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ALTERATIONS OF BRAIN METABOLISM BY THE NEUROTOXIN, KAINIC ACID

City University of New York PH.D. 1981

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ALTERATIONS OF BRAIN METABOLISM BY THE NEUROTOXIN, KAINIC ACID

by

BARBARA KRESPAN

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1981

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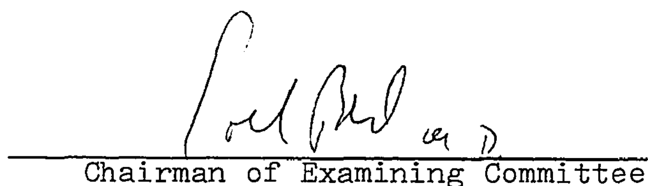

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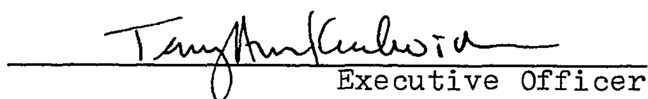

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ABSTRACT

ALTERATIONS OF BRAIN METABOLISM BY THE NEUROTOXIN, KAINIC ACID

by

Barbara Krespan

Advisor: Associate Professor William J. Nicklas

A good correlation exists between the neurotoxic and neuroexcitatory properties of glutamic acid and several of its analogs, including kainic acid. Therefore it has been suggested that excitotoxic amino acids such as kainic acid generate in affected neurons a state of continuous depolarization and increase in membrane permeability which leads to neuronal cell death, possibly by depletion of high energy intermediates. In the present study, kainic acid (1 mM) caused a maximal 30-40% decrease in ATP levels of rat cerebellar slices which could be partially reversed by reincubation in drug-free medium. Creatine phosphate levels also decreased by 30-35% in the presence of 1 mM kainic acid. ADP and AMP levels were only slightly altered by 1 mM kainic acid, thus the total content of ATP + ADP + AMP decreased by 29%. Kainic acid was also found to decrease ATP levels of cerebellar slices from weaver mice which are genetic mutants lacking granule cells, the excitatory glutamergic interneurons of the cerebellum. This is in

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contrast to evidence suggesting that glutamergic neurons are necessary for kainic acid toxicity in the striatum.

The actions of kainic acid on glutamic acid metabolism in the cerebellar slice was studied. Kainic acid caused a decrease in tissue glutamine levels (60-70%), glutamic acid and aspartic acid levels (10-20%), and no alteration in GABA levels. Kainic acid caused a large decrease of the relative specific radioactivity (RSA) of glutamine from radioactive acetate and GABA, but not from radioactive glucose. This indicates that glutamine synthesis was inhibited in the "small" pool of glutamic acid metabolism, probably preferentially localized to glia. Kainic acid did not directly inhibit glutamine synthetase activity; the decrease in glutamine synthesis may result from a depletion of ATP at that site. These studies also confirmed previous observations that transferring of slices from preincubation to incubation medium is necessary to enhance phenomena interpreted as metabolic compartmentation. In contrast to cerebellar slices, kainic acid caused no alterations of ATP levels or of radioactive acetate incorporation into amino acids in striatal slices.

Kainic acid substantially increased the levels of glutamic acid (200-350%) and aspartic acid (220-250%) in the medium of cerebellar slices, but did not alter GABA or glutamine levels. This is consistent with the hypothesis that the release of excitatory substances is involved in the neurotoxic actions of kainic acid.

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In control studies, a nonexcitatory glutamic acid uptake blocker, dihydrokainic acid (1 mM) did not alter ATP or amino acid levels. The neuroexcitant, DL-homocysteic acid (10 mM), but not N-methyl-DL-aspartic acid (1 mM), had similar effects as kainic acid.

The site from which kainic acid or veratridine released glutamic acid was compared. Veratridine, a presynaptic depolarizing agent, decreased the specific radioactivity of acetate-labelled glutamic acid in the medium by 38% and tended to increase (not significant) that of glucose-labelled glutamic acid by 16%. In addition, tetrodotoxin blocked the veratridine-induced efflux of glutamic acid, aspartic acid, and GABA. These data are consistent with veratridine causing release of presynaptic pools of glutamic acid. Kainic acid increased the specific radioactivity of glucose-labelled glutamic acid by 55% and tended to increase that of acetate-labelled glutamic acid by 43%. The release of glutamic acid and aspartic acid induced by kainic acid was not blocked by tetrodotoxin. Kainic acid also did not release glutamic acid from cortical synaptosomes. The data suggest that kainic acid released pools of glutamic acid from glia and neurons and these were different pools than veratridine released.

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Dr. Soll Berl, in whose laboratories these experiments were performed, for his support and collaboration in these experiments.

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LIST OF ABBREVIATIONS

AMP..................adenosine monophosphate

ADP..................adenosine dephosphate

ATP..................adenosine triphosphate

ATPase...............adenosine triphosphatase

Cyclic AMP...........cyclic adenosine 3'5'-monophosphate

GABA.................gamma-aminobutyric acid

GDH..................glutamate dehydrogenase

KA...................kainic acid

PCA..................perchloric acid

RSA..................relative specific radioactivity

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I. INTRODUCTION

Glutamic acid, a putative excitatory transmitter in the mammalian central nervous system, has striking neurotoxic properties. Glutamic acid and various analogs of glutamic acid (including kainic acid) cause widespread neuronal destruction and an accompanying glial proliferation with relative sparing of afferent fibers (Olney et al., 1971; Schwarcz and Coyle, 1977). The neuronal destruction caused by these agents may be related to their ability to cause neuronal discharge possibly leading to a widespread and prolonged depolarization in brain (Olney et al., 1971; Olney, 1978). It is of interest to study the metabolic effects of such drugs in order to understand the mechanism of toxins which are used as research tools in neurobiological research, and to learn more about the metabolism of the central nervous system and about the toxic properties of endogenous excitatory amino acids such as L-glutamic acid.

A. L-GLUTAMIC ACID

1. Neurotoxicity

In the 1940's, administration of L-glutamic acid was reported to have beneficial effects in the treatment of petit mal epilepsy (Price et al., 1943) and mental retardation (Albert et al., 1946). This stimulated further interest in the metabolism of glutamic acid in brain and in the clinical effects of glutamic acid. In 1957, Lucas

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and Newhouse, in the course of studying the effect of parenteral injections of L-glutamic acid on hereditary retinal dystrophy in the mouse, first observed that administration of L-glutamic acid or L-aspartic acid destroyed neurons in the inner layers of the retina. The neurotoxic action of L-glutamic acid on retinal neurons, hypothalamic neurons and neurons in developing brain was described by many investigators in the following years (for review see Olney, 1978). Neuronal degeneration induced by systemic administration of L-glutamic acid was primarily confined to brain regions which lie outside of the "blood-brain barrier" such as the retina, area postrema, and the arcuate nucleus of the hypothalamus (Olney et al., 1977; Olney, 1978). Additional toxicity associated with the systemic administration of L-glutamic acid included convulsions in rats (Bhagavan et al., 1971; Johnston, 1973) and the "Chinese Restaurant Syndrome" in humans (for review see Olney, 1978 and Kizer et al., 1978).

2. Neurotransmitter Properties

Curtis first demonstrated that L-glutamic acid, iontophoretically applied, caused a potent excitation of spinal cord neurons in the cat (Curtis et al., 1959) and, in later work, showed that several analogs of L-glutamic acid also did (Curtis and Watkins, 1963). The depolarizing action of L-glutamic acid on neurons was similar to that expected of a neurotransmitter (Curtis et al., 1960) and was associated with an increased permeability to Na^+

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(Curtis et al., 1972; Zieglansberger and Puil, 1973). L-Glutamic acid has since been suggested to be a neurotransmitter in many areas of the mammalian nervous system (Curtis and Johnston, 1974; Fonnum, 1978) and in the neuromuscular junction of the crayfish (Takeuchi and Takeuchi, 1964), the locust (Usherwood and Machili, 1968; Usherwood, 1972) and the lobster (Shank and Freeman, 1976). Similar to other putative neurotransmitters, high-affinity uptake, depolarization-induced release and receptors mediating its actions have been described for L-glutamic acid.

The uptake of L-glutamic acid and L-aspartic acid into synaptosomes, finely chopped brain tissue and glia occurs by a high-affinity sodium-dependent carrier with reported K_m values of 1 to 50 X 10^{-6} and by a low-affinity carrier with reported K_m values of 10^{-4} to 10^{-3} (for review see Cox and Bradford, 1978; Watkins, 1978). Autoradiographic (McLennan, 1976) and biochemical (Benjamin and Quastel, 1976; Okamoto and Quastel, 1972) evidence suggests that exogenously administered glutamic acid is primarily taken up by glial cells. Uptake is the proposed mechanism of inactivation of glutamic acid following its release.

Glutamic acid can be released in a Ca^{2+}-dependent manner from synaptosomes (deBelleroche et al., 1972; Bradford et al., 1973) and from brain slices (Nadler et al., 1977). A few studies have attempted to demonstrate <u>in vivo</u>

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release of glutamic acid from areas of the cerebral cortex following neural activation (Jasper and Koyama, 1969; Clark and Collins, 1976).

Until recently, glutamic acid and aspartic acid have been assumed to act at a single receptor. Similar to other putative neurotransmitters, e.g. acetylcholine and monoamines, more than one type of receptor has recently been demonstrated for glutamic acid and aspartic acid by electrophysiological and pharmacological methods. Three separate excitatory receptors have been differentiated in response to excitatory amino acids (Davies et al., 1979; Davies et al., 1980). One type of receptor, excited by N-methyl-D-aspartic acid is selectively blocked by Mg^{2+}, by D-α-aminoadipate and by D-α-aminopimelate, as well as by several other antagonists. It has been suggested that kainic acid and quisqualic acid act on a second and a third type of excitatory receptor respectively because different patterns of inhibition of the response to kainic acid and quisqualic acid are exhibited by various antagonists. Glutamic acid and aspartic acid both act on all three types of receptors to varying degrees.

B. KAINIC ACID

1. Neurotoxicity and Potency

Olney et al. (1971) studied the neurotoxic potential of glutamic acid and its analogs and of sulfur-containing amino acids and found a correlation with the

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excitatory potential of these amino acids which he thus named "excitotoxins". The order of potency for the destruction of hypothalamic (Olney et al., 1971), retinal (Schwarcz et al., 1978) and striatal (Schwarcz et al., 1978; Olney, 1978) neurons is kainic acid $>$ N-methyl-aspartic acid $>$ D,L-homocysteic acid $>$ L-glutamic acid (see Figure 1 for formulas). This parallels the order of potency for excitatory activity in spinal cord neurons (for review see Watkins, 1978). Slight alterations of the kainic acid molecule form analogs such as dihydrokainic acid and N-acetylkainic acid which are far less active as neurotoxins (Schwarcz et al., 1978) or excitants (Johnston et al., 1974).

Kainic acid is of interest because it is an extremely potent neurotoxic analog of glutamic acid. Its neurotoxicity is steeply concentration-dependent. Injection of 2 nmoles of kainic acid into the striatum causes a relatively small amount of neuronal degeneration, whereas injection of 10 nmoles causes degeneration of virtually all intrinsic striatal neurons and a proliferation of glia within ten days (Schwarcz and Coyle, 1977; McGeer et al., 1978a; Olney and de Gubareff, 1978). Many striatal neurons degenerate within 24 hours. An injection of 10 nmoles of kainic acid into the hypothalamus and most other brain regions tested causes almost complete destruction of intrinsic neurons (McGeer et al., 1978b). Certain hippocampal pyramidal neurons are extremely sensitive to

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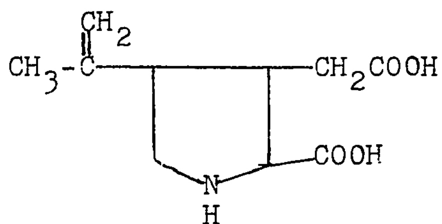
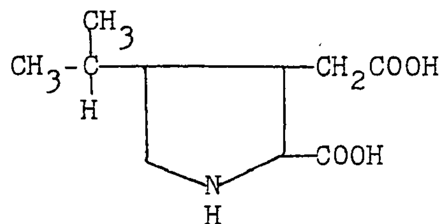
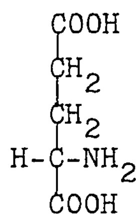


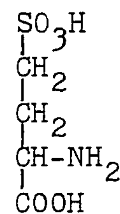
Kainic Acid



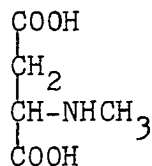
Dihydrokainic Acid



L-Glutamic Acid



D,L-Homocysteic Acid



N-Methyl-D,L-Aspartic Acid

FIGURE 1. FORMULAS OF EXCITATORY AMINO ACIDS AND DIHYDROKAINIC ACID

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the toxic effects of kainic acid and are destroyed by injections of 0.5 nmoles (Nadler et al., 1978). Conversely the granule cells in the cerebellum (Herndon and Coyle, 1978) are more resistant to the neurotoxic effects of kainic acid and are not all destroyed following 10 nmole injections of kainic acid.

Systemic injection of 10 to 40 mg/kg of kainic acid into adult rats causes convulsions and subsequent death (Olney et al., 1974). If the animals survive several hours after the injection, it can be observed that kainic acid causes neuronal destruction in the arcuate nucleus of the hypothalamus and in the hippocampus. This differs from the pattern of cells destroyed by L-glutamic acid, N-methyl-aspartic acid, or D,L-homocysteic acid after peripheral or intraventricular injections (Olney, 1978).

The nature of the brain lesion caused by excitatory amino acids after either intracerebral or peripheral injection is characterized by degeneration of neurons, with relative sparing of afferent fibers and axons of passage, and by a glial proliferation (Olney et al., 1971; Coyle and Schwarcz, 1976; Hattori and McGeer, 1977; Nadler et al., 1978).

2. Glutamergic Input and Kainic Acid Neurotoxicity

Kainic acid-induced neurotoxicity, in particular, appears to be dependent upon the integrity of excitatory inputs in some brain regions although not in others.

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It has been demonstrated both <u>in vivo</u> (McGeer et al., 1978c; Biziere and Coyle, 1978a) and in tissue culture (Whetsell et al., 1980) that kainic acid-induced neuronal degeneration in the striatum is dependent upon excitatory, probably glutamergic, innervation from the cortex. In the hippocampus, Nadler et al. (1978) find that innervation by glutamergic projections does not appear to be specifically involved in the kainic acid-induced neurotoxicity. However, hippocampal damage induced by kainic acid may be intimately related to the epileptiform activity of these neurons; thus the mechanism of toxicity may differ from that of non-epileptigenic neurons. If the excitatory input, probably glutamergic or aspartergic, to the cochlear nucleus is destroyed, cochlear neurons are not protected from kainic acid-induced neuronal damage (Bird and Gulley, 1979) although increased activity of these excitatory afferents potentiates kainic acid toxicity. Endogenous glutamergic or aspartergic neurons may be involved in the neurotoxicity of kainic acid in the cochlear nucleus.

3. Acute Morphological Alterations

Morphological alterations occur shortly after exposure to kainic acid. In the olfactory tubercle, the hippocampus and the prepyriform cortex following systemic or intracerebral injection of 4 to 15 nmoles of kainic acid, the most conspicuous acute (45 minutes) alteration observed is immense swelling of glial processes closely associated with cell bodies of neurons destined to

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degenerate (Olney and de Gubareff, 1978). In addition, the dendrites of neurons in these regions are dilated. Electron microscopic study of the olfactory tubercle has shown that the mitochondria in the swollen neuronal dendrites are grossly swollen whereas mitochondria in the swollen glial processes appear normal. No pathological changes are observed in axon terminals.

Herndon and Coyle (1977; 1978) have performed an electron microscopic study of the cerebellum after intracerebellar injection of 10 nmoles of kainic acid. At a distance of 0.3 to 1.5 mm from the injection site, the earliest (15 minutes) alteration observed is swelling of Bergmann astrocytes. Collapse and increased density of the dendrites and perikarya of all cerebellar cortical neurons except the granule cells occur within two hours. These collapsed dendrites and perikarya are surrounded by severely swollen Bergmann astrocytes. Degeneration of all cerebellar cortical neurons except the granule cells occurs in this region.

Closer to the injection site (within 0.3 mm), in addition to the other alterations, swelling of the dendrites and perikarya of granule cells and distention of the rough endoplasmic reticulum of Purkinje cells are also observed within 15 minutes. The swelling of granule cell perikarya and dendrites expands to enormous proportions by 30 minutes. All neurons in this vicinity eventually degenerate including the granule cells which are relatively

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more resistant to the effects of kainic acid. The swelling versus the shrinking reaction may reflect differences in the ionic effects of the mechanism of neurotoxicity.

4. Acute Metabolic and Electrolyte Alterations

Generally acute metabolic and electrolyte alterations caused by L-glutamic acid and kainic acid resemble those occuring after depolarization. When cerebral cortical slices are exposed to electrical stimulation or high concentrations of L-glutamic acid (Pull and McIlwain, 1975), ATP levels decrease with concomitant increases in the levels of ADP and AMP. Lactate levels (Chan et al., 1979; Cox et al., 1977), NADH levels, oxygen consumption (Cox et al., 1977), and the sodium and water content of the tissue (Bradford and McIlwain, 1966; Okamoto and Quastel, 1970) also are increased. Similarly acute increases in both glycolysis and respiration have been demonstrated following exposure to kainic acid. Glucose utilization in the striatum increases within one hour following intrastriatal injection of kainic acid as demonstrated with (^{14}C)-2-deoxyglucose autoradiography (Wooten and Collins, 1979). Oxygen consumption of cerebral cortical slices increases during incubation with 0.1 or 1.0 mM kainic acid for one hour (Cox et al., 1977)

A number of depolarizing agents decrease levels of ATP and creatine phosphate in cortical slices; such decreases in high energy intermediates have been suggested

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to be related to neuronal death (Olney, 1978). Biziere and Coyle (1978b) showed that striatal slices incubated with 10 mM L-glutamic acid likewise exhibit increases in sodium and water content and 30% decreases in ATP levels. Kainic acid similarly increases the sodium content of striatal slices but unexpectedly produces only a small decrease (10% or less) of the ATP content. However, kainic acid at 1 µM to 10 mM potentiates the decrease in ATP caused by 10 mM L-glutamic acid. The striatal slice is in a sense decorticated and cortical input, probably glutamergic, is necessary for kainic acid to cause striatal toxicity. In the present study, using cerebellar slices which contain the granule cell an intrinsic glutamergic interneuron, kainic acid is found to decrease ATP levels by 30 to 40% (Nicklas et al., 1980).

C. GLUTAMIC ACID COMPARTMENTATION

Glutamic acid in brain is the immediate precursor of glutamine, is involved in intermediary metabolism (see Figure 2) and is also an excitatory neurotransmitter. Studies on the compartmentation of glutamic acid metabolism have attempted to differentiate metabolically between the various pools of glutamic acid. Although it was suggested that release and/or inhibition of the reuptake of glutamic acid is involved in the neurotoxic action of kainic acid (McGeer et al., 1978a), kainic acid does not release glutamic acid from synaptosomes and it only moderately

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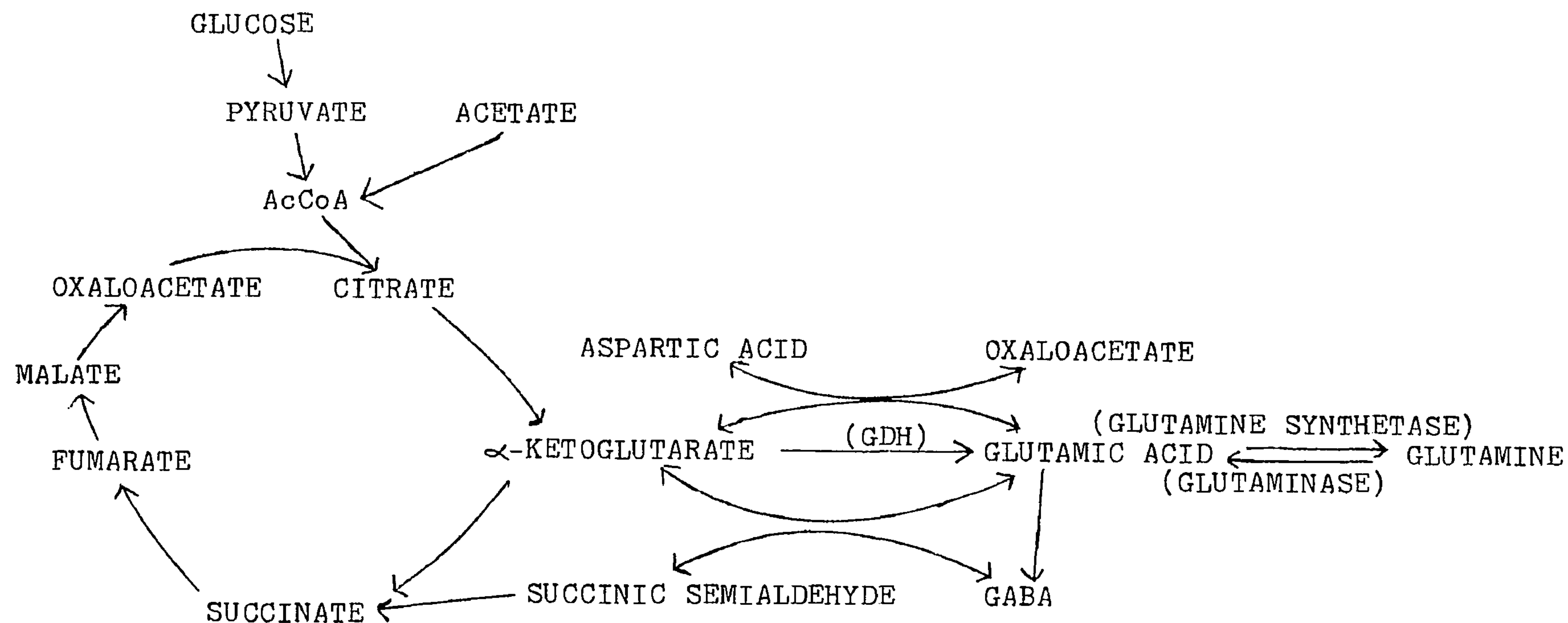

FIGURE 2. RELATIONSHIP OF GLUTAMIC ACID AND GLUTAMINE TO THE CITRIC ACID CYCLE

inhibits glutamic acid uptake into synaptosomes (Johnston et al., 1979). However, the present study shows that kainic acid increases the efflux of glutamic acid from cerebellar slices (Nicklas et al., 1980) and alters glutamic acid and glutamine metabolism. A brief description of the complex compartmentation of glutamic acid metabolism in brain tissue follows; this area has been extensively reviewed (Balazs and Cremer, 1973; Berl et al., 1975; Fonnum, 1978).

Theoretically, in a single compartment system, after administration of a single tracer dose of isotopically-labelled precursor, the specific radioactivity of the precursor will decline and that of the product will rise until the specific radioactivity of the product is equal to or slightly greater than that of the precursor pool in the tissue (Zilversmit et al., 1943). This can also occur in certain multiple compartment systems. If the specific radioactivity of the product rises rapidly to a value apparently several times larger than that of the precursor pool, the interpretation is that two or more compartments are present.

Compartmentation of glutamic acid metabolism in brain has been described using the above criteria. In brain tissue, it has been observed that total tissue glutamine, a metabolic product of glutamic acid, attains a much higher specific radioactivity than that of glutamic acid from the whole tissue after the injection of several

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labelled precursors, including acetate, glutamic acid, and gamma-aminobutyric acid (GABA). Other labelled precursors of glutamic acid, such as glucose or pyruvate, do not label glutamine with a higher specific radioactivity than that of glutamic acid. These observations have been interpreted to indicate the presence of more than one compartment as follows. One pool of glutamic acid is very highly labelled by acetate and is the precursor of a pool of very highly labelled glutamine. However, when the specific radioactivity of glutamic acid in the whole tissue is measured, the highly labelled glutamic acid is diluted by large pools of glutamic acid which are not highly labelled by acetate. Glutamine formed in such pools also has a lower specific radioactivity. However because most of the glutamine in the tissue is formed in the pool highly labelled by acetate, it is diluted to a much smaller extent by glutamine of lower specific radioactivity. Thus the specific radioactivity of glutamine from the whole tissue is greater than that of glutamic acid although in each separate compartment the specific radioactivity of glutamine does not attain higher values than that of glutamic acid. Other precursors such as glucose label all pools of glutamic acid and glutamine fairly equally. Thus a dilution effect does not occur and the specific radioactivities of glutamic acid and glutamine are similar to those expected of a precursor-product pair in a single compartment system.

It has been suggested that the observed biochemical

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compartmentation is correlated with anatomical compartmentation. That is, key enzymes of glutamic acid metabolism may be localized to certain cellular compartments and more than one citric acid cycle exists in brain which may be localized to certain cellular and subcellular compartments. There is some evidence that glutamine synthetase is localized predominantly in glial cells and that acetate utilization occurs primarily in glial cells. This was first suggested by experiments using various labelled precursors (Balazs and Cremer, 1973; Berl et al., 1975; Fonnum, 1978). More recently, glutamine synthetase was shown immunohistochemically to be preferentially localized to astrocytes (Norenberg and Martinez-Hernandez, 1979; Martinez-Hernandez et al., 1977). Nicklas et al. (1979a,b) found that in kainic acid-lesioned striata, in which glia have proliferated and neurons degenerated, glutamine synthetase activity and acetate utilization are increased. Glutamine synthetase activity has been reported to be in synaptosomes as well, however glial contamination of this preparation has not been ruled out (Dennis et al., 1980).

There is also evidence that glutamic acid "pools" of different metabolic origin, that is, labelled by different precursors, respond differently to stimulation. Minchin (1977) showed that, in spinal cord slices, the glutamic acid released by stimulation with veratridine is labelled preferentially by glucose compared to acetate. This technique offers a useful tool in elaborating which "pools"

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of glutamic acid metabolism might be altered by treatment of the tissue with toxic substances.

G. OUTLINE OF THE STUDY

Involvement of endogenous glutamic acid in kainic acid neurotoxicity has been proposed. Therefore the effects of kainic acid on ATP levels and oxygen consumption were investigated in parasagittal cerebellar slices, since the cerebellum contains an excitatory, probably glutamergic, interneuron. Also the effects of kainic acid on glutamic acid levels in cerebellar slices and medium were studied. Since it was found that kainic acid markedly decreased glutamine levels in the cerebellar slice, more detailed studies of glutamic acid and glutamine metabolism were performed. These were compared to a study on striatal slices. These studies led to work in which the metabolism of glutamic acid released by kainic acid and by veratridine was compared. In addition, the effects on ATP and amino acid levels caused by the neuroexcitants, N-methyl-aspartic acid and D,L-homocysteic acid, and by the nontoxic analog, dihydrokainic acid, were compared with the effects of kainic acid. The results were interpreted to mean that kainic acid affected both neuronal and glial sites in cerebellar slices.

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II. EXPERIMENTAL PROCEDURES

A. MATERIALS

1. Chemicals - Sources

Creatine phosphokinase - lyophilized; glucose-6-phosphate dehydrogenase from yeast - ammonium sulfate suspension; hexokinase from yeast - ammonium sulfate suspension; myokinase from hog muscle - ammonium sulfate suspension; $NADP^+$ disodium salt and pyruvate kinase from rabbit muscle were purchased from Boehringer-Mannheim Biochemicals, Indianapolis, Indiana. AMP, ADP, ATP, imidazole, kainic acid (Lot No. 117C-0044), L-aspartic acid, L-glutamic acid and phosphoenolpyruvate were purchased from Sigma Chemical Co., St. Louis, Mo. D,L-homocysteic acid, adenosine, 2-amino-4-phosphonobutyric acid, GABA, L-glutamine and tetrodotoxin were purchased from Calbiochem-Behring Corp., La Jolla, Calif. Lactate dehydrogenase was purchased from Worthington Biochemical Corp., Freehold, N.J. Veratridine and 4-aminopyridine were purchased from Aldrich Chemical Co., Milwaukee, Wisc. NADH was purchased from P-L Biochemicals, Milwaukee, Wisc. N-methyl-D,L-aspartic acid was purchased from Chemical Dynamics Corp., South Plainfield, N.J. Millipore filters (o.65 um) were purchased from Millipore Corp., Bedford, Mass. Nitrogen and 95% O_2 - 5% CO_2 gases were purchased from Union Carbide Corp. - Linde Division.

L-(U-^{14}C)glutamic acid (263 uCi/umole), L-(U-^{14}C)

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GABA (206 uCi/umole), L-(U-^{14}C)glutamine (228 uCi/umole), D-(2-^{14}C)glucose (56 uCi/umole) and (^{3}H)acetate (1.95 mCi/umole) were purchased from New England Nuclear Corp., Boston, MA. L-(U-^{14}C)glutamine and L-(U-^{14}C)GABA were passed through a column of AG 1x4 resin to remove acidic contaminants. Scintiverse was obtained from Fisher Chemical Co., Springfield, N.J.

Supplies for amino acid analysis were purchased as follows. AG 1x4 200-400 mesh chloride form and AG 50x4 200-400 mesh hydrogen ion form were obtained from Bio-Rad Laboratories, Richmond, Calif. Ninhydrin and hydrindantin were obtained from Pierce Chemical Co., Rockford, Ill. Ethylene glycol monomethyl ether (methyl cellosolve) was obtained from Fisher Chemical Co., Springfield, N.J.

All other reagents not specifically mentioned were of reagent grade purity and were obtained from Fisher Scientific Co., Springfield, N.J. or Sigma Chemical Co., St. Louis, Mo.

N-methyl-D-aspartic acid and dihydrokainic acid were generously provided by Dr. Graham Johnston, Australian National University, Australia.

2. Animals

Male Sprague-Dawley rats (150-175 grams) were obtained from Zivic Miller Laboratories and male Swiss Webster mice (20-25 grams) from Perfection Breeders. The animals were housed in the animal facilities of the Mount Sinai School of Medicine and fed Purina Laboratory Chow

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ad libitum until the time of sacrifice. Mice with the weaver (Wv) mutation B6CBA A^{WJ}/A and littermate normals were obtained from Jackson Laboratory.

B. SOLUTIONS

1. Krebs Ringer Bicarbonate (pH 7.4) gassed with CO_2

The Krebs-Ringer bicarbonate medium contained:

119 mM NaCl
4.8 mM KCl
1.7 mM $CaCl_2$
1.2 mM $MgCl_2$
5.6 mM glucose
1.2 mM KH_2PO_4
23.8 mM $NaHCO_3$

2. Krebs Ringer Phosphate (pH 7.4)

The Krebs-Ringer phosphate medium contained the same ingredients as the bicarbonate medium except that 1.2 mM KH_2PO_4 and 23.8 mM Na HCO_3 were deleted and 15.5 mM Na_2HPO_4 (adjusted to pH 7.4 with HCl) was added.

3. Krebs Ringer Tris (pH 7.4)

The Krebs-Ringer Tris medium contained:

125 mM NaCl
4.8 mM KCl
1.0 mM $CaCl_2$
1.0 mM $MgCl_2$
9.4 mM glucose
50 mM Tris•HCl

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C. METHODS

1. Tissue Preparation and Incubation Conditions for Determination of Nucleotides, Creatine Phosphate, and Amino Acids

Rats were killed by decapitation and the cerebella rapidly removed, placed on ice, and divided into quarters. Each quarter was sliced into 0.3 mm thick parasagittal slices using a McIlwain type tissue chopper. Slices (approximately 50 mg wet weight, 5 mg protein) were placed in Sorvall centrifuge tubes containing 2.5 ml of a standard Krebs-Ringer bicarbonate medium, pH 7.4. They were incubated at 37°C in a shaking water bath under an atmosphere of 95% O_2-5% CO_2. If striatal slices were used, then each striatum was rapidly removed, sliced into 0.3 mm thick slices, and incubated separately. If cerebellar slices from weaver mice or littermate controls were used, then each cerebellum was rapidly removed and sliced into 0.3 mm thick parasagittal slices. Each cerebellum was incubated separately. The Krebs-Ringer bicarbonate solution was bubbled with 95% O_2-5% CO_2 for 15 minutes prior to use. The timing and the descriptions of drug and radioactivity additions are given in legends to Figures and Tables.

If the slices were to be transferred during the incubation, this is indicated in the "Results" section. Transferring was done as follows. After a preincubation period, the slices were rapidly centrifuged in a clinical

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centrifuge, and the medium discarded. Then 2.5 ml of fresh Krebs-Ringer bicarbonate medium was added and the slices were reincubated.

At the end of the incubation period, in some cases, the tissue and medium were centrifuged for approximately 30 seconds, and the medium was immediately decanted into an ice-cold test tube. A 1 ml aliquot of the medium was then acidified to a final concentration of 0.4 N perchloric acid (PCA) and stored at -20°C until analyzed. Immediately after decanting the medium. the slices were rinsed with 2.5 ml of Krebs-Ringer bicarbonate medium, rapidly recentrifuged, and the supernatant discarded. The tubes containing the slices w re immediately put into an ice-bath, 2 ml of ice-cold 0.4 N PCA were added, and the slices were homogenized on ice using a motor driben teflon pestle. After 15-30 minutes at 4°C for thorough extraction, the homogenate was centrifuged at 12,000 x g for 10 minutes. The pellet was rehomogenized in 1 N NaOH and the protein content measured by the method of Lowry et al. (1951). If nucleotides were to be analyzed, the supernatant was immediately neutralized with 2 N K_2CO_3 with vortexing. The potassium perchlorate precipitate was removed by centrifugation at 13,000 x g for 10 minutes and nucleotides measured in the supernatant as described below. Otherwise the still acidified supernatant was frozen until amino acid analysis.

In other cases, the medium and the tissue were rapidly separated ($<$ 20 seconds) by aspiration or by

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removal of the medium with a 3 ml syringe. The tube containing the tissue was plunged into an ice-bath, 2.0 ml of ice-cold 0.4 N PCA were added to each tube, and the contents homogenized while on ice. After extracting for 15-30 minutes on ice, this homogenate was centrifuged at 12,000 x g for 10 minutes. The supernatant was neutralized as described above, and the protein content of the pellet measured (Lowry et al., 1951). If the medium was removed into a syringe, it was immediately squirted into a test tube in an ice-bath. The medium was then rapidly centrifuged with a clinical centrifuge. An 1 ml aliquot was taken and acidified to a final concentration of 0.4 N PCA. If the medium was removed by aspiration, it was not saved.

2. Determination of ATP, ADP, AMP and Creatine Phosphate Levels

ATP, ADP and AMP levels were measured fluorometrically by the method of Williamson and Corky (1969) and creatine phosphate by the method of Lamprecht et al. (1974) in aliquots of the previously described 0.4 N PCA-extracted brain slices which were neutralized to pH 6.5 to 7.0 with 2 N K_2CO_3. ATP levels were determined fluorometrically by following $NADP^+$ reduction in the following assay mixture: 5 mM glucose; 0.05 mg/ml $NADP^+$; 0.001 mg/ml glucose-6-phosphate dehydrogenase; 100-200 ul aliquot of brain extract; completed to 2 ml with 50 mM triethanolamine HCl buffer containing 10 mM $MgCl_2$ and 5 mM EDTA at pH 7.4.

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After temperature equilibration (23°C), the reaction was initiated by addition of 0.01 mg/ml of hexokinase.

Likewise ADP and AMP levels were measured fluorometrically, but by following the oxidation of NADH. The assay mixture for ADP contained: 0.01 mg/ml NADH; 0.125 mg/ml phosphoenolpyruvate, tricyclohexylamine salt; 2 µg/ml lactate dehydrogenase; 300-600 µl aliquot of brain extract; completed to a final volume of 2 ml with 1/15 M KH_2PO_4 buffer with 5 mM $MgCl_2$ at pH 7.0. The reaction was initiated by the addition of 2.5 µg/ml of pyruvate kinase. The assay mixture for AMP contained in addition to the above 5 µM ATP; the reaction was initiated by addition of 12.5 µg/ml of myokinase.

Creatine phosphate levels were determined by measuring the reduction of $NADP^+$. In a final volume of 2 ml, the following reagents were present: 25 mM triethanolamine HCl buffer at pH 7.5; 0.25 mM $NADP^+$; 3.3 mM $MgCl_2$; 0.15 mM ADP; 16.7 mM glucose; 50-150 µl aliquot of brain extract; 1.7 µg of glucose-6-phosphate dehydrogenase; 3.4 µg hexokinase. The reaction was started by the addition of 34 µg of creatine kinase.

In each case, after completion of the reaction, a second addition of the enzyme used to initiate the reaction was made as an internal enzyme blank. Also, 1 to 4 nmoles of ATP, ADP, AMP or creatine phosphate was added to each sample as an internal standard. The increase or decrease of fluorescence gave a quantitative measurement of each

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compound. The concentration of the standard (μmoles/ml) was determined on a spectrophotometer using this formula:

$$\frac{Vol_{final}}{Vol_{standard}} \quad x \quad \frac{O.D.}{extinction\ (6.22)}$$

Knowing the fluorescence change caused by a given amount of standard, the nmoles of ATP, ADP, AMP or creatine phosphate per sample could be calculated. Results were expressed as nmoles/mg protein.

3. Quantitation of Amino Acids

In some cases, the amino acid content of the medium or slice PCA extracts were measuring using the Technicon Amino Acid Autoanalyzer (TSM). The TSM automatically separates amino acids by ion-exchange chromatography using a lithium citrate buffer system and then quantitates them by reaction with ninhydrin.

Otherwise, the separation of glutamic acid, aspartic acid, glutamine and GABA by ion-exchange chromatography, their quantitation with ninhydrin, and the measurement of radioactivity were carried out by procedures described by Berl et al. (1968). Briefly, the PCA extracts of brain were neutralized to pH 6.8 to 7.0 with $KOH\text{-}K_2CO_3$. The potassium perchlorate precipitate was removed by centrifugation at 13,000 x g for 10 minutes. The supernatant was diluted to 3.5 ml and 0.5 ml of this was saved for scintillation counting. Then 3 ml was placed on a 0.7 x 20 cm Bio-Rad AG-1x4 column (200-400 mesh, chloride form,

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stored in 2N acetic acid, and washed with 0.05 N acetic acid). The neutral fraction containing glutamine and GABA was eluted with 0.05 N acetic acid; the glutamic acid-containing fraction was eluted with 0.1 N acetic acid; and the aspartic acid-containing fraction was eluted with 0.3 N acetic acid. The fractions containing glutamic acid and aspartic acid were directly quantitated with ninhydrin and counted, unless (^{3}H)acetate had been used in the experiment. In that case, the main fractions containing glutamic acid and aspartic acid were evaporated to dryness in acetic acid twice. This was done to remove all traces of ^3H_2O and (^{3}H)acetate which was primarily eluted in the glutamic acid-containing fraction. These fractions were then quantitated with ninhydrin and counted for radioactivity in a Packard TriCarb Scintillation Spectrometer.

The neutral fraction was made 2N in HCl and heated at 100°C for 2 hours to hydrolyze glutamine to glutamic acid. It was then evaporated to dryness under vacuum, redissolved in 3.5 ml distilled water, and neutralized, and 3 out of the 3.5 ml was placed onto a second AG-1 column. The neutral fraction still containing GABA was eluted as before with 0.05 N acetic acid and glutamine was eluted as glutamic acid with 0.1 N acetic acid. Glutamine (as glutamic acid) was then quantitated with ninhydrin and counted. Then 5 ml of the fraction containing GABA was acidified with 0.05 ml concentrated HCl and placed on a 0.7 x 12 cm Bio-Rad AG-50x4 column (200-400 mesh, H^+ form

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stored in 0.35 M sodium citrate buffer, pH 3.25 and washed with the same buffer). GABA was eluted with 0.35 M sodium citrate buffer, pH 5.3. The exact volume and number of washes with each buffer needed to elute each amino acid in a single peak was determined for each batch of resin. A standard with a known quantity of each amino acid and a blank were carried through the entire procedure with each set of samples.

The amino acids in each fraction were quantitated on a Technicon Analyzer by reaction with ninhydrin. The O.D. of the reaction product was measured at 570 nm, and the output was sent to a strip chart recorder Final results were expressed as µmoles/100 mg protein.

The radioactivity in ^{14}C- and ^{3}H-labelled amino acid fractions and in neutralized PCA extract was measured on a Packard liquid scintillation counter. Either 2 ml of the main amino acid fraction or 1 ml plus 1 ml of distilled water was added to 10 ml of Scintiverse and counted. The efficiencies were 27% for tritium and 55.3% for carbon-14. The crossover of ^{14}C was 9.11%. Efficiencies and crossover were determined using standard solutions of ^{3}H-toluene and ^{14}C-toluene. Specific radioactivities were calculated by dividing the radioactivity as dpm/ml by the concentration of amino acid as µmoles/ml (obtained from the ninhydrin measurements). When only single labelled-precursors were used, the data were expressed as cpm/µmole.

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4. Uptake and Release from Synaptosomes

Rats were killed by decapitation and the cortex and cerebellum rapidly removed and placed into ice-cold isolation medium (STE) containing 0.25 M sucrose, 0.01 M Tris, 0.5 mM Tris-EDTA, pH 7.4. A crude synaptosomal fraction was prepared by the method described by Nicklas et al. (1973), as follows. The tissue was minced into small pieces with scissors and washed well with cold STE. The tissue was then homogenized in $\geq$ 10 percent (w/v) cold STE in a Dounce glass homogenizer by 8 up-and-down strokes. This homogenate was diluted to 40 ml with STE and centrifuged at 2000 x g for 3 minutes in a Sorvall RC-5 refrigerated centrifuge. The supernatant from this spin was then centrifuged at 10,000 x g for 10 minutes. This pellet (the crude mitochondrial-synaptosomal pellet) was gently resuspended in 2-3 ml of cold STE to give a concentration of approximately 5-25 mg/ml.

Aliquots of this crude synaptosomal suspension (usually 0.1 ml) were added to 2.3 ml of Krebs-Ringer phosphate medium, for experiments with L-(U-^{14}C)glutamic acid), or to 1.3 ml of Krebs-Ringer Tris medium (as described under "Solutions"), for experiments with L-(U-^{14}C) glutamine. The final concentration of protein in the incubation medium was 0.5 to 1.0 mg protein/ml. L-(U-^{14}C)glutamic acid, diluted with nonradioactive L-glutamic acid, was added to give a final concentration of 10 μM L-glutamic acid, or L-(U-^{14}C)glutamine, diluted

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with nonradioactive L-glutamine, was added for a final concentration of 0.25 mM L-glutamine. When present, the concentration of kainic acid was 1 mM; veratridine was 1 µM and ouabain was 0.2 mM. Each flask was incubated at 37°C with agitation in a Dubnoff shaker bath.

In uptake measurements, the medium and an aliquot of the synaptosomal suspension, and in some cases, kainic acid or ouabain were preincubated for 5 minutes. At that time, radioactive substances were added. At 10 minutes total elapsed time, a 1 ml sample was removed, and in some experiments also studying the effect of kainic acid on veratridine-induced release, veratridine was immediately added to the incubation mixture. At 13 minutes, another 1 ml sample was removed and treated as described below.

In measurements of kainic acid-induced release, the medium and an aliquot of the synaptosomal suspension were preincubated for 5 minutes. At that time, radioactive substances were added. Then kainic acid was added to some samples at 10 minutes, and 5 minutes later, a 1 ml sample was removed and treated as described below.

As the 1 ml samples were removed, they were immediately filtered through a single Millipore filter with a pore size of 0.65 µM which had been premoistened with distilled water. Each filter was immediately washed 3 times with 1 ml of 0.154 M NaCl. The time between sampling and the final saline wash was usually less than 0.6 minutes. The whole Millipore filter was transferred to a scintilla-

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tion vial and 10 ml of Scintiverse solution added. After the filter dispersed, samples were mixed and counted on a Packard TriCarb Scintillation Counter with the window open.

Uptakes were calculated as cpm/mg protein present on the filter; and expressed as a percentage of control. The apparent net uptake was expressed as nmoles/mg protein. This was calculated from the cpm/mg protein on the filter; the cpm in the added radioactive substance; and the nmoles of glutamic acid or glutamine added to the medium. The tissue/medium ratio was defined as the cpm/mg protein on the filter divided by the cpm/ml of incubation medium. This as well as the apparent net uptake was calculated with the assumption that the total amount of radioactivity in the medium of each sample was the same and was equal to that of the standard. The standard was measured by adding an aliquot of the L-(U-^{14}C)-glutamic acid or L-(U-^{14}C)glutamine to a vial with 10 ml of Scintiverse and a moistened Millipore filter.

5. Oxygen Consumption

To measure the oxygen uptake of mouse brain cerebellar slices, mice were killed by cervical dislocation and the cerebellum rapidly removed, cut into two pieces along the sagittal plane and weighed. One-half of the cerebellum was then sliced into 0.3 mm thick parasagittal slices using a McIlwain type tissue chopper. This was incubated in the stirrer chamber of the Yellow Springs

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Instrument with stirring at 32°C in 5 ml of Krebs-Ringer phosphate buffer (pH 7.4).

The tissue was equilibrated for 45 minutes in the Ringer with stirring. Oxygen uptake was measured polarographically with a Clark-type electrode (Yellow Springs Instrument Co.) covered by a teflon membrane. At 45 minutes, a linear decline in the partial pressure of O_2 could be measured. This was monitored on a chart recorder for 5 minutes to obtain a baseline level of oxygen consumption by the tissue. Drug was then added in an aliquot of 25-50 µl. After a 30 second delay due to a perturbation in the measurement from the injection of air in the sample buffer, the oxygen consumption of the tissue was monitored for the next 5 minutes. Results are expressed as ng-atoms of oxygen/minute/mg wet weight. This is based on the solubility of O_2 in 5 ml of Ringer solution at 32°C which is 2210 ng-atoms of O_2 (Estabrook, 1967).

6. Glutamine Synthetase Activity

Glutamine synthetase activity was determined by measuring the formation of γ-glutamylhydroxamate from glutamate by a colorometric reaction as described by Nicklas et al. (1979a). Cerebellar slices were incubated in Krebs-Ringer bicarbonate medium for 45 minutes at 37°C in a shaking water bath under an atmosphere of 95% O_2-5% CO_2 with or without kainic acid. After incubation, the slices were centrifuged in a clinical centrifuge and homogenized in ice-cold 50 mM imidazole HCl buffer contain-

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ing 0.1 mM dithiothreitol, pH 7.2. Blanks with buffer instead of homogenate and cerebellar homogenates which had not been incubated were also carried through the procedure.

7. Histology

Cerebellar slices were prepared and incubated as for nucleotide measurements. After a 15 minute preincubation, kainic acid at a final concentration of 1 mM was added to some tubes. The incubation continued 30 minutes longer. At that time, the incubation medium was decanted and the slices were immediately immersed in formalin. The slices were then sectioned and stained with hematoxylin and eosin.

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III. RESULTS

A. STUDIES ON THE KAINIC ACID-INDUCED DECREMENT IN ATP LEVELS OF CEREBELLAR SLICES

Kainic acid (1 mM) caused a rapid and severe decrement in ATP levels in both "untransferred" and "transferred" (as described in "Methods") cerebellar slices incubated in Krebs-Ringer bicarbonate medium at 37°C. The effect of incubating various concentrations of kainic acid with transferred cerebellar slices for either 30 or 60 minutes is shown in Figure 3. A dose of 0.01 mM kainic acid had no significant effect on ATP levels after 30 or 60 minutes incubation; 0.1 mM kainic acid decreased ATP levels by 20% at both incubation times; 1 mM kainic acid decreased the levels of ATP by 36% after 30 or 60 minutes. ATP levels following incubation with 1 mM kainic acid or 0.1 mM kainic acid were not significantly different from each other. Such transferred cerebellar slices were primarily used for studies on glutamic acid metabolism. When untransferred cerebellar slices were incubated with 1 mM or 10 mM kainic acid for 30 minutes, a 39% and a 41% decrease in ATP levels was observed respectively, indicating that a maximal decrease in ATP levels had occurred with 1 mM kainic acid. Untransferred slices were primarily utilized in studies measuring ATP levels and related metabolites because the values of ATP

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FIGURE 3. EFFECT OF KAINIC ACID ON ATP AND CREATINE PHOSPHATE LEVELS OF CEREBELLAR SLICES AS A FUNCTION OF DOSE

Cerebellar slices 0.3 mm thick were preincubated in a Krebs-Ringer medium for 15 minutes, then transferred, and incubated for a further 30 or 60 minutes in the presence or absence of kainic acid at the concentrations indicated. After incubation, the tissue and medium were promptly separated by rapid centrifugation; and ATP and creatine phosphate levels analyzed as described in "Methods". The results are expressed as mean ± S.E.M. with the number of samples indicated in parentheses. Significance was determined using the two-tailed Students t-test for these and all other data unless otherwise indicated. Kainic acid and other drugs added to the incubation medium were first neutralized to pH 7.2-7.4 for all experiments.

* $p < 0.02$ when compared to control.

** $p < 0.005$ when compared to control.

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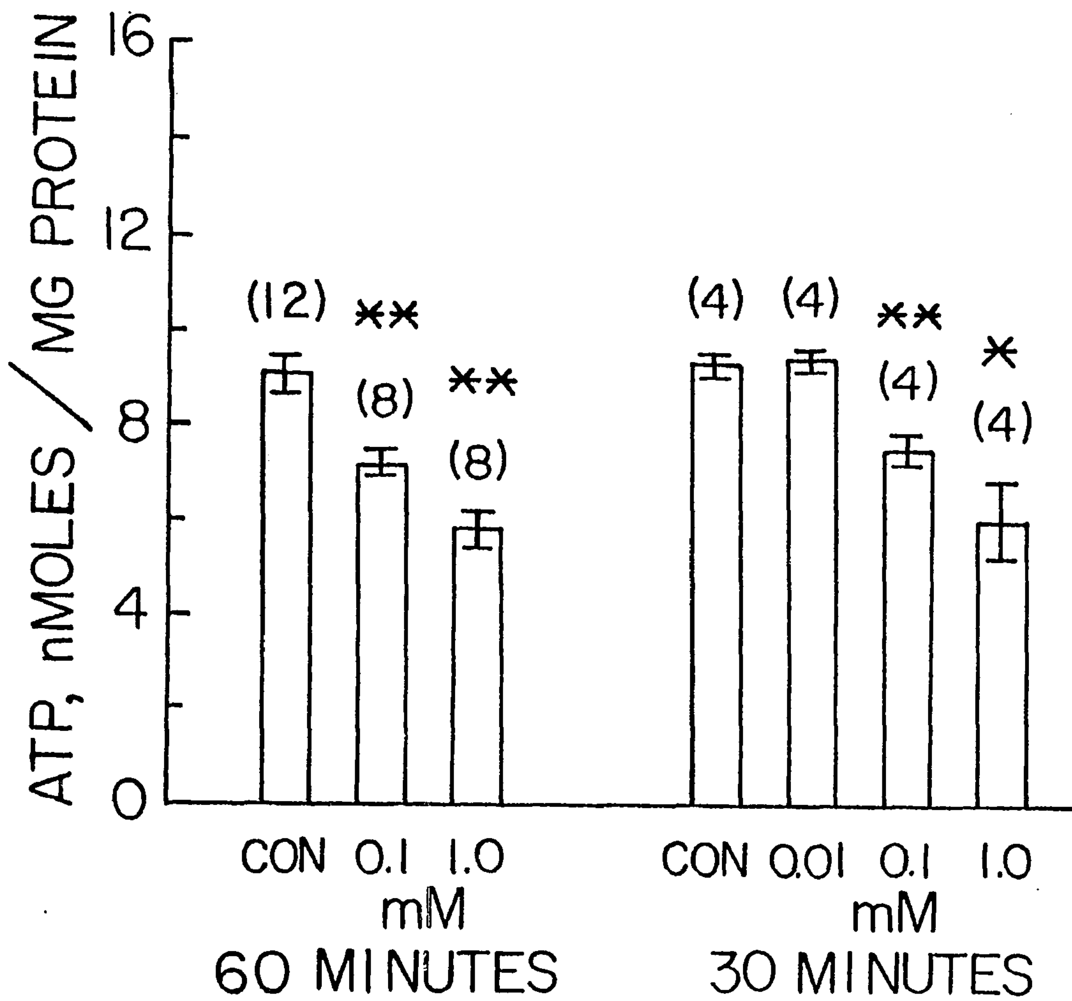

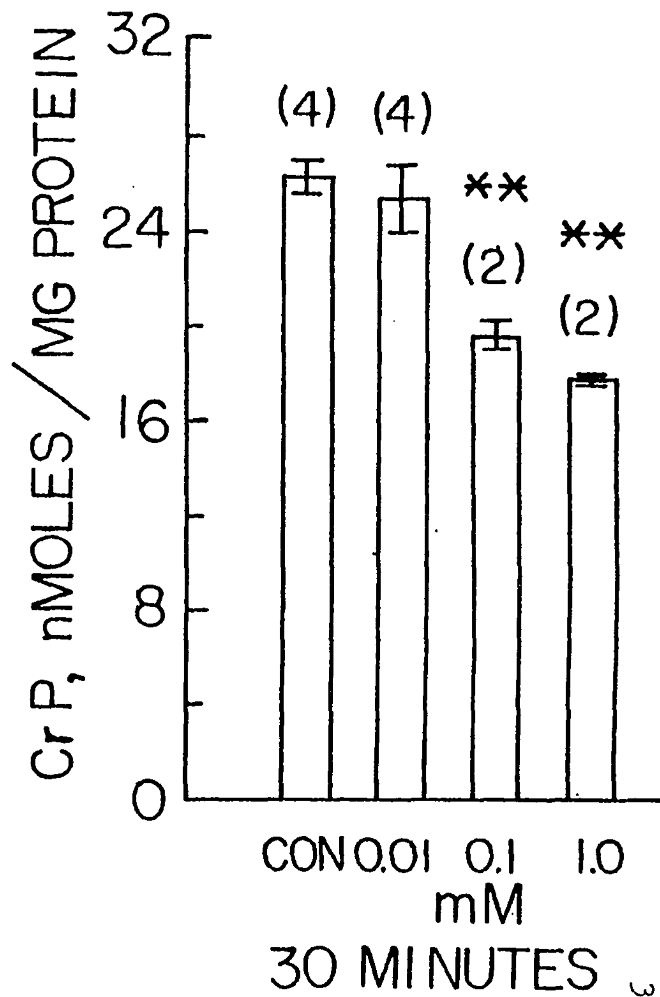

in untransferred slices at the incubation times used were somewhat higher than those of transferred slices and it was, therefore, easier to measure the 20-30% decreases in ATP levels.

Using these "untransferred" slices, the time course of the decrease in ATP levels caused by 1 mM kainic acid was studied. In control studies, it was found that when untransferred cerebellar slices were incubated in Krebs-Ringer bicarbonate medium at $37^{\circ}C$, ATP levels rose 30-40% during the initial 15-20 minutes and then remained constant for at least 45 minutes as shown in Figure 4. No such increase in ATP levels was observed if 1 mM kainic acid was present from the beginning of incubation; ATP remained at about 65% of the maximal levels. Also, if the slices were incubated for 45 minutes to generate maximal ATP levels in the tissue, and then kainic acid was added, ATP levels fell by 30% within 20 minutes.

To test whether this effect of kainic acid was reversible, tissue slices were incubated with 1 mM kainic acid for 30 minutes, transferred to fresh medium with no kainic acid and reincubated. The level of ATP in the tissue rose during this incubation (Figure 4) indicating a partial recovery.

Various attempts were made to block the action of kainic acid to decrease ATP levels. The depolarizing action of L-glutamic acid and other excitatory amino acids may result from an increase in permeability to Na^+ (Curtis

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FIGURE 4. EFFECT OF KAINIC ACID ON ATP LEVELS IN CEREBELLAR SLICES AS A FUNCTION OF INCUBATION TIME

Slices were incubated in a Krebs-Ringer bicarbonate buffer in the presence or absence of kainic acid. (●——●) Control slices; (○——○) slices incubated with 1 mM kainic acid. To other slices kainic acid was added or removed as follows. To examine if the repression of the rise in ATP was reversible, slices were incubated for 30 minutes with 1 mM kainic acid, transferred to normal medium and incubated a further 30 minutes (△- - - - - -△). To examine if kainic acid can cause a change in maximal ATP levels, 1 mM kainic acid was added to slices at 45 minutes and the slices were incubated for a further 15 minutes (□- - - - -□). The ATP levels are expressed as a % of the level in the control slices at 60 minutes (12.7 nmoles/mg protein). Each point is the mean ± S.E.M. for 3-4 slices except for control and kainic acid-treated slices at 60 minutes in which case n=12 and n=8 respectively.

* $p < 0.01$ when compared to corresponding control slices.

ǂ $p < 0.02$ when compared to corresponding kainic acid-treated slices.

Ordinate: % ATP at 1 hour.

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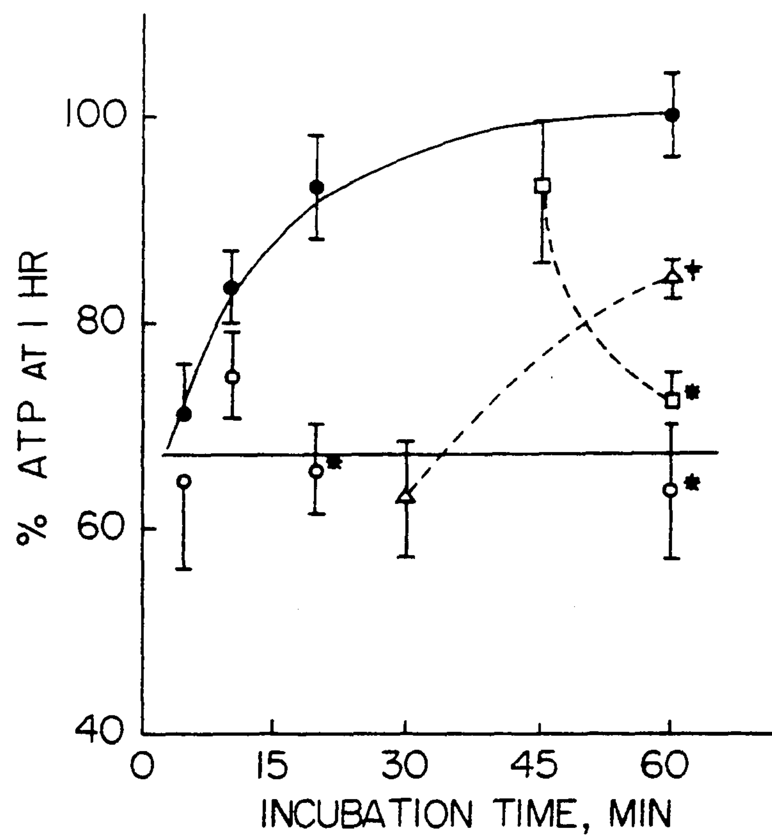

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et al., 1972; Zieglgansberger and Puil, 1973) and the ensuing ionic alterations may lead to decreases in ATP. Therefore an attempt was made to block the kainic acid-induced decreases in cerebellar ATP levels with drugs which block the conductance of Na^+ or K^+. Tetrodotoxin selectively blocks the increase in Na^+ permeability (Narahashi et al., 1964) and 4-aminopyridine blocks the increase in K^+ conductance (Meves and Pinchon, 1977) following electrical excitation. The results show that neither 3 µM tetrodotoxin (Figure 5) nor 5 mM 4-aminopyridine (Table 1), added 5 minutes prior to kainic acid, blocked the decrement in ATP levels induced by kainic acid in cerebellar slices. In contrast, the addition of 3 µM tetrodotoxin at 5 minutes prior to the addition of 10 µM veratridine did block the 50% decrease in ATP levels seen with veratridine (Figure 5).

The toxic actions of kainic acid may be mediated via a specific receptor for kainic acid. However a good antagonist of kainic acid receptors has not as yet been found. Glutamic acid diethyl ester blocks the excitations caused by L-glutamic acid in cat spinal and rat thalamic neurons (Haldeman et al., 1972; Hall et al., 1978), but does not block kainic acid-induced excitations (Hall et al., 1978; Hicks et al., 1978). D,L-2-amino-4-phosphonobutryic acid partially depresses responses to kainic acid on cat and amphibian spinal neurons and more weakly depresses responses to L-glutamic acid (Davies et al., 1979). However additions of 1 mM 2-amino-4 phosphonobutyric

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FIGURE 5. ACTION OF TETRODOTOXIN ON THE DECREMENT IN ATP CAUSED BY VERATRIDINE AND BY KAINIC ACID IN CEREBELLAR SLICES

Cerebellar slices were preincubated for 14 minutes, then transferred. Ten minutes later, 3 µM tetrodotoxin dissolved in a citrate buffer or no drug (citrate buffer control) was added. To some samples, 1 mM kainic acid or 10 µM veratridine was added 12 minutes later and the incubation continued for a further 15 minutes. The tissue and medium were then separated by aspiration and ATP and amino acid levels measured (as described in "Methods"). Results are presented as mean $\pm$ S.E.M. for the number of samples indicated in parentheses. TTX=tetrodotoxin. CIT= citrate buffer (tetrodotoxin control).

* $p < 0.01$ when compared to control (no drug).

\+ $p < 0.05$ when compared to control (no drug).

ATP levels of slices treated with veratridine vs. those treated with veratridine and tetrodotoxin are significantly different, $p < 0.001$.

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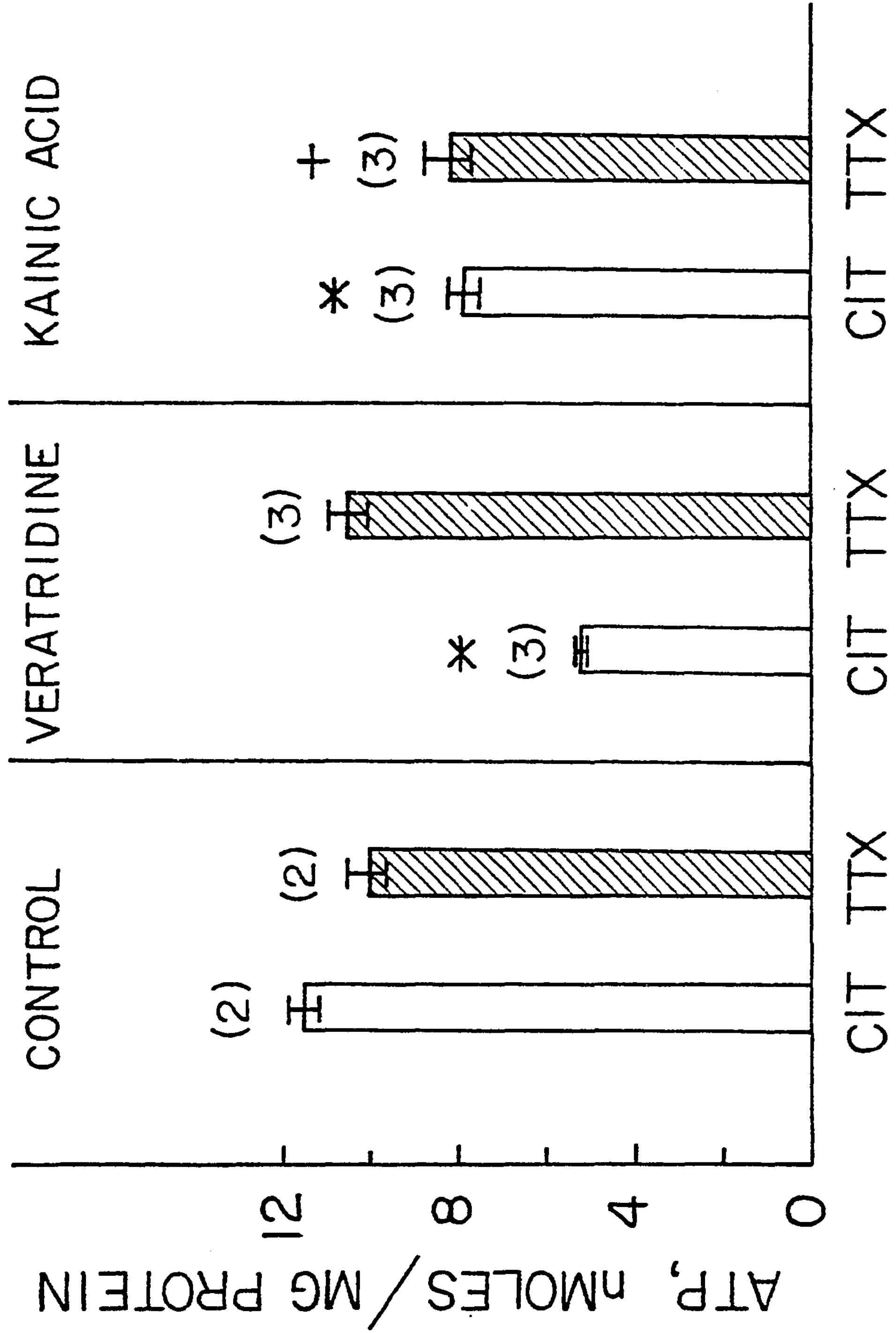

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TABLE 1

EFFECT OF VARIOUS COMPOUNDS ON ATP LEVELS AND ON THE KAINIC ACID-INDUCED DECREMENT OF ATP LEVELS

Additions or Changes of Medium	ATP (nmoles/mg protein)
0.5 mM Adenosine (4)	11.3 ± 0.4
0.5 mM Adenosine + 1 mM KA (4)	7.1 ± 0.6**
Mg^{2+}-free Medium (3)	9.3 ± 0.3
Mg^{2+}-free Medium + 10 mM N-methyl-DL-aspartic acid (3)	8.5 ± 0.4
None (2)	10.9 ± 1.0
0.6 mM KA (2)	7.8 ± 0.8
5 mM 4-Aminopyridine (2)	9.2 ± 0.3
5 mM 4-Aminopyridine + 0.6 mM KA (2)	6.5 ± 0.3*

Cerebellar slices were preincubated in the presence of adenosine, in Mg^{2+}-free medium, or with no additions from the beginning. After a 15 minute preincubation, kainic acid was added to some samples with adenosine and N-methyl-DL-aspartic acid was added to some samples in a Mg^{2+}-free buffer without transferring. These incubations continued for 30 minutes longer when ATP levels were measured. In other experiments, 4-Aminopyridine was added to samples after a 30 minute preincubation and 0.6 mM kainic acid was added 5 minutes later to samples, and the incubation continued for 20 minutes. The tissue and medium were then separated by aspiration, and ATP levels measured as described in the "Methods". Results are expressed as mean ± S.E.M. for the number of samples indicated in parentheses.

* $p<0.05$ when compared to similar samples except without kainic acid added.

** $p<0.001$ when compared to similarly treated samples without kainic acid added.

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acid did not block the decrement in ATP caused by 0.5 mM kainic acid and tended itself to cause a slight decrement in ATP (Table 2).

In contrast to cerebellar slices, when untransferred 0.3 mm thick striatal slices were incubated with 10 mM kainic acid for 30 minutes, no significant decrement in ATP levels occurred (11.1±0.7 (n=4) vs. 11.0±0.3 (n=3) nmoles/mg protein, mean ± S.E.M., for control and kainic acid-treated slices respectively).

Weaver mice, genetic mutants which lack granule cells in the cerebellum, were tested for an effect of kainic acid on cerebellar ATP levels. ATP levels were decreased in cerebellar slices from both weaver controls (normal littermates) and weaver mice (40% and 29% respectively) when incubated with 1 mM kainic acid for 30 minutes (Table 3). This is consistent with a study by Seil and Woodward (1980) which showed that granuloprival cerebellar cultures from the mouse are still sensitive to the toxic effects of kainic acid. In the mouse, the granule cells do not appear to play an essential role in demonstrating biochemically toxic effects of kainic acid. But it should be noted that Lovell and Jones (1979) have found, in contrast to rat cerebellum, Purkinje cells in the mouse are more resistant to destruction than granule cells after in vivo injections of kainic acid. In contrast, Seil et al., (1979) have found that in cultures of mouse cerebellum granule cells are more resistant than Purkinje cells to the

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TABLE 2

EFFECT OF KAINIC ACID AND 2-AMINO-4-PHOSPHONOBUTYRIC ACID ON ATP LEVELS

Additions	ATP (nmoles/mg protein)
No additions (4)	12.6 ± 0.9
1 mM APB (3)	10.4 ± 0.2
0.5 mM KA (3)	8.2 ± 0.5*
1 mM APB + 0.5 mM KA (6)	7.8 ± 0.1*

Cerebellar slices were preincubated for 10 minutes at which time 2-amino-4-phosphonobutyric acid (APB) was added to some samples, without transferring. 10 minutes later, kainic acid (KA) was added to some samples. The incubation period continued for 30 minutes longer. The tissue and medium were then rapidly separated by aspiration of the medium, and ATP levels measured as described in the "Methods". Values are the mean ± S.E.M. for the number of samples indicated in parentheses.

* $p < 0.02$ when compared to control.

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TABLE 3

EFFECT OF KAINIC ACID ON ATP LEVELS IN SLICES OF WEAVER MOUSE CEREBELLUM

	ATP (nmoles/mg protein)	
Animal	No drug	1 mM Kainic Acid
Weaver Mouse	12.5±0.4	8.9±0.6**
Normal Littermate	14.2±1.1	8.5±1.1*

Cerebellar slices (one cerebellum per tube) from weaver mice and littermate controls were preincubated for 15 minutes. Kainic acid (1 mM) was added at that time and the incubation continued for 30 minutes longer. The tissue and medium were separated by aspiration. ATP in the tissue was measured as described in the "Methods". Results are expressed as mean ± S.E.M. for two samples at each point. The mice were 34 to 51 days old when used.

* $p < 0.05$ when compared to the no drug condition.

** $p < 0.02$ when compared to the no drug condition.

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toxic effects of kainic acid.

B. EFFECTS OF VARIOUS GLUTAMIC ACID ANALOGS ON ATP LEVELS IN CEREBELLAR SLICES

The effects on ATP levels of other excitatory and nonexcitatory analogs of glutamic acid were studied. The excitatory amino acids, D,L-homocysteic acid (10 mM) and L-glutamic acid (1 and 10 mM), decreased ATP levels by 33% and 12-18%, respectively, in untransferred cerebellar slices after a 30 minute incubation (Table 4). The nonexcitatory and nontoxic analog of kainic acid, dihydrokainic acid (1 mM), did not affect ATP levels in cerebellar slices. ATP levels were also not depressed by the excitatory analog, N-methyl-D-aspartic acid (1 mM) or N-methyl-D,L-aspartic acid (10 mM). Because Mg^{2+} has been reported to antagonize excitations induced by N-methyl-D-aspartic acid (Evans et al., 1977; Davies and Watkins, 1977), the effect on ATP levels of addition of 10 mM N-methyl-D,L-aspartic acid to a Mg^{2+}-free buffer was examined. The levels of ATP were somewhat low in the Mg^{2+}-free buffer and addition of N-methyl-D,L-aspartic acid did not depress the levels of ATP any further (Table 1). If both N-methyl-D,L-aspartic acid (10 mM) and the glutamic acid uptake blocker, dihydrokainic acid (1 mM), were incubated together with cerebellar slices for 30 minutes, ATP levels were not significantly altered.

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TABLE 4

EFFECT OF VARIOUS AMINO ACIDS ON ATP LEVELS OF CEREBELLAR SLICES

Additions to Medium	Percentage of Control ATP Levels
1 mM L-glutamic acid (6)	88 ± 6*
10 mM L-glutamic acid (5)	82 ± 3*
1 mM D,L-homocysteic acid (4)	82 ± 6
10 mM D,L-homocysteic acid (4)	67 ± 3**
1 mM dihydrokainic acid (6)	97 ± 7
1 mM N-methyl-D-aspartic acid (4)	98 ± 2
10 mM N-methyl-DL-aspartic acid(2)	89 ± 6
10 mM N-methyl-DL-aspartic acid + 1 mM dihydrokainic acid (2)	84 ± 8

Cerebellar slices were preincubated for 15 minutes (35 minutes in the experiment in which 10mM N-methyl-D-aspartic acid and 1 mM dihydrokainic acid were added together) at which time the various amino acids were added to the medium, without transferring. The incubation continued for 30 minutes longer . The tissue and medium were then separated by aspiration and ATP levels measured as described in "Methods". Results are expressed as % of control ± S.E.M. for the number of samples indicated in parentheses. The control value for ATP of all the experiments combined was 11.2 nmoles/mg protein for 29 samples (S.E.M. = 0.19).
* $p < 0.05$ when compared to corresponding control.
** $p < 0.001$ when compared to corresponding control.

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C. EFFECT OF KAINIC ACID ON ADENINE NUCLEOTIDE AND CREATINE PHOSPHATE LEVELS AND ON OXYGEN CONSUMPTION IN CEREBELLAR SLICES

ADP and AMP levels were also measured to determine whether the total adenine nucleotide pool was intact after kainic acid treatment. After a 30 minute incubation with 1 mM kainic acid, the ADP levels of untransferred cerebellar slices were unaltered (1.7 $\pm$ 0.2 vs. 1.8 $\pm$ 0.1 nmoles$\cdot$mg protein^{-1}, mean $\pm$ S.E.M., control and kainic acid-treated respectively) and the AMP levels increased 20% (0.121 $\pm$ 0.002 (n=4) vs. 0.145 $\pm$ 0.004 (n=4) nmoles$\cdot$mg protein^{-1}, mean $\pm$ S.E.M., control and kainic acid-treated respectively). Thus the total content of ATP + ADP + AMP decreased 29% in the kainic acid-treated cerebellar slices. The ATP/ADP ratio decreased 36% from a control value of 6.82 to value of 4.34 in the presence of 1 mM kainic acid. Adenosine (0.5 mM) was added to the medium in an attempt to restore ATP levels. As demonstrated in Table 1, this did not inhibit the kainic acid-induced decrease in ATP levels.

The effect of kainic acid on creatine phosphate paralleled that on ATP levels. Creatine phosphate levels declined 32% and 25% after a 30 minute incubation with 1 mM and 0.1 mM kainic acid respectively (Figure 3). No decrement in creatine phosphate levels was observed with 0.01 mM kainic acid.

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Increases in the respiration of mouse cerebellar slices were noted after addition of kainic acid. The rate of oxygen uptake of mouse cerebellar slices during a 5 minute incubation at 32°C in Krebs-Ringer phosphate medium containing glucose was immediately increased by 17% after the addition of 5 mM kainic acid and 20% after the addition of 10 mM L-glutamic acid (Table 5).

D. STUDIES ON THE ALTERATIONS IN AMINO ACID LEVELS IN CEREBELLAR SLICES AND INCUBATION MEDIUM BY KAINIC ACID

Considering the possibility that an effect on glutamic acid metabolism might be involved in the neurotoxic action of kainic acid, amino acid levels in cerebellar slices and incubation medium were measured. Marked decreases (60-75%) in tissue glutamine levels were observed after incubation of transferred (Table 6) or untransferred (Table 7) cerebellar slices with 1 mM kainic acid for 30 minutes. Incubation for 60 minutes with 10 mM kainic acid did not cause any further decreases in tissue glutamine levels (Table 8). The decrease (16%) in tissue glutamine levels was smaller (3.5 $\pm$ 0.1 (n=4) vs. 3.0 $\pm$ 0.1 (n=4) umoles/100 mg protein, mean $\pm$ S.E.M., of control and kainic acid-treated slices respectively) after exposure to 0.1 mM kainic acid for 30 minutes. No decreases in tissue glutamine levels were evident after incubation with 0.01 mM

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TABLE 5

EFFECT OF KAINIC ACID AND GLUTAMIC ACID ON OXYGEN CONSUMPTION OF MOUSE BRAIN CEREBELLAR SLICES

Additions	Uptake of Oxygen (ng-atoms/mg wet wt./min.)		
	Baseline	Treated	Mean % Change
5 mM KA (7)	80.2±6.8	92.8±6.2	17.0±3.7**
10 mM L-GLU(4)	86.7±10.7	103.7±11.2	20.3±2.8**
55 mM KCl (3)	97.8±1.0	127.6±1.0	30.6±4.2*
NaCl (4)	75.7±2.5	76.8±3.5	1.1±1.7

Mouse cerebellar slices were incubated at 32°C in Krebs Ringer phosphate medium and the rate of oxygen uptake measured as described in "Methods". The concentration of KCl in the medium was increased from 5 mM to 55 mM by adding 50 ul of 5 M KCl. A similar amount of NaCl was added as a control and it increased the molarity of Na^+ in the medium from 135 to 185 mM. Values are mean ± S.E.M. for the number of experiments indicated in parentheses.

** $p < 0.005$ when compared to baseline. Paired t-test.

* $p < 0.01$ when compared to baseline. Paired t-test.

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TABLE 6

EFFECT OF KAINIC ACID ON AMINO ACID LEVELS IN TRANSFERRED CEREBELLAR SLICES AND INCUBATION MEDIUM

Tissue Amino Acid Levels (μmoles/100 mg protein)

	Control(8)	1 mM Kainic Acid (8)
Glutamic Acid	5.4±0.1	4.8±0.1*
Aspartic Acid	1.0±0.1	0.8±0.1
Glutamine	1.28±0.07	0.43±0.02**
GABA	0.92±0.02	0.92±0.06

Medium Amino Acid Levels (μmoles/100 mg protein/2.5 ml)

	Control(4)	1 mM Kainic Acid(4)
Glutamic Acid	0.31±0.02	1.44±0.05*
Aspartic Acid	0.1	0.1
Glutamine	2.82±0.08	2.54±0.09

Cerebellar slices were preincubated for 15 minutes at which time they were transferred to fresh medium and kainic acid was immediately added to some samples. After a 30 minute incubation with kainic acid, radioactive tracers were added for a period of 10 minutes. The tissue and medium were then separated by centrifugation and amino acids analyzed as described in "Methods". Results are expressed as the mean ± S.E.M. for the number of samples indicated in parentheses. GABA was not detected in the medium of any samples.

* $p < 0.02$ when compared to control.

** $p < 0.001$ when compared to control.

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TABLE 7

THE EFFECTS OF KAINIC ACID (KA), N-METHYL-D-ASPARTIC ACID (NMDA), DIHYDROKAINIC ACID (DHKA) AND D,L-HOMOCYSTEIC ACID (D,L-HCA) ON AMINO ACID LEVELS IN CEREBELLAR SLICES AND INCUBATION MEDIUM

		% Control			
		Additions to Medium			
	Tissue Amino Acid Levels (μmoles/100 mg protein)	KA	NMDA	D,L-HCA	DHKA
Glutamic Acid	7.15±0.08	80± 2**	97± 3	99± 3	97± 7
Aspartic Acid	1.71±0.02	64± 2**	107± 4	85± 4*	113± 5
Glutamine	3.16±0.07	35± 4**	98± 6	61± 3**	101± 7
	Medium Amino Acid Levels (μmoles/100 mg protein/2.5ml				
Glutamic Acid	1.58±0.07	243± 3**	112± 9	160± 6**	147±13
Aspartic Acid	0.35±0.02	334± 6**	123± 3*	181± 1**	103±13
Glutamine	6.55±0.17	96± 3	102± 2	87± 3	104± 6

After a 15 minute preincubation, cerebellar slices were incubated for a further 30 minutes without transferring with no additions or addition of 1 mM KA, 1 mM NMDA, 1 mM D,L-HCA, or 1 mM DHKA. The tissue and medium were then separated by aspiration and amino acids analyzed as described in the "Methods". Values are the mean ± S.E.M. of 8 slices for controls and 4 slices each for other conditions.

* $p < 0.05$ when compared to control.

** $p < 0.005$ when compared to control.

TABLE 8

EFFECT OF INCUBATION WITH 10 mM KAINIC ACID FOR 60 MINUTES ON AMINO ACID LEVELS IN CEREBELLAR SLICES

	Amino Acid Levels (μmoles/100 mg protein)	
	Control	10 mM Kainic Acid
Glutamic Acid	6.8±0.3	5.4±1.4*
Glutamine	4.9±2.4	1.4±0.1*
Aspartic Acid	1.2±0.1	0.8±0.03*
Threonine	0.6±0.03	0.2±0.02*
Serine	1.1±0.04	0.52±0.03*
Alanine	0.87±0.03	0.57±0.06*
GABA	0.73±0.09	0.56±0.06
Glycine	1.0±0.1	0.9±0.09
Taurine	2.3±0.3	2.0±0.4
Asparagine	0.26±0.02	0.22±0.04

After a 20 minute preincubation, kainic acid at 10 mM was added to cerebellar slices without transferring. The incubation continued for 60 minutes. The tissue and the medium were then separated by aspiration and amino acids were measured on a Technicon Amino Acid Autoanalyzer. Results are expressed as mean ± S.E.M. for 6 experiments at each point.

* $p < 0.005$ when compared with control.

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kainic acid for 30 minutes. Tissue glutamine levels were decreased by 33% within 10 minutes of addition of 1 mM kainic acid (Figure 6). The total of tissue plus medium glutamine was decreased by 18% after a 20 minute and by 25% after a 30 minute incubation but was not significantly decreased after a 10 minute incubation with 1 mM kainic acid (Figure 6). Generally tissue levels of glutamic acid and aspartic acid declined slightly (Table 6) but the decline was variable. Decreases in tissue levels of serine and threonine were also noted after a 20 minute incubation with 1 mM kainic acid (32% and 36% respectively) and larger decreases were noted after a 60 minute incubation with 10 mM kainic acid (Table 8). No changes in taurine, glycine, GABA, or asparagine levels were observed even after a 60 minute incubation with 10 mM kainic acid (Table 8).

The efflux of glutamic acid and aspartic acid into the medium substantially increased in the presence of 1 mM kainic acid whereas the levels of GABA and glutamine did not change (Tables 6 and 7). Glutamic acid levels increased in the medium from untransferred slices by approximately 150% and in the medium from transferred slices by approximately 350% after a 30 minute incubation with 1 mM kainic acid. Aspartic acid levels in the medium from untransferred slices increased by 225-245% in the presence of kainic acid. The levels of aspartic acid in the medium from transferred slices were too low to measure.

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FIGURE 6. TIME COURSE OF THE ACTION OF KAINIC ACID ON GLUTAMINE LEVELS

Cerebellar slices were preincubated for 15 minutes, then kainic acid (KA) added for a final concentration of 1 mM. The incubation was stopped at the time indicated. The tissue and medium were separated by aspiration and glutamine levels measured as described in the "Methods". Circles (●) control, (○) KA, refer to levels of glutamine in the tissue. Triangles (▲) control, (△) KA, refer to the total of tissue plus medium glutamine. The results are expressed as the mean ± S.E.M. for 4 experiments at each point.

* $p < 0.01$ when compared to control.

\+ $p < 0.05$ when compared to control.

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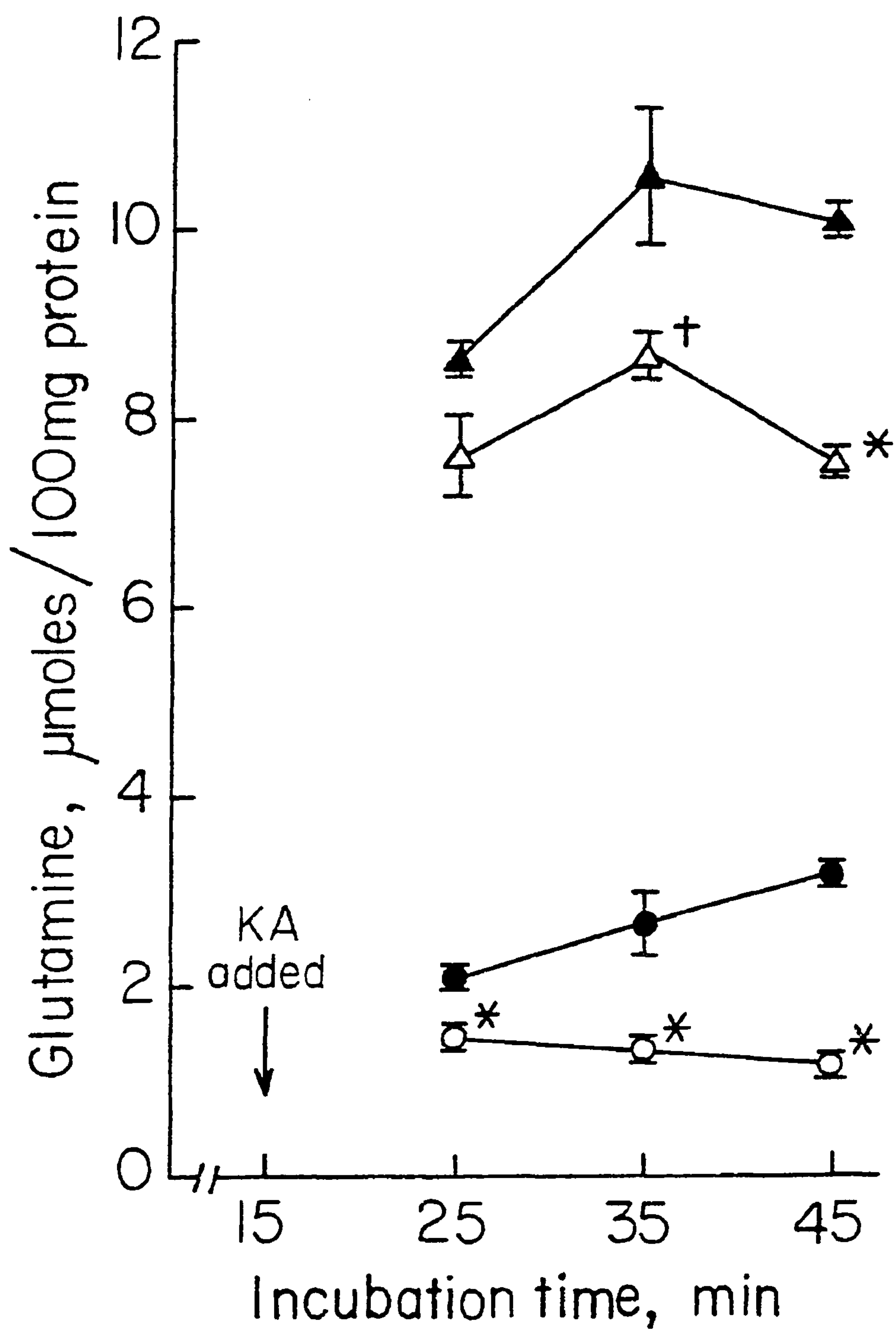

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The release of amino acids induced by kainic acid was then compared to that induced by veratridine, a presynaptic depolarizing agent. Veratridine increased the level of glutamic acid in the medium by 100%, that of aspartic acid by 45%, and that of GABA to detectable levels. Kainic acid caused a somewhat larger increase in the level of glutamic acid (by 290%) and the level of aspartic acid (by 90%) in the medium, but did not increase GABA to measurable levels during the 15 minute incubation. Tetrodotoxin, a blocker of the depolarization elicited by veratridine (Ohta et al., 1973), when added at 3 μM 5 minutes prior to the addition of 10 μM veratridine or 1 mM kainic acid, blocked the veratridine-induced release of glutamic acid, aspartic acid and GABA but did not block the release caused by kainic acid (Table 9).

E. EFFECT OF KAINIC ACID ON THE UPTAKE AND RELEASE OF GLUTAMIC ACID BY SYNAPTOSOMES

Because synaptosomal pools of glutamic acid might contribute to the increased efflux of glutamic acid into the medium from cerebellar slices which is induced by kainic acid, the effect of kainic acid on synaptosomal uptake and release of L-glutamic acid was studied. The uptake of ^{14}C-labelled L-glutamic acid into crude synaptosomal fractions prepared from rat cerebral cortex was greatly inhibited (by 55%) by 1 mM kainic acid (Table 10).

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TABLE 9

ACTION OF TETRODOTOXIN (TTX) ON THE EFFLUX OF AMINO ACIDS FROM CEREBELLAR SLICES INDUCED BY VERATRIDINE (VER) OR BY KAINIC ACID (KA)

Additions to Medium	Amino Acid Levels in the Medium (µmoles/100 mg protein/2.5 ml)			
	Glutamic Acid	Aspartic Acid	Glutamine	GABA
VER	0.72±0.06	0.16±0.01	3.07±0.23	0.17±0.02
TTX+VER	0.35±0.06**	0.11±0.01*	2.72±0.51	#
KA	1.01±0.07	0.21±0.01	3.30±0.13	#
TTX+KA	1.04±0.07	0.19±0.01	3.03±0.28	#

Citrate buffer was added to samples lacking tetrodotoxin because tetrodotoxin was added in a citrate buffer. Results are expressed as mean ± S.E.M. for 3 experiments at each point. See Figure 5 for incubation conditions.

* $p < 0.05$ when compared with similar samples not incubated with tetrodotoxin.

** $p < 0.02$ when compared with similar samples not incubated with tetrodotoxin.

GABA was not detectable in these samples.

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TABLE 10

EFFECT OF KAINIC ACID ON THE UPTAKE, THE EFFLUX, AND THE VERATRIDINE-STIMULATED EFFLUX OF L-(U-^{14}C)GLUTAMIC ACID FROM CEREBRAL CORTICAL SYNAPTOSOMES

		Percentage of Control Uptake at 10 Minutes	
		Drugs Present from the Start of the Incubation	
Sampling Time	Additions at 10 Minutes	None	1 mM Kainic Acid
10 min	None	100	45.5±0.5*
13 min	1 μM Veratridine	63.9±1.5*	34.6±2.8**
15 min	1 mM Kainic Acid	81.7±7.6	
15 min	None	107.2±3.2	

A portion of the crude synaptosomal fraction from rat brain cerebral cortex was prepared and added to Krebs-Ringer phosphate medium to give a final protein concentration of 0.9 mg protein/ml (as in "Methods"). This was preincubated for 5 minutes in the presence of absence of kainic acid at which time 100 nCi of diluted L-(U-^{14}C)glutamic acid was added to give a final glutamic acid concentration of 10 μM in the medium and a volume of 2.5 ml. The incubation was stopped and the control uptake and that measured in the presence of kainic acid were obtained 5 minutes later (10 minutes total elapsed time). Veratridine or kainic acid was added at 10 minutes, and 3 or 5 minutes later the radioactivity remaining in the synaptosomes measured. Results are expressed as the mean ± S.E.M. of the percentage of control uptake at 10 minutes for 3 samples at each point. The apparent net uptake of glutamic acid into control synaptosomes was 7 nmoles/mg protein. The tissue to medium ratio was 1.3 at 10 minutes in control synaptosomes.

* $p < 0.01$ when compared to control uptake at 10 minutes.

**$p < 0.01$ when compared to control uptake at 10 minutes and $p < 0.02$ when compared to the uptake at 10 minutes in the presence of kainic acid.

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If cortical synaptosomes were preloaded with L-(U-^{14}C)-glutamic acid, and 5 minutes later 1 mM kainic acid was added, kainic acid caused a slight nonsignificant efflux of glutamic acid (Table 10). However this small efflux is not of the same magnitude as that seen from tissue slices. During a 3 minute incubation, veratridine released 36% of the radioactive glutamic acid preloaded into control cortical synaptosomes. If the synaptosomes were preloaded with radioactive glutamic acid in the presence of 1 mM kainic acid, veratridine caused a somewhat diminished release possibly because less glutamic acid was taken up into veratridine-releaseable pools in the presence of kainic acid.

Glutamine levels are decreased by kainic acid and the amount of radioactivity in cerebellar slices incubated with (U-^{14}C)glutamine is also decreased by kainic acid (see Table 22). Since Benjamin and Quastel (1980) have recently provided evidence that active uptake of glutamine into nerve endings occurs, the active uptake of radioactive glutamine into crude cortical synaptosomal preparations was examined and was not found to be inhibited by 1 mM kainic acid (Table 11). The uptake of radioactive glutamine was inhibited by 42% by 0.2 mM ouabain, indicating that some active uptake of glutamine did occur under these conditions.

Kainic acid also had similar effects on the uptake and release of L-(U-^{14}C)glutamic acid and on the uptake of

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TABLE 11

EFFECT OF KAINIC ACID ON THE UPTAKE OF L-(U-^{14}C)GLUTAMINE BY SYNAPTOSOMES

Additions	Percentage of Control Uptake
Control	100
1 mM Kainic Acid	100.0 ± 3.5
0.2 mM Ouabain	58.4 ± 4.3*

A portion of the crude synaptosomal fraction from rat brain cerebral cortex was added to Kreb-Ringer Tris medium to give a final protein concentration of 0.5 mg protein/ ml. This was preincubated for 5 minutes at 37°C at which time 0.8 μCi of L-(U-^{14}C)glutamine was added for a final incubation medium volume of 1.5 ml. and a final concentration of glutamine of 0.25 mM. The incubation was stopped and the uptake measured after 5 minutes as described in the "Methods". Results are expressed as the mean $\pm$ S.E.M. of the percentage of control uptake. The apparent net uptake of glutamine into control synaptosomes was 9.4 nmoles/ mg protein. Samples incubated at 4^{0}C to control for passive processes had an uptake of 18% of control. The tissue to medium ratio was 1.6 at the end of the incubation, with tissue uptake determined as cpm/mg protein on the filter and the medium content expressed as cpm/ ml of incubation medium. There were 3 samples at each point.
* $p < 0.02$ when compared to control.

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L-(U-^{14}C)glutamine in a cerebellar fraction prepared in a similar way as the cortical fraction. However good cerebellar synaptosomes are especially difficult to prepare (Balazs et al., 1975) and the contents of our cerebellar fraction is unknown.

F. EFFECT OF GLUTAMIC ACID ANALOGS ON AMINO ACID LEVELS IN CEREBELLAR SLICES AND INCUBATION MEDIUM

Considering the possibility that the kainic acid-induced leakage of glutamic acid from cerebellar slices might be primarily due to an inhibition of glutamic acid uptake, the effect on amino acid levels of 1 mM dihydrokainic acid, a nonexcitatory analog of kainic acid and a potent inhibitor of glutamic acid uptake (Johnston et al., 1979), was studied. When incubated with untransferred cerebellar slices for 30 minutes, 1 mM dihydrokainic acid did not alter amino acid levels either in the slices or in the incubation medium (Table 7). To determine if an excitatory amino acid which did not also block glutamic acid uptake would alter amino acid levels, 1 mM N-methyl-D-aspartic acid was incubated with untransferred cerebellar slices for 30 minutes. Similar to its non-alteration of ATP levels, no changes in amino acid levels in the tissue or incubation medium were observed. If 1 mM dihydrokainic acid and 10 mM N-methyl-D,L-aspartic acid were incubated together with cerebellar slices, the level of glutamic acid

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and the level of aspartic acid in the medium were also not altered (Table 12).

However, the excitotoxin, D,L-homocysteic acid (10 mM), did cause similar though smaller alterations in amino acid levels; glutamine levels in the tissue declined by 39%, and glutamic acid and aspartic acid levels in the medium increased by 60% and 81% respectively (Table 7). As with kainic acid, the total tissue plus medium content of glutamine declined 19%, whereas the total amount of the other amino acids did not change if 10 mM D,L-homocysteic acid was added to the medium for 30 minutes. It should be noted that D,L-homocysteic acid also caused a large decrease in tissue ATP levels.

G. EFFECT OF KAINIC ACID AND VERATRIDINE ON THE LEVELS OF GLUTAMIC ACID AND ASPARTIC ACID IN THE MEDIUM FOLLOWING PRETREATMENT

To further discern which tissue pool of glutamic acid was released by kainic acid, cerebellar slices were preincubated with 10 µM veratridine for 10 minutes. This was done to deplete the veratridine-releasable pool of glutamic acid, presumably in nerve endings. The slices were then transferred to fresh medium and 1 mM kainic acid immediately added. After 20 minutes further incubation, glutamic acid and other amino acids in the medium were measured. As shown in Figure 7, incubation with kainic acid yielded a 47% greater efflux of glutamic acid if

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TABLE 12

EFFECT OF INCUBATION WITH 10 mM N-METHYL-D-ASPARTIC ACID (NMDA) PLUS 1 mM DIHYDROKAINIC ACID (DHKA) ON GLUTAMIC ACID AND ASPARTIC ACID LEVELS IN THE MEDIUM OF CEREBELLAR SLICES

Additions to Medium	Medium Amino Acid Levels (µmoles/100 mg protein/2.5ml)	
	Glutamic Acid	Aspartic Acid
Control	1.42±0.08	0.37±0.004
NMDA + DHKA	1.46±0.19	0.48±0.17

Cerebellar slices were preincubated for 35 minutes at which time 10 mM NMDA plus 1 mM DHKA were added to some samples, without transferring. The incubation continued for 30 minutes longer when the tissue and medium were separated by aspiration. Amino acids were analyzed as described in the "Methods". Values are the mean ± S.E.M. for 2 samples at each point.

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slices were preincubated with veratridine than if slices were preincubated in a drug-free medium. It should be noted that if slices were preincubated with veratridine, a subsequent incubation with veratridine did not cause a significant efflux of glutamic acid. Thus the increased release of glutamic acid caused by kainic acid following a preincubation with veratridine is probably not caused by a pool of glutamic acid that veratridine normally releases.

Similarly, incubation with kainic acid yielded a 42% greater level of aspartic acid in the medium if the slices were preincubated with veratridine than if the slices were preincubated in a drug-free medium (Figure 8). A preincubation with veratridine followed by incubation with veratridine also did not cause any additional release of aspartic acid as compared to a preincubation with veratridine followed by a drug-free incubation.

Conversely, in other studies, cerebellar slices were first incubated with 1 mM kainic acid for 20 minutes, transferred, and then incubated with 10 µM veratridine for 20 minutes. Similarly, incubation with veratridine caused a 93% increase in medium glutamic acid levels from slices that were preincubated with kainic acid compared to slices that were preincubated in a drug-free medium (Figure 7). Thus glutamic acid release by kainic acid was enhanced by pretreatment with veratridine and similarly glutamic acid release by veratridine was enhanced by pretreatment

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FIGURE 7. EFFECT OF KAINIC ACID AND VERATRIDINE ON GLUTAMIC ACID LEVELS IN THE MEDIUM

Cerebellar slices were preincubated for 15 minutes. At that time, as indicated by the word before the hyphen, 1 mM kainic acid (KA), 10 µM veratridine (VER) in ethanol, veratridine control (ETOH), or no drug (-) was added for 10 minutes, after which the slices were transferred to fresh medium to wash out the first drug. Then a second addition of drug was made. Kainic acid, veratridine, veratridine control, or no drug was added as indicated by the word after the hyphen. The incubation was continued 20 minutes longer. The tissue and medium were then separated by rapid centrifugation and amino acid levels in the medium were measured as described in the "Methods". Results are expressed as the mean $\pm$ S.E.M. with the number of samples indicated in parentheses. Ethanol (ETOH), is the control for veratridine.
+ $p < 0.005$ when compared to (--VER) and to (KA--).
* $p < 0.05$ when compared to (VER--) and to (ETOH--).
** $p < 0.005$ when compared to (VER-VER), (VER--), and to (ETOH--).

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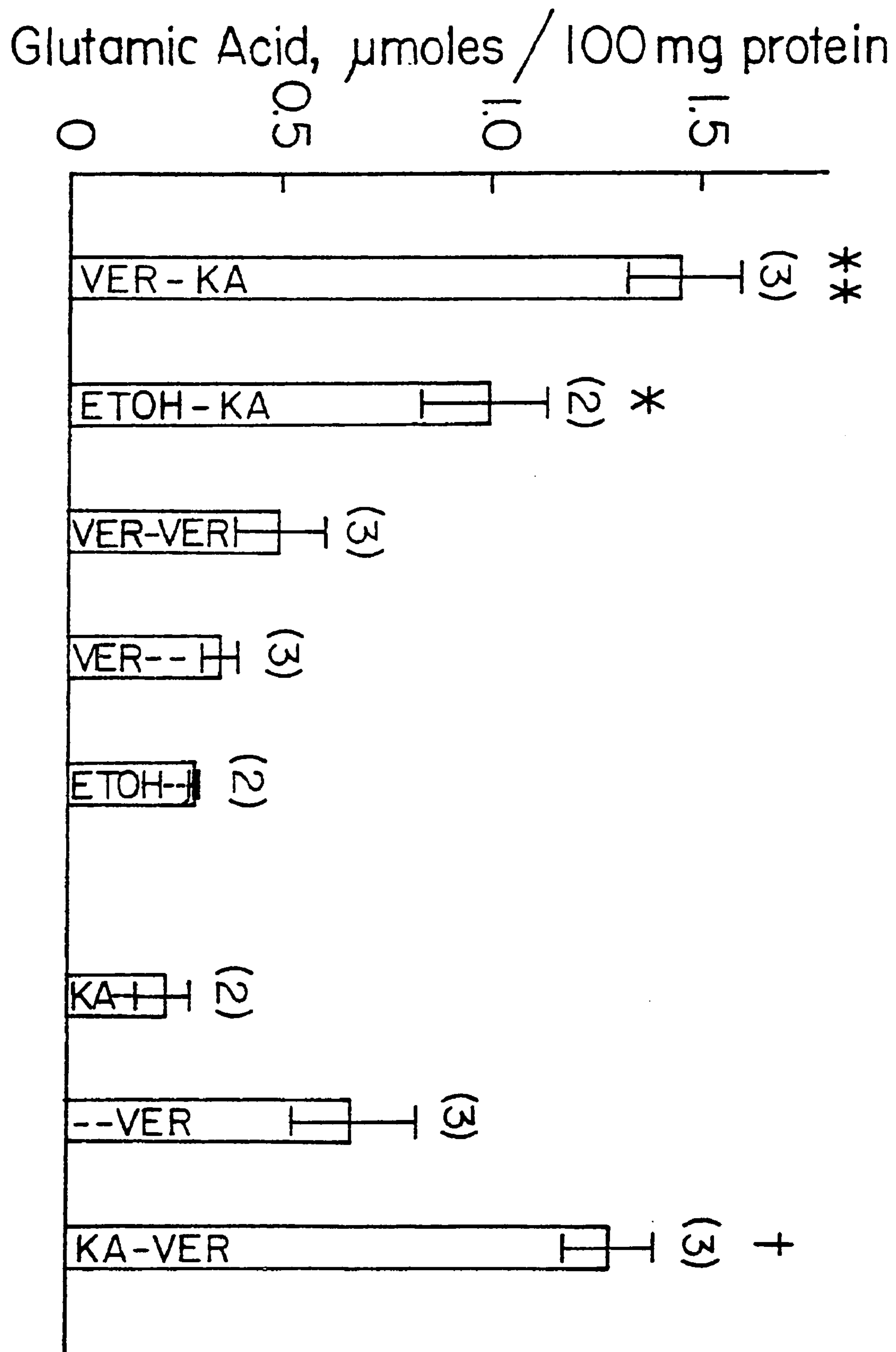

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FIGURE 8. EFFECT OF KAINIC ACID AND VERATRIDINE ON ASPARTIC ACID LEVELS IN THE MEDIUM

See Figure 7 for incubation conditions and details. Results are expressed as mean ± S.E.M. with the number of samples indicated in parentheses.

* $p < 0.02$ when compared to (VER-VER), (VER--), and (ETOH--).

** $p < 0.005$ when compared to (ETOH--), (VER-VER), (VER--), and (ETOH-KA).

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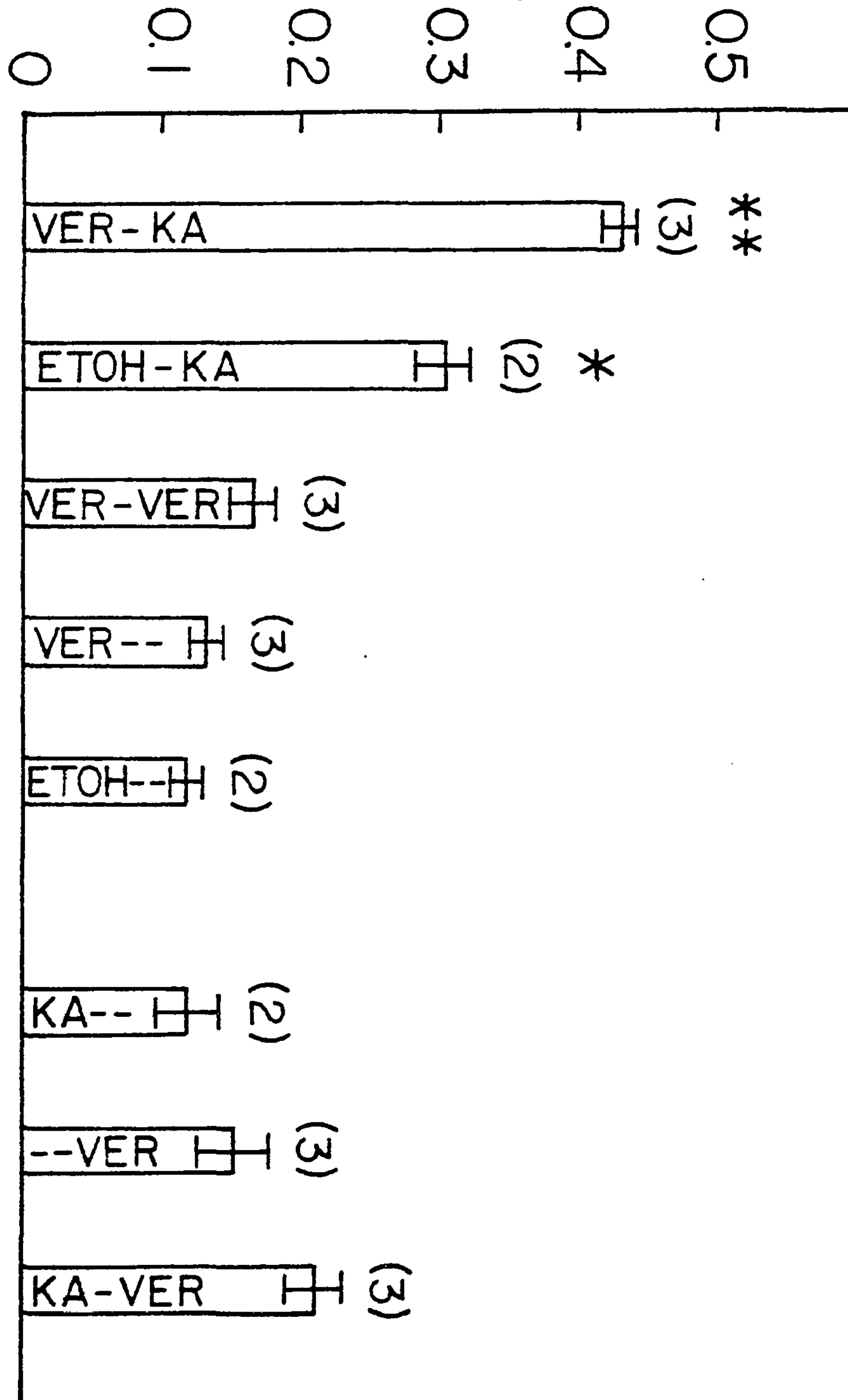

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with kainic acid. Perhaps metabolic damage due to decline in ATP or ionic alterations by veratridine and by kainic acid during preincubation made the cells more susceptible to stimulation of release during the subsequent incubation.

H. PRELABELLING STUDIES

1. Labelling of Amino Acids Released by Kainic Acid or Veratridine after Preincubation with D-(2-^{14}C)Glucose and (^{3}H)Acetate

Evidence indicates that acetate utilization occurs primarily in glia whereas glia account for < 20% of glucose utilization which is ubiquitous (see Introduction pp. 11-15). Cerebellar slices were incubated in medium containing D-(2-^{14}C)glucose and (^{3}H)acetate for 20 minutes and transferred to fresh medium. Then kainic acid or veratridine was added for 20 minutes or 6 minutes respectively and the metabolic pool of origin of the amino acids released was studied. Kainic acid (Table 13) and veratridine (Table 14) caused alterations in tissue and medium amino acid levels similar to those previously described. Kainic acid increased glutamic acid levels in the medium by 327% and aspartic acid levels by 382%. Veratridine increased glutamic acid levels in the medium by 135% and aspartic acid levels by 55%. In contrast to kainic acid, veratridine increased GABA levels in the medium to detectable levels.

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TABLE 13

EFFECT OF INCUBATION WITH 1 mM KAINIC ACID FOR 20 MINUTES ON AMINO ACID LEVELS IN CEREBELLAR SLICES AND INCUBATION MEDIUM

Tissue Amino Acid Levels (µmoles/100 mg protein)		
	Control(4)	1 mM Kainic Acid(4)
Glutamic Acid	7.9±0.4	7.1±0.4
Aspartic Acid	1.7±0.1	1.6±0.1
Glutamine	2.3±0.2	0.6±0.1*
GABA	0.9±0.1	0.9±0.1
Medium Amino Acid Levels (µmoles/100 mg protein/2.5ml)		
	Control (4)	1 mM Kainic Acid (4)
Glutamic Acid	0.26±0.03	1.11±0.04*
Aspartic Acid	0.11±0.03	0.53±0.03*
Glutamine	1.37±0.21	1.60±0.04

Cerebellar slices were preincubated for 10 minutes at which time radioactive glucose and acetate were added. Twenty minutes later, the slices were transferred to fresh Krebs Ringer bicarbonate medium and kainic acid at 1 mM was immediately added to some samples, and the incubation continued for a further 20 minutes. The tissue and medium were then separated by centrifugation and amino acids analyzed as described in "Methods". Values are the mean± S.E.M. for the number of samples indicated in parentheses. GABA was not detected in the medium of any samples.

* $p < 0.001$ when compared to control.

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TABLE 14

EFFECT OF VERATRIDINE ON AMINO ACID LEVELS IN CEREBELLAR SLICES AND INCUBATION MEDIUM

Tissue Amino Acid Levels (µmoles/100 mg protein)

	Baseline(2)	Control(3)	10 µM Veratridine(3)
Glutamic Acid	7.5±0.7	6.9±0.3	5.9±0.3
Aspartic Acid	1.8±0.2	1.4±0.1	1.3±0.08
Glutamine	1.4±0.1	1.2±0.2	1.3±0.06
GABA	1.4±0.2	1.0±0.1	1.0±0.1

Medium Amino Acid Levels (µmoles/100 mg protein/ 2.5 ml)

	Baseline(2)	Control(3)	10 µM Veratridine(3)
Glutamic Acid	0.06±0.01	0.11±0.01	0.25±0.01**
Aspartic Acid	0.05±0.01	0.05±0.005	0.08±0.01*
Glutamine	0.34±0.08	0.55±0.08	0.55±0.10
GABA	#	#	0.09±0.02

Cerebellar slices were preincubated for 13 minutes at which time radioactive glucose and acetate were added. Twenty minutes later, the slices were transferred to fresh Krebs Ringer bicarbonate medium and five minutes later 10 µM veratridine in ethanol or ethanol (5 µl) alone was added and the incubation continued for a further six minutes. The tissue and medium were then separated by centrifugation and amino acids analyzed as described in "Methods". Values are the mean ± S.E.M. for the number of samples indicated in parentheses.

** $p < 0.005$ when compared to control and to baseline.

* $p < 0.05$ when compared to control and to baseline.

GABA was not detected in these samples.

Baseline samples received no drug and were sampled at the beginning of the incubation period with veratridine (6 minutes prior to the sampling of control and veratridine-treated samples).

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A relative change in tritiated amino acid release as compared to ^{14}C-labelled amino acid release is evinced in the ratio of ^{3}H specific radioactivity/^{14}C specific radioactivity. Kainic acid caused no change in the $^{3}H/^{14}C$ specific radioactivity ratio for glutamic acid in the medium compared to control (Table 15). However the specific radioactivity of ^{3}H-glutamic acid in the medium tended to increase by 43% (not significant) and that of ^{14}C-glutamic acid increased by 55% (Table 16). Kainic acid appeared to release newly synthesized ^{3}H-glutamic acid and ^{14}C-glutamic acid. The specific radioactivity of ^{3}H- and ^{14}C-glutamic acid and of ^{3}H-glutamine in the tissue declined (Table 16). The specific radioactivity of ^{14}C-glutamine in the tissue also tended to decline.

Incubation of the slices with veratridine caused a decline of 46% in the $^{3}H/^{14}C$ specific radioactivity ratio for glutamic acid in the medium (Table 15). Veratridine tended to increase the specific radioactivity of ^{14}C-glutamic acid in the medium by 16% (not significant) and decreased that of ^{3}H-glutamic acid in the medium by 38% (Table 17). Hence veratridine preferentially released ^{14}C-glutamic acid. The specific radioactivity of ^{14}C-glutamic acid in the tissue declined slightly by 18%. In contrast to kainic acid, the specific radioactivity of ^{14}C-glutamine in the tissue tended to increase.

Kainic acid released newly synthesized ^{3}H-aspartic acid to the medium. That is, the specific radioactivity

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TABLE 15

EFFECT OF KAINIC ACID AND VERATRIDINE ON THE RATIO OF TRITIUM TO CARBON-14 SPECIFIC RADIOACTIVITY OF AMINO ACIDS IN CEREBELLAR SLICES AND INCUBATION MEDIUM AFTER PRELABELLING WITH D-(2-^{14}C)GLUCOSE AND (^{3}H)ACETATE

	Additions to Medium	Ratio of Tritium to Carbon-14 Specific Radioactivity			
		Glutamic Acid	Aspartic Acid	Glutamine	Total dpm/100 mg protein
Tissue:	Control(4)	17.9±1.7	5.4±0.6	137±13	34.7±2.7
	1 mM KA(4)	13.1±1.0*	5.3±0.4	84±6 #	21.0±1.1#
Medium:	Control(4)	90.1±9.5	-	142±19	23.0±0.9
	1 mM KA(4)	82.9±5.7	18.7±1.1	133±9	27.8±1.2*
Tissue:	Baseline(2)	13.2±0.4	4.2(1)	101±14	21.9±0.5
	Control(3)	12.1±1.8	3.8±0.6	81±8	21.6±2.8
	10 uM VER(3)	21.7±3.2	5.6±0.9	67±7	27.3±3.5
Medium:	Baseline(2)	27.0±0.2	2.7±0.3	74±1	11.1±1.2
	Control(3)	40.8±2.7	2.9±0.2	76±8	12.7±0.7
	10 uM VER(3)	22.0±3.3*	4.5±1.2	70±7	12.7±0.7

Incubation conditions are given in Table 13 for the kainic acid-treated slices and in Table 14 for the veratridine-treated slices. Values are the mean ± S.E.M. for the number of samples in parentheses.

* $p < 0.05$, # $p < 0.01$ when compared to control.

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TABLE 16

EFFECT OF KAINIC ACID ON THE SPECIFIC RADIOACTIVITY OF AMINO ACIDS IN CEREBELLAR SLICES AND INCUBATION MEDIUM AFTER PRELABELLING WITH D-(2-^{14}C)GLUCOSE AND (^{3}H)ACETATE

Tracer	Additions to Medium	Specific Radioactivity (dpm/µmole x 10^{-3}) Glutamic Acid	Aspartic Acid	Glutamine	Total dpm/100 mg protein(x10^{-3})	% of Total dpm in Amino Acids
(2-^{14}C)Glucose						
Tissue:	Control	9.5±0.1	6.3±0.1	6.3±0.5	130±8	80.2±0.7
	1 mM KA	8.6±0.2#	5.9±0.1*	4.6±0.4	101±5*	76.3±1.8
Medium:	Control	5.1±0.6	-	2.3±0.3	360±12	1.2±0.1
	1 mM KA	7.9±0.1**	5.0±0.3	2.5±0.1	391±14	4.0±0.2**
(^{3}H)Acetate						
Tissue:	Control	169±14	34.2±3.6	852±51	4455±168	76.3±1.2
	1 mM KA	113±9*	30.9±2.1	390±56**	2093±62**	54.2±1.9**
Medium:	Control	459±73	43.5±7.0(3)	338±56	8297±44	6.6±0.3
	1 mM KA	657±44	94.1±9.3*	320±32	10854±228**	11.9±0.6**

The basic incubation conditions are given in Table 13. 0.5 µCi (2-^{14}C)Glucose and 5 µCi (^{3}H)acetate were added. Values are mean ± S.E.M. for 4 samples at each point unless the number of samples is given in parentheses.

* $p < 0.05$ when compared to control.

# $p < 0.01$ when compared to control.

** $p < 0.005$ when compared to control.

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TABLE 17

EFFECT OF VERATRIDINE ON THE SPECIFIC RADIOACTIVITY OF AMINO ACIDS IN CEREBELLAR SLICES AND INCUBATION MEDIUM AFTER PRELABELLING WITH D-(2-^{14}C)GLUCOSE AND (^{3}H)ACETATE

Tracer	Additions to Medium	Specific Radioactivity (dpm/µmole x 10^{-3})			Total dpm/100 mg protein (x 10^{-3})	% of Total dpm in Amino Acids
		Glutamic Acid	Aspartic Acid	Glutamine		
(2-^{14}C)Glucose						
Tissue:	Baseline(2)	16.2±1.3		15.1±0.4	299±9	63±10
	Control(3)	15.2±0.6	10.6±0.6	15.2±0.5	203±8	73±3
	10 µM VER(3)	12.5±0.8*	8.6±0.4*	16.6±0.2	170±4*	68±2
Medium:	Baseline(2)	19.1±0.9		15.2±0.4	548±53	1.3±0.3
	Control(3)	13.7±0.6	12.7±1.7	13.6±0.6	586±30	1.6±0.1
	10 µM VER(3)	15.9±1.0	8.1±2.0	13.9±0.1	582±19	2.2±0.1*
(^{3}H)Acetate						
Tissue:	Baseline(2)	215±23		1521±241	6497±1748	64±8
	Control(3)	183±27	39.6±5.5	1236±152	4411±759	67±5
	10 µM VER(3)	266±26	47.7±6.6	1112±107	4602±490	70±2
Medium:	Baseline(2)	515±21		1127±35	6035±48	6.8±1.3
	Control(3)	558±46	35.8±2.6	1028±52	7478±781	8.4±0.6
	10 µM VER(3)	345±41*	31.2±0.3	968±88	7384±657	8.5±0.7

The basic conditions are given in Table 14. Values are mean ± S.E.M. for the number of samples given in parentheses.

* $p < 0.05$ when compared to control.

of ^{3}H-aspartic acid increased by 116% over control (Table 16). The data on ^{14}C-aspartic acid was too variable to form a conclusion. Neither kainic acid nor veratridine changed the specific radioactivity of ^{3}H- or ^{14}C-glutamine in the medium. The amount of radioactivity in GABA in the medium was too slight to measure accurately.

2. Effect of Kainic Acid and Veratridine on the Metabolism of Amino Acids after Preincubation with D-(2-^{14}C)Glucose and (^{3}H)Acetate

In prelabelled slices, the radioactivity in the tissue plus that in the medium for each amino acid should add up to control values if the drug has caused no net loss of a labelled amino acid to metabolism. The total amount of radioactivity in GABA declined during the 5 minute sampling interval between the baseline samples and the control samples, neither of which received drug, whereas the radioactivity in the other amino acids did not decrease significantly. The total content of carbon-14 radioactivity in GABA decreased 37% and the total amount of tritium in GABA decreased 31% (Table 18).

In the slices exposed to kainic acid, there was a 63% decrease in the total amount of tritium in glutamine compared to control (Table 18). There was a 53% increase in the quantity of tritium in aspartic acid and no change in glutamic acid or GABA. The total amount of tritium in the amino acids decreased by 39%. However the total

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TABLE 18

See Tables 13 and 14 for details about the incubation conditions. Results are expressed as the mean ± S.E.M. for the number of samples shown in parentheses. Control samples were treated similarly to samples to which KA (kainic acid) or VER (veratridine) were added. Baseline samples had no drug additions but were sampled at the beginning of the incubation period with veratridine (6 minutes prior to the sampling of control and veratridine-treated samples).

# $p < 0.05$ when compared to control.

** $p < 0.005$ when compared to control.

* $p < 0.05$ when compared to baseline.

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TABLE 18

EFFECT OF KAINIC ACID AND VERATRIDINE ON THE COMBINED TISSUE AND MEDIUM RADIOACTIVITY PRESENT IN GLUTAMIC ACID, ASPARTIC ACID, GLUTAMINE, GABA AND THE PCA EXTRACT AFTER PRELABELLING WITH D-(2-^{14}C)GLUCOSE AND (^{3}H)ACETATE

Tracer	Additions to Medium	Tissue plus medium radioactivity (dpm/100 mg protein x 10^{-4}) present in:				
		Glutamic Acid	Aspartic Acid	Glutamine	GABA	PCA extract
(^{3}H)Acetate						
	Control(4)	143±7	6.4±0.5(3)	238±13		1275±55
	1 mM KA(4)	152±5	9.9±0.9#	74±6**		1295±29
(2-^{14}C)Glucose						
	Control(4)	7.6±0.5	1.1±0.1(3)	1.8±0.2		49±1
	1 mM KA(4)	7.0±0.4	1.2±0.4	0.7±0.1**		49±2
(^{3}H)Acetate						
	Baseline(2)	166±32	8.5±1.6	256±11	11.0±1.0	1253±170
	Control(3)	133±21	6.0±1.2	217±51	7.7±0.8	1189±149
	10 µM VER(3)	167±21	6.6±1.1	204±26	6.8±0.1*	1199±109
(2-^{14}C)Glucose						
	Baseline(2)	12.3±2.1	2.1±0.3	2.7±0.2	1.4±0.2	85±14
	Control(3)	10.6±0.2	1.6±0.1	2.7±0.4	0.9±0.1*	79±4
	10 µM VER(3)	7.8±0.4**	1.2±0.02*#	3.0±0.1	0.9±0.1*	75±2

content of tritium in the PCA extract was unaltered. Kainic acid caused no alterations in the total content of carbon-14 radioactivity measured in the PCA extract or in any amino acid except for a decline in glutamine.

In the slices exposed to veratridine, the total amount of carbon-14 radioactivity in glutamic acid decreased by 38% and that in aspartic acid decreased by 24% (Table 18). The total content of carbon-14 radioactivity in the amino acids declined by 18%. There was no alteration in the total amount of carbon-14 present in the PCA extract. Veratridine caused no alterations in the total content of tritium measured in the PCA extract or in any of the amino acids.

I. EFFECT OF KAINIC ACID ON GLUTAMIC ACID METABOLISM IN CEREBELLAR SLICES

1. Introduction

The marked decrease in glutamine levels as well as other effects of kainic acid on glutamic acid metabolism were further examined. Cerebellar slices were preincubated for 10 to 15 minutes, then kainic acid was added to some samples with or without transferring the slices. Twenty minutes later radioactive precursor was added and the incubation continued for 10 minutes after the addition of radioactivity. The effects in both transferred and untransferred slices were examined in preliminary experiments.

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Transferred slices were then used exclusively after noting that the specific radioactivity of glutamine relative to that of glutamic acid was enhanced in the transferred cerebellar slices when using the precursors (^{3}H)acetate, D-(2-^{14}C)glucose and (U-^{14}C)GABA (Table 20). The relative specific radioactivity (RSA) values for glutamine (glutamic acid = 1) in transferred slices more closely resembled results obtained _in vivo_ (Balazs and Cremer, 1973). Transferring of slices and the temperature of preincubation were previously reported to affect the RSA of glutamine (Berl et al., 1968). The effects of kainic acid on amino acid metabolism follows. The alterations in amino acid levels that kainic acid caused during these incubations are shown in Tables 6 and 7.

2. Incorporation of D-(2-^{14}C)Glucose and (^{3}H)-Acetate in Transferred Slices

In transferred slices exposed to 1 mM kainic acid for 30 minutes, (^{3}H)acetate incorporation into amino acids decreased markedly but there was little change in (2-^{14}C)glucose incorporation. The total ^{3}H-radioactivity in the PCA extract decreased 46% compared to the control slices; the percentage of tritium in the PCA extract which was incorporated into amino acids decreased by 70% (Table 19). It is unlikely that an inhibition of (^{3}H)acetate uptake rather than an inhibition of incorporation accounted for the decrease in total acid soluble radioactivity in the PCA extract. If the amount of radioactivity in the amino

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TABLE 19

EFFECT OF KAINIC ACID ON D-(2-^{14}C)GLUCOSE AND (^{3}H)ACETATE METABOLISM IN TRANSFERRED CEREBELLAR SLICES

	Specific Radioactivity (dpm/µmole x 10^{-3})				Ratio of tritium to carbon-14 specific radioactivity	
	(2-^{14}C)Glucose		(^{3}H)Acetate			
	Control	1 mM KA	Control	1 mM KA	Control	1 mM KA
Glutamic Acid	18.5±0.7	21.9±0.3*	500±56	195±22**	26.9±2.4	8.9±1.0**
Aspartic Acid	10.6±1.0	10.0±0.6	76±13	46±9	7.2±0.7	4.5±0.6#
Glutamine	16.9±2.5	14.8±1.4	4086±872	567±85*	235.0±21.0	38.0±3.7**
GABA	10.3±0.8	11.3±0.8	123±14	108±4	12.0±0.7	7.6±1.8
Total d.p.m./100 mg protein x 10^{-3}						
	1207±62	1120±85	24114±1809	13121±1068**	20.0±1.1	11.7±0.3**
Percentage of total d.p.m./100 mg protein in amino acids						
	12.3±1.0	11.3±0.9	37.6±3.5	9.7±0.5**		

Incubation conditions are given in Table 6. 0.5 uCi (2-^{14}C)Glucose and 10 uCi (^{3}H) acetate were added. Values are mean ± S.E.M. for 4 experiments at each point.

# $p < 0.05$ when compared to control.

* $p < 0.01$ when compared to control.

** $p < 0.005$ when compared to control.

acids is subtracted from that in the PCA extract, the remaining amount of radioactivity is very nearly the same in the control and kainic acid-treated slices indicating that the ^{3}H-acetate level in the slices were the same. The rate of labelling from tritium of glutamic acid and glutamine declined. There was an 86% reduction in the specific radioactivity of glutamine and 61% decrease in that of glutamic acid. Since the specific radioactivity of glutamine was lowered to a greater extent than was that of glutamic acid, the RSA of glutamine to glutamic acid was significantly decreased from control values by 64% (Table 20). The RSA of GABA increased by 106% and that of aspartic acid by 53%.

Kainic acid caused only slight alteration in (2-^{14}C) glucose metabolism. The total ^{14}C radioactivity in the PCA extract as well as the percentage of the total PCA soluble radioactivity isolated in amino acids was unchanged (Table 19). The total ^{14}C radioactivity in glutamine decreased proportionally with the level of glutamine; therefore, the glutamine-specific radioactivity was unaffected. There was an 18% increase in glutamic acid-specific radioactivity possibly reflecting the decreased pool size. The RSAs of the ^{14}C-labelled amino acids were not altered by kainic acid (Table 20).

The relative changes in (^{3}H)acetate utilization as compared to those of (2-^{14}C)glucose were evinced by decreases in the $^{3}H/^{14}C$ ratio of specific radioactivities

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TABLE 20

Results are expressed as mean $\pm$ S.E.M. for 4 samples at each point and are the specific radioactivity (dpm/µmole or cpm/µmole) of the amino acids compared to that of glutamic acid which is taken as 1. These results are derived from the experiments listed in Tables 19, 21, 22, and 23.

* $p < 0.05$ when compared to control (no additions).

\# $p < 0.02$ when compared to control.

** $p < 0.005$ when compared to control.

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TABLE 20

RELATIVE SPECIFIC RADIOACTIVITIES (RSA) OF GLUTAMINE, GABA AND ASPARTIC ACID FROM CEREBELLAR SLICES INCUBATED WITH (^{3}H)ACETATE AND D-(2-^{14}C)GLUCOSE, (U-^{14}C)GABA, (U-^{14}C)-GLUTAMIC ACID OR (U-^{14}C)GLUTAMINE IN MEDIUM CONTAINING 1 mM KAINIC ACID

Tracer Added		Additions to Medium	Relative Specific Radioactivity		
			Glutamine	Aspartic Acid	GABA
(^{3}H)Acetate	Transferred	None	7.9±0.9	0.15±0.01	0.25+0.01
		1 mM KA	2.9±0.1**	0.23±0.02#	0.51±0.02**
	Untransferred	None	2.8±0.02	0.20±0.02	0.22±0.05
		1 mM KA	2.8±0.5	0.31±0.03*	0.41±0.07
(2-^{14}C)Glucose	Transferred	None	0.90±0.09	0.57±0.04	0.55+0.03
		1 mM KA	0.68±0.07	0.46±0.03	0.52±0.03
	Untransferred	None	0.38±0.04	0.64±0.10	0.64±0.05
		1 mM KA	0.53±0.05	0.52±0.07	0.51±0.07
(U-^{14}C)GABA	Transferred	None	1.7±0.2	10.5±0.9	63.1±4.1
		1 mM KA	0.36±0.06**	11.2±1.4	51.2+5.0
	Untransferred	None	0.50±0.02	6.9±0.3	62.1±7.7
		1 mM KA	0.27±0.03**	7.3±0.3	37.4±3.8*
(U-^{14}C)Glutamic Acid	Transferred	None	4.4±0.1	0.57±0.01	
		1 mM KA	3.2±0.5*	0.49±0.03	
(U-^{14}C)Glutamine	Transferred	None	10.1±0.3	0.48±0.02	0.32±0.02
		1 mM KA	20.3±2.8#	0.34±0.03**	0.33±0.05

for glutamic acid (67%), aspartic acid (37%), and glutamine (84%) (Table 19). The $^{3}H/^{14}C$ specific radioactivity ratio for GABA also appeared to decrease but this was not statistically significant because of the scatter in the data. The $^{3}H/^{14}C$ ratio for total acid soluble radioactivity found in the tissue decreased by 42%. As was indicated above this was due entirely to the decreased labelling of amino acids.

3. Incorporation of D-(2-^{14}C)Glucose and (^{3}H)-Acetate in Untransferred Slices

Similar decreases in the $^{3}H/^{14}C$ ratio of specific radioactivities for the amino acids and in the PCA extract were apparent in untransferred cerebellar slices. 1 mM kainic acid caused a 48% decrease in the total ^{3}H radioactivity in the PCA extract; the percentage of tritium in the PCA extract which was present in amino acids decreased by 66% (Table 21). There was a 65% reduction in the specific radioactivities of glutamine and glutamic acid. The specific radioactivities of aspartic acid and GABA were not significantly reduced, which was a reflection of the greater scatter in the data which occurred in untransferred slices. The RSA of ^{3}H-aspartic acid increased slightly. The RSA of ^{3}H-glutamine was approximately threefold lower than in transferred slices, and in marked contrast to transferred slices, kainic acid did not decrease the RSA of ^{3}H-glutamine (Table 20).

The total ^{14}C radioactivity from (2-^{14}C)glucose

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TABLE 21

EFFECT OF KAINIC ACID ON D-(2-^{14}C)GLUCOSE AND (^{3}H)ACETATE METABOLISM IN UNTRANSFERRED CEREBELLAR SLICES

	Specific Radioactivity (dpm/µmole x 10^{-3})				Ratio of tritium to carbon-14 specific radioactivity	
	(2-^{14}C)Glucose		(^{3}H)Acetate			
	Control	1 mM KA	Control	1 mM KA	Control	1 mM KA
Glutamic Acid	5.0±0.1	6.6±0.2**	398±52	135±10**	80.4±9.0	20.5±1.4**
Aspartic Acid	3.2±0.6	3.5±0.4	83±16	42±5	25.7±1.1	11.8±0.5**
Glutamine	1.9±0.2	3.4±0.5#	1084±147	394±88*	590.0±91.5	132.0±22.0**
GABA	3.2±0.3	3.4±0.5	88±22	58±13	27.4±6.1	17.9±4.1
Total d.p.m./100 mg protein x 10^{-3}						
	412±9	395±22	21445±1505	11091±632**	50.3±3.6	30.1±0.4**
Percentage of total d.p.m./100 mg protein in amino acids						
	12.8±0.2	12.6±0.6	32.7±2.6	11.0±0.4**		

Results are expressed as the mean ± S.E.M. for 4 experiments at each point. The basic conditions are given in Tables 6 & 19 except that slices were not transferred to fresh medium at the end of the 15 minute preincubation.

# $p < 0.05$ when compared to control.

* $p < 0.01$ when compared to control.

** $p < 0.005$ when compared to control.

present in the PCA extract as well as the percentage of the ^{14}C radioactivity in the PCA extract isolated in amino acids was unaltered (Table 21). There was an increase in the specific radioactivities of glutamic acid (32%) and glutamine (81%), but no alterations in the RSAs.

4. Incorporation of (U-^{14}C)GABA

Other labelling studies were performed to ascertain the site of the inhibition of labelling of glutamine. (U-^{14}C)GABA was the precursor chosen because, similar to acetate, it is a precursor of the "small" compartment of glutamic acid metabolism (see Berl et al., 1975). When transferred slices were incubated with (U-^{14}C)GABA in the presence of 1 mM kainic acid, there was a large, selective decrease (75%) in glutamine-specific radioactivity (Table 22) and the RSA of glutamine decreased by 80% (Table 20). The specific radioactivities of aspartic acid, glutamic acid, and GABA were not affected. The total ^{14}C radioactivity present in the PCA extract was also unaltered.

When untransferred cerebellar slices were incubated with (U-^{14}C)GABA in the presence of kainic acid, the specific radioactivity of glutamic acid increased by 37% and that of aspartic acid increased by 46% (Table 23). The RSA of glutamine decreased by 46% and that of GABA decreased by 40% (Table 20).

5. Incorporation of L-(U-^{14}C)Glutamic Acid

Kainic acid is a moderately potent inhibitor of glutamic acid uptake into cortical minces (Johnston et

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TABLE 22

INFLUENCE OF KAINIC ACID ON THE LABELLING OF AMINO ACIDS FROM L-(U-^{14}C)GLUTAMIC ACID, L-(U-^{14}C)GLUTAMINE AND (U-^{14}C)GABA IN TRANSFERRED CEREBELLAR SLICES

	Specific Radioactivity (cpm/µmole x 10^{-3})					
	(U-^{14}C)GABA		L-(U-^{14}C)Glutamic Acid		L-(U-^{14}C)Glutamine	
	Control	1 mM KA	Control	1 mM KA	Control	1 mM KA
Glutamic Acid	42±2	47±5	1082±44	635±38**	191±10	137±14*
Aspartic Acid	429±39	515±75	620±16	308±6**	92±4	47±6**
Glutamine	71±7	17±4**	4781±158	2022±284**	1936±139	2767±454
GABA	2592±217	2464±436	1506±314	629±50*	60±4	45±8
Glutamic Acid (medium)			109148±8685	118502±998		
Total c.p.m./100 mg protein x 10^{-3}						
	4151±557	4158±217	12316±356	4464±296**	3476±73	1851±61**
Percentage of total c.p.m./100 mg protein in amino acids						
	90.2±1.6	96.0±3.1	85.0±1.4	80.1±1.1*	85.7±0.7	86.6±3.2

Cerebellar slices were preincubated for 15 minutes and then transferred and 1 mM kainic acid was added to some samples. 20 minutes later, 1 µCi of (U-^{14}C)glutamic acid (and 45 nmoles of cold glutamic acid was added to controls) or 30 minutes later 0.2 µCi (U-^{14}C)GABA or 0.3 µCi (U-^{14}C)glutamine was added to samples. Incubation with (U-^{14}C)glutamic acid continued for 13 min. Incubation with (U-^{14}C)GABA or (U-^{14}C) glutamine continued for 10 min. Values are mean ± S.E.M. for 4 samples at each point. * $p < 0.05$; ** $p < 0.005$ when compared to control.

al., 1979) and into cortical synaptosomes (Table 10). Not surprisingly, therefore, in transferred cerebellar slices incubated with (U-^{14}C)glutamic acid and kainic acid, the specific radioactivities of glutamic acid, glutamine, aspartic acid and GABA decreased as did the total ^{14}C radioactivity in the PCA extract (Table 22). There was no change in the percentage of the ^{14}C in the amino acids. The RSA of glutamine decreased less (28%) compared to the decrease from radioactive acetate or GABA (Table 20). It should be noted that non-radioactive glutamic acid was added to the incubation medium of controls immediately prior to addition of radioactivity in order that the specific radioactivity of glutamic acid in the medium of control and kainic acid-treated slices would be the same; the measured specific radioactivity of glutamic acid in the medium at the end of the experiment was 109,148 $\pm$ 8685 cpm/nmole (n=4) in control slices and 118,502 $\pm$ 998 cpm/nmole (n=4) in kainic acid-treated slices. The control slices accumulated and metabolized much of the added glutamic acid. Glutamic acid levels in the medium at the end of the incubation were 0.37 $\pm$ 0.03 nmoles/100 mg protein, mean $\pm$ S.D., n=4, in the control slices and 1.15 $\pm$ 0.15 nmoles/100 mg protein in the kainic acid-treated slices.

6. Incorporation of L-(U-^{14}C)Glutamine

Since the decrease in glutamine levels could be due to increased glutamine breakdown, transferred

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TABLE 23

EFFECT OF KAINIC ACID ON THE LABELLING OF AMINO ACIDS FROM (U-^{14}C)GABA IN UNTRANSFERRED CEREBELLAR SLICES

	Specific Radioactivity (cpm/µmole x 10^{-3})	
	(U-^{14}C)GABA	
	Control	1 mM KA
Glutamic Acid	231±18	317±10*
Aspartic Acid	1594±121	2321±118*
Glutamine	115±13	84±9
GABA	14105±1300	11810±1183
Total c.p.m./100 mg protein x 10^{-3}		
	17396±1467	19062±1815
Percentage of total c.p.m./100 mg protein in amino acids		
	85.6±1.9	89.7±0.9

Cerebellar slices were preincubated for 15 minutes when 1 mM kainic acid was added to some samples. 30 minutes later 0.5 µCi (U-^{14}C)GABA was added and the incubation continued for 15 minutes. Values are the mean ± S.E.M. for 4 samples at each point.

* $p < 0.01$ when compared to control.

cerebellar slices were incubated with (U-^{14}C)glutamine. The specific radioactivity of glutamine was slightly but not significantly elevated in the kainic acid-treated slices. However the specific radioactivities of glutamic acid and aspartic acid decreased by 29% and by 50% respectively (Table 22). The RSA of glutamine increased by 100% and that of aspartic acid decreased by 29% (Table 20). There was a 47% decrease in ^{14}C radioactivity in the PCA extract, but no change in the percentage of the ^{14}C radioactivity in the PCA extract which was incorporated into the amino acids. In cortical synaptosomes kainic acid did not significantly inhibit glutamine uptake (Table 11).

J. EFFECT OF KAINIC ACID ON GLUTAMINE SYNTHETASE

Because glutamine levels were markedly decreased and the labelling data indicated that this was due to a decrease in glutamine synthesis, the effect of kainic acid on glutamine synthetase activity was tested as described by Nicklas et al. (1979b). Kainic acid at 1 mM did not directly alter the glutamine synthetase activity of either homogenates of rat cerebellum or of cerebellar slices incubated with kainic acid for 45 minutes prior to homogenization and assay.

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K. LIGHT MICROSCOPY OF CEREBELLAR SLICES

Garthewaite et al. (1979) have reported that $\geq 90\%$ of cells in chopped cerebellar slices appeared pyknotic and that much vacuolation was present, whereas in hand-sliced cerebellar slices, no pyknosis and little vacuolation was evident. Therefore, a light microscopic study was performed on chopped cerebellar slices which had been incubated for 45 minutes as in the biochemical studies. Some slices were exposed to kainic acid (1 mM) for 30 minutes. As demonstrated in Figures 9 and 10, the majority of the cells in the control or kainic acid-treated slices were not pyknotic. It is easiest to observe that most Purkinje cells are not pyknotic and that little vacuolation is present. Also, in the kainic acid-treated slices, no gross neuronal toxicity is evident during thistime of incubation.

L. ACTION OF KAINIC ACID ON THE INCORPORATION OF D-(2-^{14}C)GLUCOSE AND (^{3}H)ACETATE INTO AMINO ACIDS OF STRIATAL SLICES

In contrast to cerebellar slices, when striatal slices were incubated with 1 mM kainic acid for 30 minutes, the level of glutamine decreased slightly by 18% and aspartic acid decreased by 26% (Table 24). No alteration in the level of glutamic acid or GABA in the tissue was observed.

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FIGURE 9. APPEARANCE OF PARASAGITTAL CEREBELLAR SLICES INCUBATED FOR 45 MINUTES IN KREBS-RINGER BICARBONATE MEDIUM

Molecular (m), Purkinje (p), and granular (g) layers are indicated. Magnifications A) x600. B) x1080. The slices were stained with hematoxylin and eosin.

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FIGURE 10. APPEARANCE OF PARASAGITTAL CEREBELLAR SLICES INCUBATED WITH KAINIC ACID

Molecular (m), Purkinje (p), and granular (g) layers are indicated. Magnifications A) x600. B) x1080. The slices were stained with hematoxylin and eosin.

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B

A

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Incorporation of D-(2-^{14}C)glucose and (^{3}H)acetate in the presence and absence of 1 mM kainic acid was studied. (^{3}H)acetate incorporation into amino acids was not altered for glutamine, glutamic acid, aspartic acid or GABA. Slight alterations in the incorporation of (2-^{14}C)glucose occurred. The total ^{14}C radioactivity in the PCA extract declined by 18% (Table 25) which was not accounted for by amino acids. The ^{14}C specific radioactivity of glutamic acid increased slightly by 11% and that of aspartic acid increased by 47%. The specific radioactivity of glutamine decreased by 24% and that for GABA decreased by 29%. In contrast to cerebellar slices, the RSA of ^{3}H-glutamine was unaltered but the RSA of ^{14}C-glutamine decreased slightly (by 33%)(Table 26). The ratio of ^{3}H specific radioactivity to ^{14}C specific radioactivity for glutamine tended to increase in the presence of kainic acid.

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TABLE 24

EFFECT OF KAINIC ACID ON AMINO ACID LEVELS IN STRIATAL SLICES

	Amino Acid Levels (μmoles/100 mg protein)	
	Control	1 mM Kainic Acid
Glutamic Acid	3.4±0.5	3.1±0.2
Aspartic Acid	1.7±0.1	1.3±0.1**
Glutamine	0.46±0.01	0.38±0.03**
GABA	0.8±0.1	0.9±0.1

Striatal slices were preincubated for 15 minutes in Krebs-Ringer bicarbonate medium in the presence or absence of kainic acid. Radioactivity was then added and the incubation continued for a further 15 minutes. The tissue and medium were separated by filtration and the tissue was analyzed for amino acids and radioactivity as described in the "Methods". Results are expressed as the mean ± S.E.M. for 4 samples at each point.
**$p < 0.05$ when compared to control.

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TABLE 25

EFFECT OF KAINIC ACID ON D-(2-^{14}C)GLUCOSE AND (^{3}H)ACETATE METABOLISM IN STRIATAL SLICES

	Specific Radioactivity (dpm/µmole x 10^{-3})				Ratio of tritium to carbon-14 specific radioactivity	
	(2-^{14}C)Glucose		(^{3}H)Acetate			
	Control	1 mM KA	Control	1 mM KA	Control	1 mM KA
Glutamic Acid	12.7±0.3	14.0±0.5*	715±13	747±26	56.6±0.9	52.1±1.3
Aspartic Acid	4.2±0.4	6.1±0.4**	61±3	74±4	13.2±0.4	11.6±0.3
Glutamine	13.2±0.9	10.0±0.9*	6591±430	7282±246	509.0±51.5	679.0±65.5
GABA	3.2±0.2	2.2±0.3**	185±8	145±17	55.6±1.0	62.7±2.3
Total d.p.m./100 mg protein x 10^{-3}						
	242±4	198±17*	7806±198	6549±547	32.3±0.4	33.1±0.6
Percentage of total d.p.m./100 mg protein in amino acids						
	26.4±0.3	28.5±2.0	75.7±0.8	78.1±3.0		

Striatal slices were preincubated for 15 minutes in the presence or absence of 1 mM kainic acid. 0.5 µCi (2-^{14}C)Glucose and 10 µCi of (^{3}H)acetate were then added to the medium and the incubation continued for 15 minutes and amino acids were analyzed as described in "Methods". Values are mean ± S.E.M. for 4 experiments at each point.

* $p<0.05$ when compared to control

** $p<0.02$ when compared to control.

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TABLE 26

RELATIVE SPECIFIC RADIOACTIVITIES OF GLUTAMINE, GABA AND ASPARTIC ACID IN STRIATAL SLICES INCUBATED WITH (^{3}H)ACETATE AND D-(2-^{14}C)GLUCOSE IN MEDIUM CONTAINING 1 mM KAINIC ACID

		Relative Specific Radioactivity		
Tracer Added	Additions to Medium	Glutamine	Aspartic Acid	GABA
(^{3}H)Acetate	None	9.33±0.64	0.09±0.003	0.25±0.02
	1 mM Kainic Acid	9.74±0.15	0.10±0.004	0.20±0.02
(2-^{14}C)Glucose	None	1.06±0.08	0.36±0.002	0.26±0.01
	1 mM Kainic Acid	0.71±0.04*	0.44±0.020	0.16±0.03*

Values are the mean ± S.E.M. for 4 experiments at each point with the specific radioactivity of glutamic acid taken as 1. The incubation conditions are given in Tables 24 and 25.

* $p < 0.02$ when compared to control.

IV. DISCUSSION

A. EFFECTS OF KAINIC ACID ON ATP LEVELS

Based on evidence that cortical input is necessary for kainic acid neurotoxicity in the striatum (McGeer et al., 1978a; Biziere and Coyle, 1978a; Whetsell et al., 1980), McGeer postulated that the neurotoxicity of kainic acid involves a release and/or inhibition ofthe uptake of glutamic acid. Olney (Olney, 1978; Olney et al., 1971) postulated that excitotoxic amino acids including kainic acid generate a state of continuous depolarization in affected neurons and an increase in membrane permeability which leads to neuronal cell death, presumably by depletion of energy reserves. Indeed, several excitatory amino acids decrease levels of creatine phosphate (Bradford and McIlwain, 1966) and ATP (Pull and McIlwain, 1975) in cerebral cortical slices. However, kainic acid, in striatal slices, has no effect on ATP levels in concentrations up to 3 mM (Biziere and Coyle, 1978b). The present <u>in vitro</u> studies were performed using cerebellar slices because evidence indicates that the cerebellum has an intrinsic glutamergic interneuron, the granule cell (Young et al., 1974; McBride et al., 1976; Tran and Snyder, 1979).

In these studies, kainic acid at concentrations of 0.1 to 1.0 mM incubated with cerebellar slices did indeed cause significant decreases in creatine phosphate and ATP

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levels. Kainic acid did not decrease ATP levels by directly altering oxidative-phosphorylation of brain mitochondria (Nicklas, unpublished observations). However, since Na^+-K^+ ATPase activity is sensitive to changes in Na^+ concentration (Skou, 1957; Schwartz et al., 1962), kainic acid might decrease ATP levels by stimulation of Na^+-K^+ ATPase activity following a depolarization-induced increase in Na^+ permeability. Various excitatory amino acids including L-glutamic acid, DL-homocysteic acid, L-aspartic acid and N-methyl-D-aspartic acid exert a strong depolarizing action associated with an increased permeability to Na^+ *in vivo* (Curtis et al., 1972; Zieglgansberger and Puil, 1973) and *in vitro* (Bradford and McIlwain, 1966). The present findings show that L-glutamic acid and DL-homocysteic acid also decreased ATP levels. However N-methyl-D-aspartic acid which was also reported to increase Na^+ permeability did not significantly affect ATP levels. The particularly large decrease in ATP caused by kainic acid probably involves actions in addition to a postsynaptic stimulation of Na^+-K^+ATPase activity.

In our studies, kainic acid depressed ATP levels without causing a corresponding increase in the amount of ADP plus AMP present. In cerebellar slices from the rat, Schmidt et al. (1977; 1976) have reported 400 fold increases in the level of cyclic AMP in the presence of kainic acid, possibly mediated by kainic acid-induced release of adenosine. Thus activation of adenylate cyclase could account

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for some of the decrease in ATP levels. Also, exposure of cortical slices to L-glutamic acid and to electrical stimulation has been reported to increase tissue adenosine levels and to release adenine derivatives into the medium (Pull and McIlwain, 1975; Newman and McIlwain, 1977).

Tetrodotoxin selectively blocks excitable sodium channels thus it blocks the depolarization and the influx of $^{22}Na^{+}$ associated with electrical stimulation (McIlwain et al., 1969; Narahashi et al., 1964) and it blocks veratridine-induced depolarizations (Ohta et al., 1973). In contrast, tetrodotoxin does not block depolarizations elicited by L-glutamic acid (Zieglansberger and Puil, 1972) or DL-homocysteic acid (Curtis et al., 1972) and only slightly blocks the influx of $^{22}Na^{+}$ induced by L-glutamic acid (Chang and Michaelis, 1980; McIlwain et al., 1969).

In the present studies, tetrodotoxin did not block the decrease in ATP levels caused by kainic acid, but did inhibit the decrease in ATP caused by veratridine, a presynaptic depolarizing agent. This supports the notion that veratridine causes a drop in neuronal ATP levels. The results with kainic acid, although consistent with the electrophysiological data on similar excitatory amino acids, does not provide information on the site of the depression in ATP levels.

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B. EFFECT OF KAINIC ACID ON OXYGEN CONSUMPTION

It has been suggested that the increase in respiratory rates caused by depolarizing amino acids in cortical slices (Cox et al., 1977) is primarily a neuronal phenomenon. Presumably, increases in postsynaptic ion fluxes following depolarization lead to increased ion pump activity and an increased oxidation of glucose to provide energy in neurons. Our data are consistent with that possibility. That is, kainic acid and glutamic acid increased the respiratory rate of cerebellar slices. Kainic acid increased the specific radioactivity of glutamic acid and aspartic acid when labelled from D-(2-^{14}C)glucose (which reflects primarily neuronal metabolism) (Table 19), whereas no increase in the specific radioactivity of glutamic acid or aspartic acid was observed when "small" compartment precursors such as (U-^{14}C)GABA, L-(U-^{14}C)-glutamic acid or (^{3}H)acetate were used (Tables 19 and 22). Watkins (1971a,b) similarly found that the incorporation of ^{14}C-glucose was enhanced and that of ^{14}C-acetate was decreased into glutamic acid and aspartic acid when N-methyl-D-aspartic acid was injected into nembutalized mice. However it has been shown that the concentration of K^+ in the medium increases immediately following the application of excitatory amino acids to the spinal cord of the frog (Evans, 1980). Hertz et al. (1973) have found that elevated concentrations of K^+ increase oxygen consump-

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tion in glia but not in neurons. Thus the increase in oxygen consumption may indirectly occur in glia also.

C. EFFECTS OF KAINIC ACID ON AMINO ACID METABOLISM - EVIDENCE FOR GLIAL ALTERATIONS

An unexpected finding in these studies was the 60-70% depression of glutamine levels in the kainic acid-treated cerebellar slices which was not accounted for by a concomitant increase in the medium. To further investigate this, labelling studies were performed which can be interpreted by recognizing that several glutamic acid pools exist in nervous tissue each associated with distinct citric acid cycles and that at least one glutamic acid pool is associated with glutamine synthesis (Berl et al., 1975; Fonnum, 1978). There are several precursors of the heterogeneous "small" compartment of glutamic acid metabolism which yield a relative specific radioactivity (RSA) of glutamine $>$ 1 (glutamic acid = 1) and which can be demonstrated both *in vivo* and *in vitro*. About 10% of the glutamic acid in brain tissue is estimated to readily mix with this compartment. Its precursors include GABA, acetate and exogenous glutamic acid. Precursors of the citric acid cycle associated with the "large" pool of glutamic acid metabolism, which is estimated to contain 80-90% of glutamic acid in nervous tissue, include glucose and pyruvate. Such precursors yield a RSA of glutamine

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approximately equal to 1. The "small" pool of glutamic acid has been suggested to be primarily associated with glia and the "large" pool of glutamic acid to be associated with neurons. These considerations can be used to explain anomalies observed in labelling from various precursors.

For example, one possible reason for the low RSA of glutamine from radioactive acetate and GABA observed in the untransferred control slices (Table 20) is that the levels of glutamic acid in the medium are approximately 25 μM due to spontaneous leakage compared to about 3-4 μM in the medium of transferred control slices. Because 25 μM is approximately the K_m for the high affinity transport system for glutamic acid (see Cox and Bradford, 1978), glutamic acid from the medium will be actively taken up into the slices. A greater uptake of glutamic acid from the medium into the pool of glutamic acid that synthesizes glutamine than into other pools of glutamic acid would result in a relatively greater dilution of the specific radioactivity of the precursor pool of glutamic acid for glutamine synthesis and thus a lower RSA of glutamine. Indeed, there is evidence that glial cells, where a large proporation of glutamine synthesis may occur, have an avid affinity for exogenous glutamic acid (Benjamin and Quastel, 1976; Hertz et al., 1978). The preferential uptake of exogenous glutamic acid by glial cells may also explain the fact that exogenous L-glutamic acid acts as a "small" compartment precursor.

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When transferred cerebellar slices were incubated in the presence of kainic acid, glutamine synthesis appeared to be inhibited in pool(s) of glutamine labelled by acetate, GABA and glutamic acid. This inhibition presumably occurs in glia although other compartments may also be involved. That is, most evidence supports a glial localization for glutamine synthetase activity and for acetate utilization via acetate thiokinase (Berl et al., 1975; Norenberg and Martinez-Hernandez, 1979; Nicklas et al., 1979a,b). However some glutamine synthetase (Dennis et al., 1980) and acetate thiokinase (Lai et al., 1977) activity has been reported in synaptosomal fractions or in mitochondria derived from synaptosomal preparations, although synaptosomal preparations may be contaminated with glial fragments or with free mitochondria. A large proportion of GABA is also proposed to be metabolized in the same compartment in which acetate thiokinase and glutamine synthetase are localized (Van den Berg et al., 1975).

The RSA of glutamine was less markedly reduced by kainic acid when radioactive L-glutamic acid was the precursor than from radioactive GABA or acetate (Table 20). This could be explained if kainic acid preferentially inhibits L-glutamic acid uptake into nerve terminals rather than into glia or if kainic acid decreases the quantity of glutamic acid in the "small" pool possibly by releasing it; thus the "small" pool of glutamic acid (precursor for glutamine) would be labelled to a higher specific

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radioactivity than the "large" pool of glutamic acid. An inhibition of glutamic acid transport to the site of glutamine synthesis probably does not occur to explain the inhibition of glutamine synthesis. In other experiments, kainic acid did not inhibit glutamine synthetase directly. The decrease in glutamine synthesis more likely results from an accompanying decline in ATP at the same site.

Isolated acetate thiokinase can be inhibited by high concentrations of Na^+ (Von Korff, 1953). Chan and Quastel (1967) found that the rate of formation of $^{14}CO_2$ from ^{14}C-acetate by cortical slices was decreased by electrical stimulation and that decline was blocked by tetrodotoxin. Watkins (1971a) found that the *in vivo* labelling of glutamine, glutamic acid, aspartic acid and GABA from (U-^{14}C)acetate in nembutalized mice was decreased upon co-injection with L-glutamic acid, aspartic acid or N-methyl-D-aspartic acid. However Watkins did not measure the specific radioactivities of the amino acids; and the effect of nembutal on the labelling of amino acids from acetate is unknown. In the present studies, kainic acid decreased the total incorporation of label from radioactive acetate into glutamic acid, glutamine, and aspartic acid, but interestingly not into GABA.

It has been suggested that depolarization-induced changes in Na^+ permeability alter neuronal utilization of acetate via inhibition of acetate thiokinase (Watkins, 1971a; Chan and Quastel, 1967). Inhibition of acetate

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incorporation does not necessarily occur in response to increased uptake of Na^+. Although 1 mM kainic acid increased Na^+ uptake into striatal slices (Biziere and Coyle, 1978b), in our hands, 1 mM kainic acid did not inhibit acetate incorporation into amino acids (Table 25) and it did not decrease ATP levels in striatal slices. It may be that depletion of the pool of ATP required for the conversion of acetate to acetyl CoA caused inhibition of acetate incorporation. Possibly the enhanced uptake of Na^+ into striatal slices caused by kainic acid did not occur in the compartment containing acetate thiokinase.

In guinea pig cortical slices, 27 mM K^+ (Berl et al., 1968) alters amino acid metabolism differently from the alterations observed with kainic acid in the rat. However, ouabain, an inhibitor of Na^+-K^+ ATPase at the plasma membrane which increases intracellular sodium and decreases intracellular potassium (Swanson, 1968), caused strikingly similar effects on "small" compartment glutamic acid metabolism (Berl et al., 1970a) as kainic acid. Ouabain inhibits both glial Na^+-K^+ ATPase which may cause its "small" compartment effects and nerve terminal Na^+-K^+ ATPase (Grisar et al., 1979). The electrophysiological effect of kainic acid on glia has not been studied. Although glutamic acid does not depolarize glia directly (Krnjevic and Schwartz, 1967), it depolarizes glia indirectly perhaps through the release of neuronal K^+ or other ions (Hosli et al., 1979). Kainic acid, L-glutamic acid

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and other excitatory amino acids increase the level of K^+ in the medium containing frog spinal cords, and also depolarize nerve terminals in the same preparation (Evans, 1980). Kainic acid may alter other ions in addition and thus indirectly cause depolarization or ionic alterations at sites which lead to decreases in ATP, decreases in glutamine synthesis and decreases in acetate utilization.

D. EFFECTS OF KAINIC ACID AND VERATRIDINE ON GLUTAMIC ACID RELEASE - EVIDENCE FOR NONPRESYNAPTIC RELEASE BY KAINIC ACID

The neurotoxicity of kainic acid may involve a release and/or an inhibition of the uptake of glutamic acid. In the present studies, kainic acid elevated glutamic acid levels (Tables 6 and 7) in the medium of cerebellar slices both by inhibiting its uptake (Table 22) and by releasing glutamic acid. Simply inhibiting the uptake of glutamic acid with 1 mM dihydrokainic acid, a glutamic acid uptake inhibitor (Johnston et al., 1979) which lacks neuroexcitatory or neurotoxic actions, did not increase glutamic acid levels in the medium of cerebellar slices (Table 7).

Veratridine presumably releases the nerve terminal pool of glutamic acid. Veratridine released about 2% of the tissue glutamic acid content after 5 minutes (Table 14)

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and about 6% after 15 minutes. Kainic acid released about 12% of the tissue content of glutamic acid after 15 minutes and 20% after 30 minutes (Table 6), approximately twice as much glutamic acid as veratridine released. The veratridine-induced release was sensitive to tetrodotoxin whereas the kainic acid-induced release was not sensitive to tetrodotoxin.

An attempt was made to deplete the presynaptic glutamic acid pool by pretreatment of the tissue with veratridine. The tissue was then exposed to kainic acid to examine if kainic acid-induced release of glutamic acid was reduced, and vice versa. However, kainic acid or veratridine pretreatment potentiated the subsequent glutamic acid and aspartic acid release caused by the other (Figures 7 and 8). Possibly metabolic damage or ionic changes caused by one enhanced the depolarizing effect of the other. Depolarizing and toxic actions of kainic acid may similarly be enhanced <u>in vivo</u> by excitatory events.

Minchin (1977) reported that, from spinal cord slices, veratridine elevated the efflux of glucose-labelled glutamic acid but not of acetate-labelled glutamic acid. However 50 mM K^+ elevated the efflux of both acetate- and glucose-labelled glutamic acid. Minchin concluded that veratridine and 50 mM K^+ released glucose-labelled glutamic acid from nerve terminals but that 50 mM K^+ in addition released acetate-labelled glutamic acid from glial pools. In the present studies, kainic acid tended to leak

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newly synthesized acetate- and glucose-labelled glutamic acid (Table 16) whereas veratridine preferentially released glucose-labelled glutamic acid (Tables 15 and 17). The acetate-labelled glutamic acid leaked by kainic acid is unlikely to come from nerve endings, since veratridine did not enhance release of acetate-labelled glutamic acid. Indeed, kainic acid did not release exogenously loaded glutamic acid from synaptosomal preparations from cortex whereas veratridine did (Table 10). Kainic acid may release some glutamic acid from presynaptic pools indirectly, however the bulk of the released glutamic acid is probably from glia and perhaps neuronal perikarya.

The site of release might be further clarified by measuring the labelling of glutamic acid released from glial cells by depolarizing stimuli such as high K^+ when using radioactive acetate, glucose and glutamine as precursors. Bradford et al. (1978) found that glutamic acid released from synaptosomes was more highly labelled by glutamine than by glucose, and that acetate did not label amino acids in synaptosomes well enough to measure. This is consistent with the present interpretation.

These studies indicate that kainic acid probably caused a good deal of glial release of glutamic acid from cerebellar slices, whereas lesion studies in the striatum have previously indicated that a presynaptic glutamergic input was involved. Possibly the source of released glutamic acid induced by kainic acid differs in

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the cerebellum and the striatum. In the mouse, Seil and Woodward (1980) found that granuloprival cerebellar cultures were still sensitive to the toxic actions of kainic acid. Our data demonstrating that kainic acid decreased ATP levels in cerebellar slices from weaver mice which are depleted of granule cells is consistent with that of Seil and Woodward (1980). Similar studies on the rat cerebellum depleted of granule cells would be useful because the sensitivity of various neurons to kainic acid toxicity may be different in the mouse (Lovell and Jones, 1979) and the rat (Herndon and Coyle, 1978).

DL-homocysteic acid but not N-methyl-D-aspartic acid also increased the release of glutamic acid and aspartic acid from cerebellar slices (Table 7). N-methyl-D-aspartic acid also did not mimic the actions of kainic acid on ATP or glutamine levels in cerebellar slices. Interestingly, kainic acid and N-methyl-D-aspartic acid probably act on different receptors (Davies et al., 1979), and possibly the biochemical alterations that kainic acid has caused are receptor mediated.

E. SUMMARY

Thus, to summarize the data, a hypothetical model of the actions of kainic acid in the cerebellum is presented in Figure 11. Information on the site of the ATP reduction, location of kainic acid receptors, the relationship of

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glutamic acid release to the decrease of glutamine in the tissue, the biochemical effects caused by glial swelling, and on the manner in which kainic acid can affect glia are needed to refine this model.

The suggested sequence of events follows:

1) Kainic acid depolarizes neurons postsynaptically via a specific receptor, causing an efflux of K^+ and an influx of Na^+.
2) These alterations lead to activation of Na^+-K^+ ATPase postsynaptically and to decreases in the levels of creatine phosphate and ATP and to increases in glycolysis in the neuronal perikarya.
3) Glia are also depolarized, although indirectly, perhaps by the efflux of K^+ and other ions into the medium from neurons.
4) Glial ion pumps are activated and ATP levels decrease, and glia swell with the uptake of water and ions.
5) The loss of ATP, required for glutamine synthesis, inhibits the synthesis of glutamine in glia and perhaps the incorporation of acetate into amino acids.
6) Inhibition of glutamine synthesis may lead to an accumulation and leakage of glutamic acid, or depolarization of glia and neurons may lead to an efflux of glutamic acid into the medium.
7) Glutamic acid in the medium then acts to potentiate the actions of kainic acid to depolarize neurons via a postsynaptic receptor.

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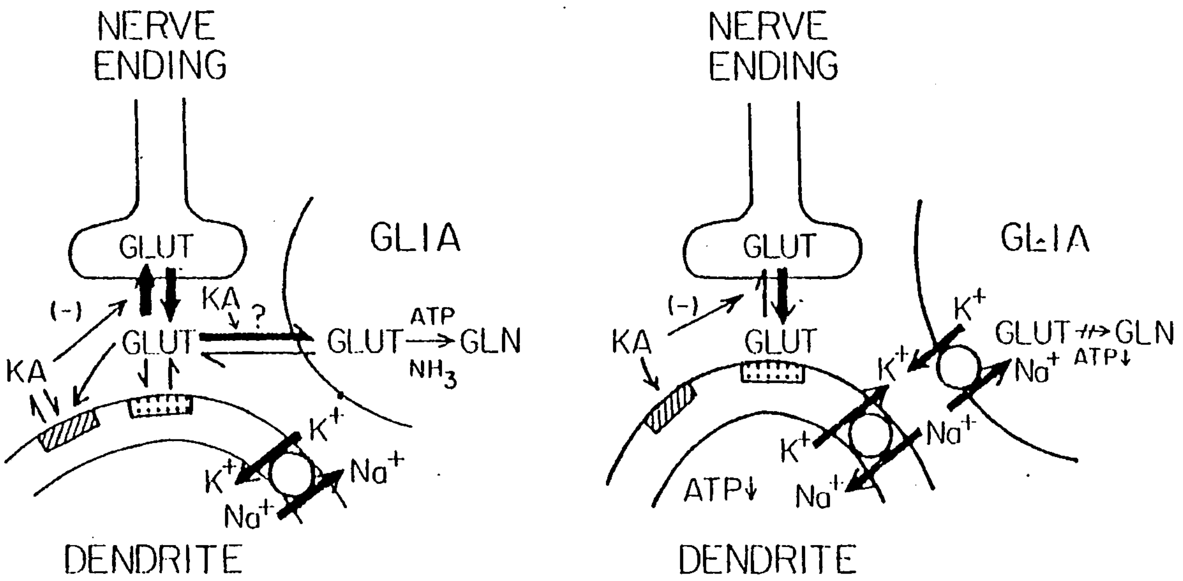

FIGURE 11. PROPOSED MODEL

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