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SUBCELLULAR LOCALIZATION OF GLUTAMIC ACID AND OTHER ^{3}H-AMINO ACIDS IN CEREBRAL CORTICAL SLICES OF THE RAT

by

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ABSTRACT

The subcellular localization of glutamic acid, other ^{3}H-amino acids and ^{14}C-norepinephrine in rat cerebral cortex has been examined. Slices of rat cerebral cortex were incubated with tritiated compounds, homogenized and subjected to subcellular fractionation. Some suspected neurotransmitters (glutamic acid, glycine, and norepinephrine), the basic amino acids (arginine, lysine, ornithine and histidine) and the small neutral amino acids (serine, alanine and threonine) were localized in particulate fractions more than were others. Differential centrifugation showed that several of these amino acids were predominantly in a particulate fraction (P2) enriched with pinched off nerve endings ("synaptosomes") and mitochondria, and were in osmotically sensitive compartments. When P2 fractions were centrifuged on continuous density gradients, ^{3}H-glutamic acid, ^{3}H-glycine, ^{3}H-leucine, ^{14}C-norepinephrine, and ^{14}C gamma-aminobutyric acid were localized tc the same fractions as potassium, a marker for cytoplasm occluded within synaptosomes, and were separable from fractions containing the highest levels of monoamine oxidase activity, a mitochondrial marker. The amino acid fractions also displayed the highest frequency of synaptosomal profiles upon electron micro-

scopic investigation of the density gradients. The distribution of the amino acids in sucrose gradients were broader than that of norepinephrine, and this difference was enhanced by incomplete equilibrium sedimentation. In contrast to the synaptosomal localization of ^{3}H-glutamic acid, endogenous glutamic acid was primarily localized in the soluble supernatant fraction. However, the particulate moieties of exogenous and endogenous glutamic acid had identical patterns of distribution in continuous sucrose gradients, implying a mixing of exogenous with endogenous pools. All of the ^{3}H-amino acids examined were mostly unmetabolized in the tissue slices, although metabolites of glutamic acid accumulated in the medium.

To my wife, Joan,
and to Dr. Martin D. Appleton
and Dr. Solomon H. Snyder,
teachers and friends.

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INTRODUCTION

The discovery that various amino acids when applied electrophoretically were active on neurones in the mammalian central nervous system suggested that these compounds might possibly be involved in central neurotransmission (Curtis and Watkins, 1960; Curtis et al., 1961). The actions of glutamate and aspartate were excitatory, while glycine, alanine, and gamma-aminobutyric acid were inhibitory. Even though there is no direct proof that any of these compounds are neurotransmitters, increasing evidence seems to insure an important role for at least some of them. This investigation concerns the uptake and subcellular distribution of glutamic acid in rat cerebral cortical slices, and its possible role as a central neurotransmitter.

Some commonly employed criteria for identifying a neurotransmitter substance are: (1) The substance itself, or its synthesizing enzymes should be present in nerve terminals; (2) The substance should be released from nerve endings by presynaptic stimulation; (3) An inactivation mechanism is necessary; (4) When electrophoretically applied at the postsynaptic membrane, the substance should produce ionic permeability changes identical to those produced by the natural transmitter. Attempts to establish such criteria for glutamate at the arthropod neuromuscular junction and in

and mammalian CNS are reviewed below.

Arthropod Neuromuscular Junction. A. Takeuchi and N. Takeuchi (1963, 1964) have shown, using an isolated walking leg of the crayfish (cambarus clarkii) with nerve and muscle intact, that L-glutamic acid depolarized the nerve-muscle junction area. The glutamate sensitive spots on the muscle coincided with areas where natural excitatory junction potentials were recorded, and the depolarization was stereospecific, that is, the muscle was 250 times more sensitive to L- than to D-glutamate.

With another species of crab (carcinus maenas), it was shown that a ninhydrin positive substance identified as glutamic acid was released into perfusion fluid from a stimulated nerve-muscle preparation (Kerkut et al., 1965). The amount of substance released was proportional to the frequency and duration of nervous stimulation. However, this demonstration of glutamic acid release due to nerve stimulation does not indicate release at the synapse. In fact, some workers have evidence that glutamate is an organic ion exchanged along the axon during an action potential (Wheeler et al., 1965).

In the locust (Schistocerca gregaria) nerve-muscle preparation P.N.R. Usherwood and P. Machilli (1966, 1968) showed: (1) potentiation of nerve stimulated contractions by small amount of glutamic acid (6×10^{-12}M - 6×10^{-9}M); (2) a reduction of the mechanical response due to nerve stimulation by perfusing the preparation with relatively

low concentrations of glutamic acid decarboxylase (5×10^{-7}); (3) potentiation of these same mechanical responses by perfusion with low concentrations of a relatively nonspecific enzyme inhibitor, phenylhydrazine hydrochloride; (4) a large release of glutamate, along with smaller amounts of alanine, glycine, and aspartate into the bathing medium upon nerve stimulation (alanine and glycine are not active when electrophoretically applied and aspartate is 100 times less active than glutamate); (5) that serotonin, which blocks nerve-muscle transmission, increased the amount of glutamate appearing in the bathing fluid, suggesting that the glutamate postsynaptic receptors were also responsible for the destruction of glutamate; (6) that increasing the concentration of Mg^{++} in the medium depressed transmission of the impulse and the amount of glutamate released, while increasing the concentration of Ca^{++} in the medium increased the amount of glutamate released to the medium by neural stimulation. All of the above findings are consistent with the proposals that glutamic acid is an excitatory transmitter at the neuromuscular junction, and that enzymatic destruction is a possible mechanism of inactivation. However, the investigation also found that the application of small amounts of glutamate to the end-plate region increased the frequency of miniature end-plate potentials, suggesting that glutamate's action was only to facilitate the release of the excitatory transmitter. Also the evidence for inactiva-

tion by enzymatic decarboxylation is weak, but their other findings are very interesting. If it is shown that glutamate can be synthesized and/or stored in the terminals of excitatory axons, then the evidence in this case may be fairly convincing. These same authors have shown that very high concentrations of amino acids are found in the blood of these locusts.

Similar results have been found with the cockroach, (Periplaneta americana) (Kerkut et al., 1965a, 1965b, 1967; Kerkut and Walker, 1966). When the nerve in an isolated perfused nerve-muscle preparation was stimulated, a ninhydrin positive substance identified as glutamic acid by thin layer chromatography, appeared in the perfusate. The concentration of L-glutamate that showed slight threshold contractions was 5×10^{-7}M, while acetylcholine, D-glutamic acid and L-aspartic acid required higher concentrations, 5×10^{-6}, 5×10^{-6}, and 6×10^{-3}M respectively. Gamma-aminobutyric acid easily inhibited the contractions caused by glutamic acid. However, it was again found that L-glutamate increased both the amplitude and frequency of the miniature end-plate potentials, suggesting that glutamic acid is not the neurotransmitter at all, but a compound that facilitates the release of the true transmitter substance.

Takeuchi and Takeuchi (1964) found that glutamate applied iontophoretically to the crayfish muscle did not increase the frequency of the miniature end-plate potentials.

Also, iontophoretic application of acetylcholine does not affect the frequency of the miniature endplate potentials (Del Castillo and Katz, 1956). Clearly, more work is needed before we may understand the precise mechanism of action of glutamate at the insect neuromuscular junction.

Mammalian Central Nervous System (CNS). Because of anatomical considerations the mammalian central nervous system does not lend itself easily to investigation. Neurons of various types and glia are densely packed so that a well defined experimental situation is almost impossible. But circumstantial evidence that glutamic acid may have a synaptic function is steadily accumulating.

Glutamic acid concentration in mammalian brain exceeds that of any other amino acid (10 μmoles/gm tissue) (Berl and Waelsch, 1958). Although the subcellular localization of glutamic acid will be discussed in detail below, it should be mentioned now that there seems to be enough glutamic acid asscciated with nerve endings and vesicles to function as an excitatory neurotransmitter (Krnjevic and Whittaker, 1966).

Glutamic acid is active when electrophoretically applied to many kinds of cells in the mammalian CNS. D. R. Curtis, and others, found that glutamate, aspartate, and cysteate had excitatory actions on interneurons, motor-neurons, and Renshaw cells in the lumbar cord of the cat, while β-alanine, gamma-aminobutyric acid, and taurine were inhibitory (Curtis and Watkins, 1960, 1961; Curtis

et al., 1960). In contrast to the case for the locust, glutamate decarboxylase inhibitors did not potentiate the action of iontophoretically applied glutamic acid. In fact, application of various inhibitors of all the known enzymes utilizing glutamate as a substrate failed to show any effect on its depolarization. Moreover, since Renshaw cells were thought to have cholinergic synapses, these early workers concluded that the excitatory action of glutamate was nonspecific and unrelated to neurotransmission.

K. Krnjevic and J. W. Phillis (1963) showed that glutamate had powerful excitatory actions on neurons in the cerebral and cerebellar cortex of cats, rabbits, and monkeys. The effect of L-glutamate was stereospecific; L glutamate was more effective than L or D-aspartate and D-glutamate, and had a rapid onset and a rapid termination. However, almost all neurones were affected by glutamate and it was again suspected that the action was nonspecific.

Cuneate cells in anesthesized cats were strongly excited by L-glutamate and somewhat less by D-glutamate. Again, the effect was quick and reversible (Galindo et al., 1967).

H. McLennan (1968) examined neurons in the feline thalamus. Cells that were probably within the nucleus ventro-oralis posterior were much more sensitive to L-glutamate than were other neurons, while there was no such differential sensitivity to N-methylaspartic acid or

homocysteic acid. The author suggests that two kinds of receptors are involved; perhaps one type is nonspecific and responds to many excitatory amino acids while the other type is specific for L-glutamate and found on some neurons in the nucleus ventro-oralis posterior.

The ionic basis for the action of glutamic acid on nervous tissue has been investigated (Bradford and McIlwain, 1966; Harvey and McIlwain, 1968). L-Glutamate depolarized guinea pig cortical cells in vitro, lowered the phosphocreatin content and initiated intracellular concentration changes of Na^+ and K^+. Further, glutamate increased the tissues' permeability to sodium relative to that of potassium five-fold and the authors suggested that the glutamate induced Na^+ influx would be enough to depolarize the tissue in a short time. After electrical stimulation of the cortex for 10 minutes, the changes in intracellular Na^+ and K^+ were similar to the changes produced by exposure of cortex slices to glutamate for 10 minutes.

The subcellular localization of glutamic acid in guinea pig brain was examined by J. L. Mangan and V. P. Whittaker (1966) who found no specific localization of glutamate in pinched off nerve endings, or "synaptosomes," but rather a distribution similar to cytoplasmic markers. This discovery mitigated against a unique synaptic function but certainly did not exclude one since a small "transmitter" pool of glutamic acid in nerve endings would have escaped detection. Indeed, it has been established that

there is more than one pool of glutamate in the brain (Van den Berg, 1969; Berl et al., 1969). Moreover, while even a smaller percentage of total glutamate was found associated with synaptic vesicles, a rough calculation implied that there was much more glutamic acid than acetylcholine per vesicle. Other electrophoretically active amino acids, such as glycine and alanine were also found to be associated with vesicles, although in smaller amounts than glutamic acid (Whittaker, 1968). Glutamic acid extracted from these fractions was capable of producing excitation effects on single cortical neurons of the guinea pig brain (Krnjevic and Whittaker, 1965). Thus, while the distribution of glutamic acid in the guinea pig is not striking from a neurotransmitter's point of view, it does not preclude the possibility that glutamic acid has such a role.

L. Salganicoff and E. DeRobertis (1965) studied the subcellular distribution of enzymes of the glutamic acid cycle in rat brains. They found several related enzymes in nerve endings, many of them associated with the mitochondria within the synaptosomes. The enzymes studied included alanine aminotransferase, aspartate aminotransferase, glutamic acid decarboxylase, glutamic acid dehydrogenase, glutaminase, glutamine synthetase and α-oxoglutamate dehydrogenase. Another similar study examined the distribution of glutamic acid decarboxylase and aspartate transaminase in subcellular fractions of rat and

guinea pig brain (Fonnum, 1968). Glutamic acid decarboxylase was released from synaptosomes by hypo-osmotic treatment, and aspartate transaminase was found both in the cytoplasm and mitochondria of nerve endings. The presence of glutamic acid decarboxylase in nerve endings has significance since its product, gamma-aminobutyric acid, is a putative inhibitory neurotransmitter, and the presence of the other enzymes not only insure the presence of synthetic components, but may suggest multiple roles for glutamic acid at nerve endings.

There have been few extensive studies of the release of glutamate from nerve endings after nervous stimulation in mammals. One investigation, utilized cervical or midbrain sectioning in the cat to produce "aroused" or "sleeping" electroencephalograms respectively (Jasper, et al., 1965). Results indicated that glutamic acid released from brain tissue was reduced significantly during the sleeping state while the release of gamma-aminobutyric acid increased significantly. The relevance of this work is in doubt since "release" meant that the compounds were found in the exudate collected from the surface of the brain, and it is not known how the composition of this exudate relates to compounds released at nerve endings.

Transport systems into nerve terminals have been described for several putative neurotransmitters including norepinephrine (Iversen, 1967), serotonin (Blackburn et al., 1967) and gamma-aminobutyric acid (Iversen and Neal, 1968).

Such transport may serve to terminate the synaptic action of these compounds.

An uptake system for acetylcholine in mammalian brain has been demonstrated when acetylcholinesterase was inhibited by organophosphates (Polak, 1969; Liang and Quastel, 1969). A choline transport system has been found in the isolated perfused superior cervical ganglia; choline present in the perfusion media was incorporated into the acetylcholine and free choline pools of the ganglia, while very little labeled acetylcholine was taken up (Collier and MacIntosh, 1969). Synthesis and subsequent acetylcholine release was slowed if choline was not present, or if hemicholineum base was present along with choline (Birks and MacIntosh, 1961). It is unclear whether the acetylcholine uptake in mammalian brain involves a unique mechanism or is identical to the choline transport system, and whether acetylcholine is accumulated into nerve endings, axons, cell bodies or glia.

If glutamic acid were a neurotransmitter, it is conceivable that an analogous uptake system would transport exogenous glutamic acid into nerve terminals. Amino acid transport in brain slices has been examined (Blasberg and Lajtha, 1965; Blasberg, 1967) and an uptake system specific for both glutamic acid and aspartic acid has been found. The objective of the experiments presented in this dissertation is to examine the subcellular distribution of exogenous and endogenous glutamic acid in rat cerebral cortex.

METHODS AND MATERIALS

Tissue Preparation. Sprague-Dawley male rats (150-200 g) were killed be decapitation, their brains were rapidly removed and dissected according to the method of Glowinski and Iversen (1966). The cerebral cortex was dissected with a sharp razor blade on a dental wax plate on ice, into slices of tissue approximately cube shaped (about 2.5 mm on a slide) and weighing 20 to 30 mg. Tissue slices were immersed in 20 ml beakers containing 2 ml of Krebs-Henseleit (1932) bicarbonate medium with glucose and were shaken in a Dubnoff metabolic incubator at 37°C under at atmosphere of 95% O_2-5% CO_2. After 10 minutes of preincubation, radioactive amino acids or norepinephrine were added (0.1 μM), and the incubation was continued. The cerebral cortex slices were quickly removed from the medium, rinsed in ice cold 0.32 M sucrose, blotted lightly and weighed at room temperature.

Tissue/Medium Ratios. After homogenizing incubated tissue slices in 2 ml of 0.4 N perchloric acid or 70% ethanol and obtaining a supernatant fluid by centrifuging at 1000 x g for 10 minutes at 25°C, the tissue to medium ratios were calculated as counts per minute per gram weight of tissue to counts per minute per milliliter of medium. The radioactivity in 0.1 ml of supernatant fluid as well as

that in 0.1 ml of medium from each beaker was measured by transferring the aliquots to a vial containing 3 ml of absolute ethanol and 10 ml of toluene phosphor (0.4% 2,4-diphenyloxazole and o.01% β-bis-(2-phenyl-oxazole) benzene in toluene). A Nuclear Chicago Unilux liquid scintillation spectrometer (tritium counting efficiency = 15%, carbon-14 counting efficiency = 40%) was used in all radioactivity measurements, except for the data presented in Figures 5, 6 and 7, which were determined with a Packard Model 3375 liquid scintillation spectrometer (tritium counting efficiency = 25% and carbon-14 counting efficiency = 60%).

Subcellular Fractionation Procedure. Tissue slices, prepared and incubated with radioactive compounds by the above procedure, were homogenized in 2 ml of ice cold 0.32 M sucrose (Mallinckrodt) in a Potter-Elvehjem glass homogenizer with a teflon pestle (0.004"-0.006" clearance). The homogenates were poured into centrifuge tubes, and the homogenizers were rinsed with 1 ml of 0.32 M sucrose which was added to the homogenates. After centrifugation at 1,000 x g for 10 minutes at 4°, the supernatant fluid was decanted. The pellet was resuspended in 3 ml of 0.32 M sucrose and centrifuged again under the same conditions and the pellet designated P1. The combined supernatant fluid (6 ml total) was centrifuged at 17,000 x g for 20 minutes to form a pellet (P2). In some experiments, the supernatant fluid from P2 was centrifuged at 100,000 x g for 60 minutes to form a pellet (P3). This procedure was a modification of

the method of Gray and Whittaker (1962).

In some experiments, cerebral cortical homogenates were centrifuged directly for 1 hr at 100,000 x g to obtain total particulate and soluble supernatant fractions.

Pellets were homogenized with chilled 0.4N perchloric acid to extract radioactivity, centrifuged at 1,000 x g for 10 minutes and radioactivity in a 0.2 ml aliquot of the supernatant fluid was measured. After the pellets were resuspended and recentrifuged in 0.4 N perchloric acid, radioactivity in the resulting supernatant fraction was not significant.

Leakage of tritiated amino acids from particulate fractions was examined. If certain amino acids leaked out of particulate compartments more than others, one would find misleading particulate/supernatant ratios. After homogenizing tissue previously incubated with ^{3}H-glutamic acid, ^{3}H-glycine, and ^{3}H-leucine (0.1 μM concentrations) total particulate fractions were obtained by centrifuging the homogenates at 100,000 x g for 60 minutes. If the particulate fractions were resuspended in 0.32 M sucrose for 4 hours, and recentrifuged at 100,000 x g, radioactivity in particulate fractions was reduced by 35%, 25% and 5% for pellets from tissue previously incubated with ^{3}H-glutamic acid, ^{3}H-glycine and ^{3}H-leucine respectively. This indicates that the amino acids most concentrated in particulate fractions tended to have the highest leakage.

Continuous linear sucrose gradients (5 ml, 17 ml,

40 ml) were prepared utilizing a triple outlet mixer (Buchler 2-5104) and a peristaltic pump (Buchler 2-6100). Three gradients were made at the same time by diluting 1.46 M sucrose (Mallinckrodt) linearly with 0.32 sucrose.

P2 pellets were obtained from cerebral cortex stripped of white matter that had been dissected and incubated in the usual way. The pellets were suspended in 1 ml of 0.32 M sucrose and layered carefully on gradients that were stored for 18 hours at 4°C prior to use, although there was no difference in results if the gradients were used immediately after preparation. The gradients were then centrifuged for the desired time and speed with either an SW50.1 or SW27 rotor in a Spinco L2-65 ultracentrifuge. Fractions were obtained by piercing the bottom of the centrifuge tubes and collecting drops. The ^{14}C and ^{3}H content of the fractions were measured after adding 15 ml of Bray's phosphor (Bray, 1960) to aliquots in scintillation vials.

Estimation of Endogenous Glutamic Acid. Glutamic acid was extracted from tissue pellets by suspension in cold 70% ethanol. Aliquots of sucrose gradient fractions were added to cold absolute ethanol to a final concentration of 70% ethanol, and the same procedure was followed with aliquots of supernatant fluid from subcellular centrifugation. The extracts obtained after centrifuging these samples at 1,000 x g were used in the following procedure which is a modification of the method of Graham and

Aprison (1966).

Aliquots (25-200 µl) were added to a mixture containing 1.5 ml of 0.2 M hydroxylamine HCl buffer (pH 8.6) and 25 µl of 10 mM NAD solution. The fluorescence at 460 mµ (340 exciting wavelength) was recorded for this mixture using an Aminco-Bowman spectrophotofluorometer and served as the blank reading. Twenty five µl of L-glutamic acid dehydrogenase (Calbiochem, bovine liver, amonium sulfate free, in 50% glycerol, A grade) was then added, and each sample was stirred and maintained at 25° for 1 hour. The fluorescence was measured again at 340/460 mM, and blanks were subtracted. Internal standards of 10×10^{-10} mole of L-glutamic acid added to samples indicated negligible quenching of fluorescence. Glutamic acid added to homogenates was recovered to the extent of 95-100%. Fluorescence was proportional to L-glutamic acid concentration in the range $1\text{-}30 \times 10^{-10}$ moles of L-glutamic acid.

Lactic Acid Dehydrogenase (LDH) Assay. Tissue pellets were suspended in 2 ml of 0.1 M glycine/NaOH buffer (pH 10) to which was added 20 µl of 10% Triton-X-100. After the suspensions were maintained at 4° for 2 hours to insure lysis of subcellular compartments, they were centrifuged at 1,000 x g, and aliquots of the supernatant fluid were used in the procedure described below. Supernatant fractions obtained from sub-cellular fractionation procedures were assayed directly. This procedure is a modification of the method of Neilands (1957).

Aliquots of 200 µl or less were added to a previously stirred mixture of 0.8 ml of 0.1 M glycine/NaOH (pH 10) buffer and 100 µl of 0.02 M NAD and 100 µl of 0.5 M Na^+ DL-lactate. Tissue extracts were added directly to a quartz cuvette containing the mixture, and the fluorescence at 450 mµ (350 mµ exciting wavelength) was recorded continuously on a Varian Model F-80A X-Y recorder connected to an Aminco-Bowman spectrophotofluorometer.

Potassium Determination. Pellets were suspended in 2 ml of 0.4 N perchloric acid and maintained at 4° for 2 hours to precipitate macromolecules. Equal volumes of 0.8 N perchloric acid were added to aliquots of supernatant fluids and gradient fractions, stirred, and also put in the cold. The supernatant fluid obtained by centrifuging each of these samples was analyzed for K^+ content on a Techtron atomic absorption spectrophotometer.

Monoamine Oxidase (MAO) Assay. MAO activity was assayed by a modification of the method of Wurtman and Axelrod (1963). Portions (200 µl) of each gradient fraction were added to glass stoppered centrifuge tubes containing 0.1 µmole of ^{14}C-tryptamine (40,000 cpm) in a volume of 0.1 ml and 0.7 ml of 0.1 M sodium phosphate buffer (pH 7.8). After a 20 minute incubation at 37°C, 0.5 ml of 1 N HCl and 7 ml of toluene were added and samples were agitated in a mechanical shaker for 10 minutes. After centrifuging at 1,000 x g, 5 ml of the organic phase were added to 10 ml of toluene phosphor in a scintillation vial. Heated enzyme

blanks were subtracted and MAO activity was expressed as nmole of ^{14}C-indoleacetic acid formed per 20 minutes.

Metabolism Studies. Incubated tissue slices were homogenized in 70% ethanol and centrifuged to obtain a supernatant fluid which was evaporated to dryness under a stream of N_2. The residue was dissolved in a small volume of water and applied to Whatman #1 paper, and cochromatographed with authentic amino acids for 18 hours in a descending manner at 25°. At that time, the solvent fronts had moved about 15 to 20 inches. Solvents used were phenol:water (100:20 v/v) for glutamate and its metabolites, and propanol: ethanol:Tartaric acid buffer (pH 3.4) (62.5:37.5:25) and propanol:1 N acetic acid (3:1) for other amino acids. In some experiments, high voltage paper electrophoresis (90 v/cm) on Whatman 3 MM paper with 1.5 N Formic acid: 2 M acetic acid (1:1) pH 2.0, was performed.

After drying the paper, two inch wide strips containing the samples were cut from the chromatograms, stained with 1% ninhydrin in acetone solution and R_f's of the authentic amino acids were noted. The Phenol:water (100: 20 v/v) solvent was routinely used to separate glutamate and its metabolites, and the R_f's of glutamate, GABA, glutamine, aspartate and glutathione were 0.31, 0.74, 0.57, 0.19 and 0.12 respectively. The R_f's of the other amino acids in the various solvents were the same as those reported by Block et al. (1953). Every amino acid was examined in more than one solvent.

The strips were cut into 1 cm wide portions, soaked in vials containing 2 ml ethanol and 2 ml methanol for 15 minutes, and radioactivity was measured after adding 10 ml of toluene phosphor.

Electron Microscopy. Five ml gradients were prepared with P2 pellets from 25 mg of tissue incubated in the presence of ^{3}H-glutamic acid. Thirty-drop fractions were collected and the sucrose concentrations were adjusted to 0.5 M sucrose. During the collection procedure, every tenth drop was measured for radioactivity content. Five volumes of cold potassium permanganate (1% in veronal-acetate buffer, pH 7.4) were added as fixative, and the suspension kept at 4° for 30 minutes. The particulate matter was sedimented at 10,000 x g for 10 minutes and the pellets resuspended in 0.5 ml of the fixative and recentrifuged at 11,000 x g in a microfuge (Beckman) for 5 minutes. The resulting pellet was quickly dehydrated in ice-cold acetone, transferred to propylene oxide and embedded in Araldite. Sections were cut on a Porter-Blum MT-1 ultramicrotome and viewed stained in 1:25 lead hydroxide, or unstained in a RCA EMU 3-F electron microscope.

A total of 3 gradients were prepared in the above manner and sampled for electron microscopy. From 6 to 20 plates were taken from 2 or more grids prepared from each fraction. Prints (37,800 x) were analyzed independently by two observers. Synaptosomes were defined as membrane-bound sacs containing 300-500 Å synaptic vesicles, sometimes

with one or more mitochondria, and occasionally an intact synaptic cleft with an adherent post-synaptic element.

Materials. L-^{3}H valine (2.15 c/mmol), L-^{3}H-alanine (2.1 c/mmol), L-^{3}H-threonine (2.25 c/mmol), L-^{3}H-proline (5.26 c/mmol), L-^{3}H-arginine (1.21 c/mmol), ^{3}H-glycine (3.5 c/mmol), L-^{3}H-aspartic acid (2.15 c/mmol), DL-^{3}H-glutamic acid (27 c/mmol), L-^{3}H-histidine (6.24 c/mmol), L-^{3}H-serine (3.73 c/mmol), DL-^{3}H-leucine (5.0 c/mmol), ^{14}C-taurine, DL-^{14}C-norepinephrine (46 mc/mmol), DL-^{3}H-norepinephrine (6.6 c/mmol), L-^{3}H-isoleucine (1.64 c/mmol), ^{14}C-tryptamine (10.2 mc/mmol), ^{3}H-GABA (2.0 c/mmole) and ^{14}C-GABA (4.45 mc/mmole), DL-^{3}H-ornithine (2.38 c/mmol), ^{3}H-water (4.5 mc/mmol) and ^{14}C-urea (10 mc/mmol) were purchased from the New England Nuclear Corporation. ^{3}H-methionine (3.1 c/mmol), L-^{3}H-tryptophan (2.2 c/mmol), L-^{3}H-lysine (7.0 c/mmol) and L-^{3}H-tyrosine (8.0 c/mmol) were purchased from Schwarz Bioresearch, Inc. Non radioactive amino acids were obtained from Calbiochem and were regarded as authentic samples.

RESULTS

Accumulation of ^{3}H-amino acids into cerebral cortical slices. Single cerebral cortical slices were incubated for 30 minutes with 0.1 μM concentrations of each of eleven ^{3}H-amino acids and ^{3}H-norepinephrine. Tissue and medium were assayed for tritium content and the tissue to medium ratio of tritium determined (Table 1). There was considerable variation in the accumulation of various amino acids with a 5-fold range from highest to lowest. Glutamic acid and glycine showed the greatest accumulation with tissue to medium ratios of 5.3 and 4.5 while lysine was accumulated least efficiently with a tissue to medium ratio of 0.9. The tissue to medium ratios of the other amino acids varied between two and four. At 0°, tissue to medium ratios for all amino acids were about 0.8 at 30 minutes and about 1.0 at 60 minutes. Similar experiments were performed with slices of medulla oblongata, hypothalamus, midbrain, striatum and hippocampus (Table 2). The tissue to medium ratios were similar to those shown in Table 1, except for the medulla and midbrain slices, which were less than those of the other areas.

The tissue/medium ratios of ^{3}H-glutamic acid, ^{3}H-glycine and ^{3}H-leucine were determined after 15, 30, 45, 60 and 90 minute incubations (Table 3). The accumulation

TABLE 1

TISSUE/MEDIUM RATIOS OF ^{3}H COMPOUNDS

IN RAT CEREBRAL CORTICAL SLICES

^{3}H-COMPOUNDS	TISSUE/MEDIUM RATIO		^{3}H-COMPOUNDS	TISSUE/MEDIUM RATIO	
DL-^{3}H-NOREPINEPHRINE	3.92	0.17	L-^{3}H-TYROSINE	2.67	0.19
DL-^{3}H-GLUTAMIC ACID	5.27	0.14	L-^{3}H-HISTIDINE	2.09	0.06
^{3}H-GLYCINE	4.58	0.18	L-^{3}H-METHIONINE	1.96	0.16
L-^{3}H-TRYPTOPHAN	4.04	0.08	DL-^{3}H-LEUCINE	1.89	0.09
L-^{3}H-SERINE	3.86	0.34	L-^{3}H-ASPARTIC ACID	1.80	0.15
L-^{3}H-PROLINE	3.69	0.59	L-^{3}H-LYSINE	0.91	0.02

Single cerebral cortical slices were incubated for 30 minutes with tritiated compounds (0.1μ M), blotted lightly, weighed and homogenized in 70% ethanol. The radioactivity in the supernatant fluid obtained by centrifuging the homogenate, as well as the radioactivity in the incubation medium was measured. The tissue to medium ratio was calculated as counts per minute per gram wet weight of tissue to counts per minute per ml of medium. Results are the mean of four experiments $\pm$ standard error of the mean.

TABLE 2

TISSUE/MEDIUM RATIOS OF ^{3}H-GLUTAMIC ACID IN SLICES OF VARIOUS REGIONS OF RAT BRAIN

REGION	TISSUE/MEDIUM RATIO
MEDULLA	1.3
HYPOTHALAMUS	5.9
MIDBRAIN	3.3
STRIATUM	4.0
HIPPOCAMPUS	5.0

Single slices of various regions of rat brain were incubated for 30 minutes with ^{3}H-glutamic acid (0.1μM), blotted lightly, weighed and homogenized in 70% ethanol. The radioactivity in the supernatant fluid obtained by centrifuging the homogenate, as well as the radioactivity in the incubation medium was measured. The tissue to medium ratio was calculated as counts per minute per gram wet weight of tissue to counts per minute per ml of medium. Results presented are the average of two experiments with less than 5% variation between them.

TABLE 3

TISSUE/MEDIUM RATIOS OF ^{3}H-GLUTAMIC ACID, ^{3}H-GLYCINE, AND ^{3}H-LEUCINE IN RAT CEREBRAL CORTICAL SLICES AT DIFFERENT TIMES

^{3}H-AMINO ACID	TISSUE/MEDIUM RATIO				
	15 MIN	30 MIN	45 MIN	60 MIN	90 MIN
DL-^{3}H-GLUTAMIC ACID	3.7 ± 0.2	5.4 ± 0.2	5.6 ± 0.1	5.1 ± 0.3	4.9 ± 0.3
^{3}H-GLYCINE	3.2 ± 0.6	4.7 ± 0.2	6.0 ± 0.5	6.6 ± 0.2	9.5 ± 0.5
DL-^{3}H-LEUCINE	1.4 ± 0.2	2.0 ± 0.1	2.6 ± 0.2	2.6 ± 0.2	2.8 ± 0.2

Single cerebral cortical slices were incubated for different times with tritiated compounds (0.1 μM), blotted lightly, weighed and homogenized in 70% ethanol. The radioactivity in the supernatant fluid obtained by centrifuging the homogenate, as well as the radioactivity in the incubation medium was measured. The tissue to medium ratio was calculated as counts per minute per gram wet weight of tissue to counts per minute per ml. of medium. Results are the mean of four experiments ± standard error of the mean.

of ^{3}H-glutamic acid and ^{3}H-leucine did not significantly change after 30 minutes, while the accumulation of ^{3}H-glycine continued even after 60 minutes.

Although these experiments were conducted with concentrations of amino acids considerably lower than the Km values (Blasberg, 1967), the tissue to medium ratios were similar to those reported using 2 mM concentrations of the amino acids (Blasberg and Lajtha, 1965). Also, exchange processes were not measured in the presented experiments.

To obtain an estimate of radioactivity remaining in the precipitate, the pellets were repeatedly suspended in 0.1 N perchloric acid and centrifuged until no radioactivity was found in the supernatant fluid. The entire pellet was suspended in toluene phosphor which seemed to dissolve the pellet and the radioactivity was measured. After 90 minute incubation of single cerebral cortical slices with ^{3}H-glutamic acid, ^{3}H-glycine and ^{3}H-leucine (0.1 μM concentrations), the percent of total tissue radioactivity in acid washed pellets were 0.4, 0.8, and 8.4% respectively (Table 4). Increase in pellet radioactivity was approximately linear in time. When incubations of tissue slices were performed at 0^{o}, pellet radioactivity was not significantly different from background counts.

Metabolism of ^{3}H-glutamic Acid in Brain Slices. Cerebral cortical slices were incubated with DL-^{3}H-glutamic acid (0.1 μM) for 30 minutes. After incubation, slices were

TABLE 4

PERCENT OF TOTAL TISSUE RADIOACTIVITY IN ACID WASHED PELLETS

^{3}H-AMINO ACID	TISSUE/MEDIUM RATIO	PERCENT OF TOTAL TISSUE RADIOACTIVITY FOUND IN PELLET
DL-^{3}H-GLUTAMIC ACID	5.9 ± 0.3	0.4 ± 0.1
^{3}H-GLYCINE	10.2 ± 0.5	0.8 ± 0.1
DL-^{3}H-LEUCINE	3.2 ± 0.2	8.4 ± 0.3

Single cerebral cortical slices were incubated for 90 minutes with tritiated compounds (0.1 μM), blotted lightly, weighed and homogenized in 0.4 N perchloric acid. The radioactivity in the supernatant fluid obtained by centrifuging the homogenate, as well as the radioactivity in the incubation medium was measured and the tissue/medium ratio was calculated. The pellet was repeatedly suspended in 0.4 N perchloric acid and centrifuged until no radioactivity was found in the supernatant fluid. The entire pellet was suspended in toluene phosphor and the radioactivity was measured. Results are the mean of four experiments ± standard error of the mean.

homogenized in 70% ethanol, centrifuged and the supernatant fluid evaporated to dryness under a stream of nitrogen. The residue was dissolved in a small volume of water and chromatographed on Whatman #1 paper in a descending manner using phenol:water, propanol:ethanol:tartaric acid buffer and propanol:acetic acid solvents as described in Methods. Aliquots of the medium were applied directly to paper and chromatographed in the same systems (Table 5). More than 85% of the radioactivity in the tissue slices were unchanged glutamic acid. There were small amounts of ^{3}H-gamma-aminobutyric acid, ^{3}H-glutamine and a very small amount of ^{3}H-aspartic acid. In the medium, ^{3}H-glutamic acid accounted for 67% of total tritium. No ^{3}H-aspartic acid or ^{3}H-gamma-aminobutyruc acid could be detected in the medium.

Tissue slices were incubated at 37° for 30 minutes with ^{3}H-glycine, L-^{3}H-tryptophan, L-^{3}H-serine, L-^{3}H-proline, L-^{3}H-tyrosine, L-^{3}H-histidine, L-^{3}H-methionine, DL-^{3}H-leucine, and L-^{3}H-lysine at 0.1 μM concentrations, and extracts were chromatographed as described above. After incubation with all of these amino acids, more than 85% of the radioactivity within the slice was unchanged ^{3}H-amino acid. These results are similar to those obtained with 2 mM concentrations of these amino acids (Blasberg and Lajtha, 1965).

In some experiments, tissue slices were incubated at 37° for 60 minutes with ^{3}H-glutamic acid, ^{3}H-glycine, and ^{3}H-leucine (0.1 μM) and extracts were chromatographed as

TABLE 5

METABOLITES OF ^{3}H-GLUTAMIC ACID IN RAT CEREBRAL CORTICAL TISSUE SLICES AND INCUBATION MEDIUM

^{3}H-COMPOUND	TISSUE	MEDIUM
^{3}H-GLUTAMIC ACID	85.6 ± 2.5	67.0 ± 1.5
^{3}H-GLUTAMINE	5.5 ± 1.6	33.0 ± 1.5
^{3}H-ASPARTIC ACID	1.6 ± 0.2	0
^{3}H-GAMMA-AMINOBUTYRIC ACID	7.2 ± 2.7	0

Slices of rat cerebral cortex were incubated with ^{3}H-glutamic acid for 30 minutes and homogenized in 70% ethanol. The homogenates were centrifuged, and the supernatant fluids evaporated to dryness under a stream of N_2. The resulting residue was dissolved in a small volume of water and transferred, as were qliquots of the incubation medium, to Whatman #1 chromatography paper. The samples were cochromatographed with authentic amino acids in a descending manner for 18 hours, as described in Methods. Results are the mean of six experiments ± standard error of the mean, and are expressed as percent of total radioactivity in tissue or medium.

described above. Results indicated that 80-90% of the radioactivity within the slice was unchanged amino acid.

Particulate/supernatant ratios of ^{3}H-amino acids in rat cerebral cortex. In order to obtain a rough approximation of the subcellular localization of ^{3}H-glutamic acid and other amino acids, the following experiments were performed. Cerebral cortical slices were incubated with 17 ^{3}H-amino acids, ^{14}C-taurine, DL-^{3}H-norepinephrine, ^{3}H-H_2O, or ^{14}C-urea at 0.1 μM concentrations. After incubation for 30 minutes at 37°, slices were homogenized in 2 ml of ice cold 0.32 sucrose. Homogenates were centrifuged at 100,000 x g for 1 hour and radioactivity in the particulate fraction (P) and soluble supernatant fluid (S) were determined (Table 6). Of amino acids which have been implicated as putative neurotransmitters, glutamic acid and glycine showed P/S ratios of 0.84 and 0.62 respectively, similar to the P/S ratio for norepinephrine, while aspartic acid had a much lower P/S ratio of 0.18. The basic amino acids, arginine, lysine, ornithine, and histidine showed high P/S ratios as did the small neutral amino acids, serine, threonine, and alanine. The P/S ratios for other amino acids, including proline, tryptophan, tyrosine, methionine, taurine, leucine, valine and isoleucine were lower. ^{3}H-water and ^{14}C-urea had P/S ratios less than 0.1.

When incubations were performed at 0°, the P/S ratios for glutamic acid and glycine were 0.27 and 0.22 respectively. If ^{3}H-glutamic acid, ^{3}H-glycine and ^{3}H-ly-

TABLE 6

PARTICULATE/SUPERNATANT FLUID RATIOS OF ^{3}H-AMINO ACIDS IN RAT CEREBRAL CORTICAL SLICES

COMPOUNDS	P/S	COMPOUNDS	P/S
PUTATIVE NEUROTRANSMITTERS:		OTHER:	
DL-^{3}H-NOREPINEPHRINE	0.99 ± 0.04	L-^{3}H-PROLINE	0.39 ± 0.03
DL-^{3}H-GLUTAMIC ACID	0.82 ± 0.02	L-^{3}H-TRYPTOPHAN	0.42 ± 0.01
^{3}H-GLYCINE	0.62 ± 0.03	L-^{3}H-TYROSINE	0.41 ± 0.01
L-^{3}H-ASPARTIC ACID	0.18 ± 0.01	L-^{3}H-METHIONINE	0.32 ± 0.01
BASIC AMINO ACIDS:		^{14}C-TAURINE	0.23 ± 0.04
L-^{3}H-ARGININE	1.07 ± 0.13	DL-^{3}H-LEUCINE	0.20 ± 0.09
L-^{3}H-LYSINE	1.07 ± 0.01	L-^{3}H-VALINE	0.19 ± 0.02
DL-^{3}H-ORNITHINE	0.81 ± 0.86	L-^{3}H-ISOLEUCINE	0.19 ± 0.0001
L-^{3}H-HISTIDINE	0.65 ± 0.05	^{3}H-H_2O	0.08 ± 0.01

TABLE 6 (CON'T.)

COMPOUNDS	P/S	COMPOUNDS	P/S
SMALL NEUTRAL AMINO ACIDS:		OTHER (CON'T.)	
L-^{3}H-ALANINE	0.50 ± 0.005	^{14}C-UREA	0.08 ± 0.0001
L-^{3}H-SERINE	0.60 ± 0.02		
L-^{3}H-THREONINE	0.81 ± 0.01		

Cerebral cortical slices (4 slices, 20 mg each) were incubated with tritiated compounds (0.1 μM) and homogenized in 0.32 M sucrose. Homogenates were centrifuged at 100,000 x g for 1 hour to form a total particulate fraction (P) and a soluble supernatant fluid (S). P/S ratios were calculated as counts per minute in particulate to counts per minute in soluble supernatant fluid. Results are expressed as the mean of four experiments ± standard error of the mean.

sine were added to homogenates of previously incubated tissue, the P/S ratios were 0.06, 0.04, and 0.1 respectively (Table 7). These data indicate that the uptake into particulate compartments is an active process, and that non-specific adsorption and redistribution during homogenization did not significantly influence the apparent P/S ratios.

In some experiments, tissue slices were incubated with tritiated compounds at 37° at different concentrations (0.1 μM, 10 μM, 1 mM). P/S ratios for ^{3}H-glutamic acid, ^{3}H-glycine, and ^{3}H-leucine declined slightly as the concentration in the medium increased (Table 8).

It is possible that anatomical barriers may hinder access of amino acids to certain parts of neurons during brief incubations and thus affect the apparent subcellular localization. Accordingly, in some experiments, tissue slices were incubated with ^{3}H-glutamic acid, ^{3}H-glycine and ^{3}H-leucine at 37° (0.1 μM concentrations) for 60 minutes, a time at which equilibrium has taken place between tissue and medium. Under these conditions P/S ratios were 1.1, o.7, and 0.2 respectively, slightly higher than the P/S ratios found using 30 minute incubation periods (Table 9).

Release of ^{3}H-amino acids from particulate fractions of cerebral cortex by hypo-osmotic shock. Some amino acids were shown to be stored in osmotically sensitive compartments in the following experiments. Cerebral cortical slices were incubated with ^{3}H-glutamic acid, ^{3}H-histidine,

TABLE 7

PARTICULATE/SUPERNATANT FLUID RATIOS OF ^{3}H-AMINO ACIDS AFTER 0° INCUBATION AND AFTER ADDITION TO HOMOGENATES

	P/S RATIOS	
^{3}H-AMINO ACID	0° INCUBATION	^{3}H-AMINO ACIDS ADDED TO HOMOGENATES
^{3}H-GLUTAMIC ACID	0.27 ± 0.03	0.06 ± 0.01
^{3}H-GLYCINE	0.22 ± 0.02	0.04 ± 0.01
^{3}H-LYSINE	-	0.1 ± 0.01

Cerebral cortical slices (4 slices, 20 mg each) were incubated with tritiated glutamic acid and glycine at 0° for 30 minutes. P/S ratios were determined in the same way as for Table 4.

Also, cerebral cortical slices were incubated without ^{3}H-compounds for 30 minutes at 37° and homogenized in 0.32 M sucrose. Tritiated glutamic acid, glycine and lysine were added to the homogenates (0.2 nmoles), stirred and maintained at 4° for 10 minutes. The homogenates were centrifuged at 100,000 x g for 60 minutes, and the P/S ratios were determined.

Results are expressed as the mean of four experiments ± standard error of the mean.

TABLE 8

P/S RATIOS IN RAT CEREBRAL CORTICAL SLICES AFTER INCUBATION WITH VARYING CONCENTRATIONS OF ^{3}H-COMPOUNDS

^{3}H-COMPOUNDS	0.1 μM	10 μM	1 mM
DL-^{3}H-GLUTAMIC ACID	0.84 ± 0.02	0.79 ± 0.06	0.66 ± 0.03
^{3}H-GLYCINE	0.62 ± 0.03	0.65 ± 0.02	0.60 ± 0.02
DL-^{3}H-LEUCINE	0.20 ± 0.09	0.19 ± 0.02	0.15 ± 0.01
DL-^{3}H-NOREPINEPHRINE	0.99 ± 0.04	0.95 ± 0.04	---

Cerebral cortical slices (4 slices, 20 mg each) were incubated with tritiated compounds of varying concentrations for 30 minutes, and homogenized in 0.32 M sucrose. Homogenates were centrifuged at 100,000 x g for 1 hour to form a total particulate fraction (P) and a soluble supernatant fluid (S). P/S ratios were calculated as counts per minute in particulate to counts per minute in soluble supernatant fluid. Results are expressed as the mean of four experiments ± standard error of the mean.

TABLE 9

P/S RATIOS OF ^{3}H-COMPOUNDS IN RAT CEREBRAL CORTICAL SLICES AFTER 30 AND 60 MINUTE INCUBATIONS

^{3}H-COMPOUNDS	30 MINUTES	60 MINUTES
DL-^{3}H-GLUTAMIC ACID	0.84 ± 0.02	1.09 ± 0.06
^{3}H-GLYCINE	0.62 ± 0.03	0.72 ± 0.001
DL-^{3}H-LEUCINE	0.20 ± 0.09	0.17 ± 0.001

Cerebral cortical slices (4 slices, 20 mg each) were incubated with tritiated compounds (0.1 μM concentrations) for 30 and 60 minutes. P/S ratios were determined in the same way as for Table 8. Results are expressed as the mean of four experiments ± the standard error of the mean.

^{3}H-glycine, and ^{3}H-lysine at 37° for 30 minutes. Pellets were obtained by centrifuging homogenates of the slices in 0.32 M sucrose at 100,000 x g for 60 minutes. P/S ratios were determined for some samples, while others were resuspended in water or 0.32 M sucrose, maintained at 4° for 2 hours, and recentrifuged at 100,000 x g for 60 minutes. Treatment with water reduced P/S ratios to extremely low values ranging from 0.02 to 0.10, while resuspension in 0.32 M sucrose did not markedly lower the P/S ratios. This indicates that hypo-osmotic shock released the amino acids from particulate fractions to the soluble supernatant fluid (Table 10).

Subcellular localization of ^{3}H-amino acids in four fractions obtained by differential centrifugation. More detailed subcellular distribution studies were performed using differential centrifugation in the following experiments. Cerebral cortical slices were incubated with one of five ^{3}H-amino acids or with ^{3}H-norepinephrine at 37° for 30 minutes. Slices were homogenized in 0.32 M sucrose and P1 (1,000 x g for 10 minutes), P2 (17,000 x g for 20 minutes) and P3 (100,000 x g for 60 minutes) pellets as well as a soluble supernatant fraction were obtained and assayed for tritium as described in Methods (Table 11). For all compounds examined, much more tritium was retained in the P2 fraction, which contains mitochondria and synaptosomes (DeRobertis, 1967; Whittaker, 1965), than in the other particulate fractions. The retention of tritium in P2 was

TABLE 10

RELEASE OF ^{3}H-AMINO ACIDS FROM PARTICULATE FRACTIONS OF RAT CEREBRAL CORTEX BY HYPO-OSMOTIC SHOCK

^{3}H-AMINO ACID	P/S	P/S AFTER RESUSPENSION FOR 2 HOURS IN WATER
DL-^{3}H-GLUTAMIC ACID	0.84 ± 0.017	0.04 ± 0.002
L-^{3}H-HISTIDINE	0.65 ± 0.052	0.03 ± 0.0001
^{3}H-GLYCINE	0.62 ± 0.033	0.02 ± 0.0009
L-^{3}H-LYSINE	1.07 ± 0.077	0.10 ± 0.003

Some of the pellets obtained by centrifuging isotonic sucrose homogenates at 100,000 x g for 60 minutes were resuspended in water and maintained at 4° for 2 hours. The suspensions were centrifuged again at 100,000 x g for 60 minutes and the resulting supernatant fluid was added to the original supernatant fraction. P/S ratios were calculated as counts per minute in particulate to counts per minute in the soluble supernatant fluid. Results are the means of four experiments ± standard error of the means.

TABLE 11

THE SUBCELLULAR DISTRIBUTION OF TRITIATED COMPOUNDS IN HOMOGENATES OF RAT CEREBRAL CORTEX AFTER DIFFERENTIAL CENTRIFUGATION

^{3}H-COMPOUND	P1	P2	P3	S
DL-^{3}H-NOREPINEPHRINE	5.26 ± 0.28	44.1 ± 2.8	15.5 ± 1.18	35.1 ± 1.7
DL-^{3}H-GLUTAMIC ACID	4.27 ± 0.16	28.11± 2.4	8.63± 0.58	59.01± 2.85
^{3}H-GLYCINE	4.31 ± 0.4	31.03± 3.3	8.25± 0.18	56.39± 3.34
L-^{3}H-ASPARTIC ACID	2.74 ± 0.42	15.53± 1.2	5.86± 0.11	75.85± 1.75
DL-^{3}H-LEUCINE	2.46 ± 0.10	15.5 ± 0.63	5.76± 0.27	76.2 ± 0.57
L-^{3}H-LYSINE	6.71 ± 0.56	38.5 ± 1.5	11.3 ± 1.7	43.6 ± 0.75

Tissue slices (4 slices, 20 mg each) were incubated with tritiated compounds (0.1 μM) and homogenized in 0.32 M sucrose. The P1 pellet was obtained by washing the pellet formed after centrifuging the homogenate at 1000 x g for 10 minutes. The P2 pellet was formed by centrifuging the supernatant fluid at 17,000 x g for 20 minutes and a P3 pellet was obtained by centrifuging the resulting supernatant fluid at 100,000 x g for 60 minutes. Results are the mean of four experiments ± standard error of the mean and are expressed as percent of total content of the homogenates.

greater with norepinephrine, glutamic acid, glycine and lysine than with aspartic acid and leucine.

Subcellular localization of endogenous and exogenous glutamic acid, ^{3}H-norepinephrine and cytoplasmic markers. Cerebral cortical slices were incubated with ^{3}H-glutamic acid or ^{3}H-norepinephrine (0.1 M) at 37° for 30 minutes. After homogenization in 0.32 M sucrose, P1 (1,000 x g for 10 minutes) P2 (17,000 x g for 20 minutes) and a supernatant fraction (S) were obtained as described in Methods. Subcellular fractions were assayed for tritium, endogenous glutamic acid, lactic acid dehydrogenase activity, and potassium content (Table 12). The subcellular localization of endogenous glutamic acid was similar to that of the cytoplasmic markers LDH and potassium. The concentration of ^{3}H-glutamic acid in the P2 fraction, however, was double that of the endogenous glutamic acid and somewhat lower than that of ^{3}H-norepinephrine.

Subfractionation of P2 pellets using sucrose gradients. The P2 fraction contains mitochondria and myelin fragments, as well as synaptosomes (Whittaker, 1965). To resolve further the particulate localization of ^{3}H-glutamic acid, sucrose gradient centrifugation was performed. Cerebral cortical slices were incubated with ^{3}H-glutamic acid (0.1 μM) for 30 minutes at 37°. After homogenization in 0.32 M sucrose, P1 (1,000 x g for 10 minutes) and P2 (17,000 x g for 20 minutes) pellets were prepared. The P2 pellet was resuspended in 1 ml of 0.32 M sucrose and centrifuged on a

TABLE 12

THE SUBCELLULAR LOCALIZATION OF EXOGENOUS AND ENDOGENOUS GLUTAMIC ACID, ^{3}H-NOREPINEPHRINE, LACTIC ACID DEHYDROGENASE AND POTASSIUM IN RAT CEREBRAL CORTEX

	P1	P2	S
ENDOGENOUS GLUTAMIC ACID	3.1	14.2	82.8
^{3}H-GLUTAMIC ACID	4.3	28.1	67.6
^{3}H-NOREPINEPHRINE	5.3	44.1	50.6
LACTIC ACID DEHYDROGENASE	5.0	16.0	79.0
POTASSIUM	3.8	16.7	79.5

Tissue slices were incubated with tritiated compounds (0.1 μM) and homogenized in 0.32 M sucrose. The P1 pellet was obtained by centrifuging the homogenate at 1000 x g for 10 minutes and washed by resuspending in 0.32 sucrose and recentrifuging. The resulting supernatant fluid was centrifuged at 17,000 x g for 20 minutes to form a pellet (P2) and supernatant fluid (S). Results are the average of two experiments and are expressed as percent of total content of the homogenate.

linear continuous sucrose gradient (1.46-0.32 M) at 100,000 x g for 80 minutes. About 30 fractions were obtained after punching a hole in the bottom of the tube and assayed for monoamine oxidase activity, potassium content and tritium as described in Methods (Figure 1 and Table 13). ^{3}H-glutamic acid and potassium showed a peak concentration at a level corresponding to a density of 1.0 M sucrose. Monoamine oxidase activity, a mitochondrial enzyme, showed a peak activity four or five fractions lower than the peak activity for glutamic acid or potassium, a further indication that the gradient centrifugation employed separated free mitochondria from synaptosomes.

In some experiments the subcellular localization of ^{3}H-glutamic acid or ^{3}H-glycine was compared with that of ^{14}C-norepinephrine, a synaptosomal marker (Potter and Axelrod, 1963; Glowinski et al., 1965). Cerebral cortical slices were incubated with ^{3}H-glutamic acid (0.1 μM) and ^{14}C-norepinephrine (0.1 μM) or ^{3}H-glycine and ^{14}C-norepinephrine at 37^{o} for 30 minutes. The P2 pellet obtained by differential centrifugation was suspended in 0.32 M sucrose, layered on the continuous sucrose gradient described above and 29 fractions were obtained and assayed for tritium as described in Methods. ^{3}H-glutamic acid showed a peak localization at a level corresponding to about 1.0 M sucrose as did ^{3}H-glycine. The peak of ^{14}C-norepinephrine occurred at the same or adjacent fractions of the gradient as that of ^{3}H-glutamic acid and ^{3}H-glycine. However, ^{14}C-

FIGURE 1. Subcellular distribution of ^{3}H-glutamic acid, potassium, and monoamine oxidase (MAO) in a continuous sucrose gradient.

Tissue slices incubated with ^{3}H-glutamic acid (0.1 μM) were homogenized in 0.32 M sucrose and P2 pellets (see Methods) were obtained by differential centrifugation. After suspension in 0.32 M sucrose the P2 pellets were centrifuged on 0.32 to 1.46 M continuous sucrose gradients. MAO, potassium, and tritium were determined on the same gradients, and the results shown represent a typical one of three gradients. MAO activity is expressed as nmoles of Indoleacetic acid/20 minutes. 100,000 x g for 80 minutes.

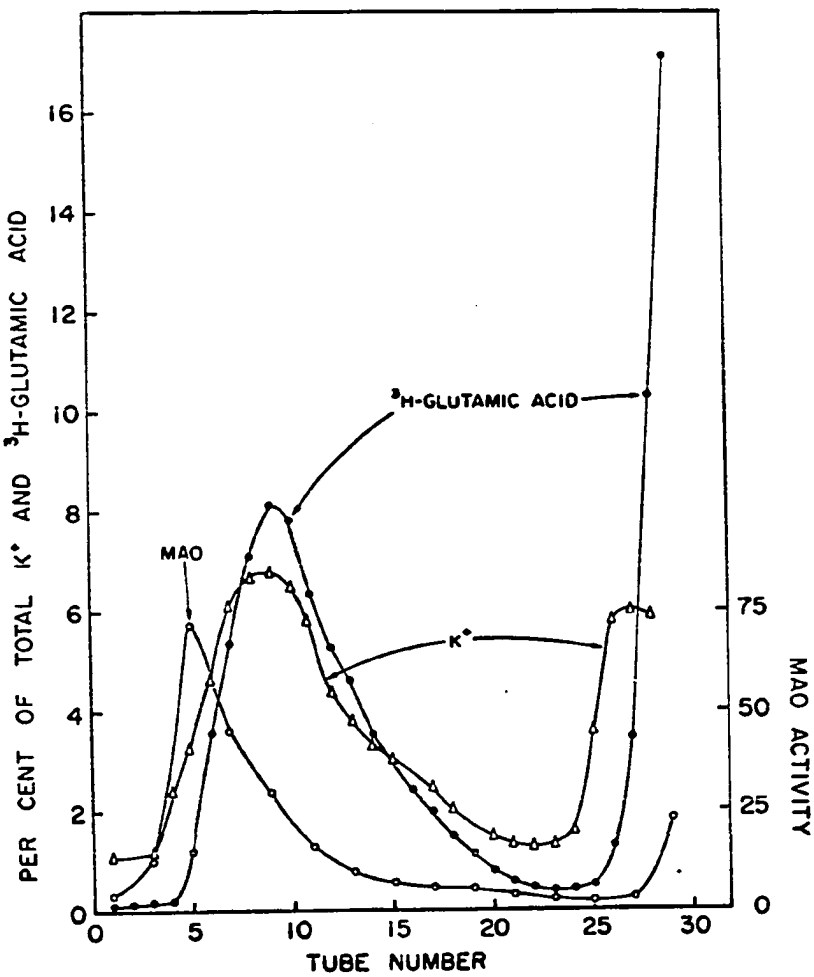

TABLE 13

POSITIONS OF PEAKS AND HALF-HEIGHTS OF ^{3}H-GLUTAMIC ACID, POTASSIUM AND MONOAMINE OXIDASE ACTIVITY (MAO) IN A CONTINUOUS SUCROSE GRADIENT

	EXPT. NO.:	LEFT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:	PEAK FRACTION NO.:	RIGHT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:
	1	3 - 4	5	7 - 8
MAO	2	3 - 4	5	8 - 9
	3	4 - 5	7	8 - 9
	1	6 - 7	8	13 - 14
^{3}H-GLU	2	6 - 7	9	13 - 14
	3	7 - 8	9	14 - 15
	1	5 - 6	9	15 - 16
K	2	5 - 6	9	14 - 15
	3	5 - 6	9	14 - 15

This table is a summary of three experiments, the first of which is presented in Figure 1.

norepinephrine was distributed in a narrower band than the two amino acids, and was confined to the denser region of synaptosomal distribution (Figures 2,3; Tables 14,15). Similar experiments were performed with ^{3}H-leucine and ^{14}C-norepinephrine and the distribution of ^{3}H-leucine in the gradients was found to be the same as that of ^{3}H-glycine and ^{3}H-glutamic acid.

The subcellular localization of the endogenous and exogenous glutamic acid of the P2 pellet was compared using sucrose gradients. Cerebral cortical slices were incubated with ^{3}H-glutamic acid (0.1 μM) at 37° for 30 minutes, and the P2 pellet obtained after homogenization and differential centrifugation was resuspended and centrifuged on a sucrose gradient as described in Methods. The pattern of distribution of endogenous and exogenous glutamic acid in the synaptosomal region was identical (Figure 4 and Table 14).

The differences between the profiles of norepinephrine and the amino acids in the gradients were more apparent when gradients were prepared in the same way as for figures 2 and 3, but centrifuged for shorter times. Centrifugation at 100,000 x g for 15 minutes resulted in two distinct peaks of radioactivity (Figure 5 and Table 16).

Particle bound isotope versus total isotope in gradient fractions. To rule out the possibility that the radioactivity profiles in the sucrose gradients were due to radioactive compounds leaking out of the synaptosomes the

FIGURE 2. Subcellular distribution of ^{3}H-glutamic acid and ^{14}C-norepinephrine in a continuous sucrose gradient.

Tissue slices incubated with ^{3}H-glutamic acid (0.1 μM) and ^{14}C-norepinephrine (0.1 μM) were homogenized in 0.32 M sucrose and P2 pellets were obtained by differential centrifugation (See Methods). The P2 pellets were suspended in 0.32 M sucrose and centrifuged on 0.32 to 1.46 M continuous sucrose gradients. Tritium and carbon-14 were determined on the same gradients, and the results shown represent a typical one of three gradients. 100,000 x g for 80 minutes.

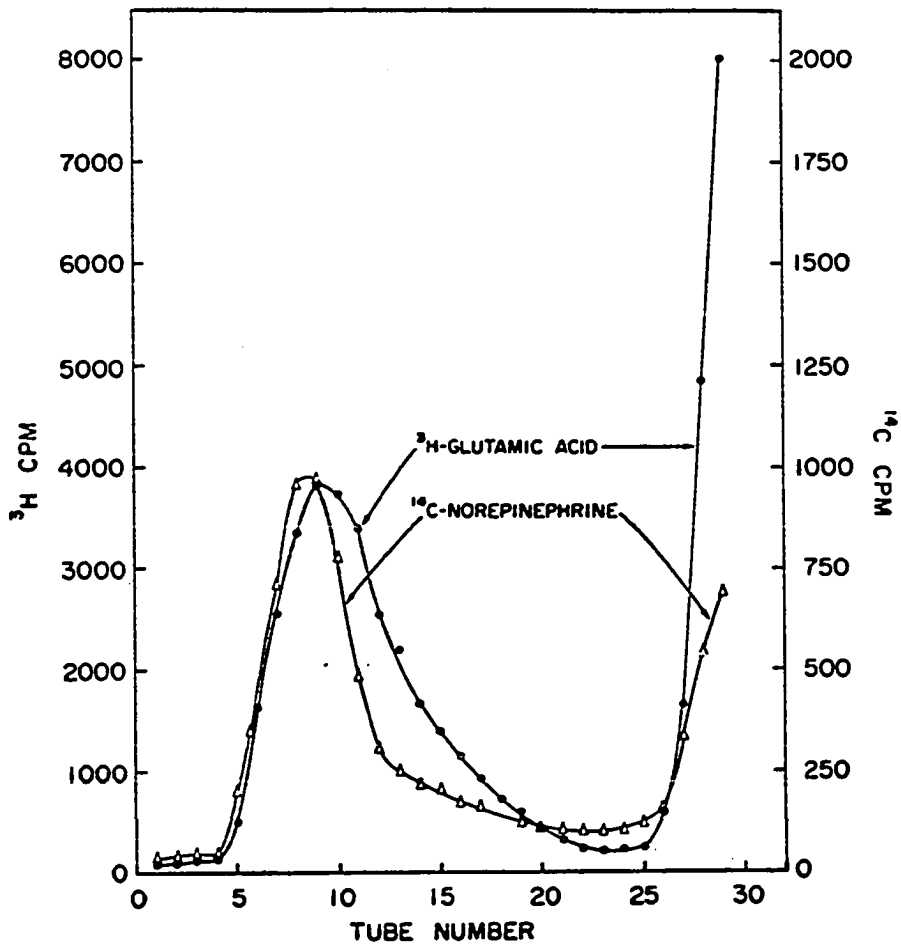

FIGURE 3. Subcellular distribution of ^{3}H-glycine and ^{14}C-norepinephrine in a continuous sucrose gradient.

Tissue slices incubated with ^{3}H-glycine (0.1 μM) and ^{14}C-norepinephrine (0.1 μM) were homogenized in 0.32 M sucrose and P2 pellets were obtained by differential centrifugation (see Methods). After suspension in 0.32 M sucrose, the P2 pellets were centrifuged on 0.32 to 1.46 M continuous sucrose gradients. Tirtium and carbon-14 were determined on the same gradients, and the results shown represent a typical one of three gradients. 100,000 x g for 80 minutes.

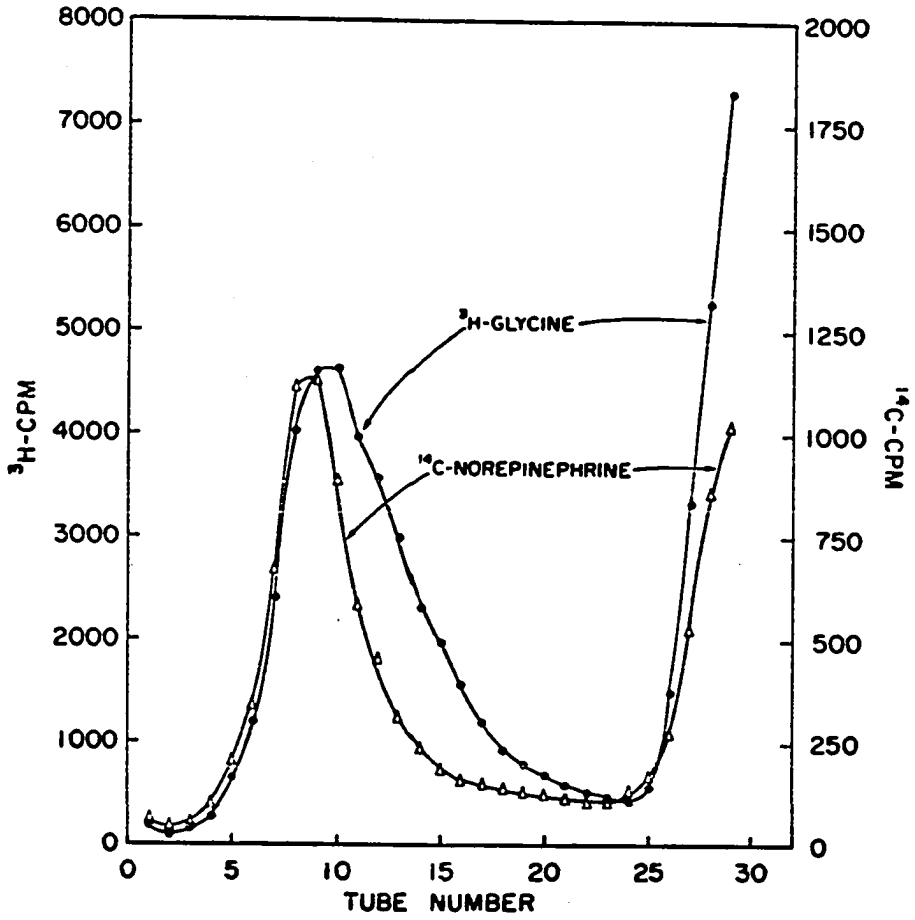

TABLE 14

POSITIONS OF PEAKS AND HALF-HEIGHTS OF ENDOGENOUS GLUTAMIC ACID, ^{3}H-GLUTAMIC ACID AND ^{14}C-NOREPINEPHRINE IN A CONTINUOUS SUCROSE GRADIENT

	EXPT. NO.:	LEFT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:	PEAK FRACTION NO.:	RIGHT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:
	1	6 - 7	8	13 - 14
^{3}H-GLU	2	5 - 6	9	13 - 14
	3	6 - 7	9	14 - 15
	1	6 - 7	9	13 - 14
ENDOGENOUS GLU	2	5 - 6	9	13 - 14
	3	6 - 7	9	14 - 15
	1	6 - 7	8	10 - 11
^{14}C-NE	2	5 - 6	8	9 - 10
	3	6 - 7	9	10 - 11

This table is a summary of three experiments, the first of which is shown in figures 2 and 4.

TABLE 15

POSITIONS OF PEAKS AND HALF-HEIGHTS OF ^{3}H-GLYCINE AND ^{14}C-NOREPINEPHRINE IN A CONTINUOUS SUCROSE GRADIENT

	EXPT. NO.:	LEFT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:	PEAK FRACTION NO.:	RIGHT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:
^{3}H-GLY	1	6 - 7	9	13 - 14
	2	6 - 7	9	13 - 14
	3	5 - 6	10	14 - 15
^{14}C-NE	1	6 - 7	9	10 - 11
	2	6 - 7	9	10 - 11
	3	5 - 6	9	10 - 11

This table is a summary of three experiments, the first of which is shown in figure 3.

FIGURE 4: Subcellular distribution of exogenous and endogenous glutamic acid.

Tissue slices incubated with ^{3}H-glutamic acid were homogenized in 0.32 M sucrose and P2 pellets were obtained by differential centrifugation (See Methods). After suspension in 0.32 M sucrose, P2 pellets were centrifuged on 0.32 to 1.46 M continuous sucrose gradients. Exogenous and endogenous glutamic acid were determined on the same gradients, and the results shown represent a typical one of three gradients. 100,000 x g for 80 minutes.

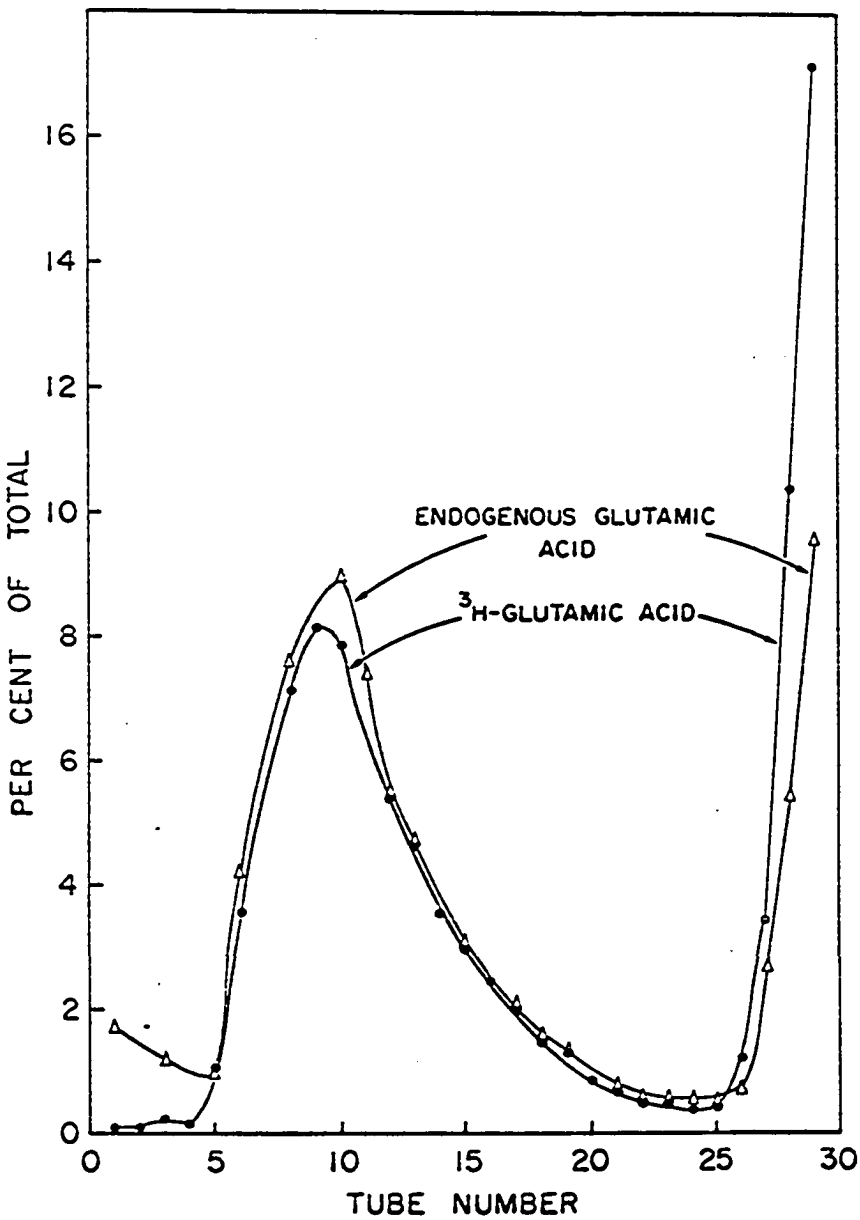

FIGURE 5. Subcellular distribution of ^{14}C-norepinephrine and ^{3}H-glutamic acid in a continuous sucrose gradient after a centrifugation of short duration.

Tissue slices incubated with labelled norepinephrine and glutamic acid were homogenized and P2 pellets were obtained in the same way as for figure 4. After suspension in 0.32 M sucrose, the P2 pellets were centrifuged on linear gradients at 100,000 x g for 15 minutes. Tritium and carbon-14 were determined in the same gradients, and the results shown represent a typical one of three experiments.

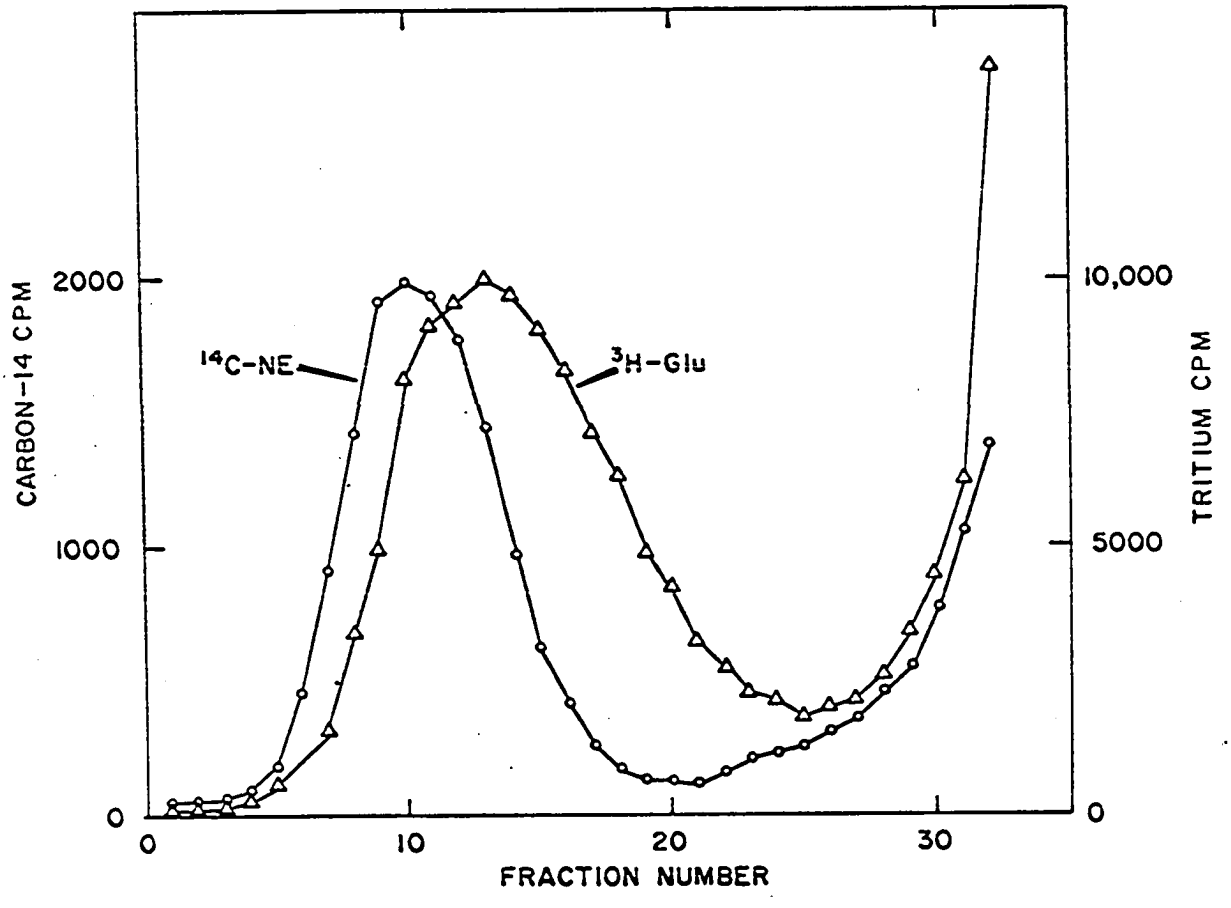

TABLE 16

POSITIONS OF PEAKS AND HALF-HEIGHTS OF ^{14}C-NOREPINEPHRINE AND ^{3}H-GLUTAMIC ACID IN A SUCROSE GRADIENT AFTER A CENTRIFUGATION OF A SHORT DURATION

	EXPT. NO.:	LEFT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:	PEAK FRACTION NO.:	RIGHT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:
	1	9	12	18 - 19
^{3}H-GLU	2	8 - 9	13	19 - 20
	3	8 - 9	13	18 - 19
	1	7 - 8	9	13 - 14
^{14}C-NE	2	7 - 8	10	13 - 14
	3	7 - 8	9 - 10	14 - 15

This table is a summary of three experiments, the first of which is shown in figure 5.

following experiments were performed. P2 pellets were prepared after incubating cortical slices (200 mg) with either ^{3}H-norepinephrine or ^{3}H-glutamic acid. The resuspended pellet was layered on a 40 ml continuous sucrose gradient and centrifuged at 100,000 x g for 20 minutes. Eighteen 2 ml fractions were obtained by eluting from the bottom of each tube and total tritium was determined in an aliquot of each fraction. The remainder of each fraction was diluted with 10 ml of 0.32 M sucrose and centrifuged at 50,000 x g for 30 minutes. Particulate tritium was determined in the resulting pellet from each of these fractions (Figure 6 and Table 17).

Results indicate that total tritium had virtually the same profile as particulate tritium except for the most dilute section of the gradient which would be expected to contain soluble isotope. The soluble isotope might originate by leaking out of synaptosomes during resuspension.

Subcellular localization of ^{3}H-glutamic acid and ^{14}C-GABA in continuous sucrose gradients. Cerebral cortical slices were incubated with labelled glutamate and GABA at 37° for 30 minutes in the presence of amino-oxyacetic acid (10 μM), a compound that inhibits the metabolism of GABA. P2 pellets were obtained after homogenization in 0.32 M sucrose and centrifuged on continuous sucrose gradients at 100,000 x g for 15 minutes. Tritium and carbon-14 were determined in each fraction, and it was found that glutamate, the immediate biochemical precursor of GABA,

FIGURE 6. Comparison of tissue-bound tritium with total tritium in continuous sucrose gradients.

Tissue slices (200 mg) incubated with either ^{3}H-norepinephrine or ^{3}H-glutamic acid were homogenized and a P2 pellet was obtained in the same way as for figure 4. The resuspended pellet was layered on a 40 ml continuous sucrose gradient and centrifuged at 100,000 x g for 20 minutes. Eighteen 2 ml fractions were obtained by eluting from the bottom of each tube and total tritium was determined in an aliquot of each fraction. The remainder of each fraction was diluted with 10 ml of 0.32 M sucrose and centrifuged at 50,000 x g for 30 minutes. Tissue bound tritium was determined in the resulting pellet from each of the fractions. The experiment was performed 3 times.

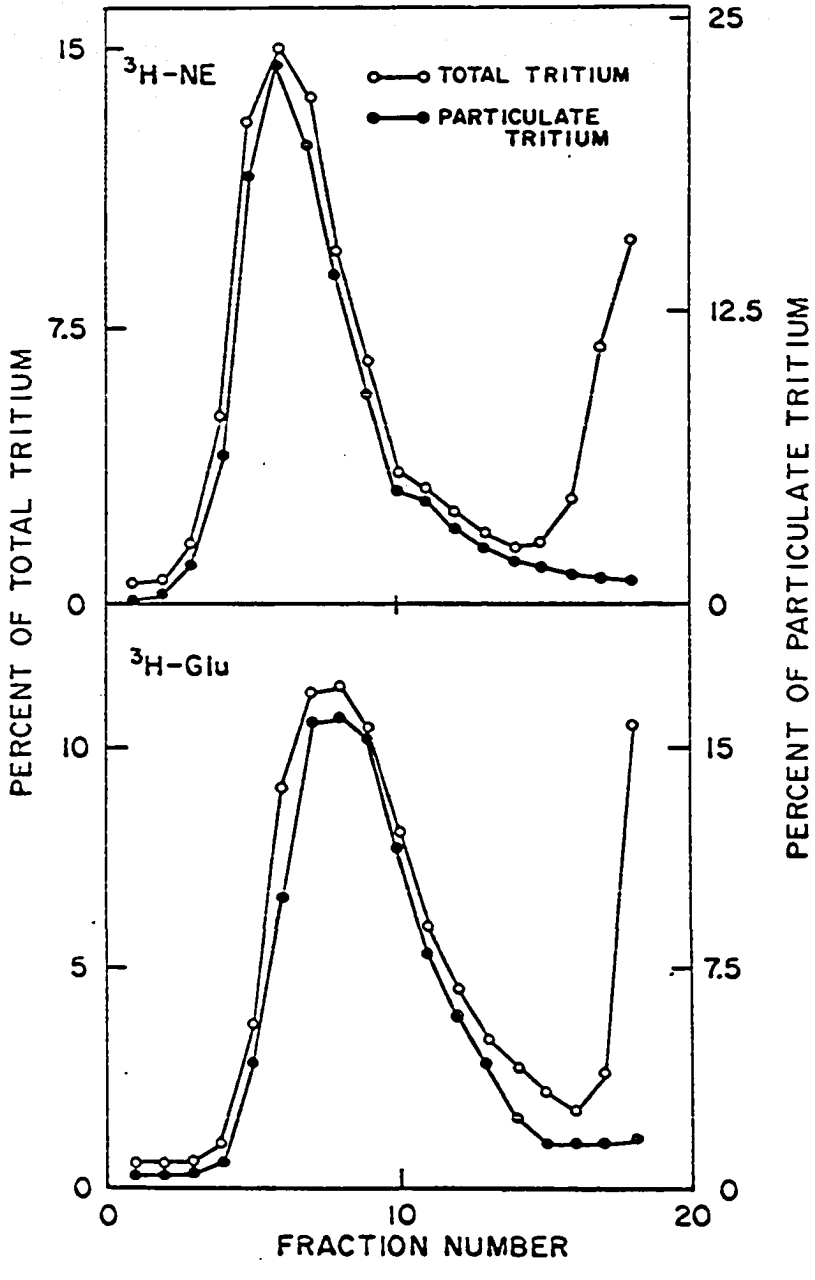

TABLE 17

POSITIONS OF PEAKS AND HALF-HEIGHTS OF TISSUE BOUND TRITIUM AND TOTAL TRITIUM IN SUCROSE GRADIENTS

	EXPT. NO.:	LEFT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:	PEAK FRACTION NO.:	RIGHT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:
	1	4 - 5	6	8 - 9
TOTAL ^{3}H-NE	2	4 - 5	6	8 - 9
	3	4 - 5	6	8 - 9
	1	4 - 5	6	8 - 9
BOUND ^{5}H-NE	2	4 - 5	6	8 - 9
	3	4 - 5	6	8 - 9
	1	5 - 6	8	10 - 11
TOTAL ^{3}H-GLU	2	5 - 6	8	10 - 11
	3	5 - 6	8	11 - 12

TABLE 17 (CON'T.)

	EXPT. NO.:	LEFT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:	PEAK FRACTION NO.:	RIGHT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:
BOUND ^{3}H-GLU	1	5 - 6	8	10 - 11
	2	5 - 6	8	10 - 11
	3	5 - 6	8	10 - 11

This table is a summary of three experiments, the first of which is shown in figure 6.

had the same distribution as GABA in the gradients (Figure 7 and Table 18).

Electron microscopic findings. Twelve 30-drop fractions were eluted from gradients prepared in the same way as for Figures 5 and 7, but only containing ^{3}H-glutamic acid. During the elution procedure, every tenth drop was assayed for radioactivity and its profile was identical to those shown in Figures 5 and 9. The contents of each fraction were fixed with 1% $KMnO_4$ and embedded in Araldite as described in Methods.

A brief analysis of the electron micrographs (Figure 8) revealed frequent synaptosomal profiles in the fractions that contained significant amounts of radioactivity. The fractions containing the densest sucrose had no tissue, while the fractions containing lightest sucrose had only microsomes and membrane fragments with very few synaptosomes.

FIGURE 7. Subcellular localization of ^{14}C-GABA and ^{3}H-glutamic acid in continuous sucrose gradients after a centrifugation of short duration.

Tissue slices incubated with labelled GABA and glutamic acid were homogenized and P2 pellets were obtained in the same way as for figure 4. After suspension in 0.32 M sucrose, the P2 pellets were centrifuged on linear gradients at 100,000 x g for 15 minutes. Tritium and carbon-14 were determined in the same gradients, and the results shown represent a typical one of three experiments.

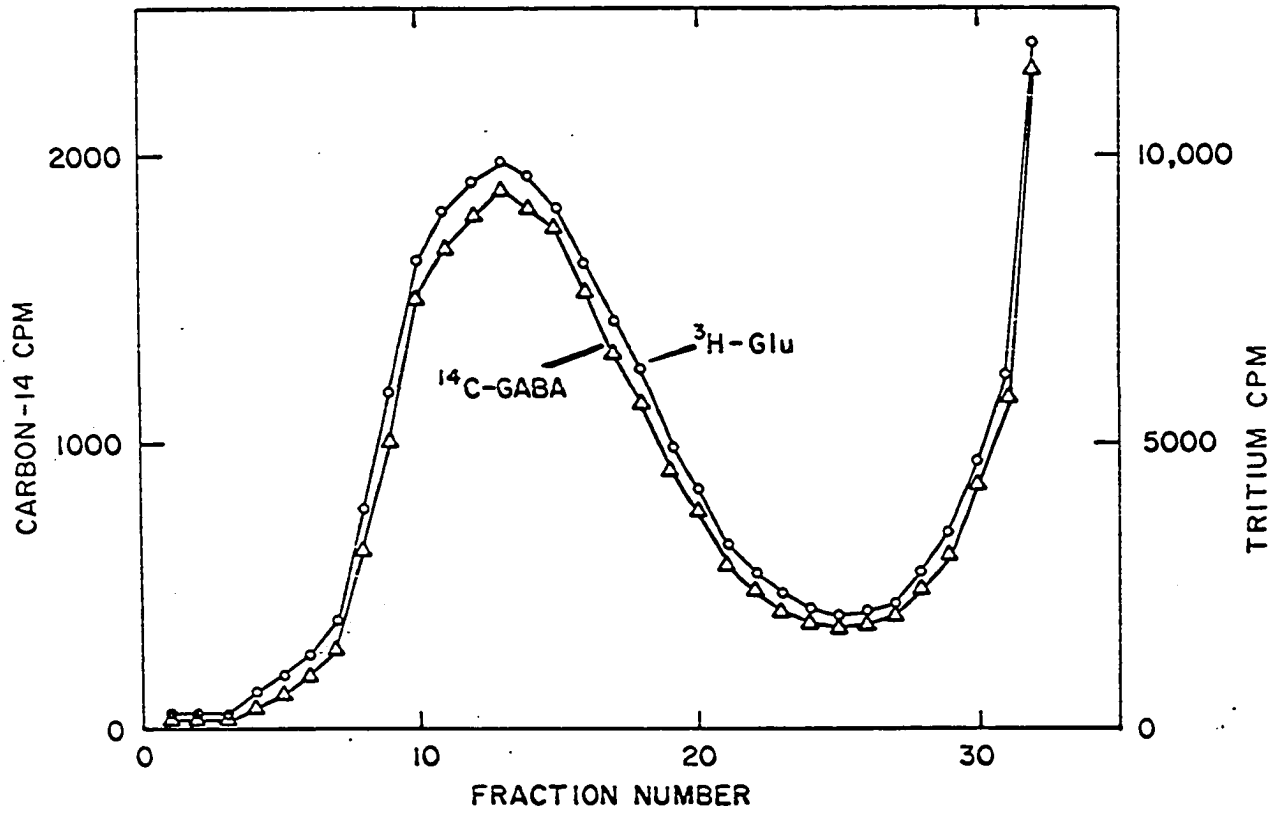

TABLE 18

POSITION OF PEAKS AND HALF-HEIGHTS OF ^{14}C-GABA AND ^{3}H-GLUTAMIC ACID IN CONTINUOUS SUCROSE GRADIENTS AFTER A CENTRIFUGATION OF SHORT DURATION

	EXPT. NO.:	LEFT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:	PEAK FRACTION NO.:	RIGHT SHOULDER HALF-HEIGHT OCCURRED BETWEEN FRACTION NOS.:
^{14}C-GABA	1	8 - 9	13	18 - 19
	2	8 - 9	14	21 - 22
	3	9 - 10	14	21 - 22
^{3}H-GLU	1	8 - 9	13	18 - 19
	2	8 - 9	14	21 - 22
	3	9 - 10	14	22 - 23

This table is a summary of three experiments, the first of which is shown in figure 7.

FIGURE 8. Electron microscopic examination of continuous sucrose density gradients. Gradients were prepared and 12 fractions eluted in the same way as for figure 5. The contents of each fraction were fixed in 1% $KMnO_4$ and embedded in Araldite. Synaptosomes from gradient fractions numbered (upper, left to right): 7-8-9, 10-11-12; (lower, left to right): 13-14-15, 7-8-9, 16-17-18; (numbers correspond to fraction in figures 5 and 7).

SYNAPTOSOMES FROM RAT CEREBRAL CORTEX

DISCUSSION

Synaptosomal Uptake. In the present study, it was found that of a variety of ^{3}H-amino acids accumulated by cerebral cortical slices, some tended to localize in particulate fractions more than others. Several workers (Blasberg, 1967; Blasberg and Lajtha, 1965; Smith, 1967; Lajtha et al., 1969; Kandera et al., 1968) have shown that various amino acids are accumulated into brain slices by specific uptake mechanisms. Of the compounds that have been implicated as putative neurotransmitters (Curtis et al., 1960; Krnjevic and Phillis, 1963; Werman et al., 1968), glutamic acid, norepinephrine, and glycine tended to localize in particulate fractions while aspartic acid was largely confined to the supernatant fluid. Basic amino acids as well as small neutral amino acids, also localized in particulate fractions. The particulate/supernatant ratios for other amino acids tended to be lower.

The incorporation of amino acids into particulate fractions was temperature dependent, and the particulate/supernatant ratios for water and urea, which are freely diffusible, were very low. The tissue/medium ratio for accumulation of amino acids into whole slices was also temperature dependant, indicating that the accumulation of

labeled amino acids is an active process.

While there is no direct evidence that the tritiated amino acids found in the P2 pellet are within synaptosomes, the indirect evidence is very strong. The amount of tritiated amino acids found in particulate fractions was sensitive to the incubation medium temperature and hypo-osmotic shock, and the tritium in sucrose gradients was coincident with potassium, a marker for cytoplasm occluded within synaptosomes, different from MAO, a mitochondrial marker, and coincident with synaptosomal profiles found in electron micrographs of gradient fractions. Direct evidence for the accumulation of tritiated amino acids into synaptosomes could be obtained, in theory, with autoradiography of nerve endings under the electron microscope. However, this is not possible with existing methods, since amino acids leak out of compartments during fixation (E. Gfeller, personal communication; G. Aghajanian, personal communication).

Since the incubations were performed with whole tissue slices, one might wonder if the accumulation into subcellular compartments is direct or indirect. For example, are the amino acids first taken up by glia, and then transferred to nerve endings, or first taken up by cell bodies and then transported down the axons by axonal flow? This latter possibility may be supported by the fact that the P/S ratios increased slightly, although not significantly, in time. However, Navon and Lajtha (1969) incubated nu-

clear and mitochondrial fractions from brain homogenates with glutamate, glycine and leucine (1 mM concentrations), and they found that subcellular particles were capable of direct uptake of amino acids.

Coyle and Snyder (1969) showed, by purification in sucrose gradients, that isolated synaptosomes were capable of direct uptake of catecholamines. However, on the basis of the data presented here, it is not possible to say if the subcellular localization of amino acids is influenced by indirect transport.

Uptake of amino acids as a reflection of metabolic requirements of nerve endings. It is possible that the relative accumulation of amino acids into nerve endings may provide an indication of the biochemical requirements of nerve endings. Amino acid transport may be related to the synaptosomes' capability of synthesizing protein (Gordon and Deanin, 1968; Autilio et al., 1968; Austin and Morgan, 1967). It is possible that serine, threonine and glycine are transported to serve as precursors for phospholipid synthesis, which takes place in synaptosomes (Durell and Sodd, 1966).

The basic amino acids showed the highest localization in particulate fractions of any amino acids examined, a finding for which no simple explanation is readily apparent. Although on one has yet reported that these amino acids cause electrical activity when applied to mammalian neurons, perhaps they are involved in some undiscovered

aspect of synaptic function. The particulate localization of amino acids with no known neurophysiological role may also indicate that such a localization is not relevant to synaptic function.

Different populations of synaptosomes. A significant finding is that the pattern of distribution of ^{14}C-norepinephrine, a synaptosomal marker, was less broad than that of ^{3}H-glutamic acid or potassium. Thus while glutamic acid appears to be uniformly distributed in the total population of synaptosomes, norepinephrine appears to be confined to a limited population. ^{3}H-glycine and ^{3}H-leucine showed the same broad distribution as glutamic acid and potassium. These data indicate that the norepinephrine storing synaptosomes represent a limited portion of the total synaptosomal population, which appears to be heterogeneous (Whittaker, 1965; Iversen and Snyder, 1968; Green et al., 1969). The apparent separation of synaptosomal populations was enhanced by incomplete equilibrium sedimentation. To be sure that the isotope within the gradients labeled particulate material, rather than existing freely, it was shown that the profiles of particulate isotope and total isotope were identical.

Glutamic acid as a neurotransmitter. One of the important criteria to establish a chemical as a putative neurotransmitter is localization in nerve endings. Of the amino acids heretofore implicated as possible neurotransmitters, exogenous glutamate and glycine had the highest

localization in nerve endings. Endogenous glycine was not measured.

In studies of the metabolism of ^{3}H-glutamic acid more than 85% of the radioactivity within the slice was unchanged glutamic acid, while about 1/3 of the radioactivity in the medium was ^{3}H-glutamine. This suggests that glutamine formed within the slice is not bound and leaks out into the medium. As with glutamic acid, the other ^{3}H-amino acids were largely unmetabolized within the slice, a finding which is consistent with data obtained by other workers (Blasberg and Lajtha, 1965; Margolis and Lajtha, 1968).

^{3}H-glutamic acid showed a greater degree of localization in particulate fractions, than did endogenous glutamic acid whose subcellular distribution pattern was similar to that of a cytoplasmic marker. When P2 fractions were centrifuged on sucrose gradients, ^{3}H-glutamic acid was localized in the synaptosomal fraction and its distribution was identical with endogenous glutamic acid. Exdogenous gamma-aminobutyric acid and norepinephrine also have the same distribution in gradients as endogenous gamma-aminobutyric acid and norepinephrine (Green et al., 1969; Neal and Iversen, 1969).

The fact that endogenous glutamic acid is not highly localized to synaptosomes mitigates against a neurotransmitter role. However, there is a large amount of glutamic acid in the brain and perhaps only a small portion of this

is involved in neurotransmission. Indeed, there was sufficient glutamic acid in synaptosomal extracts to produce effects on single cortical neurons of the guinea pig (Krnjevic and Whittaker, 1965; Whittaker, 1968).

Gamma-aminobutyric acid, a suspected neurotransmitter, has the same synaptosomal distribution as glutamic acid, its biochemical precursor. Neal and Iversen (1969) found that glutamate decarboxylase had the same synaptosomal localization as GABA. Thus, the evidence suggests that a small pool of glutamic acid is available for decarboxylation to form GABA.

Transport systems into nerve terminals have been described for a variety of putative neurotransmitters including acetylcholine, norepinephrine, serotonin, and gamma-aminobutyric acid. Such a transport system appears to be responsible for inactivation of norepinephrine released at synapses (Iversen, 1967; Axelrod, 1965). It is possible that the transport of glutamic acid into synaptosomes described here could represent an analogous uptake system for the putative "neurotransmitter" pool of glutamic acid, which presumably would constitute only a small portion of the endogenous glutamic acid.

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VITA

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In June, 1968, the author began thesis research under Dr. Solomon H. Snyder in the Department of Pharmacology and Experimental Therapeutics, the Johns Hopkins School of Medicine.

One June 8, 1969, he was married to the former Joan Wendy Barenburg in Baltimore, Maryland.