sacrifice.

Thiamine deficiency was induced in Rats 227 and 228 by feeding a test diet obtained from Nutritional Biochemical Corporation. This diet was fed for 30 days before the labeling experiments were conducted. The rats continued to gain weight for the first 10 days on this diet and then steadily lost weight. They had lost approximately 30 per cent of their peak body weight when the labeled glucose was administered. These data are shown in Table II.

TABLE II

BODY WEIGHT OF RATS FED A THIAMINE DEFICIENT DIET

Rat No.	Initial Weight	Peak Weight	Weight when killed
	g	g	g
227	213	228	166
228	196	216	152

This diet supported growth when control rats were injected intraperitoneally with 20 µg of thiamine hydrochloride per day. Blood sugar concentrations in Rats 227 and 228 were 56 mg per cent when they were killed.

Six rats were treated with $LD_{99.9}$ doses of ammonium acetate (34). Rats 203 and 204 were injected intraperitoneally with ammonium acetate (10.8 mmole/kg body weight) dissolved in water 20 and 12 minutes, respectively, before the labeled glucose was injected. The same size doses were injected into Rats 206, 213 and 232 immediately after the labeled glucose had been given. Rat 221, which received no anesthetic, was treated with ammonium acetate 10 minutes before he was killed.

After, or in conjunction with the previously described treatments, 0.05 to 0.50 ml of a saline or water solution of the labeled compound was injected into the rat with a heparinized syringe. Information concerning the size of the rats prior to injection of the labeled compound, size of the brain obtained, length of fasting period, route of injection, kind and amount of compound injected, and duration of the experiment is presented in Table III. The animals were decapitated with a guillotine and a small volume of blood was collected in a heparin-coated container for blood sugar and/or total radioactivity analysis (49-51). The brain was excised and blotted on filter paper to remove the blood. The brain in this case refers mainly to the cerebrum and cerebellum because most of the midbrain and the medulla oblongata was not excised. As quickly as possible, usually less than 1 minute after the animals were killed, the excised brains of Rats 189, 190, 191, 193, 200, 201 and 202 were placed on wax paper over crushed ice. The brains of all the other rats tested were placed in liquid nitrogen.

TABLE III

SUMMARY OF ANIMAL EXPERIMENTS

Rat Number	Rat Weight	Brain Weight	Time Fasted	Compound Administered	Route of Injection	Volume Injected	Weight Injected	Activity Injected	Duration of Labeling Experiment
	g	g	hr			ml	mg	μc	min
189[2]	232	1.40	72	L-Lactic acid-2-C^{14}	Heart puncture	0.5	0.30	18	30
190[2]	290	1.80	48	L-Lactic acid-2-C^{14}	Heart puncture	0.5	0.30	18	30
202[3]	220	1.55	0	L-Lactic acid-2-C^{14}	Vena cava	0.4	0.45	27	45
212	192	1.70	0	L-Lactic acid-2-C^{14}	Femoral vein	0.2	0.30	18	10
218A[2]	147	1.43	48	L-Lactic acid-2-C^{14}	Femoral vein	0.3	0.45	27	30
191[2]	270	1.72	48	D-Lactic acid-2-C^{14}	Heart puncture	0.5	0.30	18	30
193[2]	218	1.55	48	D-Lactic acid-2-C^{14}	Carotid artery	0.4	0.45	27	30
216[3]	210	1.45	0	D-Lactic acid-2-C^{14}	Vena cava	0.3	0.45	27	45
219[2]	194	1.50	48	D-Lactic acid-2-C^{14}	Femoral vein	0.5	0.45	27	30

TABLE III (Continued)

Rat Number	Rat Weight g	Brain Weight g	Time Fasted hr	Compound Administered	Route of Injection	Volume Injected ml	Weight Injected mg	Activity Injected μc	Duration of Labeling Experiment min
200	210	1.55	0	D-Glucose-2-C^{14}	Femoral Vein	0.1	0.83[1]	11	10
201	215	1.80	0	D-Glucose-2-C^{14}	Femoral Vein	0.1	0.83[1]	11	10
208	125	1.50	24	D-Glucose-2-C^{14}	Femoral vein	0.1	0.83[1]	11	10
209[4]	185	1.60	0	D-Glucose-2-C^{14}	Femoral vein	0.1	0.83[1]	11	10
214[4]	210	1.60	0	D-Glucose-2-C^{14}	Vena cava	0.1	0.83[1]	10	10
227[5]	166	1.57	0	D-Glucose-2-C^{14}	Femoral vein	0.1	0.83[1]	10	10
228[5]	152	1.45	0	D-Glucose-2-C^{14}	Femoral vein	0.1	0.83[1]	10	10
203[6]	156	1.35	24	D-Glucose-2-C^{14}	Femoral vein	0.1	0.83[1]	11	10
204[6]	156	1.55	24	D-Glucose-2-C^{14}	Femoral vein	0.1	0.83[1]	11	10
206[6]	125	1.55	24	D-Glucose-2-C^{14}	Femoral vein	0.1	0.83[1]	11	10

TABLE III (Continued)

Rat Number	Rat Weight	Brain Weight	Time Fasted	Compound Administered	Route of Injection	Volume Injected	Weight Injected	Activity Injected	Duration of Labeling Experiment
	g	g	hr			ml	mg	μc	min
213[6]	170	1.65	24	D-Glucose-2-C^{14}	Femoral vein	0.1	0.83[1]	11	10
221[6]	179	1.54	24	none					
232[6]	215	1.53	0	D-Glucose-2-C^{14}	Intracisternal	0.05	0.83[1]	10	10
205	200	1.63	0	L-Glutamic acid-2-C^{14}	Femoral vein	0.1	2.1	5.3	10
207	135	1.40	0	L-Glutamic acid-2-C^{14}	Femoral vein	0.2	4.2	10.6	10
215	215	1.65	0	L-Glutamine-U-C^{14}	Femoral vein	0.15	0.91[1]	12.7	10
220	215	1.47	0	L-Glutamine-U-C^{14}	Femoral vein	0.15	0.91[1]	12.7	10
223	238	1.55	0	Sodium butyrate-3-C^{14}	Femoral vein	0.15	0.91[1]	14	10
229	210	1.55	0	Sodium butyrate-1-C^{14}	Femoral vein	0.15	0.20[1]	4.8	10
225	228	1.48	0	Glycerol-2-C^{14}	Femoral vein	0.1	0.15[1]	12	10

TABLE III (Continued)

Rat Number	Rat Weight g	Brain Weight g	Time Fasted hr	Compound Administered	Route of Injection	Volume Injected ml	Weight Injected mg	Activity Injected μc	Duration of Labeling Experiment min
230	240	1.55	0	Glycerol-2-C^{14}	Femoral vein	0.08	0.12[1]	9.5	10
211	195	1.50	0	Sodium pyruvate-2-C^{14}	Femoral vein	0.1	0.18[1]	6.5	10
217	180	1.60	0	DL-Aspartic acid-3-C^{14}	Femoral vein	0.15	0.25[1]	13	10
224	242	1.50	0	DL-Malic acid-3-C^{14}	Femoral vein	0.15	0.03[1]	1.5	10
226	250	1.63	0	Citric acid-1,5-C^{14}	Femoral vein	0.08	0.13[1]	13	10

1 Taken from data supplied by manufacturer.
2 Received 2 g of glucose by stomach tube 30 minutes prior to the labeled lactate.
3 Hepatic artery and portal vein ligated prior to administration of labeled lactate.
4 Received insulin (150 Units/kg) prior to injection of labeled glucose.
5 Maintained on a thiamine deficient diet 30 days before labeling experiment was conducted.
6 Received ammonium acetate (10.8 mmole/kg) 10 to 30 minutes before sacrifice.

C. Isolation and Assay of Brain Free Amino Acids

The ice cold or frozen brain tissue was homogenized with 1.3 ml of 0.6 N perchloric acid per gram of tissue (53). The precipitate was removed by centrifugation in the cold and rehomogenized with a volume of 0.33 N perchloric acid equal to that of the first supernatant solution. The protein fraction was again removed by centrifugation. The supernatant fluids were combined, neutralized with 2 N potassium hydroxide, refrigerated overnight, and centrifuged to remove potassium perchlorate.

The neutralized, protein-free, brain filtrate was passed over a Dowex 1-X8 (100 to 200 mesh) acetate column (0.5 X 12 cm) at the rate of approximately 10 ml per hour. The column was next washed slowly with about 50 ml of distilled water. After this wash the free glutamic and aspartic acids were eluted with 0.5 N acetic acid as described by Hirs, Moore, and Stein (54). Collection of 2 ml fractions proved to be very satisfactory for the resolution of glutamic and aspartic acids. After the aspartic acid peak had been eluted with 0.5 N acetic acid, the column was treated with approximately 30 ml of 3 N hydrochloric acid to remove the remaining compounds.

The volume of water effluent was reduced to approximately 15 ml by low pressure evaporation and the solution was made 3 N by the addition of an equal volume of 6 N hydrochloric acid. Then both the acidified water effluent and the hydrochloric acid eluant were autoclaved (15 lb/sq in) from 3 to 12 hours. The hydrochloric acid was removed by distillation *in vacuo*. The hydrolysates were taken up in 5 to 10 ml of water and neutralized with 0.5 N potassium hydroxide.

The neutralized water effluent hydrolysate was passed over a Dowex-1 acetate column like that previously described. The water effluent from this column was saved for analysis of the neutral and basic amino acids

which is to be done at a later date. The glutamic acid and aspartic acid moieties of glutamine and asparagine were eluted from the column with 0.5 N acetic acid as previously described.

The neutralized hydrochloric acid eluant hydrolysate was passed over a Dowex-1 acetate column and eluted with acetic acid as above to separate and isolate the glutamic and aspartic acid moieties of glutathione and N-acetylaspartic acid. A diagram of this isolation scheme is presented in Figure 1.

The glutamic and aspartic acid-containing fractions from the above mentioned columns were identified by spotting aliquots of each fraction collected on pH 7.0 phosphate buffered filter paper and developing with ninhydrin. The fractions found to contain the glutamic acid or aspartic acid were pooled and made up to a known volume with distilled water.

The amino acids were quantatively determined by the ninhydrin method as described by Rosen (55). This method was modified slightly in this laboratory. Instead of 0.01 M sodium cyanide, 0.01 M potassium cyanide was used for preparation of the cyanide-acetate solution. Also the volumes of the amino acid containing solution and the reagents used in this laboratory differed from those used by Rosen as shown in Table IV.

TABLE IV

COMPARISON OF VOLUMES USED BY ROSEN (55) AND THIS LABORATORY FOR NINHYDRIN ASSAYS

Item	Rosen	This Laboratory
	ml	ml
Amino acid solutions	1.0	1.5
Cyanide-acetate buffer	0.5	0.25
Ninhydrin	0.5	0.25
Isopopyl alcohol	5.0	1.0

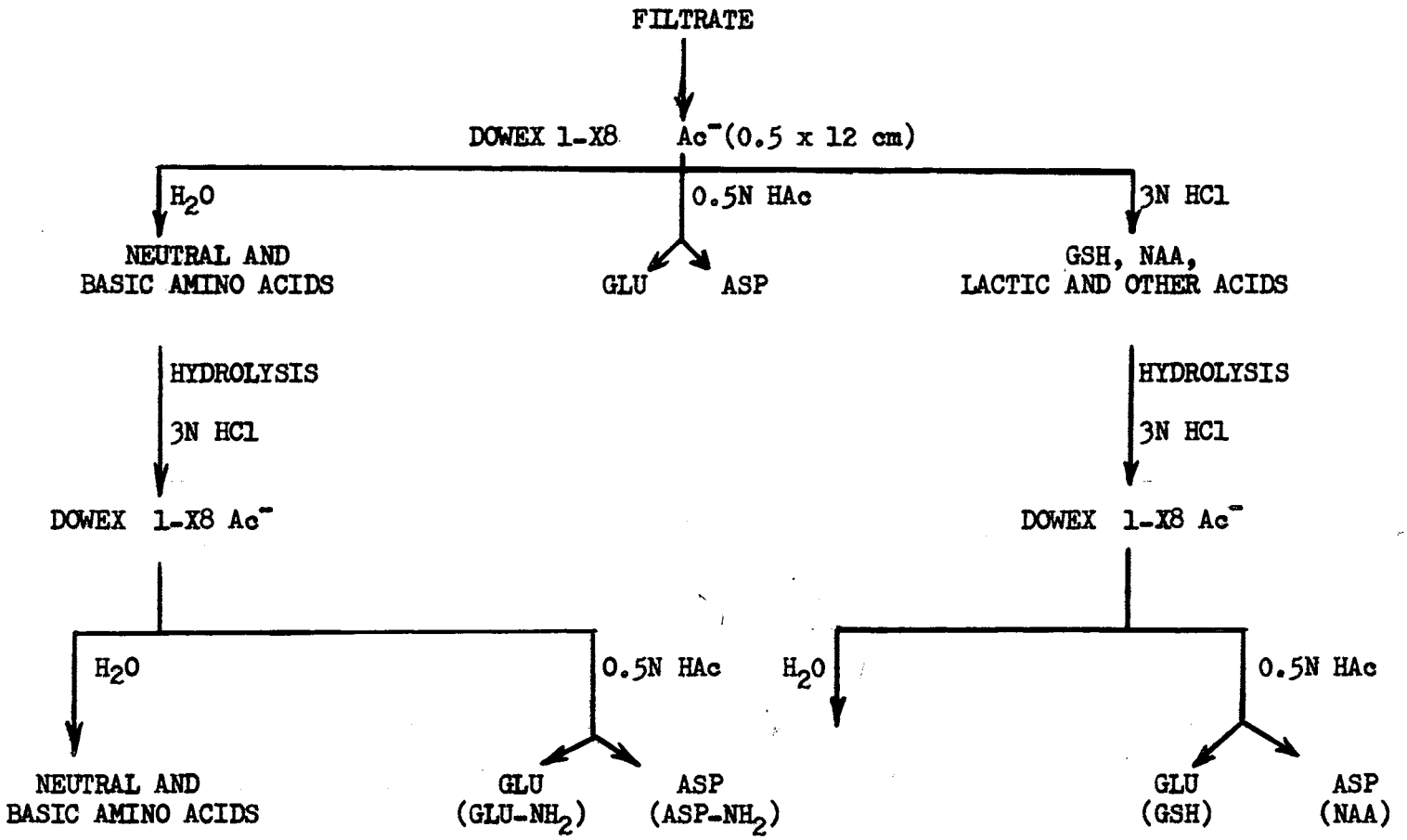

Figure 1

Scheme for Isolation of Brain Free Glutamic Acid, Aspartic Acid and Their Moieties in Glutamine, Asparagine, Glutathione and N-Acetylaspartic Acid

Aliquots of the isolated glutamic acid and aspartic acid estimated to contain 5 to 20 μg were assayed in duplicate. Standard L-glutamic acid samples were run with each group of unknown samples. The quantities in the unknown samples were calculated from the standard L-glutamic acid curves obtained at the same time the unknown samples were analyzed. It was noted while using this procedure that the standard L-glutamic acid should not be stored at very low concentrations and that the cyanide-acetate buffer should not be prepared more than 3 or 4 days prior to use.

Aliquots of the isolated glutamic and aspartic acid were dried under a stream of filtered air on a steam hot plate. Known amounts of carrier were added and the samples were combusted and assayed for radioactivity as previously described in section A, Chapter II.

D. Degradation Procedures

The degradation of glutamic acid was carried out by the method described by Mosbach, Phares, and Carson (47, 56, 57), as modified by Koeppe and Hill (51). Aliquots of the free glutamic acid or glutamic acid obtained from glutamine were diluted with carrier glutamic acid before being degraded. The glutamic acid of Rat 228 was crystallized with carrier as described in section A, Chapter II before the degradation was carried out. In most cases the degradation was stopped when ethylamine was obtained. Wet combustions were run on the ethylamine hydrochloride to determine the total radioactivity in carbons 2 and 3.

In some experiments aliquots of the free glutamic acid or glutamic acid obtained from glutamine were diluted with carrier and treated with hydrazoic acid to convert carbon 5 to carbon dioxide (58). The C^{14} content of carbon 1 of glutamic acid and of carbons 1 and 4 of aspartic acid was determined by decarboxylation with ninhydrin as described by Van Slyke et al. (59).

CHAPTER III

RESULTS AND DISCUSSION

A. Isolation of Brain Free Amino Acids

Numerous methods for the isolation of the free amino acids of brain have been described. Most of these methods involve some type of chromatography. Roberts et al. (14) used ion-exchange columns to remove contaminants and then isolated the amino acids by paper chromatography. Glutamic acid and aspartic acid were isolated by Dowex-1 carbonate column chromatography by Vrba et al. (19). Barkulis et al. (15) isolated brain glutamic acid and aspartic acid by the use of Dowex-1 acetate columns. Berl et al. (5, 36) used alumina columns and later Dowex-1 chloride-acetate and Dowex-50 sodium-hydrogen columns for isolation of glutamic acid, aspartic acid, glutamine, glutathione and N-acetylaspartic acid. Tallan, Moore and Stein (1, 2) used ion-exchange chromatography for complete cat brain amino acid analysis. Koeppe and Hahn (24) isolated brain free glutamic acid by adding carrier glutamic acid to rat brain perchloric acid filtrates and then crystallizing out the glutamic acid.

Some of the above mentioned methods for isolation of the brain free amino acids were not available at the beginning of this study, others were considered too time consuming, and still others too limited or inaccurate. Therefore it was necessary to develop a quick, accurate, relatively simple method for isolation of brain free glutamic acid, aspartic acid, glutamine, glutathione and N-acetylaspartic acid.

All the experiments conducted in the development of the isolation

method were done with synthetic mixtures of the compounds tested. With the exceptions of the N-acetylaspartic acid and pyrrolidone carboxylic acid, which were prepared by Dr. R. E. Koeppe, all the compounds were obtained from the California Corporation for Biochemical Research.

A very suitable method for the isolation of glutamic acid and aspartic acid was being used in this laboratory before this problem arose. Good separation of these amino acids was obtained by gradient elution with acetic acid from Dowex-1 acetate columns. This method was first considered for isolation of glutamic acid and aspartic acid in this study. In order to keep the procedure as simple as possible, an attempt was made to isolate glutamine and N-acetylaspartate from the Dowex-1 acetate columns used for the separation of glutamic acid and aspartic acid. To get glutamine to stay on the anion exchange resin it was converted to pyrrolidone carboxylic acid by the procedure described by Greenstein and Winitz (60). Although glutamic acid and aspartic acid were eluted from these columns very easily by the acetic acid gradient procedure, pyrrolidone carboxylic acid and N-acetylaspartic acid could not be eluted from these columns by increasing the acetic acid concentration to 4 N. After several failures it was noted that by changing from an acetic acid to a formic acid gradient immediately after the last aspartic acid fraction, pyrrolidone carboxylic acid and N-acetylaspartic acid could be eluted from the column with good separation.

To simplify the method the gradient procedures were discontinued. Glutamic acid and aspartic acid were eluted with 0.5N acetic acid as described by Hirs et al. (54). Following the elution of aspartic acid, pyrrolidone carboxylic acid and N-acetylaspartic acids were eluted with 0.35N formic acid. Even though good separation of these compounds was obtained by this method, when lactic acid and glutathione were added to

this mixture of compounds they appeared in the same fraction as did pyrrolidone carboxylic acid. Also, attempts to convert glutamine to pyrrolidone carboxylic acid in a mixture of all these compounds and under conditions that would not alter the other compounds, gave somewhat less than quantitative results. Since glutamine and glutathione were brain components of interest in this study and the brain was known to contain significant amounts of lactic acid, another method for isolating glutamine and glutathione had to be used.

The next approach taken was to wash the glutamine through the Dowex-1 acetate column with water, hydrolyze it with hydrochloric acid, and then isolate the glutamic acid moiety from another Dowex-1 acetate column by elution with 0.5N acetic acid. This method for the isolation of glutamine appeared to be the method of choice because nearly quantitative yields of glutamic acid were obtained from prepared samples of glutamine. Also, when the Dowex-1 acetate columns were eluted with 0.5 N acetic acid after the glutamine had been washed through, no glutamic acid was obtained. This indicated that very little hydrolysis of glutamine was occurring during the isolation procedure.

The first attempt at separating glutathione and lactic acid after the method for isolation of glutamine had been changed involved pooling all the fractions containing lactic acid and glutathione which had been eluted with 0.35 N formic acid from the Dowex-1 acetate column, reducing the volume and passing over a Dowex-50 hydrogen column. The lactic acid was washed through this column with water and the glutathione eluted with 4N hydrochloric acid. This method proved to be satisfactory for the separation of lactic acid and glutathione, but the recovery of glutathione was not satisfactory.

This poor recovery of glutathione coupled with the fact that the

recovery of N-acetylaspartic acid was also poor, even though it was separated from the other compounds by elution from the Dowex-1 acetate columns with 0.35N formic acid, required that another method for the isolation of glutathione and N-acetylaspartic acid be used. The isolation of these compounds was next attempted by removing them from the Dowex-1 acetate columns with 4N formic acid or 3N hydrochloric acid. The glutathione and N-acetylaspartic acid-containing fractions were pooled, hydrolyzed and passed over another Dowex-1 acetate column. This column was developed with 0.5N acetic acid to elute the glutamic acid and aspartic acid moieties of glutathione and N-acetylaspartic acid. The recovery when the glutathione and N-acetylaspartic acid were eluted with 3N hydrochloric acid was very close to theory. The recovery when 4N formic acid was used was not quite so high.

The method of choice for the isolation of the brain free glutamic acid, aspartic acid, glutamine, glutathione and N-acetylaspartic acid is described in section C, Chapter II. Some recovery data of synthetic mixtures of these compounds isolated from perchloric acid filtrates which were carried through this isolation scheme are presented in Table V. These data show that, with the exception of glutamine in two experiments and glutathione in one case, the recovery of these compounds approached the theoretical value. Since as already mentioned the low recovery of glutamine was not thought to be due to hydrolysis, the possibility was suggested that the glutamine sample used when the recovery was low contained less than the calculated amount. This possibility was substantiated by the fact that when a sample was hydrolyzed and the glutamic acid moiety isolated from a Dowex-1 acetate column, the recovery was only 86 per cent of theory. When a new sample was prepared, passed over the column, hydrolyzed and the glutamic acid moiety isolated, the recovery was 96 per cent of theory.

TABLE V

RECOVERY OF GLUTAMIC ACID, ASPARTIC ACID, GLUTAMINE, GLUTATHIONE AND N-ACETYLASPARTIC ACID FROM SYNTHETIC MIXTURES SEPARATED BY DOWEX-1 ACETATE COLUMNS

Compound	Date			
	10-8-62	10-9-62	10-22-62	1-31-63
	%	%	%	%
Glutamic acid	100		103	102
Aspartic acid	96		100	100
Glutamine[1]		80	80	96
Glutathione[1]	83		108	
N-Acetylaspartic acid[2]	92		100	

[1] Based on the glutamic acid moiety isolated.
[2] Based on the aspartic acid moiety isolated.

B. Animal Experiments

Data relating to the concentrations of the compounds isolated from rat brain are presented in Table VI. The concentrations of the compounds isolated from the normal-treated rats are in fairly good agreement with values in the literature (4, 5, 7, 20, 27, 30, 31, 61). In normal treatment the animals ate ad libitum a stock laboratory diet prior to injection of the anesthetic and labeled precursors were injected intravenously ten minutes prior to decapitation. The fact that these concentrations agree with those in the literature indicates that the isolation procedure used was satisfactory. This agreement also shows that the injection of the small amounts of radioactive precursors tested had little effect on the levels of the brain amino acids. With the exception of the radioactive glucose and lactate, the small amounts of labeled precursors injected were estimated to have increased the blood levels of the compounds considerably. In the case of injected L-glutamic acid and DL-aspartic acid these data agree with those of others (7, 8) which showed that no net uptake by the brain of these amino acids occurred, even though the blood levels were increased several fold. However, the data from rats injected with L-glutamine, quantities estimated to have increased the blood level two-fold, do not agree with those of other workers (7-9) who showed that brain glutamine levels increased after the administration of glutamine. A probable explanation for this difference is that much larger amounts of glutamine were administered in their experiments than in those reported here. Failure of glutamic acid, aspartic acid and glutamine to rapidly traverse the blood-brain barrier may explain why injection of these compounds did not affect the brain concentrations, even though the blood levels were assumed to be considerably higher than normal. The reason for the unusually low levels of aspartic acid and

TABLE VI

CONCENTRATION OF THE FREE GLUTAMIC ACID, ASPARTIC ACID, GLUTAMINE, ASPARAGINE, GLUTATHIONE AND N-ACETYLASPARTIC ACID IN RAT BRAIN

Rat Number	Treatment	Compound Injected	Glutamic Acid	Aspartic Acid	Glutamine	Asparagine	Glutathione	N-Acetyl-aspartic Acid
			μmole/g tissue					
189[1]	Fasted 72 hr	L-Lactic acid-2-C^{14}	8.5	1.6	3.4		1.3	5.0
190[1]	Fasted 48 hr	L-Lactic acid-2-C^{14}	6.4	1.1			1.4	4.8
202	Liver ligated	L-Lactic acid-2-C^{14}	4.7	6.2	1.9	0.3	1.2	5.6
212	Normal	L-Lactic acid-2-C^{14}	8.8	2.5	4.3	0.4		
218A[1]	Fasted 48 hr	L-Lactic acid-2-C^{14}	10.2	2.2				
191[1]	Fasted 48 hr	D-Lactic Acid-2-C^{14}	7.2	1.3	3.6		1.8	4.9
193[1]	Fasted 48 hr	D-Lactic acid-2-C^{14}	4.6	1.0	1.8		1.2	3.9
216	Liver ligated	D-Lactic acid-2-C^{14}	8.7	2.2	4.4	0.5		
219[1]	Fasted 48 hr	D-Lactic acid-2-C^{14}	10.0	2.6	5.2			
200	Normal	D-Glucose-2-C^{14}	9.9	1.6	2.6		1.3	4.8
201	Normal	D-Glucose-2-C^{14}	7.2	2.2			1.5	5.6
208	Fasted 24 hr	D-Glucose-2-C^{14}	7.7	2.5	4.5	0.3		
209	Insulin	D-Glucose-2-C^{14}	7.7	3.4	2.3	0.3		

TABLE VI (Continued)

Rat Number	Treatment	Compound Injected	Glutamic Acid	Aspartic Acid	Glutamine	Asparagine	Glutathione	N-Acetyl-aspartic Acid
			μmole/g tissue					
214	Insulin	D-Glucose-2-C^{14}	4.2	5.5	1.8			
227	B_1 deficient	D-Glucose-2-C^{14}	8.9	2.3	4.6			
228	B_1 deficient	D-Glucose-2-C^{14}	9.6	2.6	4.8			
203	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	6.4	1.0	5.7	0.9	1.6	5.5
204	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	7.0	1.1	5.3	0.3	1.5	5.6
206	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	8.1	1.6	6.4	0.4		
213	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	7.4	1.6	5.4	0.5		
221	Fasted 24 hr, ammonia	None	8.8	1.4	5.5			
232	Ammonia	D-Glucose-2-C^{14}	8.5	2.5	5.8			
205	Normal	L-Glutamic acid-2-C^{14}	8.2	2.2	3.5	0.3	1.4	5.6
207	Normal	L-Glutamic acid-2-C^{14}	7.8	2.0	4.6	0.4		
215	Normal	L-Glutamine-U-C^{14}	8.2	2.3	4.5	0.4		

TABLE VI (Continued)

Rat Number	Treatment	Compound Injected	Glutamic Acid	Aspartic Acid	Glutamine	Asparagine	Glutathione	N-Acetyl-aspartic Acid
					μmole/g tissue			
220	Normal	L-Glutamine-U-C^{14}	9.0	2.2	5.0			
223	Normal	Sodium butyrate-3-C^{14}	11.6	2.8	3.7			
229	Normal	Sodium butyrate-1-C^{14}	8.8	2.7	4.3			
225	Normal	Glycerol-2-C^{14}	9.0	2.5	4.8			
230	Normal	Glycerol-2-C^{14}	9.0	2.4	4.6			
211	Normal	Sodium pyruvate-2-C^{14}	6.6	2.4	4.4	0.4		
217	Normal	DL-Aspartic acid-3-C^{14}	8.9	2.4	4.7			
224	Normal	DL-Malic acid-3-C^{14}	10.1	2.5	4.4			
226	Normal	Citric acid-1,5-C^{14}	8.2	2.3	4.8			

[1] Received 2 g glucose by stomach tube 1 hour before sacrifice.

glutamine found in the brain of Rat 200 was not obvious. Poor recovery during the isolation seems to be the most reasonable explanation for these low results.

In contrast to results reported by Ferrari (38), thiamine deficiency did not lower the level of brain glutamic acid. The brain glutamic acid, aspartic acid and glutamine levels of thiamine deficient rats were comparable with those of normal rats.

Results obtained with Rat 208, which was fasted for 24 hours, indicated that fasting did not affect the amino acid concentrations of brain. However, the brain aspartate levels of three (Rats 189, 190, and 191) of the five rats which were fasted for 48 or 72 hours and were given two g of glucose one hour before sacrifice were lower than those observed in normal rats. One possible explanation for these results is that the brain free aspartate levels were reduced by the longer periods of starvation and in Rats 189, 190 and 191 had not returned to the normal level one hour after the fast had been broken by the administration of glucose. Another possible explanation is that aspartate might have been partially used by the tissue before enzymatic action was stopped because the brains of these animals were not frozen in liquid nitrogen immediately after removal, whereas those of the other fasted rats were.

The brain components determined in fasted, glucose-treated rats were lower in Rat 193 than in other similarly treated rats. One explanation for this result is that the levels of these compounds are lower in females than in males. Since this was the only female rat used, this explanation lacks confirmation.

Insulin, 3 hours after it was administered, resulted in marked changes in the levels of the brain amino acids of Rat 214. The aspartic acid concentration was doubled and that of glutamic acid and glutamine

reduced to one-half the normal level. The results with insulin were not as striking in Rat 209, but there was an increase in the brain aspartic acid concentration and a decrease in that of glutamine 2 hours and 35 minutes after the insulin had been injected. The glutamic acid content appeared to be normal. These results, especially those obtained with Rat 214, are in good agreement with the results of other workers (27-32).

After the hepatic artery and portal vein of Rat 202 had been ligated for approximately 1 hour and 45 minutes, the brain amino acid concentrations were similar to those observed in insulin-treated Rat 214. However, in Rat 216 the brain amino acid concentrations 45 minutes after ligation of the hepatic artery and portal vein were similar to those of normal rats.

The insulin-treated rats and Rat 202 which had the hepatic artery and portal vein ligated, in addition to having high levels of brain aspartic acid and low levels of glutamic acid and glutamine, also had very low levels of blood sugar, 12 to 35 mg per cent, at the time of sacrifice. These results with the insulin-treated rats are in good agreement with those of DeRopp and Snedeker (30) who suggested that the brain amino acid concentrations were related to blood sugar levels. The changes in brain amino acid concentrations of Rat 202 further support their conclusion because the possibility of a direct effect on the brain amino acid concentrations by insulin had been eliminated. These workers also showed that even though the blood sugar levels were reduced by insulin in 1 hour, it took an additional 2 hours for the major concentration changes of the brain amino acids to occur. Thus the fact that Rat 216 had a low blood sugar level, 30 mg per cent, and normal brain amino acid concentrations 45 minutes after the liver had been tied off can be explained by assuming the low blood sugar level had not existed long enough to cause major

changes in the brain amino acid concentrations.

A possible mechanism for the changes in levels of the brain amino acids during hypoglycemia was proposed by Baxter and Roberts (62). They proposed that the amount of acetylcoenzyme A funneling into the tricarboxylic acid cycle by way of condensation with oxalacetate would be less than normal. More oxalacetate would thus become available for transamination reactions. Glutamic acid could transaminate with oxalacetate to form aspartate and α-ketoglutarate. The α-ketoglutarate would be oxidized via the tricarboxylic acid cycle to oxalacetate ready to transaminate with more glutamic acid. Thus the glutamic acid level would decrease at the same rate the aspartic acid level increased.

The concentration of N-acetylaspartic acid in the brain of Rat 202, which had the liver tied off, was comparable to that of normal rats. Since the changes in the levels of the brain amino acids of insulin-treated rats were attributed to reduced levels of blood sugar, and since our data for Rat 202, which had a low blood sugar level, agreed with those of other workers (20, 30) who had shown that insulin treatment did not affect the brain concentration of N-acetylaspartate, this compound was not isolated from the brain of the insulin-treated rats.

The injection of $LD_{99.9}$ doses (34) of ammonium acetate into rats raised the level of brain glutamine. These observations agree with those of a number of other workers (34-36). The fact that the brain glutamic acid level was not reduced by the ammonia treatment and that intravenously injected glutamine or glutamic acid did not affect the brain level of these compounds supports the proposal of Flock et al. (33) that de novo synthesis of glutamine is the brain's method for the detoxification of ammonia.

The brain aspartic acid levels of all the ammonia-treated rats,

except Rat 232, were lower than those observed in normal rats. All the ammonia-treated rats, except Rat 232, were fasted 24 hours before the ammonia treatment and the intravenous injection of glucose-2-C^{14}. Rat 232 was not fasted before the ammonia treatment and was given the glucose-2-C^{14} intracisternally; therefore, the failure of ammonia treatment to reduce the brain aspartate level of Rat 232 could have been due to these differences. An explanation for the reduced brain aspartate levels observed is that the increased use of citric acid cycle intermediates for the de novo synthesis of glutamine following ammonia treatment could result in such a low level of these intermediates in the brain that normal amounts of aspartic acid could not be maintained. The roll of ammonia treatment in this explanation for reduced brain aspartate levels is supported by the work of Clark and Eiseman (35) who showed that ammonia caused a reduction in the brain level of α-ketoglutarate. This explanation supports Bessman's theory of citric acid cycle depletion as a mechanism of ammonia intoxication (63).

A second explanation for these reduced levels of brain aspartate is that in the fasted, ammonia-treated rat the aspartate was being used for the synthesis of asparagine faster than it could be replaced. However, this explanation was not considered very probable after the brain asparagine levels were observed to be essentially unchanged following ammonia treatment of the fasted rats. Since the brain is known to be able to synthesize urea, another explanation for these reduced brain aspartate levels is that the aspartate was being used in the synthesis of urea faster than it could be replaced in the fasted, ammonia-treated rats (64). The results of Berl et al. (36) however suggest that urea formation does not play a very significant role in removal of ammonia from the brain.

In agreement with the data of Berl et al. (36), our results show that the brain levels of glutathione and N-acetylaspartic acid were not changed by ammonia treatment.

The radioactivity in the isolated brain glutamic acid, aspartic acid, their moieties of glutamine, asparagine, glutathione and N-acetylaspartic acid and of the whole blood of rats which were injected with labeled precursors is presented in Table VII. The brain glutamic acid, aspartic acid and glutamine of rats given lactate and glucose had much higher specific activities than did the brain glutathione and N-acetylaspartic acid isolated from some of these same rats. These results confirm the conclusions of others (16, 36) that brain glutathione and N-acetylaspartic acid turn over rather slowly. Because of this fact the isolation of glutathione and N-acetylaspartic acid was omitted in most subsequent experiments.

Considerable amounts of radioactivity from both the D and L isomers of lactate-2-C^{14} were incorporated into the brain glutamate, aspartate and glutamine. The specific activity of the glutamate was usually the highest, that of aspartate next and that of glutamine approximately one-half that of glutamate. The distribution of radioactivity in brain glutamate of some of the lactate rats is shown in Table VIII. The high percentage of total radioactivity in carbon 5 of the brain glutamate of Rat 193 and the low percentage randomization in carbon 4 of the brain glutamate of Rat 202 show, based on the conclusions of Koeppe and Hahn (24), that most of the lactate entered the brain without prior conversion to glucose by the liver. This observation in Rat 202 further supports their explanation, involving liver gluconeogenesis, for high randomization in carbon 4 of brain glutamate of rats given pyruvate-2-C^{14} and alanine-2-C^{14} because the lactate was administered after the hepatic

TABLE VII

RADIOACTIVITY IN RAT BLOOD AND ISOLATED BRAIN COMPONENTS

Rat Number	Treatment	Compound Injected	Activity Injected	Blood	Glutamic Acid	Aspartic Acid	Glutamine	Glutathione	N-Acetylaspartic Acid
			μc	mμc/ml			μc/mmole		
189	Fasted 72 hr	L-Lactic acid-2-C^{14}	18		1.3	1.0	1.4	0.2	0.07
190	Fasted 48 hr	L-Lactic acid-2-C^{14}	18		1.1	1.4		0.2	0.00
202	Liver ligated	L-Lactic acid-2-C^{14}	27	95	2.1	1.8	1.2	0.1	0.03
212	Normal	L-Lactic acid-2-C^{14}	18	44	2.3	1.7	1.4		
218A	Fasted 48 hr	L-Lactic acid-2-C^{14}	27	65	3.5	2.6			
193	Fasted 48 hr	D-Lactic acid-2-C^{14}	27		2.6	2.3	1.6	0.2	0.05
216	Liver ligated	D-Lactic acid-2-C^{14}	27	30	1.5	0.9	1.1		
219	Fasted 48 hr	D-Lactic acid-2-C^{14}	27	88	1.6	1.1	0.9		
200	Normal	D-Glucose-2-C^{14}	11	52	2.0	1.9	1.0	0.2	0.04

TABLE VII (Continued)

Rat Number	Treatment	Compound Injected	Activity Injected	Activity Blood	Glutamic Acid	Aspartic Acid	Glutamine	Glutathione	N-Acetylaspartic Acid
			μc	mμc/ml			μc/mmole		
201	Normal	D-Glucose-2-C^{14}	11	77	2.7	2.2		0.1	0.04
208	Fasted 24 hr	D-Glucose-2-C^{14}	11	104	2.1	1.7	1.0		
209	Insulin	D-Glucose-2-C^{14}	11	17	5.7	4.9	3.4		
214	Insulin	D-Glucose-2-C^{14}	10	23	7.1	5.1	2.3		
227	B_1 deficient	D-Glucose-2-C^{14}	10	53	1.7	1.4	0.9		
228	B_1 deficient	D-Glucose-2-C^{14}	10	58	2.5	1.9	1.3		
203	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	11	70	2.4	3.1	0.9	0.1	0.03
204	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	11	95	4.3	5.2	2.2	0.2	0.05
206	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	11	54	1.2	0.8	0.6		
213	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	10	59	2.3	1.4	1.2		

TABLE VII (Continued)

Rat Number	Treatment	Compound Injected	Activity Injected	Blood	Glutamic Acid	Aspartic Acid	Glutamine	Glutathione	N-Acetylaspartic Acid
			μc	mμc/ml			μc/mmole		
232	Ammonia	D-Glucose-2-C^{14}	10	91	4.2	2.7	2.8		
205	Normal	L-Glutamic acid 2-C^{14}	5.3	10	0.1	0.1	0.2		
207	Normal	L-Glutamic acid 2-C^{14}	10.6	12	0.2	0.2	0.3		
215	Normal	L-Glutamine-U-C^{14}	12.7	19	0.3	0.2	0.9		
220	Normal	L-Glutamine-U-C^{14}	12.7	20	0.2	0.1	0.9		
223	Normal	Sodium butyrate-3-C^{14}	14	45	0.6	0.3	1.7		
229	Normal	Sodium butyrate-1-C^{14}	4.8	56	0.3	0.3	1.1		
225	Normal	Glycerol-2-C^{14}	12	60	1.5	1.1	0.7		
230	Normal	Glycerol-2-C^{14}	9.5	55	1.1	1.0	0.6		
211	Normal	Sodium pyruvate-2-C^{14}	6.5	441	0.5	0.3	0.2		
217	Normal	DL-Aspartic acid-3-C^{14}	13	16	0.2	0.3	0.1		

TABLE VII (Continued)

Rat Number	Treatment	Compound Injected	Activity Injected	Blood	Glutamic Acid	Aspartic Acid	Glutamine	Glutathione	N-Acetylaspartic Acid
			μc	mμc/ml			μc/mmole		
224	Normal	DL-Malic acid-3-C^{14}	1.5	5	0.1	0.1	0.1		
226	Normal	Citric acid-1,5,C^{14}	13	42	0.1	0.1	0.1		

TABLE VIII

DISTRIBUTION OF RADIOACTIVITY IN BRAIN GLUTAMIC ACID AND GLUTAMINE

Rat Number	Treatment	Compound Injected	Compound	Distribution of C^{14} in Compound					Randomization in Carbon 4 (24)
				Total	Percentage of Total in Carbon Atoms				
					1	2&3	4	5	
				μc/mmole					%
202	Liver ligated	L-Lactic acid-2-C^{14}	GLU[1]	2.1	16	17	5	62	15
218A	Fasted 48 hr	L-Lactic acid-2-C^{14}	GLU	3.5	21	23	10	35	45
193	Fasted 48 hr	D-Lactic acid-2-C^{14}	GLU	2.6				70	
219	Fasted 48 hr	D-Lactic acid-2-C^{14}	GLU	1.6		15	13	53	40
227	B_1 deficient	D-Glucose-2-C^{14}	GLU	1.7				74	
228	B_1 deficient	D-Glucose-2-C^{14}	GLU	2.5	12	5	3	75	8
			$GLU\text{-}NH_2$[2]	1.3		22	5	60	15
204	Ammonia	D-Glucose-2-C^{14}	GLU	4.3		9	6	78	14
			$GLU\text{-}NH_2$	2.2			1	60	

TABLE VIII (Continued

Rat Number	Treatment	Compound Injected	Compound	Distribution of C^{14} in Compound				Randomization in Carbon 4 (24)
				Total	Percentage of Total in Carbon Atoms			
					1	2&3	4	5
				μc/mmole				%
223	Normal	Sodium butyrate-3-C^{14}	GLU	0.6				70
			$GLU\text{-}NH_2$	1.7				75
229	Normal	Sodium butyrate-1-C^{14}	GLU	0.3				74
			$GLU\text{-}NH_2$	1.1				77
225	Normal	Glycerol-2-C^{14}	GLU	1.5				76
			$GLU\text{-}NH_2$	0.7				59
230	Normal	Glycerol-2-C^{14}	GLU	1.1				79
			$GLU\text{-}NH_2$	0.6				50

[1] GLU refers to brain free glutamic acid.

[2] $GLU\text{-}NH_2$ refers to brain free glutamine.

artery and portal vein had been ligated. However, in lactate Rats 218A and 219 the randomization in carbon 4 of the brain glutamate was over 40 per cent. This indicates that in these rats some of the lactate was converted to blood glucose prior to entry of the brain. The C^{14} distribution in brain glutamate of Rats 193 and 202 accompanied by the relatively high specific activities of the isolated brain components suggests that blood lactate enters brain tissue quite readily.

From 0.2 to 1.0 per cent of the total radioactivity injected as D-glucose-2-C^{14} was found in the brain glutamate, glutamine and aspartate (Table VII). This further emphasizes the conclusions of others (12-20) that glucose is an excellent precursor of the non-essential brain amino acids. Fasting 24 hours or thiamine deficiency did not greatly alter the above rate of incorporation.

As shown in Table VIII, over 70 per cent of the total C^{14} in the brain glutamate of thiamine-deficient Rats 227 and 228 was in carbon 5 ten minutes after injection of glucose-2-C^{14}. This very high labeling found in carbon 5 of the glutamate as well as the low labeling found in carbons 2 and 3 of Rat 228 brain glutamate indicates that most of the brain glucose was metabolized via acetylcoenzyme A (41). Since thiamine deficiency did not appear to reduce the amount of glucose-2-C^{14} incorporated into brain glutamate and metabolism of glucose via acetylcoenzyme A involves decarboxylation of pyruvate, these data indicate that severe thiamine deficiency did not alter the ability of brain to decarboxylate pyruvate. Failure of thiamine deficiency to reduce pyruvate decarboxylation in intact animals was also indicated by the results of Jones and de Angeli (40) and Koeppe et al. (41). Neither the results of these experiments nor those of the others cited support the conclusion of Peters (39) that one of the primary defects of thiamine deficiency is

the reduced ability of the animal to decarboxylate pyruvate. However, Peters' conclusion was based on results from thiamine-deficient pigeons and he had difficulty obtaining similar results from thiamine-deficient rats. Thus it appears that this defect caused by thiamine deficiency is not prominent in all species.

Insulin treatment seemed to enhance incorporation of the C^{14} from the injected glucose-2-C^{14} into the brain amino acids isolated (Table VII). This is explained by assuming that the blood glucose available to the brain had a higher specific activity in the insulin-treated rats than in the other glucose animals. The smaller amount of blood sugar in the insulin-treated rats diluted the injected glucose to a lesser extent.

In the case of Rats 204 and 232, ammonia treatment appeared to enhance the incorporation of C^{14} from glucose-2-C^{14} into the brain amino acids (Table VII). However, in the other ammonia-treated rats the amounts of C^{14} incorporated into the brain amino acids were comparable to those incorporated by normal animals. The fact that the labeled glucose was injected intracisternally into Rat 232 may have increased the specific activity of the glucose available to the brain, therefore resulting in a greater amount of C^{14} being incorporated into the brain amino acids. The specific activity of the brain aspartate of ammonia-treated Rats 203 and 204 was considerably higher than that of the brain glutamate and glutamine of these rats. This relatively high aspartate specific activity is unusual in rats given glucose-2-C^{14}. The fact that these rats received the ammonia several minutes (12 and 20) before the glucose was injected instead of in conjunction with it may have resulted in increased rates of turnover of brain aspartate during the time the labeled glucose was being metabolized.

The high labeling in carbon 5 and low labeling in carbons 2 and 3

of brain glutamate from ammonia-treated Rat 204 indicate that a very small percentage of the glucose-2-C^{14} was metabolized via oxalacetate (Table VIII). Since the metabolism of glucose via oxalacete is necessary for the net synthesis of citric acid cycle intermediates, these data suggest that very little glucose-2-C^{14} was used to replace the intermediates used for the de novo synthesis of glutamine following ammonia injection. Assuming that brain citric acid cycle intermediates are synthesized largely from glucose and that glutamine is not synthesized from a special compartment of glutamate, a possibility mentioned later, this observation further supports Bessman's theory of citric acid cycle depletion as a mechanism of ammonia intoxication (63). Labeling patterns, similar to that of Rat 204 brain glutamate, of the brain glutamates of both ammonia-treated and normal rats given pyruvate-2-C^{14} intracisternally were interpreted by McMillan and Mortensen (25) to refute Bessman's theory. They reasoned that a depletion of citric acid cycle intermediates due to ammonia would result in increased metabolism of pyruvate via oxalacetate. This would be reflected in brain glutamate by higher carbon 2 and 3 labeling. However, they did not consider the fact that these intermediates would have to be synthesized more rapidly in the ammonia-treated rats to prevent their depletion by de novo synthesis of glutamine. Therefore, ammonia would be expected to increase the amount of pyruvate metabolized via oxalacetate if normal levels of the citric acid cycle intermediates were to be maintained. Since their data show this did not happen, it can also be interpreted to support Bessman's theory.

The amounts of C^{14} incorporated into the brain glutamate, aspartate, and glutamine following intravenous injection of L-glutamic acid-2-C^{14} were relatively small compared to the amounts of C^{14} incorporated into these amino acids from similar amounts of radioactivity injected as

glucose-2-C^{14} (Table VII). The labeling in carbon 1 of brain glutamate and glutamine, isolated from Rat 207 given L-glutamic acid-2-C^{14}, was 18 and 16 per cent, respectively, of the total radioactivity found in these compounds. The percentage of labeling in carbon 1 of brain glutamate is almost identical to that found in carbon 1 of carcass glutamate when the precursor was DL-glutamate-2-C^{14} (65). However, it is much lower than the percentage of C^{14} found in carbon 1 of brain glutamate isolated 1 hour after DL-glutamate-2-C^{14} had been injected intraperitoneally (24). This low percentage labeling observed in carbon 1 of brain glutamate indicates that the intravenously injected L-glutamate-2-C^{14} probably entered the brain without prior conversion to blood glucose-3,4-C^{14} which is in contrast to the proposed entry of C^{14} from the intraperitoneal injected DL-glutamate-2-C^{14} (24). These data, suggesting entry of small amounts of blood glutamate into the brain, agree with the results of others (14, 26). The fact that the brain glutamine of glutamate-injected rats had higher specific activities than the brain glutamic acid suggests that the glutamic acid may have been converted to glutamine prior to or during entry into the brain.

Labeled blood glutamine entered the brain very slowly even though the blood levels were estimated to have been doubled by the amounts of glutamine injected (Table VII). The fact that the specific activity of the brain glutamine was higher than that of the brain glutamic acid suggests that the labeled glutamine entered the brain as glutamine.

The brain amino acids did not have as much C^{14} incorporated into them following the injection of sodium butyrate-3-C^{14}, sodium butyrate-1-C^{14}, or glycerol-2-C^{14} as when labeled glucose was used as the precursor (Table VII). The brain glutamate isolated from these rats had high percentages of labeling in carbon 5 (Table VIII). This high labeling in

carbon 5 of glutamate is consistent with the metabolism of these labeled precursors in the tricarboxylic acid cycle. It indicates that the glycerol-2-C^{14} was metabolized primarily through acetylcoenzyme A and that the labeled butyrates entered the brain without prior conversion to blood glucose-3,4-C^{14} (24). Also of note is the fact that following labeled butyrate injection the brain glutamine had three-fold higher specific activities than the brain glutamate (Table VII).

The amounts of radioactivity incorporated into the brain free amino acids of rats following the injection of labeled pyruvate, aspartate, malate and citrate were very small. This low rate of incorporation of pyruvate-2-C^{14} into brain glutamate agrees with the work of Koeppe and Hahn (24). The small amount of C^{14} incorporated in the brain amino acids following the intravenous injection of these precursors or of labeled glutamic acid and glutamine can be explained by assuming the blood-brain barrier restricts their entry into the brain. This explanation has been used by others (24, 26) to account for the small amounts of radioactivity incorporated into the brain free amino acids following the administration of such precursors as acetate-1-C^{14}, pyruvate-2-C^{14}, glutamic acid-2-C^{14}, glutamic acid-U-C^{14} and glutamine-U-C^{14}.

The radioactivity in the blood 10 minutes after the precursors had been injected indicates that with the exception of sodium pyruvate-2-C^{14} all the precursors were taken up by the tissues quite rapidly (Table VII).

The data presented in Table VII are the observed specific activities of the brain amino acids isolated from different size rats which had been given different doses of the labeled precursors. Direct comparisons of these specific activities can lead to false conclusions concerning which compounds are the better precursors. By definition, the better precursor is

the one of which the most molecules are converted to the compound in question. Thus, if glucose is a better precursor of brain glutamate than glycerol, more glucose molecules are converted to brain glutamic acid than are glycerol molecules. If C^{14} incorporation data are to be used for comparing precursors according to such a criterion, the specific activities of the compounds in question should be corrected for the differences in specific activities of the precursors presented to the system, in this case, brain. This correction was made by multiplying the observed specific activities of the isolated brain amino acids by the reciprocal of the estimated specific activity of the precursor in the blood at zero time.

The estimated specific activities of the precursors in the blood were determined by dividing the microcuries of C^{14} injected by the mmoles of precursor injected plus the mmoles of that compound assumed to be present in the blood. Thus the correction factor includes variation in animal size, dilution by components in the blood and the specific activity of the compound injected. Blood volume was assumed to be 6 per cent of the body weight. The blood glucose concentration used was 100 mg per cent except in Rats 209, 214, 227, and 228 where it had been determined analytically. The blood glutamate, glutamine and aspartate concentrations used were 2.0, 8.0 and 0.08 (66) mg per cent, respectively. The blood pyruvate and lactate levels used were 2 and 13 mg per cent, respectively (67). The blood concentrations of all the other precursors tested were assumed to be so low that the injected compounds were virtually undiluted.

The corrected specific activities of the brain glutamate, aspartate and glutamine are presented in Table IX. Only the data from rats receiving the labeled precursors 10 minutes prior to sacrifice were

TABLE IX

CORRECTED[1] SPECIFIC ACTIVITY OF BRAIN GLUTAMIC ACID, ASPARTIC ACID AND GLUTAMINE

Rat Number	Treatment	Compound Injected	Specific Activity of Precursor in Blood[2]	Glutamic Acid	Aspartic Acid	Glutamine
			mc/mmole		μc/mmole	
212	Normal	L-Lactic acid-2-C^{14}	0.92	2.6	1.9	1.6
200	Normal	D-Glucose-2-C^{14}	0.13	15.4	14.6	7.7
201	Normal	D-Glucose-2-C^{14}	0.13	20.8	16.9	
208	Fasted 24 hr	D-Glucose-2-C^{14}	0.22	9.6	7.7	4.5
209	Insulin	D-Glucose-2-C^{14}	0.59	9.6	8.4	5.7
214	Insulin	D-Glucose-2-C^{14}	0.64	11.0	8.0	3.5
227	B_1 deficient	D-Glucose-2-C^{14}	0.28	6.1	5.2	3.3
228	B_1 deficient	D-Glucose-2-C^{14}	0.30	8.2	6.4	4.3
203	Ammonia	D-Glucose-2-C^{14}	0.18	13.3	17.2	5.0
204	Ammonia	D-Glucose-2-C^{14}	0.18	23.9	28.9	12.2
206	Ammonia	D-Glucose-2-C^{14}	0.22	5.5	3.6	2.7
213	Ammonia	D-Glucose-2-C^{14}	0.16	14.1	8.9	7.7
205	Normal	L-Glutamic acid-2-C^{14}	0.33	0.2	0.3	0.5

TABLE IX (Continued)

Rat Number	Treatment	Compound Injected	Specific Activity of Precursor in Blood[2]	Glutamic Acid	Aspartic Acid	Glutamine
			mc/mmole		μc/mmole	
207	Normal	L-Glutamic acid-2-C^{14}	0.36	0.4	0.6	0.8
215	Normal	L-Glutamine-U-C^{14}	0.98	0.3	0.2	0.9
220	Normal	L-Glutamine-U-C^{14}	0.98	0.2	0.1	0.9
223	Normal	Sodium butyrate-3-C^{14}	1.7	0.3	0.2	1.0
229	Normal	Sodium butyrate-1-C^{14}	2.7	0.1	0.1	0.4
225	Normal	Glycerol-2-C^{14}	7.0	0.2	0.2	0.1
230	Normal	Glycerol-2-C^{14}	7.0	0.2	0.1	0.1
211	Normal	Sodium pyruvate-2-C^{14}	2.0	0.2	0.1	0.1
217	Normal	DL-Aspartic acid-3-C^{14}	6.7	0.03	0.05	0.02
224	Normal	DL-Malic acid-3-C^{14}	6.8	0.01	0.01	0.01
226	Normal	Citric acid-2,5-C^{14}	1.9	0.02	0.07	0.05

[1] Corrected by multiplying the observed specific activity of the isolated compound by the reciprocal of the specific activity of the precursor in the blood.

[2] Estimated by assuming the labeled precursor was completely mixed with that component of blood shortly after injection.

corrected, since the time a precursor was available to the brain would also probably affect the amount of C^{14} incorporated into the amino acids. When compared on this basis, none of the other compounds tested approached glucose as a precursor of the brain amino acids. Lactic acid appeared to be a better precursor than any of the other compounds tested, but it was still far less effective than glucose.

Recently evidence presented by Waelsch and colleagues (36, 68) has suggested compartmentation of brain glutamic acid and glutamine. In some work with C^{14}-labeled glutamate, glutamine and aspartate, they showed that regardless of which labeled compound was injected into the brain a short time later brain glutamine consistently had a higher specific activity than that of brain glutamic acid (68). In other experiments using N^{15}-ammonia, they found a much greater incorporation of label into the α-amino group of brain glutamine than into that of glutamic acid (36). They suggested that these results indicated brain glutamine was formed from a small and metabolically very active compartment of glutamic acid which was not in rapid equilibrium with the total tissue glutamic acid.

Some of the results from these experiments support such a compartmentation concept of brain glutamic acid and glutamine. The distribution of radioactivity in the brain glutamate of Rats 204 and 228 (given glucose-2-C^{14}) and Rats 225 and 230 (given glycerol-2-C^{14}) appeared to be different from that in brain glutamine of the same rats (Table VIII). The glutamate had over 75 per cent of the total radioactivity in carbon 5, whereas the glutamine had 60 or less per cent in carbon 5. Assuming these differences are real and not due to analytical error, this could mean that glutamine was formed from a source of glutamic acid which was not in equilibrium with the total tissue glutamic acid. The data from

animals given labeled butyrate or glutamate also support the compartmentation concept because the brain glutamine of these rats always had higher specific activities than the glutamic acid (Table VII). However, other data from rats given glucose, glycerol, pyruvate or aspartate do not support such a concept. Following the injection of these precursors brain glutamate always had a higher specific activity than did brain glutamine. We do not have a suitable hypothesis to explain all of these data and those of Waelsch and co-workers at present.

The distribution of C^{14} in the brain aspartate is presented in Table X. In every case the carboxyl carbon of the aspartate isolated from rats given lactate-2-C^{14}, glucose-2-C^{14}, glycerol-2-C^{14}, pyruvate-2-C^{14}, butyrate-1-C^{14} or butyrate-3-C^{14}, contained more than 70 per cent of the total radioactivity. With the exceptions of Rats 218A and 219 which had relatively low labeling in carbon 5 of glutamate, the high carboxyl labeling in the aspartate is in accord with the high labeling found in carbon 5 of the corresponding brain glutamate.

TABLE X

DISTRIBUTION OF RADIOACTIVITY IN BRAIN ASPARTIC ACID

Rat Number	Treatment	Compound Injected	Total Radioactivity μc/mmole	Percentage in Carboxyl Carbon
189	Fasted 72 hr	L-Lactic acid-2-C^{14}	1.0	84
190	Fasted 48 hr	L-Lactic acid-2-C^{14}	1.4	75
202	Liver ligated	L-Lactic acid-2-C^{14}	1.8	83
212	Normal	L-Lactic acid-2-C^{14}	1.7	96
218A	Fasted 48 hr	L-Lactic acid-2-C^{14}	2.6	83
193	Fasted 48 hr	D-Lactic acid-2-C^{14}	2.3	96
216	Liver ligated	D-Lactic acid-2-C^{14}	0.9	83
219	Fasted 48 hr	D-Lactic acid-2-C^{14}	1.1	79
200	Normal	D-Glucose-2-C^{14}	1.9	85
201	Normal	D-Glucose-2-C^{14}	2.2	84
208	Fasted 24 hr	D-Glucose-2-C^{14}	1.7	87
209	Insulin	D-Glucose-2-C^{14}	4.9	92
214	Insulin	D-Glucose-2-C^{14}	5.1	98
227	B_1 deficient	D-Glucose-2-C^{14}	1.4	92
228	B_1 deficient	D-Glucose-2-C^{14}	1.9	89

TABLE X (Continued)

Rat Number	Treatment	Compound Injected	Total Radioactivity μc/mmole	Percentage in Carboxyl Carbon
203	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	3.1	72
204	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	5.2	81
206	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	0.8	84
213	Fasted 24 hr, ammonia	D-Glucose-2-C^{14}	1.4	81
232	Ammonia	D-Glucose-2-C^{14}	2.7	73
207	Normal	L-Glutamic acid-2-C^{14}	0.2	73
223	Normal	Sodium butyrate-3-C^{14}	0.3	100
229	Normal	Sodium butyrate-1-C^{14}	0.3	71
225	Normal	Glycerol-2-C^{14}	1.1	79
230	Normal	Glycerol-2-C^{14}	1.0	74
211	Normal	Sodium pyruvate-2-C^{14}	0.3	81
217	Normal	DL-Aspartic acid-3-C^{14}	0.3	26

CHAPTER IV

SUMMARY

The brain free glutamic acid, aspartic acid, glutamic acid from glutamine and, in some instances, the glutamic acid from glutathione and the aspartic acid from asparagine and N-acetylaspartic acid, of normal, fasted, thiamine-deficient, insulin-treated and ammonia-treated rats were isolated, and assayed for amino acid content and radioactivity after intravenous injection of glucose-2-C^{14}. These brain components were isolated and analyzed also from rats which had been given various other labeled precursors. The results of these experiments indicate the following:

1. The isolation procedure used appeared to be satisfactory based on comparison of concentrations of the compounds in the brains of normal animals with values in the literature and recovery from standard samples of these compounds.

2. Although lactic acid is known to leave the brain, blood lactate appeared to enter the brain tissue quite readily without prior conversion to blood glucose.

3. Glucose was a much better precursor of the non-essential brain amino acids than any of the other compounds tested. From 0.2 to 1.0 per cent of the total radioactivity injected as D-glucose-2-C^{14} was found in the brain glutamate, glutamine and aspartate.

4. Neither fasting nor thiamine deficiency altered the concentrations of the brain amino acids. Thiamine deficiency did not appear to

reduce the amount of pyruvate decarboxylated by the brain.

5. Either insulin or ligation of the hepatic artery and portal vein increased the brain aspartate levels and decreased the glutamate and glutamine levels. Insulin increased the rate of incorporation of C^{14} from glucose-2-C^{14} into the brain amino acids. These effects appeared to be related to the low blood sugar levels caused by the treatments.

6. Injection of $LD_{99.9}$ doses of ammonium acetate resulted in increased levels of brain glutamine, decreased levels of aspartate and no changes in the levels of glutamate. This increase in glutamine appeared to result from de novo synthesis in brain.

7. Blood glutamic acid, glutamine and aspartic acid did not enter the brain at a very rapid rate. Increased blood levels did not produce net increases of these compounds in the brain.

8. Only very small amounts of radioactivity from labeled sodium pyruvate, sodium butyrate, glycerol, malic acid and citric acid were incorporated into the brain amino acids when these compounds were given intravenously.

9. Data in some experiments but not in others supported the concept that glutamine may be formed from a metabolically small but active compartment of glutamic acid which is not in rapid equilibrium with the total tissue glutamic acid.

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