THE EFFECT OF ELECTROCONVULSIVE SHOCK AND 5-HYDROXYTRYPTAMINE ON PROTEIN SYNTHESIS IN THE CENTRAL NERVOUS SYSTEM

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This work is lovingly dedicated to my wife, Judith, and my daughter, Karen.

ABSTRACT

The effects of electroconvulsive shock (ECS) and intracranial injection of 5-hydroxytryptamine (5-HT) on protein synthesis in the central nervous system of the mouse were examined and compared. ECS produced considerable inhibition (35-60%) of the incorporation of labeled amino acids into proteins of the whole brain. The inhibition appears to result from a reduced rate of protein synthesis, rather than from changes in cellular or regional permeability or from the isotopic dilution effect that might result from electroconvulsive shock. The inhibition is of a brief duration, lasting only about 15 minutes after the administration of the shock. Regional studies revealed that the cerebral cortex is the most affected brain region. An evaluation of the mechanism by which ECS inhibits protein synthesis indicated the following: 1) The brain energy reservoir state may govern the rate of protein synthesis within the central nervous system. 2) Electroconvulsive shock resulted in a decreased ATP/ADP+AMP ratio which may cause the reduced efficiency of the protein synthesizing system. This effect may be attributed to the inhibitory properties of ADP and AMP. It appears that the inhibition occurred somewhere during polypeptidation, but not during amino acid activation. It is therefore concluded that the decreased level of ATP is not responsible *per se* for the inhibitory effect of the ECS.

Observations related to subcellular localization of the electroshock effect support the notion that there are several independent sites for protein synthesis within the neuron. The principal site is within the ribosomal system of the perikaryon; subsidiary sites are within the ribosomal system of the mitochondria and in the nerve terminals. Electroconvulsive shock was noted to have a differential effect upon the synaptosomal system and the ribosomal system, inhibiting the former to a greater extent than the latter.

An evaluation was made of the hypothesis that increased levels of 5-HT may mediate the inhibitory effect of ECS. The following facts suggest that such mediatory effect may be relevant to the synaptosomal site, but not to the ribosomal site: 1) Elevated brain 5-HT levels induced by monoamine oxidase (MAO) inhibitors did not change the incorporation of the labeled amino acid into the whole brain protein. 2) Intracranial injection of 5-HT, which produces 15-20% inhibition of the cerebral protein synthesis, caused electrical changes that might be related to the inhibitory effect on protein synthesis. 3) The concentrations of 5-HT needed to produce 30% inhibition of protein synthesis with microsomes *in vitro*, are above those expected in the ribosomal vicinity *in vivo*. 4) The mechanism by which 5-HT inhibits microsomal protein synthesis *in vitro* indicated amino acylation as the stage at which inhibition occurs. A different

stage was suggested for the inhibitory effect of ECS. 5) Autoradiographic studies revealed dispersion of intracranially injected 5-HT around but not within the perikaryon. 6) Concentrations of 5-HT are expected to be higher in the synaptosomal site than in the ribosomal site [128]. 7) Intracranial injection of 5-HT produced a greater amount of protein synthesis inhibition in the synaptosomal fraction than in the ribosomal fraction. 8) It has been reported that 5-HT is a strong inhibitor of protein synthesis in synaptosomes while a weak inhibitor in microsomes in vitro.

Inference was made that ECS and 5-HT are capable of producing retrograde amnesia through inhibition of protein synthesis. The possibility that protein synthesis at the nerve terminal plays a role in the consolidation process is considered and discussed in the present work.

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LIST OF ABBREVIATIONS

aa-tRNA	Aminoacyl-tRNA
A.Ch.	Acetylcholine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
cm	Centimeter
CNS	Central nervous system
CPM	Counts per minute
Cr.P.	Creatine phosphate
DA	Dopamine
DNA	Deoxyribonucleic acid
ECS	Electroconvulsive shock
EDTA	Ethylenediamine tetracetate
EEG	Electroencephalogram
g	Gram
GABA	γ aminobutyric acid
GTP	Guanosine triphosphate
hrs.	Hours
homog.	Homogenate
5-HT	5-hydroxytryptamine (serotonin)
IC	Intracranial injection
inhib.	Inhibition

IP	Intraperitoneal injection
l	Liter
λ	Microliter
LDH	Lactic dehydrogenase
μ	Micron
ma	Milliampere
MAO	Monoamine oxidase
m eq	Milliequivalent
met-tRNA	Methionyl-tRNA
μc	Microcurie
mg	Milligram
μg	Microgram
μ mole	Micromole
mic.	Microsomes
min.	Minutes
mit.	Mitochondria
ml	Milliliter
mm	Millimeter
m mole	Millimole
myel.	Myelin
NE	Norepinephrine
nm	Nanometer
P_i	Orthophosphate
P_2	Crude mitochondrial pellet (see method section)
PCA	Perchloric acid
poly U	Polyuridylic acid

post mit sup	Postmitochondrial supernatant
prot	Protein
RA	Retrograde amnesia
RNA	Ribonucleic acid
S_{100}	Cell sap (see method section and reference 82)
S_2	Cell sap (see method section and reference 84)
S_{30}	Postmitochondrial supernatant (see method section)
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
sec	Seconds
SS	Soluble fraction of the synaptosome
Synapt.	Synaptosome
TCA	Trichloroacetic acid
tRNA	transfer RNA

INTRODUCTION

The study of the protein synthesis machinery of protokaryotic as well as eukaryotic cells has rapidly advanced within the last two decades. Most of the recent progress in our understanding of the biochemistry of protein synthesis was achieved by the use of cell-free systems. The first such system was discovered in 1948 by Winnick et al [1]. Following that discovery it was shown by Zamecnik and Keller [2] that the microsomal fraction of rat liver, supplemented by soluble enzymes and ATP, was capable of actively incorporating amino acids into proteins. The ribosomes were the portion of the microsomal fraction responsible for protein synthesis. Most of the key experiments have been peformed with three major systems: a) A cell-free system from rabbit reticulocytes which has the advantage of completing (and perhaps initiating) a well-defined protein, hemoglobin. b) A cell- free system prepared from liver in which the activity is determined to a large extent by the previous *in vivo* conditions such as regenerating liver or activation of the microsomal enzymes by drugs, etc. c) A cell-free system from E. coli which has the advantage that under appropriate conditions it retains the flow of information, DNA $\longrightarrow$ RNA $\longrightarrow$ protein.

The general scheme of protein synthesis was elaborated in such studies and described in several reviews [3,4,5]. This scheme may be outlined as follows:

a) Activation of amino acids and formation of aminoacyl-tRNA molecules.

b) Formation of a component complex between ribosomes and mRNA.

c) Binding of aminoacyl-tRNA to this complex.

d) Formation of peptide bonds in the complex.

e) Release of completed chains from the complex.

Although the systems which were described above were investigated in detail, relatively little information on the mechanism of polypeptide synthesis in the brain has been obtained. Considerable interest in brain protein synthesis developed in the early 1960's. Zomzely et al [6] first described the ribosomal system from the rat brain. Independently, a cell-free microsomal system from guinea pig brain was characterized by Satake et al [7]. A number of investigators later described cell-free systems from the brain which incorporate amino acids into protein [8-13]. In some of these studies, and especially in the study of Campbell et al [11], the uniqueness of the brain system was elaborated. Two distinct cell-free protein synthetic systems have been isolated from the immature rat brain, one localized in purified ribosomes and one localized in crude mitochondrial preparations. The brain mitochondrial system

has an unusually active protein synthesizing system. It was suggested that this activity was of mitochondrial origin, despite the fact that these crude mitochondrial preparations contain other particles such as nerve endings, myelin fractions and probably some microsomes. Several studies suggested that mitochondria probably contribute to the overall rate of protein synthesis to varying extents in different tissues. The presence of DNA, RNA, and ribosomes within the mitochondria from a number of different cell types is well documented [14,15,16]. In some cells the density of the mitochondrial DNA differs from that of the nuclear DNA [17]. Hybridization experiments revealed that the base sequence of nuclear DNA and mitochondrial DNA are different [18]. The mitochondrial ribosomes probably differ in their sedimentation properties from their microsomal counterpart [19]. Enzymes, the synthesis of which was directed by the mitochondrial DNA, are probably different from the equivalent bacterial or cytoplasmic enzymes in their ability to recognize the homologous substrate. Thus, specificity of the mitochondrial Met-tRNA transformylase for the mitochondrial t-RNA was shown when it was compared with the equivalent enzyme from E. coli [20]. It is apparent that mitochondria possess an independent apparatus for protein synthesis which is different from that of the microsomal system. Mitochondrial protein

synthesis in the liver appears to be directed principally toward the formation of the inner membrane proteins [21]. However, the situation may be different in the nervous system. It was suggested that in the brain, functional proteins such as the myelin protein are synthesized by glial mitochondria. Structural proteins of the vesicle walls, on the other hand, are thought to be synthesized by synaptic mitochondria [11]. Some support for this hypothesis came from a study in which the composition of the proteolipid fraction of white matter from beef brain was shown to be different from that of similar fractions of nonneuronal origin [22].

The principal cells in the central nervous system (not including the glandular structures) are the neurons and the glia. The morphology of such cells, particularly of neurons, reveals an extended structure, the axon, which originates from the cell body and which can vary in length from a few millimeters to decimeters. Proteins in the axon terminal may therefore be far removed from the neuron nucleus or ribosomes of the neuron. The difficulties of supplying essential protein components through the axon toward the nerve terminal present a considerable problem. This problem could be solved by two different mechanisms: 1) local protein synthesis within the axon and the axon terminal; 2) efficient transport along the axon.

In all species thus studied it has been found that axonal proteins are synthesized mainly in the neuronal perikaryon and then migrate progressively down the axon [23-25]. There is conflicting evidence about whether or not a small amount of protein is also formed locally in the axon or in nerve terminals. Although ribonucleoprotein granules are completely absent from the axoplasm [26], local protein synthesis may still take place at this site. It has been shown by Koenig [27,28], that at least acetylcholinesterase may be synthesized locally within the axon. This study was based on irreversible inactivation of a major proportion of acetylcholinesterase by an organophosphorus anticholinesterase compound. The rate of enzyme restoration was then studied along segments of peripheral nerve. The rate of the restoration and its uniformity suggested that local synthesis of this enzyme is responsible for the restoration. A fast rate of axoplasmic flow, which was established later by Ochs [29] as 400 mm/day may explain the rate of the restoration of acetylcholinesterase without hypothesizing local synthesis within the axon. The cytochemical localization of this enzyme [30] revealed that the enzyme was associated with those structures which may be involved in the fast transport within the axon, namely the external membrane of the axon and the microtubules.

More direct evidence for local protein synthesis within the nerve terminal has been supplied by several studies. Austin and Morgan [31] used chopped brain which was fractionated after incubation in a medium containing C^{14}-leucine. They demonstrated that significant amounts of radioactivity were incorporated into the synaptosomal protein even after a short period of incubation. The results of this experiment, however, may be ambiguous. The possibility that proteins which were found in the synaptosomal fraction may have been transported by the fast axoplasmic flow through short axons was not ruled out. Another alternative explanation is contamination of the synaptosomal fraction by labeled proteins originating from the soluble fraction of the perikaryon, which were bound to the synaptosomal fraction during the preparation. The alternative mechanisms mentioned above do not preclude the possibility of additional local protein synthesis within the axon. In vitro studies by Autilio et al [32] were done with isolated synaptosomes. They demonstrated that isolated synaptosomes may incorporate labeled amino acids into protein. The incorporation is linear for 20 minutes and insensitive to RNAase. Chloroamphenicol which does not affect protein synthesis by microsomes, partially inhibits this incorporation. In attempts to demonstrate that bacterial contamination did not contribute significantly to the apparent incorporation, bacteria were isolated and incubated in tryptone broth

containing C^{14}-leucine. The kinetics of the incorporation showed 5 hours of linearity after a lag period of 20 minutes. Labeled bacteria added to a homogenate which was fractionated subsequently did not contribute significant label to the synaptosomal fraction. However, tryptone medium is different from the medium in which synaptosomes were incubated and could change the characteristic of the incorporation of the labeled amino acid into the bacterial protein. Furthermore, even a small amount of bacteria in the synaptosomal fraction could contribute a significant amount of radioactivity to the apparent incorporation because of the heavily labeled precursor that had been used in this experiment. Therefore, the possibility that the results were influenced by bacterial contamination has not been ruled out entirely.

Recently, new evidence has been provided to support local protein synthesis within the synaptosomal membrane [33]. This evidence is based on the ability of the membrane fragments prepared from lysed synaptosomes, to incorporate labeled amino acids in a cell-free preparation. The labeled proteins extracted from this preparation and separated on gel electrophoresis were different in their mobility along the gel from the newly synthesized proteins extracted from the microsomal fraction. The incorporation was inhibited by cyclohexamide and puromycin, but was insensitive to chloramphenicol. Other studies dealing with protein synthesis in the central nervous system and specifically synaptosomal protein synthesis

have been recently reviewed and need not be repeated here [34-36].

The study of brain protein synthesis and its regulation became even more attractive when findings indicated that protein synthesis may play an important role in what has been called the memory consolidation process [37-39]. There is now considerable evidence that inhibitors of cerebral protein synthesis interfere with memory storage (For review see 40). The use of memory disruptive techniques such as injection of protein synthesis inhibitors and electroconvulsive shock (ECS) provide a biochemical and biophysical approach with which to examine the concepts of memory consolidation, short term memory and long term memory. Treatments, such as ECS or injection of drugs that inhibit protein synthesis, given immediately after a learning experience, produce marked impairment of memory when the animal is subsequently tested, while treatments given hours after a learning experience generally have no effect on subsequent retention. Studies of this type suggest that the memory process is not completed by the end of training. The period during which the animal is susceptible to memory disruptive treatments is called the "labile phase of memory" [41]. Within this period memory consolidation takes place. Experiments utilizing drugs which inhibit protein synthesis have not been productive in elucidating the site of memory consoli-

dation, the kind of proteins which may play a role in this process, or the critical period during which inhibition of protein synthesis causes loss of memory. The difficulty of obtaining results with this approach is due to the slow diffusion of drugs in the central nervous system and to the non-selective nature of these drugs. In most cases inhibition of protein synthesis was over 90% without preferential inhibition for proteins or sites. Electroconvulsive shock (ECS) seems to be a more promising approach because of its selective site of action.

Observations from earlier studies [42,43] have indicated that ECS is capable of producing retrograde amnesia. The greater the delay in administration of ECS after a learning experience, the less the effect on subsequent memory. ECS is administered in a very brief duration and the problems of diffusion which occur with drugs do not exist. Although these effects have been studied for over two decades little is known of their nature. Studies dealing with the intensity of the current needed to produce retrograde amnesia are controversial. Whereas McGaugh suggested that the passage of current through the brain is not a sufficient condition for producing retrograde amnesia until it reaches seizure threshold [44], Essman found that convulsions are not essential to the production of amnesia and suggested that the passage of the current alone is sufficient to produce retrograde amnesia [45,46].

ECS has been widely used clinically as therapy for mental depression in man as well as in the laboratory. Literature reviews concerning biochemical and physiological alterations produced by ECS are numerous. Those that are relevant to the present work are given in the references section [43,47,48]. The most prominent symptom produced by ECS is the convulsion itself. ECS when given in intensities above 7.5 ma produces a maximal and easily characterizable convulsion [43]. The convulsion is developed after a single treatment. However, some metabolic changes such as increased monoamine oxidase activity and changes in the turnover rate of NE occur only when several consecutive treatments are given. Unfortunately, in many studies no attempt has been made to distinguish between the neurobiological effect of a single ECS versus multiple ECS's.

Several biochemical events which are affected by ECS have been described. Cerebrovascular permeability is increased for several compounds such as norepinephrine or even macromolecules which under normal conditions have limited permeability through the blood brain barrier [49,50]. The concentrations of glycogen, glucose, and ATP in the brain seemed to be reduced as a consequence of convulsive activity [51]. Moreover, the brain energy reservoir is reduced as a consequence of ECS [52,53]. Changes in brain acetylcholine (ACh) concentrations have also been noted. It has been shown that ACh concentration is markedly reduced during the

convulsion period produced by electrical stimulation [54]. On the other hand, biogenic amines such as serotonin (5-HT) are significantly elevated within the brain as a consequence of a single ECS [55]. There are controversial reports with respect to the above phenomenon. While Garattini et al [55] showed an increase of brain 5-HT levels after ECS, no change was found by Feighner et al [56]. However, there is agreement with respect to the turnover changes of 5-HT following ECS [57]. The activity of monoamine oxidase (MAO) was reported to be increased after multiple treatments of ECS [58]. The discrepancies between the results concerning the level of the 5-HT after ECS can be explained by the time at which the determination of the 5-HT concentrations was made, or by differences in the species tested and in the conditions under which ECS was given. An increased turnover of NE, coupled with a significant increase in brain tyrosine hydroxylase was also noted after multiple ECS treatments [59].

Among the changes in small molecules occurring after ECS, the shift and the changes in ionic concentrations cannot be ignored. It has been shown by Woodbury that after a single convulsion the total concentration of Na+ was increased with no change in K^+ concentration [60]. In a different study, Escueta and Appel [61] reported that repeated ECS decreased K^+ content within isolated nerve terminals by enhancing its passive leakage.

Macromolecular changes associated with ECS have also been investigated. It has been shown that administration of a single ECS leads to a reduction in total RNA concentration [62]. The decrease in RNA concentration has also been shown for other convulsive agents [63]. Based upon subcellular studies it was suggested that the RNA changes occur in the nuclear fraction. Increase in brain weight [64] and in the DNA content [65] have been shown after chronic ECS administration. The increase in DNA content was accompanied by corresponding RNA changes. It has been suggested that the number of neuroglia cells had increased as a consequence of multiple ECS. Several studies have elaborated some aspects of the interrelationship between serotonin, RNA, and ECS [66-68]. This interrelationship has been based upon the observation of an inverse relationship between the concentration of brain serotonin and brain ribonucleic acid content. It was shown that ECS resulted in a regional depletion in RNA concentration in the brain, and that this depletion is paralleled by an increase in brain serotonin at these same regions [69]. It has further been shown by Bittman et al [70] that 5-HT and RNA may interact and it was suggested that this interaction may modulate other processes in the central nervous system such as protein synthesis and memory consolidation [71].

The findings that ECS depletes the energy reservoir in the CNS and also depletes RNA content suggested that protein

synthesis which is RNA dependent and which is an energy consuming process may be inhibited as a consequence of ECS. The effect of ECS on brain protein synthesis has been studied by several laboratories [72-79], and controversial results have been reported. While Dingman et al [72] found that multiple ECS had no effect on the incorporation of C^{14}-proline and Bailey and Heald [74] observed no qualitative changes in the cytoplasmic proteins resolved by starch gel electrophoresis, Geiger et al [73] observed a triphasic effect in the perfused cat brain with C^{14}-glucose as a precursor. The latter group found that an increase during the convulsive phase was followed first by a dramatic inhibition and then by a subsequent increase in protein synthesis activity. Minard and Richter [76] showed a slight decrease in the labeling of the protein in the brain of convulsed animals. However, in all these studies, effects may have been masked due to long periods of incorporation and the use of indirect precursors such as glucose. Methods of low sensitivity have also been employed in some experiments. A study which may indicate the potential inhibitory effect of multiple ECS on protein synthesis was reported by Vesco and Giuditta [75]. They showed that the number of free ribosomes was increased by approximately 80% along with a comparable decrease in the population of polysomes as a consequence of ECS. Recently three independent laboratories have shown that the effect of a single ECS on protein synthesis in the central nervous system is much greater than was previously observed for

multiple ECS [77-79]. It is one of the purposes of this present work to evaluate and define this type of inhibition.

The inverse relationship between 5-HT and RNA, and the relationship of the changes in these two substances to ECS provide a clue of a possible mediating effect of 5-HT in protein synthesis inhibition produced by ECS. Possible differential effects in different subcellular fractions produced by ECS are suggested by the localization of the 5-HT within the neuron and the potentially inhibitory effect of 5-HT on synaptosomal protein synthesis in vitro [80].

The purposes of the present work are: 1) To define and specify the inhibitory effect of ECS on protein synthesis in the central nervous system. 2) To correlate this effect with the elevated level of 5-HT produced by ECS. 3) To find the mechanism by which ECS may affect protein synthesis inhibition, and 4) To characterize the proteins which are affected by ECS and/or 5-HT at different sites in which inhibition of protein synthesis takes place. Based upon the above findings, an evaluation will be made of the possibility of local protein synthesis at the synaptic site.

MATERIALS AND METHODS

A. Reagents and substrates

The 5-hydroxytryptamine was obtained from Sigma Chemical Company. In most of the experiments an oxalate salt was used. The dose for injection was calculated for the free amine. In some experiments creatinine salt was used and gave basically the same results. The 5-hydroxy-tryptamine derivatives were supplied by Sigma Chemical Company. The 1-methyl, 5-methoxytryptamine was locally synthesized and kindly contributed by Dr. R. Bittman. Labeled amino acids were purchased from New England Nuclear. The C^{14}-amino acids were uniformly labeled. The tritiated leucine was L-Leucine-4,5-H^3. Isotopic dilution to the proper specific activity was made with cold L-Leucine obtained from Sigma. DEAE cellulose (0.88 meq/g) was supplied by Sigma Chemical Company. Reagents for the acrylamide gel electrophoresis were purchased from Canalco. All other reagents were in analytical grade and were purchased through routine laboratory order from City Chemical Corporation of New York, Sigma Chemical Company, Fisher Scientific Company and Baker Analyzed Reagents.

B. Animals

Mice used in these experiments were male CF-1s strain obtained from Carworth, Inc., New City, New York. In most of the experiments, and especially in the behavioral studies, animals weighing 18-21 g were used. Albino guinea

pigs were bred in the laboratory and were used when they weighed approximately 400 g.

C. Treatment of Animals

1) Administration of ECS: Single electroconvulsive shock was administered transcorneally, using electrode cream supplied by Bendix Corporation, Cincinnati, Ohio. The shock was delivered for 0.2 sec. from a high voltage (400 V) power supply. The intensity used in the present work was 20 ma. The duration of the clonic-tonic convulsion under these conditions is approximately 10 sec.

2) Intracranial injections: 5-HT or labeled amino acids were administered intracranially by injection with a Hamilton microliter syringe fitted with a 27 gauge needle in an adaptor to control the depth of the needle penetration through the skull into the brain tissue. The injections were given 1 mm lateral to the midline and 5 mm posterior to a line between the eyes. The penetration was adjusted to 3 mm in depth. The volume injected was always 10 μl. In a histological study, India ink diluted to a 25% solution when injected under comparable conditions was found to be localized in the lateral ventricles.

3) Behavioral procedures: For passive avoidance, animals were trained in an apparatus designed to establish a conditioned passive avoidance response [81]. The apparatus consisted of a small transparent chamber fixed to a hole opening into a larger dark chamber. The floor of the dark chamber consisted of stainless steel grids through which a

scrambled footshock (5.9 ma) was delivered. The interval between the placement of the animal in the outer chamber and its entry into the larger chamber was measured (response latency). Immediately upon entrance a shock with intensity of 5.9 ma was delivered through the paws of the animal for 5-10 sec. The animal was then removed from the dark chamber. The acquisition of the passive avoidance response was measured 24 hrs. later by placing the animal in the outer chamber and measuring the response latency. Individual animals which showed a response latency longer than 2 minutes were considered as trained animals and removed from the outer chamber. Ten animals were usually used in one group when the effects of certain treatments were measured.

Active avoidance was measured in the following way: The animal was placed in a box consisting of a stainless steel grid floor and an open top. The measurements of the box were 21 X 21 cm with depth of 11 cm. Ten minutes after placement of the animal in the box, a footshock was delivered with intensity of 5.9 ma. The time required for the animal to escape from the box was measured. The experiment was repeated 10 times with fixed inter-trial intervals of 15 sec. The curve describing the escape latencies as a function of number of trials is the learning curve.

D. Preparation

1) Dissection of brain regions: Animals were sacrificed by cervical dislocation and brains were removed and placed on ice in a Petri dish. The dissection of the

regions was always done on ice. When labeled amino acid was injected prior to the preparation, brains were soaked in 0.32 M sucrose containing 0.75 g/l unlabeled amino acid. During the preparation the brains were moistened with the same medium. After placing the brain in the Petri dish, the hemispheres were separated from the underlying corpus callosum and the mantle of each cerebral hemisphere was reflected laterally. The undersurface of the cerebral cortex was thus exposed from the frontal to the occipital poles and laterally to the ventral surface of the temporal lobes. The basal ganglia which were reflected with the cerebral cortex were subsequently dissected free. The associated white matter was removed by scraping in those cases in which subcellular fractionation was carried out. Visual examination revealed the presence of negligible amount of white matter. After removal of the cerebral cortex and the corpus callosum, the structures composing the diencephalon were visible. Although it was not possible to determine the precise boundaries of the diencephalic structures, these structures were then dissected out, using a conservative criterion as to the nature of the tissue involved. That is to say, some non-diencephalic matter may be included in the removed tissue, but the majority would be the diencephalic matter. The basal ganglia already excised were combined with other elements of the diencephalon.

The entire cerebellum was removed intact and contained only cerebellar structures (both cortex and medulla).

2) Preparation of postmitochondrial supernatant: Animals were sacrificed by cervical dislocation and the tissue was quickly removed and placed into a cold medium A (see page 28). The subsequent operations were carried out keeping the material at 0-4°C. The tissue was minced, and then homogenized in medium A (1 g tissue with 2 ml medium A), with a Potter-Elvehjem teflon-glass homogenizer. The clearance which was allowed was about 1 mm. Motor speed was adjusted to 1500-2000 RPM and homogenization continued for 30 sec. The homogenate was centrifuged for 10 minutes at 30,000 xg in a refrigerated centrifuge adjusted to 2°C. The supernatant was collected and filtered through absorbent cotton in order to remove lipid residues. This supernatant is the post-mitochondrial supernatant (S_{30}) [82].

3) Preparation of microsomes and cell sap: The S_{30} was centrifuged for one hour in the ultracentrifuge (Beckman model L-2) at 105,000 xg. The supernatant was collected and used as the cell sap - the soluble fraction (S_{100}). The pellet was washed and resuspended in medium A to a final concentration of about 50 mg protein/ml. The suspension was used as microsomes [82].

4) Preparation of ribosomes: The ribosomes were prepared according to Korner [83]. The S_{30} was added to a 5% solution of sodium deoxycholate in medium A in sufficient amount to yield a final concentration of 0.5%. The suspension was centrifuged for one hour in an ultracentrifuge at 105,000 xg. The supernatant was removed and the pellet was washed, then resuspended in medium A and recentrifuged. The pellet col-

lected after the second centrifugation was resuspended in medium A to yield a final concentration of 2 to 5 mg RNA/ml.

5) Preparation of various subcellular fractions from the brain: Animals were sacrificed and brains were quickly removed and homogenized in 0.32 M sucrose (1 g tissue with 9 ml sucrose) with a Potter-Elvehjem teflon-glass homogenizer. Subcellular fractions were prepared from this homogenate according to the method of Whittaker et al [84]. The homogenate was separated into P_1 (nuclei, large myelin fragments, tissue debris), P_2 (mitochondria, synaptosomes, small myelin fragments, some microsomes), S_2 (postmitochondrial supernatant), P_3 (microsomes) and S_3 (soluble fraction). The P_2 was washed three times and then subfractionated on a discontinuous sucrose gradient of 1.2 M and 0.8 M sucrose. The fractions obtained from this procedure are: fraction A, small myelin fragments (interphase between 0.32 M and 0.8 M sucrose); fraction B, synaptosomes (interphase between 0.8 M and 1.2 M sucrose); and fraction C, extrasynaptosomal mitochondria (pellet).

6) Preparation of synaptosomal subfractions: Synaptosomes were washed twice with 0.32 M sucrose and then resuspended in H_2O and disrupted by freezing and thawing. The disrupted synaptosomes were subfractionated on a discontinuous sucrose gradient of 0.4 M and 1.2 M by centrifugation for 90 minutes at 51,000 xg. The fractions found between 0.4 and 1.2 M sucrose corresponded to external synaptosomal membranes as was determined by marker enzymes. The pellet corresponded to intrasynaptosomal mitochondria. The fraction

from the top of the gradient was collected and considered as a soluble fraction of the synaptosomes.

7) Extraction of tRNA from various fractions of the cerebral cortex: For the extraction of the tRNA the following fractions were used: a) pH5 fraction prepared from the soluble fraction extracted from brain homogenate (S_{100}), b) pH5 fraction prepared from the soluble fraction of the synaptosomes (SS).

The synaptosomes that were used for the preparation of the above fractions were prepared by a modified procedure as follows: RNAase (50 μg/ml) was added to the P_2 which was then incubated for 10 minutes at 30°C. The P_2 was washed four times with cold 0.32 M sucrose and then placed on a sucrose gradient as previously described. Fraction B was collected from the gradient after centrifugation and 1% bentonite was then added. The bentonite was removed by centrifugation at 3000 xg for 10 minutes and an equal volume of water was added to the suspension to yield 0.5 M sucrose. The synaptosomes were then sedimented by centrifugation at 105,000 xg for 30 minutes. The synaptosomal pellet was washed three times with 0.32 M sucrose and then was disrupted as described above. The soluble fraction was collected from the supernatant after one hour of centrifugation of the disrupted synaptosomes at 105,000 xg. The tRNA was extracted from these fractions by the procedure described by Moldave [85].

8) Preparation and purification of aminoacyl-tRNA synthetase: The fractions from which the synthetases were extracted were prepared as described in Sections 3 and 6

above. The soluble fraction of the perikaryon (S_{100}) and the soluble fraction of the synaptosome were used for the enzyme purification procedure. The fractions were adjusted to 0.02 M β-mercaptoethanol, 0.01 M $MgCl_2$ and 10% glycerol and applied to a DEAE-cellulose column [86] and eluted with a 0.25 M KH_2PO_4, pH6.5, containing 0.02 M β-mercaptoethanol and 10% glycerol. A fraction exhibiting amino acid activation was eluted immediately after the void volume.

E. Incorporation of labeled amino acid into protein.

1. In vivo: The labeled amino acid was injected intracranially and five minutes later the animals were sacrificed and the brains were rapidly removed and frozen by dipping the brains in cold (-20°C.) Freon. In a standard experiment mice were divided into groups of three to five animals per group; each experimental variable was repeated with one or two groups resulting in a range of 9-12 animals utilized per experimental variable. The brains of each group were pooled for the radioactive determination, a procedure which obviated observation of the variability among individual animals and resulted in a single observation for each group. Since inferential statistical analysis was inappropriate, the maximum variability among the groups receiving the same treatment was considered as the probable variability resulting from the groups receiving different treatments, if there was no effect involved. Greater differences were considered as an effect of the relevant treatment. This procedure involved injection of 10 λ of 0.001 M leucine containing 0.06 μc of C^{14}. The

brains were homogenized in medium A (described on page 28), containing 0.75 g/l cold leucine and aliquots of the homogenate were precipitated in 5 ml TCA 5% for radioactive determination. The precipitate was washed once with 5% TCA and hydrolyzed in 5% TCA for 20 minutes at 90°C. The precipitate was then washed twice and extracted twice with ethanol: ether (2:1). The remaining precipitate was dissolved in 99% formic acid and transferred to a scintillation vial containing 15 ml scintillation liquid for the radioactive determination.

2. In vitro: The standard incubation mixture contained 50 mM tris-HCl pH7.5, 100 mM KCl, 5 mM $MgSO_4$, 2 mM ATP, 0.1 mM GTP, 15 mM creatine phosphate, 50 μg/ml creatine phosphokinase (30 units/mg), 0.1 μ mole/ml C^{14}-leucine or C^{14}-phenylalanine (3 μc/μ mole), and 0.3 ml S_{30} or 5 mg microsomal protein with 0.25 ml S_{100} fraction. The final volume of the incubation mixture is 0.5 ml. When the incorporation of the labeled amino acid into tRNA was measured no source of ribosomes was added to the incubation mixture. The incubation was carried out at 37°C. for 10-15 minutes and terminated with 5 ml of 5% TCA. The incorporated radioactivity was determined as described for the in vivo experiments.

F. Physiological and chemical measurements and determinations.

1. Electroencephalographic recording: EEG recording was made with a 4 channels Grass Model 78 EEG and Polygraph, using 7P511 amplifier. The recording was done with bilateral stainless steel electrodes made from insect pins with 50 μ tip inserted through holes which were drilled in the skull at the

frontal area of the cortex. The recording was taken against a reference electrode connected to the bone or to the skin. A solution of serotonin in saline or saline was injected through a cannula, made from a 22 gauge needle, inserted into the lateral ventricle of the left hemisphere. The apparatus used in these experiments was designed and made in this laboratory. It is described in Figure 15. The animals were immobilized by stereotaxic apparatus during the recording.

2. Polyacrylamide Gel Electrophoresis: The fractions used for the electrophoretic separation were obtained from animals that previously were injected with H^3-leucine (10 μc per animal) and pretreated with either sham ECS or ECS. Three animals were used per each group and the brains of these animals were combined for the preparation of the fractions. The procedure of Davies [87] was used for the electrophoretic separation of proteins from the various fractions obtained from the cerebral cortex. Each gel was composed of 7.0% acrylamide, 0.03% N,N,N',N'-Tetramethylethylenediamine, 0.20% N,N'-Methylene-bis-acrylamide, and was buffered with 4.5% Tris base and enough HCl to obtain pH8.1. Polymerization was initiated with ammonium persulfate 0.14% (w/v) and then exposed to fluorescent light for 30 minutes until polymerization was completed. The upper and the lower buffer consisted of 0.3 g Tris base and 1.44 g glycine per liter (dissolved in H_2O) with or without SDS as indicated for each specific experiment. The electrophoresis was carried out at room temperature but the buffer used was refrigerated prior to the electrophoresis. The current transferred through the gels was 3-4 ma per gel. The samples were

used for the electrophoretic separation either without additional treatment or after preincubation with SDS. The preincubation was carried out in the presence of 1% SDS, 5 mM dithiothreitol, 0.01 M Tris pH8.1, and 2 mM EDTA, for 30 minutes at 37°C. The protein was then precipitated with 5 volumes ethanol and redissolved in 10 mM dithiothreitol, 2 mM EDTA, 0.01 M Tris HCl pH8.1 and 1% SDS by boiling for 1 minute. The samples were applied on the basic gel using a stacking gel consisting of 2.5% acrylamide, 0.5% riboflavin, 0.06% Temed and buffered with 0.75% Tris HCl pH8.1. The stacking gel was polymerized as described for the basic gel. After electrophoresis the gels were transferred to 12% TCA for 45 minutes and then washed with H_2O and stained with Coomasie blue for 4 hours. After staining, the gels were destained in 20% TCA or in 7% acetic acid. The gels were cut into slices of 1 mm thickness and the slices were then transferred to scintillation vials containing 0.5 ml formic acid 99%. The radioactivity of each slice was counted by a Beckman scintillation counter using 15 ml of the same scintillation liquid as described below (see solutions).

3. Autoradiography: Solutions of H^3-5-HT and H^3-leucine in saline were injected intracranially as described before. At the proper time, as indicated for each specific experiment, animals were sacrificed by cervical dislocation and the brains were quickly removed and transferred into 10% formaldehyde and 70% ethanol. After the labeled amino acid was introduced into the tissue it could be located by inspection of the photographic reaction that it produced on a photosensitive emulsion superimposed on the tissue. The silver grains produced

in this way were visible under the light microscope and were localized inside and outside the cells. The cells were visualized by a staining technique. In the present experiment the tissue was stained with hematoxylin and eosin. The exposure time in which the histological slices were exposed to the photosensitive emulsion was two weeks.*

4. Enzymatic determinations:

a. ATPase: The fraction of which ATPase activity was measured was incubated in 50 mM Tris HCl pH7.5, 100 mM KCl, 10 mM Mg^{++}, and 2 mM Na-ATP. The incubation was carried out at 37°C. for 5 and 10 minutes. The incubation was terminated by adding 5% TCA or 0.5 M PCA. The phosphate which was released during the incubation period as well as the ATP which remained after the termination of the incubation were then determined. (See section 7, page 27.)

b. Lactic dehydrogenase: The activity of this enzyme was measured according to Johnson [88]. The fraction in which the activity was to be measured was incubated in the presence of 50 mM Tris HCl, pH7.5, 0.001 M NADH and 0.001 M Na-pyruvate. The activity was measured both before and after the addition of triton X-100, by recording the changes in absorbance at 340 nm as a function of time.

c. Succinic dehydrogenase: Activity of this enzyme was determined according to the spectrophotometric method described by Bonner [89]. The incubation was carried out in the presence of 0.01 M KCN, 0.001 M $K_3Fe(CN)_6$, 0.02 M Na-succinate,

*The developing of the autoradiography was done in Dr. M. Hamburgh's laboratory at City College of the City University of New York. The author wishes to thank Dr. Hamburgh for his help in this part of the work.

and 0.01 M phosphate buffer pH7.2. The absorbance at 400 nm was followed as a function of time.

d. 5' nucleotidase: Activity of this enzyme was determined according to the method of Heppel and Hilmore [90]. The fraction in which the activity was tested was incubated with 0.1 M glycine - NaOH buffer pH8.5, 0.01 M $MgCl_2$, and 0.003 M 5' - AMP. Incubation period was 15 minutes at 37°C. The reaction was stopped by 5% TCA and the phosphate liberated during the incubation period was then determined. (See section 8, page 28.)

5. RNA determination: The Orcinol method described by Mejbaum [91] was used for RNA determination.

6. Protein determination: The method described by Lowry et al [92] was used for protein determination.

7. ATP determination: The endogenous content of the ATP in the whole brain was determined as follows: Animals with an exposed skull were soaked in cold Freon immediately after treatment and brains were quickly removed (10 sec.) and transferred to 5 ml solution of NaF (0.15 M) in which homogenization was carried out. One ml of the homogenate was transferred to 4 ml of boiling water and kept boiling for 5 minutes. The supernatant obtained after precipitating the denatured proteins was used for the ATP determination, which was done according to the Luciferin-Luciferase method [93]. When ATP concentrations were determined for <u>in vitro</u> experiments, the neutralized supernatant obtained after the addition of PCA to the incubation medium was used. The PCA was neutralized with $KHCO_3$. Perchlorates were removed by centrifugation after the solution was kept at -20°C. for 20 minutes.

8. Phosphate determination: Phosphate concentration was determined according to Fiske and SubbaRow [94].

9. 5-HT: The method described by Welch and Welch [95] was used for the determination of the 5-HT.

10. Determination of endogenous ATP, ADP and AMP concentrations: The same supernatant that was used for ATP determination was also used for that of the three adenine nucleotides. The concentrations were determined by measuring the absorbance at 260 nm of the eluted bands corresponding to ATP, ADP and AMP after chromatographic separation. The preparation for the chromatographic separation involved adsorption of the nucleotides on activated charcoal (50 mg/ml) and elution with 50% ethanol containing 1% ammonium hydroxide after being washed twice with 5% TCA and three times with H_2O. The chromatographic separation was done on a Whatman No. 3 paper using isobutyric acid: H_2O :25% NH_4OH (66:33:1) as a solvent. The bands were identified with the help of markers.

G. Solutions

1. Medium A: 0.25 M sucrose, 0.05 M Tris HCl pH7.5, 0.025 M KCl, 0.001 M $MgSO_4$.

2. Scintillation liquid 0.3% Diphenyl Oxazol (PPO) and 0.01% 1,4 bis (4-methyl, 5 phenyl oxazolyl) Benzene (POPOP) in toluene: ethanol 7:3.

Other specific procedures were described as footnotes in the related figure or table.

RESULTS

Experiments were designed to answer several major questions:

1. Does ECS cause inhibition of protein synthesis in the central nervous system?

2. Is the inhibitory effect specific with respect to various regions of the brain, subcellular fractions or certain proteins?

3. What mechanism could be accounting for the inhibitory effect?

4. Is it possible that 5-HT mediates the inhibition of protein synthesis induced by ECS?

5. Do elevated levels of 5-HT in the CNS produce retrograde amnesia?

6. Are there relationships between retrograde amnesia induced by post training ECS, elevated levels of 5-HT, and inhibition of protein synthesis in the CNS?

The experiments were divided into three major groups: 1) In vivo experiments using mice as experimental subjects; 2) In vitro experiments using cell-free systems; and 3) Behavioral studies.

In vivo experiments:

1. Characterization of the in vivo system

The rate of the incorporation of C^{14}-leucine into the protein of the whole brain homogenate is described in Figure 1.

In this experiment two routes of injection were tested: intraperitoneal injection (IP) and intracranial injection (IC). During the first 5 minutes of the incubation, both slopes are linear. Ten minutes after the injection the rate of the incorporation resulting from the IC injection departed from linearity, whereas the rate of the incorporation resulting from the IP injection was still linear. These results are consistent with the finding described by Lajtha [96], in which 50% of the free radioactive leucine administered subarachnoidally was shown to disappear within 14 minutes. As can be seen in Figure 1, the rate of the incorporation resulting from IC injection is much greater than the rate resulting from the IP injection, but the period of the linearity is shorter, probably because of the fast disappearance of the labeled leucine from the brain under the condition of IC injection. In addition, in order to obtain the same or even higher specific activity of the brain proteins, much less radioactive amino acid is required when IC injection is used than when IP injection is used.

The distribution of the radioactive leucine after IC administration was examined by autoradiography. Rapid distribution in all brain regions was demonstrated. (Figure 2) In the cerebral cortex the number of silver grains found in slices prepared from animals treated with ECS were about the same as those found in the control animals. The number of silver grains found within the cellular area (as indicated

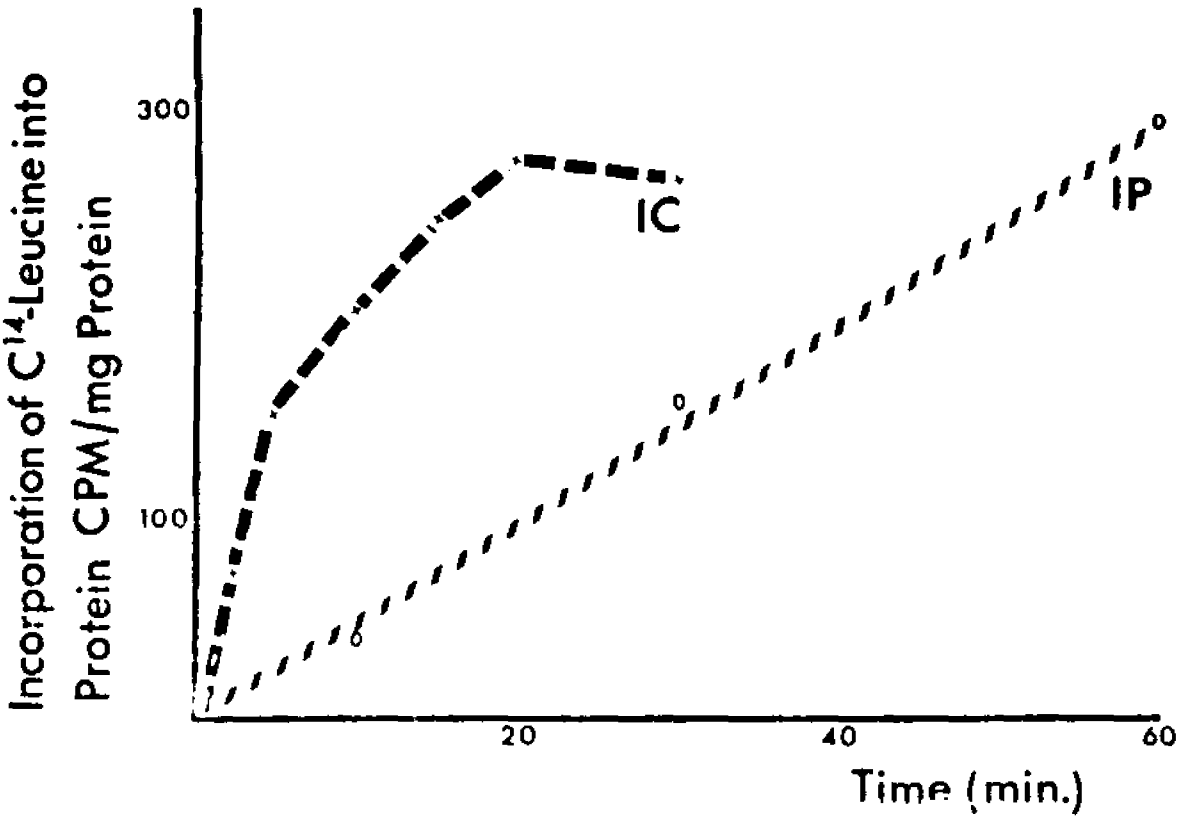


Figure 1: The kinetics of the incorporation of C^{14}-leucine into whole brain protein after intracranial and intraperitoneal injections.

Intracranial injection (IC) was given as described in the method section. For the intraperitoneal injection (IP) 1 µc was administered per 10g body weight. Each point on the graph represents combined tissue from at least 6 animals.

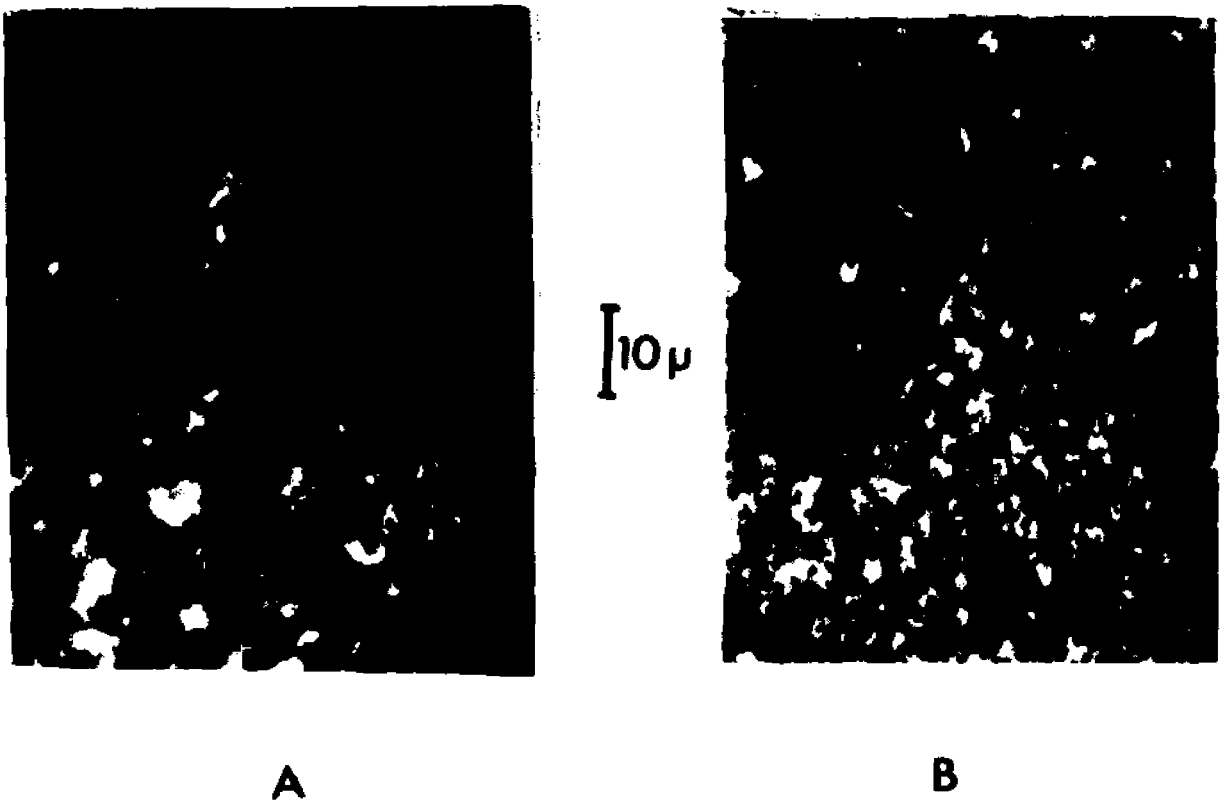


Figure 2: Autoradiograph of the cerebral cortex and cerebellum labeled with H^3-leucine before and after ECS.

H^3-leucine was injected intracranially to animals receiving either ECS or sham ECS ($\overline{ECS}$). Five minutes later the animals were sacrificed and the autoradiograph was prepared as described in the method section. The following brain areas were observed:
A) Cerebral cortex from $\overline{ECS}$ treated animals
B) Cerebral cortex from ECS treated animals
C) Cerebellar cortex from $\overline{ECS}$ treated animals
D) Cerebellar cortex from ECS treated animals
The area within the cerebral cortex which was observed was between the corpus callosum and the upper surface of the cortex approximately halfway between the olfactory bulbs and the occipital pole. The area observed within the cerebellar cortex was between the granular inner layer surrounded by the Purkinje cells and the surface area of the cerebellar cortex. The magnification was 625X. $\overline{ECS}$ represents sham treatment and ECS represents actual treatment.

FIG. 2 C

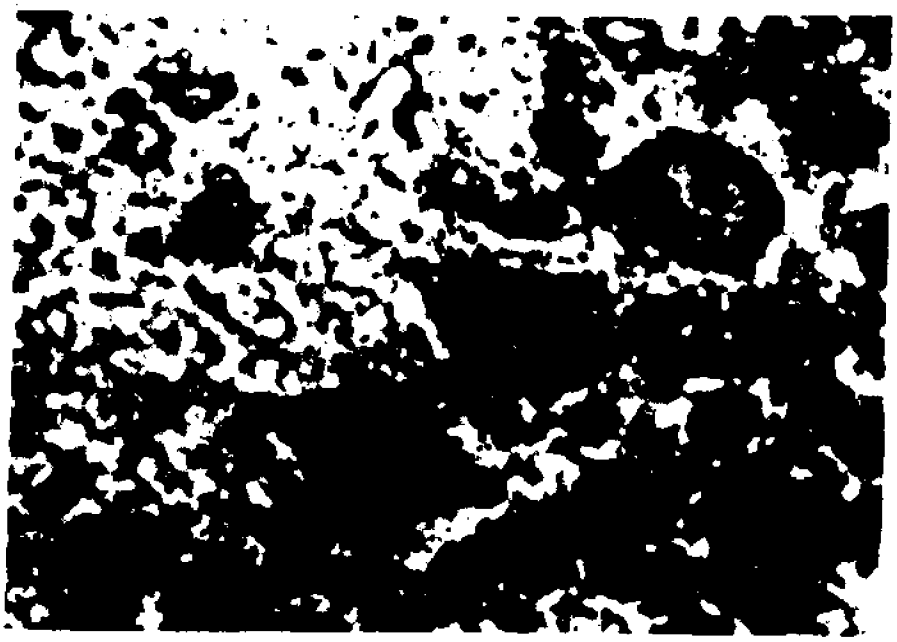


FIG. 2 D

by the staining technique) were similar in both treatments, ECS and control. On the other hand, for the cerebellar cortex the number of silver grains produced within the cellular area seems to be greater for the control animals (an average of 12 per cell) when compared to ECS treated animals (an average of 7 per cell).

An intracranial injection of labeled amino acid might change the endogenous concentration of this same amino acid. Therefore, several concentrations of C^{14}-leucine ranging from 0.0002 μ moles, which is much below the whole brain content, to 0.06 μ mole, which is double the whole brain content [97], were injected and the incorporation of the amino acid into protein was examined. The results of this experiment are presented in Table 1. It seems that the

Amount of C^{14}-leucine intracranially injected μ moles	Incorporation of C^{14}-leucine into whole brain protein CPM/mg protein/5 min.
0.0002 (300 μc/ mole)	172
0.001 (60 μc/ mole)	178
0.01 (6 μc/ mole)	162
0.06 (1 μc/ mole)	96

Table 1: The effect of increasing amounts of intracranially injected C^{14}-leucine on the incorporation of the labeled amino acid into the whole brain protein.

A constant amount of radioactivity with increasing leucine concentration was injected (10 λ per animal) intracranially as described in the method section. Each value represents an average from five animals.

incorporation is not concentration dependent. Only those concentrations which are close to the endogenous levels (0.01 and 0.06 μ moles) changed the specific activity of the whole brain proteins. This effect could be accounted for by the isotopic dilution which is apparent at the higher concentrations of the amino acid injected. The specific activity of the whole brain proteins resulting from the incorporation of several amino acids are described in Table 2. Leucine and proline showed the same rate of

Amino acid injected	Incorporation of the labeled amino acid into whole brain protein CPM/mg protein/5 min.
l-leucine	173
l-proline	180
l-phenylalanine	129

Table 2: Incorporation of several amino acids injected intracranially.

The amino acids were labeled with C^{14}. 0.06 μc of each amino acid was injected intracranially. Each number represents combined tissue from five animals.

incorporation; phenylalanine was incorporated to a lesser extent. Based on the results described above, intracranial injection of labeled leucine (10 μ l of 0.001 M containing 0.06 μ c) with incubation period of five minutes were chosen for the experiments in the present work.

2. The effect of ECS on the incorporation of labeled amino acids into the brain proteins

The effect of ECS on the incorporation of C^{14}-leucine into the whole brain protein at different intervals between ECS and the injection of the labeled amino acid is described in Figure 3. The effect appears to be brief. Full recovery to pre-treatment levels of incorporation usually has been observed about 15 minutes following treatment. An isotopic dilution effect due to an increase in the endogenous pool of the free amino acid [78] was excluded by increasing the amount of leucine which was intracranially injected (with a constant amount of radioactivity). As shown in Table 3, the extent of the inhibition was not affected by increasing the concentration of the labeled amino acid injected. Differential

Amount of C^{14}-leucine injected intracranially (μmoles)	Percent inhibition induced by ECS
0.0002 (300 μc/ mole)	36% ($\pm$3)
0.001 (60 μc/ mole)	37% ($\pm$4)
0.01 (6 μc/ mole)	30% ($\pm$4)
0.06 (1 μc/ mole)	39% ($\pm$3)

Table 3: The inhibitory effect of ECS on the incorporation of the labeled amino acid into the whole brain protein in presence of varying amounts of C^{14}-leucine.

Conditions were the same as those described in Figure 3. Each percent represents an average between two groups given the same treatment. The numbers in parentheses are indices of variability.

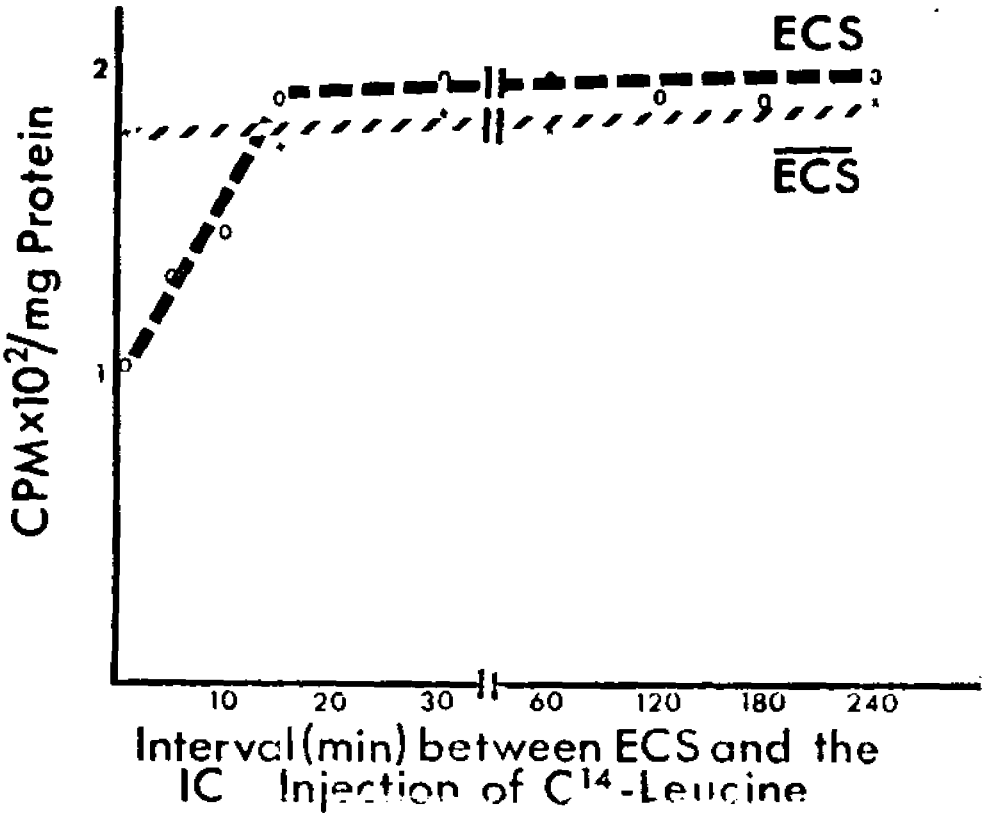


Figure 3: The effect of ECS on the incorporation of C^{14}-leucine into the whole brain protein in vivo.

Each value represents an arithmetic mean of two groups, each of which consisted of 5 animals. $\overline{ECS}$ represents sham treatment (current was not transfered through the electrodes). ECS represents actual treatment.

distribution of the injected amino acid as a result of ECS can be excluded as indicated in the autoradiography studies (Figure 2).

Regional studies of the inhibitory effect of ECS are shown in Table 4. The greatest amount of inhibition was

Brain region	Incorporation of C^{14}-leucine into protein CPM/mg protein		Percent inhibition by ECS
	$\overline{ECS}$	ECS	
cerebral cortex	190 (±5)	84 (±8)	56
diencephalon	571 (±31)	379 (±19)	33
cerebellum	468 (±22)	343 (±24)	27

Table 4: The effect of ECS on the incorporation of C^{14}-leucine into the protein of various regions of mouse brain.

Each number represents an average between two groups. Equivalent brain regions from five animals were combined and treated as a group resulting in two groups totalling 10 animals per treatment per brain region. Numbers in parentheses represent the maximum variability. $\overline{ECS}$ represents sham treatment and ECS represents actual treatment. Brain regions were prepared as described in the method section.

For the calculation of the percent inhibition see Appendix Ia.

observed in the cerebral cortex. The cerebellum on the other hand was characterized by the smallest amount of inhibition. The amount of the radioactivity which was incorporated into the cerebellar proteins together with rapid distribution of the radioactivity intracranially injected, shown in the autoradiographic studies (see page 32A) indicate that the

low inhibition in the cerebellum is not due to a lack of labeled substrate availability at the cerebellar site.

Results obtained with subcellular fractions of the cerebral cortex following ECS are summarized in Table 5.

Subcellular fraction	Incorporation of C^{14}-leucine into protein CPM/mg protein		Percent inhibition by ECS
	$\overline{ECS}$	ECS	
whole homogenate	190 (±5)	84 (±8)	56
microsomes (P_3)	565 (±21)	232 (±11)	59
mitochondria (C)	64 (±2)	30 (±2)	53
synaptosomes (B)	50 (±2)	14 (±1)	72
soluble fraction (S_3)	201 (±4)	90 (±6)	55

Table 5: The effect of ECS on the incorporation of C^{14}-leucine into the protein of various subcellular fractions from the cerebral cortex of a mouse brain.

Each value represents an average taken from two groups, each group consisting of five animals, the brains of which were pooled and developed for radioactive determination as described in the method section. Numbers in parentheses represent variabilities between duplicates. $\overline{ECS}$ represents sham treatment and ECS represents the actual treatment. It should be noted that variations of this experiment were performed at three other times through the course of the present work and the same general tendency was shown in each case.

The greatest inhibition occurred in the synaptosomal fraction as compared with the microsomal or the mitochondrial fractions. The presence of the synaptosomes in fraction B was assessed by enzyme markers (Table 6). Electron micrograph of the synaptosomal fraction obtained in this laboratory using the same procedure which is described in the present dissertation,

revealed relatively pure synaptosomes with minimal contamination from mitochondria and myelin fragments [98]. In order

Subcellular fraction	Enzyme activity μ moles/hour/mg protein			
	ATPase	Succinic dehydrogenase	Lactic dehydrogenase -triton X100	Lactic dehydrogenase +triton X100
whole homog.	10.4	12.0	20.1	26.4
mic. (P_3)	19.7	0.0	0.9	1.4
light myel. fragments(A)	11.2	0.2	1.5	2.0
synapt.(B)	21.2	31.0	4.9	95.0
mit.(C)	5.9	82.0	11.4	19.7
soluble fractions (S_3)	2.1	0.0	181.2	173.0

Table 6: Activities of marker enzymes in various subcellular fractions from the cerebral cortex of a mouse brain.

The enzyme's activities were determined as described in the method section. The following amounts of the various fractions expressed in mg protein were taken for the enzymatic determinations. For ATPase, whole homogenate: 1mg, P_3:0.5mg, A:0.2mg, B:0.2mg, C:2mg, S_3:2mg were used. For succinic dehydrogenase, whole homogenate: 1mg, P_3:1mg, A:0.5mg, B:0.1mg, C:0.5mg, S_3:2mg were used. For lactic dehydrogenase, whole homogenate: 0.1mg, P_3:0.5mg, A:0.2mg, B:0.05mg, C:0.1mg, S_3:0.05mg were used.

to obtain a significant amount of radioactivity in the synaptosomal fraction, massive amounts of the labeled precursor were administered. Therefore, even little microsomal contamination in the synaptosomal fraction would result in a considerable amount of radioactivity in this fraction. To test further for the possibility of microsomal contamination in the synaptosomal fraction, unlabeled cortex was homogenized in labeled post-

mitochondrial supernatant (S_2) and refractionation of the homogenate was then performed. Evidence for some microsomal contamination was found (Table 7), but this contamination clearly is not sufficient to explain the higher labeling that was observed in this fraction when radioactive amino acid was injected into the brain and labeled cortex was then fractionated.

Subcellular fraction	Fractions prepared from labeled homogenate		Fractions prepared from unlabeled homogenate and labeled S_2	
	CPM/mg protein	% radioactivity of whole homog.	CPM/mg protein	% radioactivity of whole homog.
whole homog.	1418	100.0	605	100.0
postmit. sup. (S_2)	4254	79.0	2366	96.2
small myel. fragments (A)	411	0.4	207	0.5
synapt. (B)	421	8.8	98	2.5
mit. (C)	493	9.7	77	1.6

Table 7: Specific activities and percent labeling which appeared in subcellular fractions extracted from animals injected with labeled leucine and in fractions extracted from unlabeled homogenates supplemented with labeled S_2.

Six animals were injected intracranially with H^3-leucine (38 c/mM, 5 μc/animal). Five minutes after the injection animals were sacrificed and fractions were then prepared from the pooled cerebral cortex. Five additional animals which did not receive injections were sacrificed and the brains were homogenized in 9.8 ml of the labeled S_2 extracted from the labeled brains. The homogentates were then fractionated and radioactivity was determined in the subcellular fractions and expresseed per mg. protein. The total radioactivity in each fraction was measured and expressed as a percent of the total radioactivity of the whole homogenate.

The inhibition of protein synthesis in the synaptosomal fraction as a function of time is shown in Figure 4. The inhibition is of a very short duration and recovery to the control levels of incorporation occurred after five minutes. However,

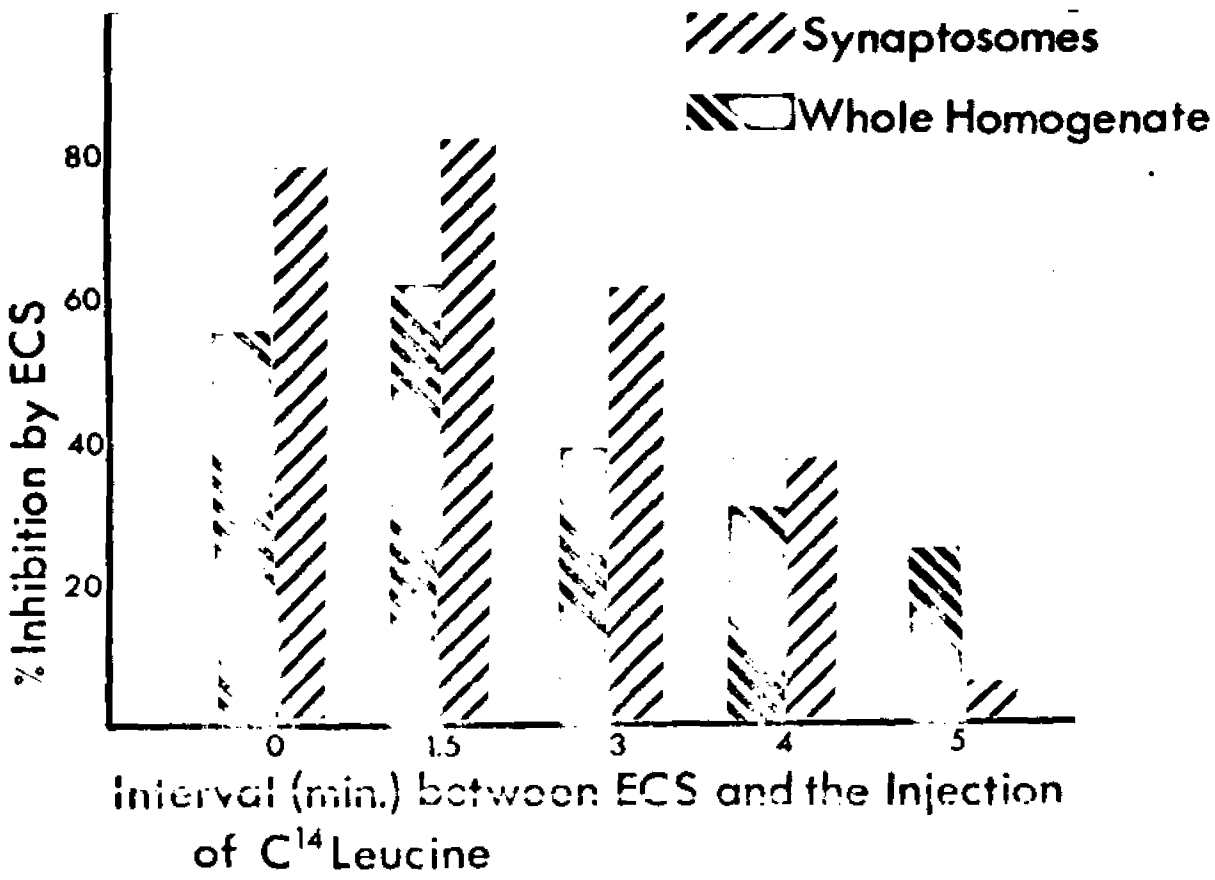


Figure 4: The effect of ECS on the incorporation of C^{14}-leucine into the protein of the synaptosomal fraction and the whole homogenate after varying intervals between ECS and the injection of C^{14}-leucine.

Five animals per group were injected with H^3-leucine intracranially at different periods after the administration of the ECS. Five minutes later the animals were sacrificed and the brains were subfractionated. Radioactivity was determined in the whole homogenate and in the synaptosomal fraction. Percent inhibition by ECS was calculated for both fractions as shown in Appendix I-a.

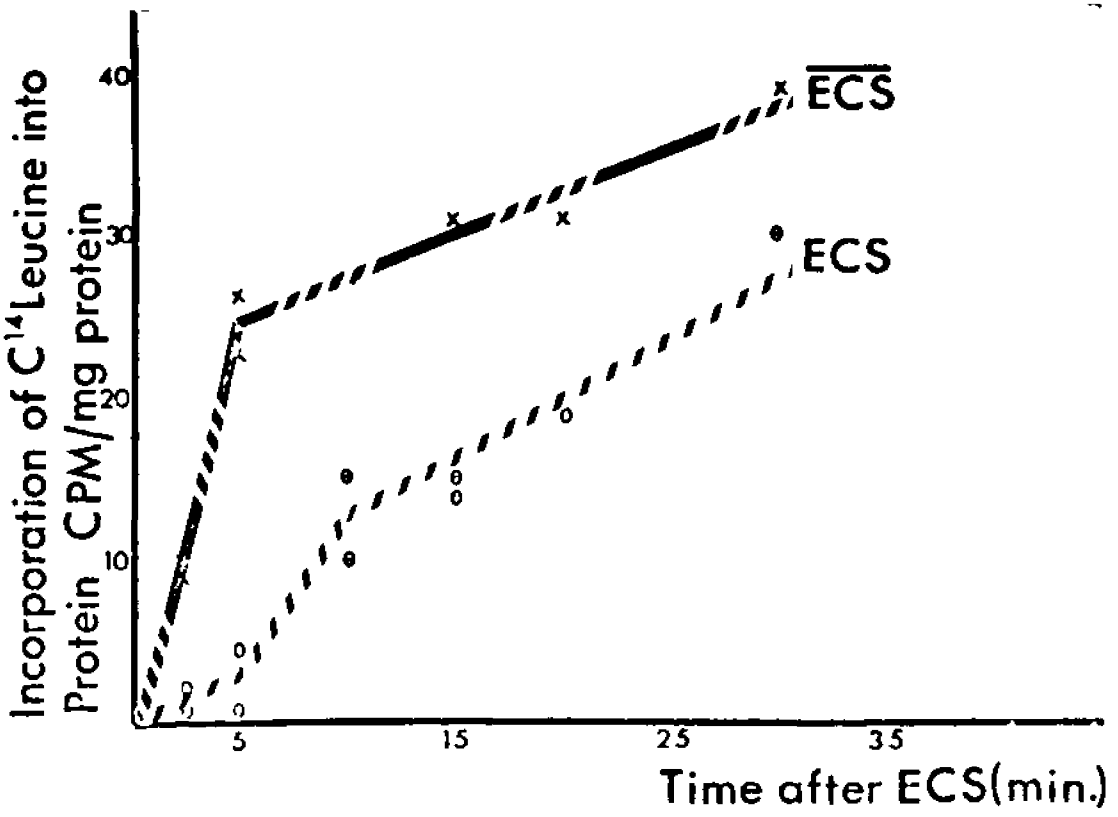


Figure 5: The kinetics of the incorporation of H^3-leucine into the synaptosomal protein with and without ECS.

C^{14}-leucine was injected simultaneously with the administration of ECS or sham ECS. At different periods after the ECS administration, animals were sacrificed and synaptosomes were prepared as described in the method section. Each point on the graph represents results obtained from three animals. Observations at several periods were duplicated and are indicated where obtained. ECS represents sham treatment and ECS represents actual treatment.

when the amino acid was injected intracranially immediately after ECS and the incorporation was then determined at varying periods, a triphasic curve was shown for the ECS treated animals, whereas a biphasic curve was indicated for the control animals (Figure 5). A small amount of inhibition in the synaptosomal fraction was still observed in this case even thirty minutes after the injection. The rate of the incorporation at thirty minutes after the treatment was similar to that of the control animals.

In order to locate the synaptosomal site (i.e., outer membrane, intra-synaptosomal mitochondria, or the soluble fraction of the synaptosome) into which the labeled amino acid was incorporated, synaptosomes were disrupted and subfractionation was then carried out. Most of the label was located on the fraction derived from the interphase of 0.4 M and 1.2 M sucrose (Table 8). On the basis of enzyme markers (LDH as a

Fraction	Incorporation of H^3-leucine into protein	
	CPM/mg protein	% radioactivity
whole synaptosome	94	100.0
soluble fraction of the synaptosome (top of gradient)	21	0.3
external synaptosomal membrane (interphase between 0.4 and 1.2 M sucrose)	143	70.5
intrasynaptosomal mitochondria (pellet)	43	27.7

Table 8: The distribution of the labeled proteins within the synaptosomal subfraction after a short pulse with H^3-leucine.

H^3-leucine was injected intracranially. Five minutes later animals were sacrificed and fractions prepared from cerebral cortex (see method section). Percent of radioactivity in each fraction was calculated as described in Appendix Ib.

marker for the soluble fraction, 5' nucleotidase as a marker for the outer membrane and succinic dehydrogenase as a mitochondrial marker) this fraction was identified as synaptosomal membrane, and possibly some partially disrupted synaptosomes (Table 9). It is clear from these results that the specific activity of the intrasynaptosomal mitochondrion is much smaller than the specific activity of the synaptosomal membrane. The significance of this finding will be considered in the Discussion section.

Origin of fraction	Enzyme activity μ moles/hours/mg protein			
	ATPase	LDH	Succinic dehydrogenase	5' Nucleotidase
top of the gradient	5.2	82	--	--
interphase between 0.4 and 1.2M sucrose	23.4	7	12	++
pellet	9.1	5	152	--

Table 9: Activities of marker enzymes in various subfractions extracted from disrupted synaptosomes.

Fractions were prepared as described for Table 8. Conditions for enzymatic determination were the same as those described in the method section and for Table 6. About 92% of the succinic dehydrogenase was found in the pellet whereas 8% was found in the interphase between 0.4 and 1.2M succrose. No detectable activity was found on the top of the gradient. The sign -- represents the cases where activity was not detectable by the method used. The sign ++ was used in those cases where considerable amount of activity was detected.

Electrophoresis studies which were done with several subcellular fractions contributed relevant information to several pertinent questions: 1) Is the synthesis of specific proteins affected by ECS while the synthesis of other proteins is not

affected? 2) Is the observed differential inhibitory effect of ECS on microsomal and synaptosomal fractions due to different sedimentation properties of the labeled protein resulting from ECS? 3) Is the electrophoretic mobility changed as a consequence of ECS? The electrophoretic pattern of several fractions prepared from animals treated with ECS and sham ECS ($\overline{ECS}$) are shown in Figure 6. It is evident that ECS does not affect the mobility of the constituent proteins of S_2, P_2, and synaptosomal membrane. Figure 7 shows the distribution of the labeling along the bands electrophoretically separated, using fraction S_2 with and without the action of sodium deodecyl sulfate (SDS). When fraction S_2 was examined without pretreatment with SDS (Figure 7-A) most of the radioactivity was found at the origin. After ECS the peak at the origin showed the largest percent decrease in incorporated radioactivity. The proteins which migrated are probably the soluble proteins. Among these proteins the inhibition was relatively small and homogeneously distributed along the bands (Figure 7-A). When S_2 was pretreated with SDS, rapid migration of the radioactivity was indicated. Under these conditions, the inhibition was noted principally for those proteins which showed higher migration (Figure 7-B). These are probably polypeptides of lower molecular weight.

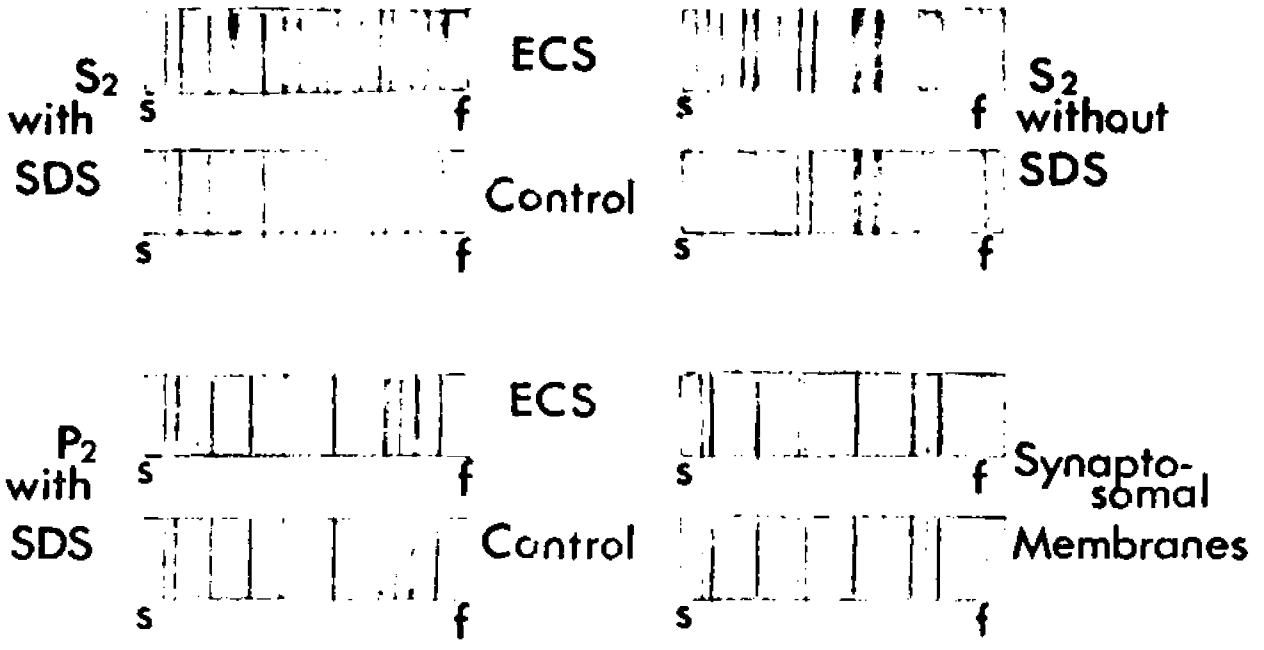


Figure 5: The electrophoretic distribution of proteins and polypeptides from various fractions extracted from animals pretreated with ECS or sham ECS.

Fractions were prepared for electrophoresis as described in the method section. The bands were stained with coomassie blue. The tracking dye was bromophenol blue. f represents the front where the tracking dye was located at the end of the electrophoresis. S represents the starting point (the border between the stacking gel and the basic gel). Electrophoresis was from cathode to anode.

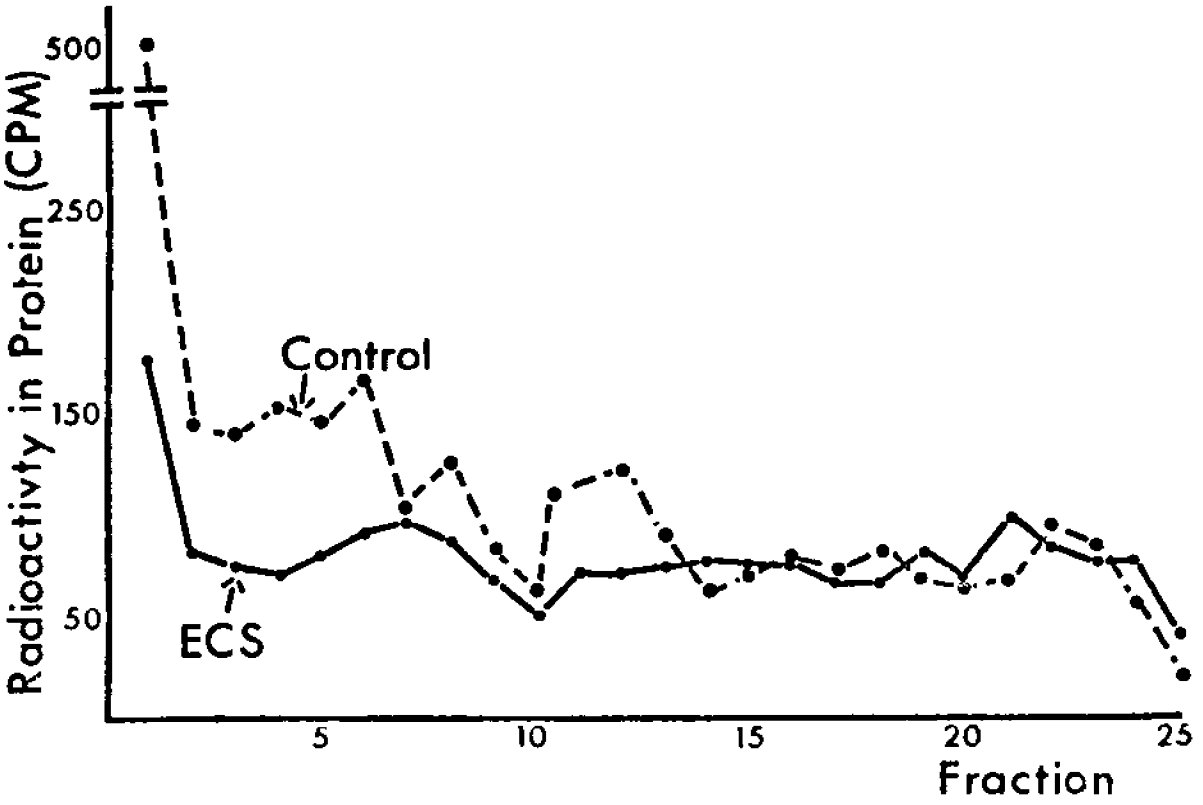


Figure 7: The effect of ECS on the incorporation of H^3-leucine into the postmitochondrial supernatant proteins separated by polyacrylamide gel electrophoresis.

(A) Polyacrylamide gel electrophoresis without sodium dodecyl sulfate (SDS)

Fractions were prepared for electrophoresis as described in the method section. After the electrophoresis and the fixing procedure the gels were cut and radioactivity of each fraction was determined and corrected by subtracting the background. Each fraction represents 1.5-2mm of gel. Each point on the graph represents the mean obtained from two similar experiments.

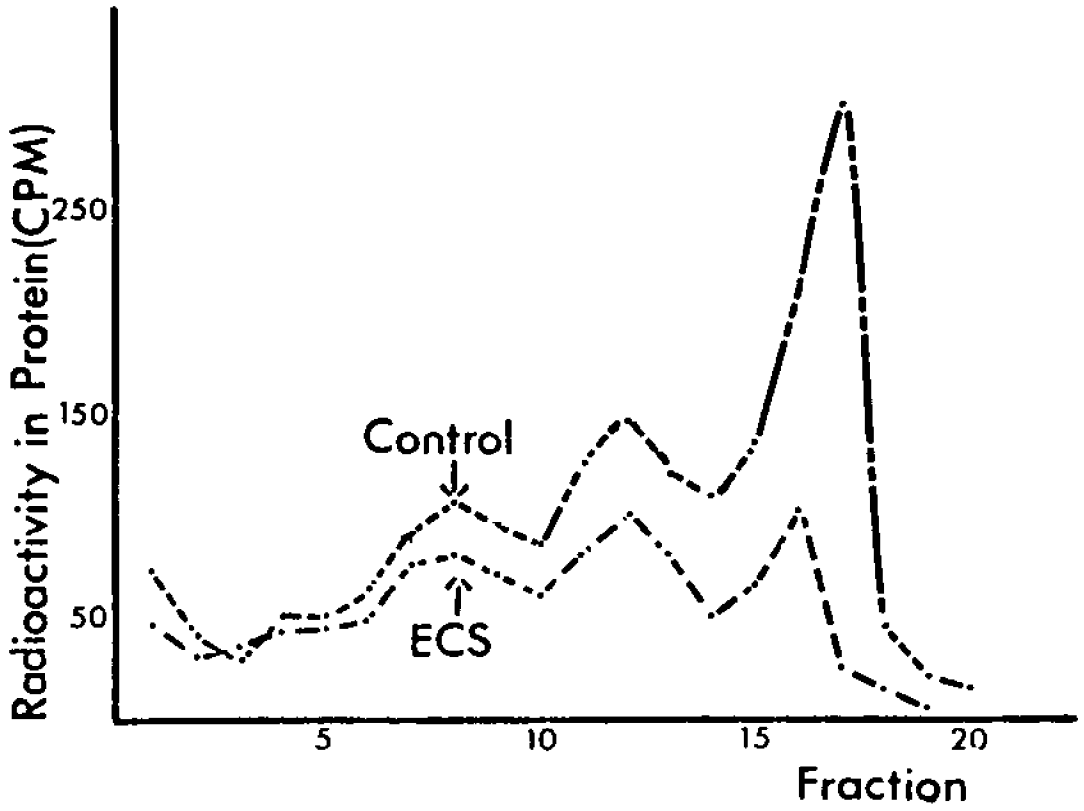


Figure 7(B): Polyacrylamide gel electrophoresis of S_2 after pretreatment with SDS; the effect of ECS on the distribution of the radioactivity in the electrophoretically separated proteins from S_2.

The distribution of the labeling along the bands electrophoretically separated from P_2, which was pre-treated with SDS, is shown in Figure 8. Whereas the inhibition induced by ECS was shown for faster migrating protein when S_2 was used for the electrophoretic separation, P_2 showed greater inhibition which was located on a very slowly migrating band. The distribution of the labeling among the bands electrophoretically separated from synaptosomal fraction and synaptosomal membranes are described in Figure 9. The results reveal a slowly migrating protein for both the synaptosomes and the synaptosomal membrane, the synthesis of which is apparently highly inhibited by ECS. This protein may be considered as a membrane constituent.

It has been indicated by Orrego and Lipman [99] that protein synthesis is inhibited in brain slices, but not in kidney slices, after electrical stimulation. They based their interpretation of such inhibition upon a fall in ATP level as a consequence of the electrical stimulation. ECS may cause a similar decrease in whole brain ATP level [100]. The results summarized in Figure 10 indicated that there is no strict correlation between the ATP drop and the course of the inhibition of the incorporation of labeled amino acid into protein after ECS. While the inhibition of protein synthesis lasted for 10 - 15 minutes, ATP returned to the normal level one to two minutes after the administration of

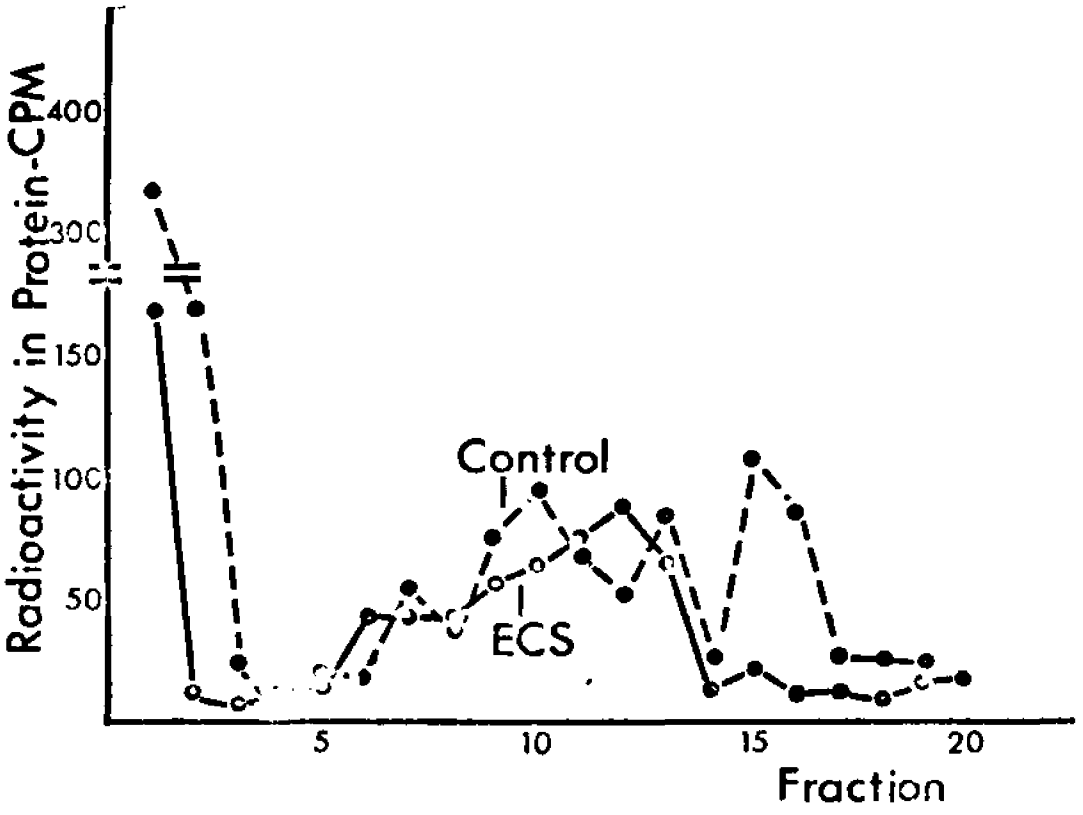


Figure 8: The effect of ECS on the incorporation of H^3-leucine into the crude mitochondrial (P_2) proteins separated by polyacrylamide gel electrophoresis in SDS.

For procedure and legend, see Figure 7.

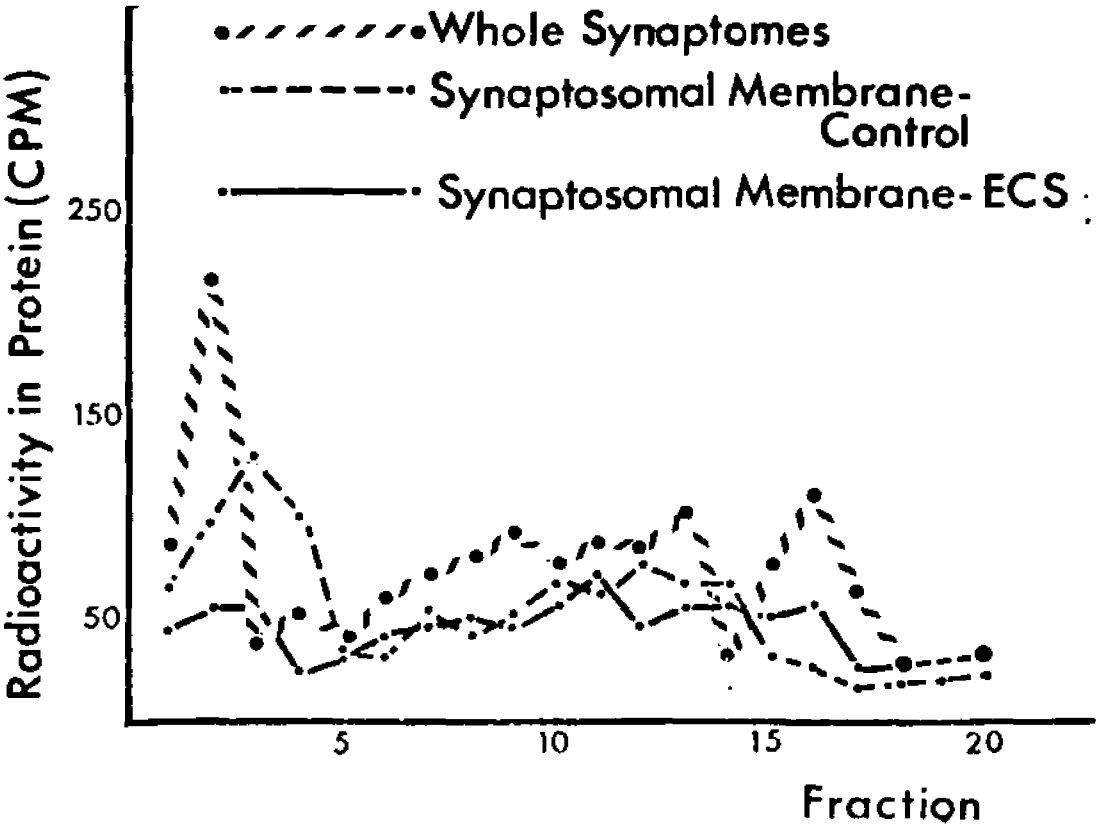


Figure 9: Polyacrylamide gel electrophoresis in SDS of labeled proteins and peptides of the synaptosomal and synaptosomal membrane fractions with and without ECS.

The synaptosomal membrane fraction was extracted from animals pretreated with ECS or sham ECS. The whole synaptosomal fraction was extracted only from control animals which were injected with H^3-leucine (3Sc/mC; 10 μc/animal). Other procedures were as described for Figure 7.

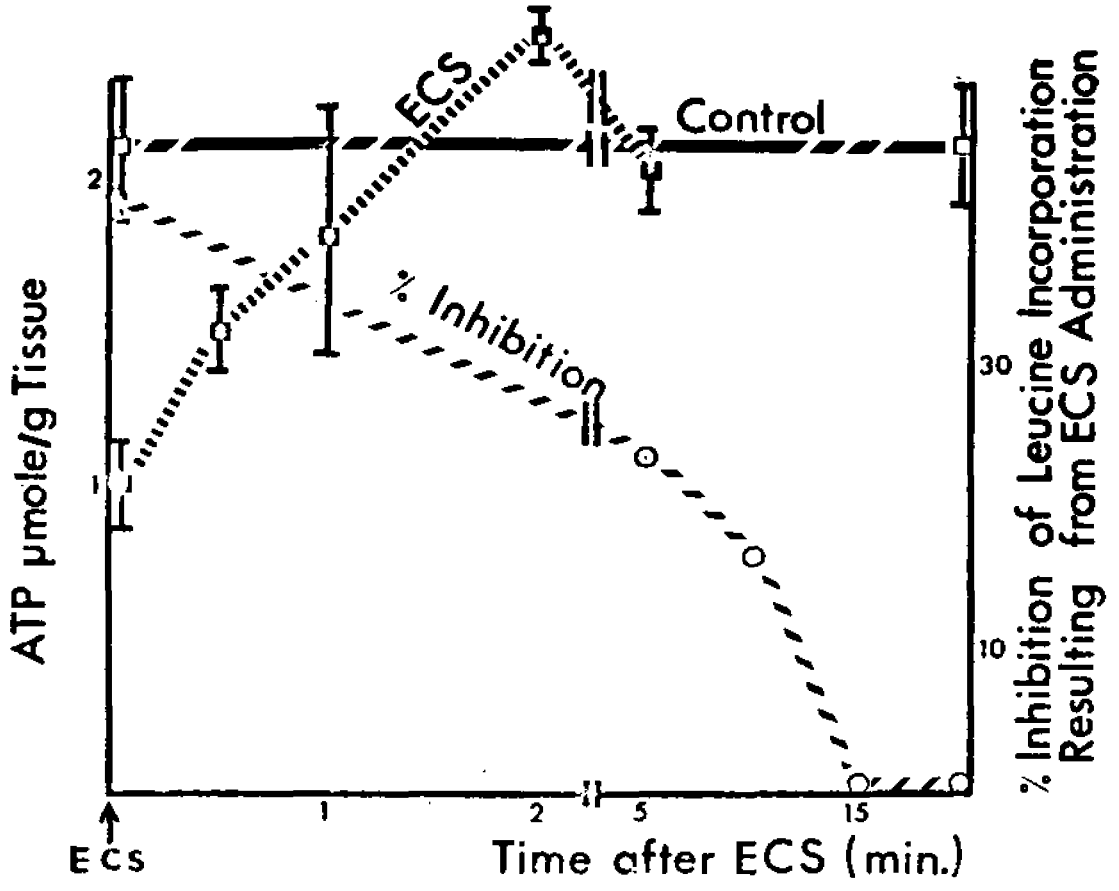


Figure 19: The changes in ATP levels and the recovery time of protein synthesis inhibition after ECS.

ATP was determined using five animals per group. Determinations were made individually for each animal. Each point on the graph represents the mean value obtained from five animals ± S.D. The percent inhibition was determined in a separate experiment and was calculated as described in Appendix I-a.

the shock. A more prolonged effect was found for ADP as well as for AMP levels. These results are summarized in Figure 11. A correlation between the recovery of the inhibition of protein synthesis and the return of ATP/ADP+AMP ratio to normal is shown in Figure 12.

A possible relationship between ECS, 5-HT and protein synthesis inhibition was considered in the next experiment. Mianserine, which has been recently suggested as a central 5-HT antagonist [101], was injected IP and the incorporation of labeled leucine into whole brain protein was tested at the peak of the antagonistic effect. As can be seen from Table 10, mianserine did not affect the degree of the inhibition produced by ECS. Further consideration of these results will be given in the Discussion section.

Treatment	Incorporation of H^3-leucine into whole brain protein	
	CPM/g tissue	% inhibition
control	7840 (825*)	--
control and mianserine	7320 (950*)	7
ECS	5020 (880*)	36
ECS and mianserine	4940 (785*)	33

Table 10: The effect of ECS on the incorporation of H^3-leucine into whole brain protein in animals pretreated with mianserine.

The measurement of the incorporated radioactivity was done for each animal individually. For each experimental variable 10 animals were used. The values represent the mean of 10 animals $\pm$ S.D. (S.D. is given in parentheses.) *p 0.05.

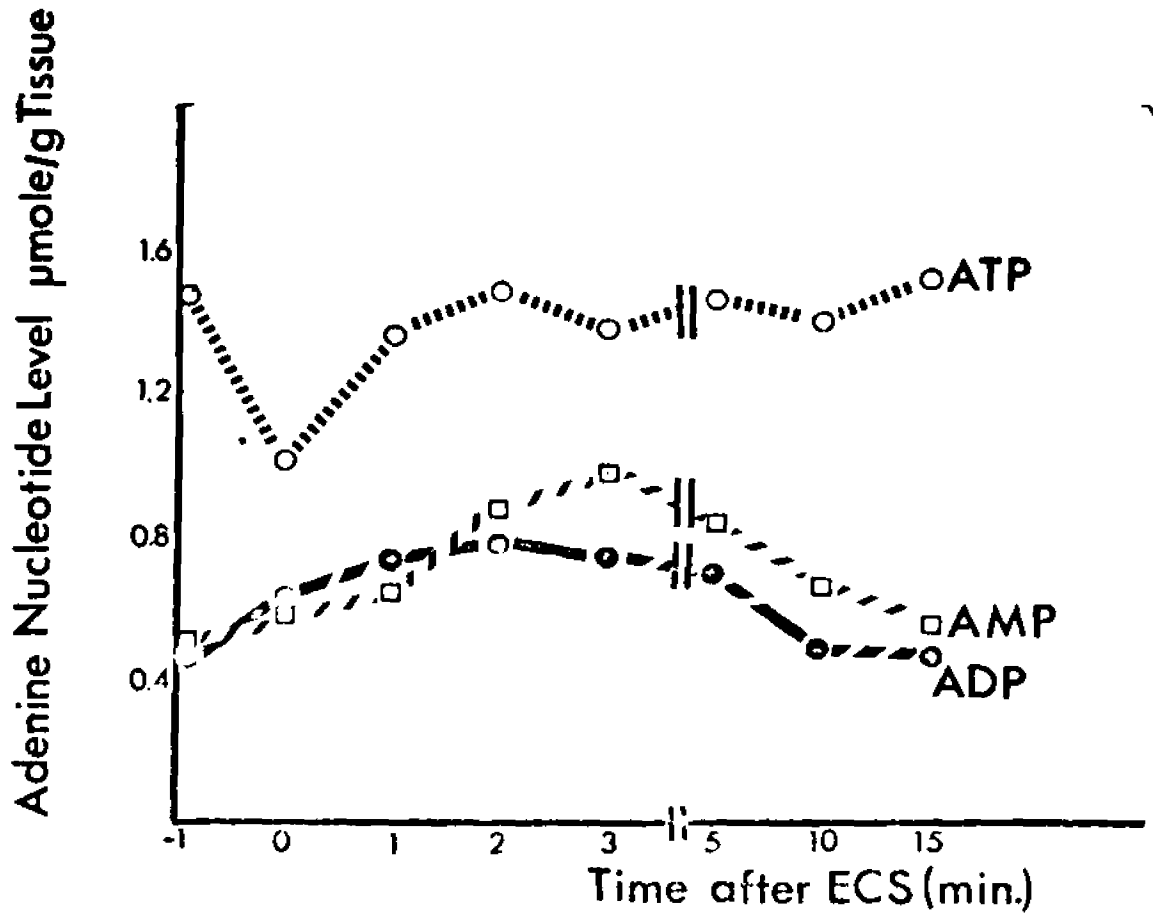


Figure 11: Adenine nucleotide levels in the whole brain after ECS.

Determinations of the nucleotide levels were made after chromatographic separation by measuring the absorbance at 260 nm. Each point on the graph represents the arithmetic mean of a duplicate obtained from 4 animals (2 animals for each determination).

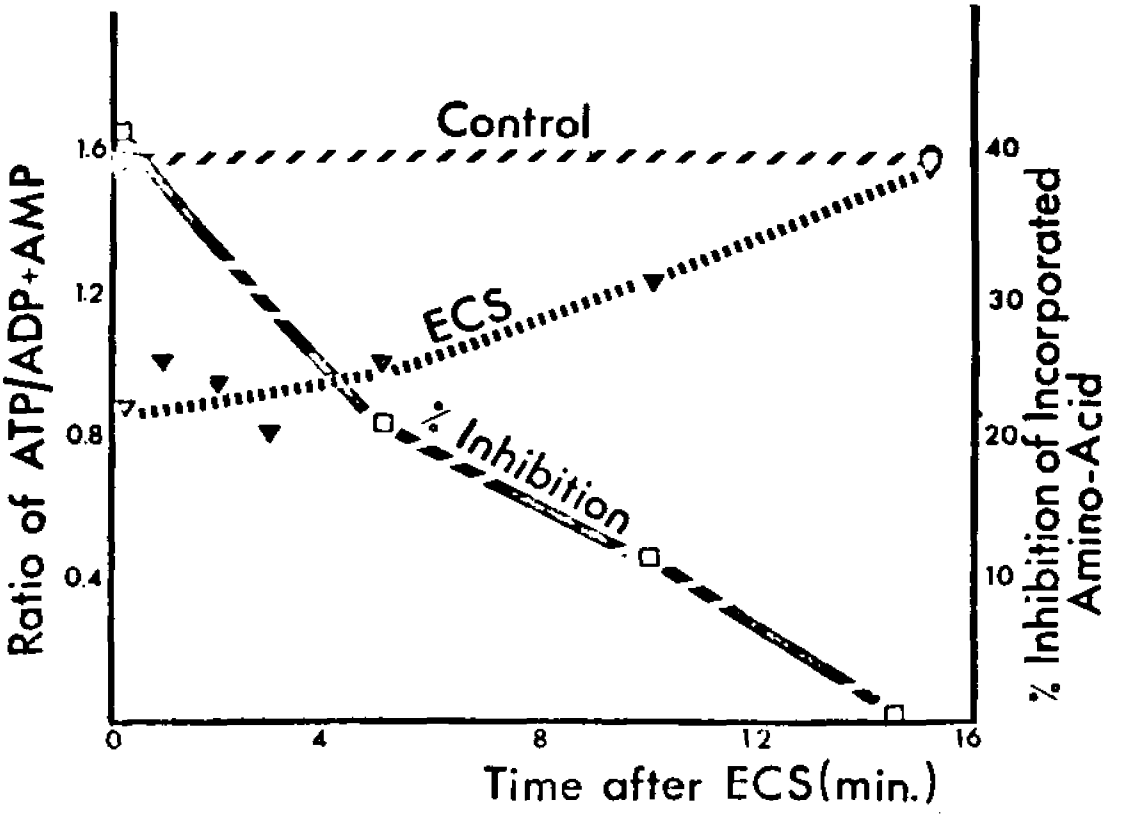


Figure 12: The changes in ATP/ADP+AMP ratio and the recovery time of protein synthesis inhibition after ECS.

Data from Figure 11 were used for the calculation of the ATP/ADP+AMP ratio. For detailed calculation procedure see Appendix I-c.

3. The effect of 5-HT on the incorporation of labeled amino acids into the brain proteins

In order to further correlate an elevated level of 5-HT with inhibition of protein synthesis in the brain, an MAO inhibitor (Pargyline) was injected IP and the incorporation of labeled leucine into whole brain proteins was then examined. The results are described in Table 11. It is clear that even at the maximum level of 5-HT, no significant inhibition of protein synthesis was indicated. A comparison of IP and IC injections did not show a significant difference between pargyline treated and the control animals.

MAO inhibitors are not specific in their action and may affect several enzymes beside MAO [102]. Furthermore, MAO inhibitors may not affect neuronal transmission through the inhibition of MAO but through a different mechanism [102]. In addition, it is reported that the release of NE from vesicles is inhibited by MAO inhibitors [102]. By analogy, the same may be applied to 5-HT. Therefore, a different method to elevate brain 5-HT was tested. 5-HT was injected IC and the whole brain 5-HT levels were then determined at several consecutive periods. The results are shown in Table 12. Two μg of 5-HT were found to produce an elevation of 5-HT comparable in total concentration to that produced by ECS. Most of the 5-HT injected intracranially was not recovered, probably due to a rapid degradation caused by the action of MAO *in vivo* and during homogenization.

Route of the injection of the labeled amino acid	Treatment	Whole brain 5-HT levels at various times (min.) after the injection of pargyline (μg/g tissue)			Incorporation of H^3-leucine into the whole brain protein CPM/g tissue
		0	90	150	
IP	control	0.79±0.10	--	0.61±0.16	3566± 633
	pargyline	0.91±0.15	1.11±0.10	1.17±0.12	3999± 502
IC	control	--	--	--	7796± 727
	pargyline	--	--	--	8373±1170

Table 11: The effect of pargyline on the incorporation of H^3-leucine into the whole brain protein and on the whole brain 5-HT levels.

The incorporated radioactivity was individually measured for each animal. Each group consisted of five animals. The values represent the mean from five animals ± S.D.

IP signifies intraperitoneal injection and IC signifies intracranial injection. 5-HT was determined individually for each animal according to the procedure described in the method section.

Treatment	5-HT levels (μg/g tissue) Time after IC injection (minutes) 0	10	30
saline	0.48 (0.06)	0.39 (0.03)	0.40 (0.05)
0.5 μg 5-HT	--	--	0.65 (0.10)
1.0 μg 5-HT	--	--	0.80 (0.10)
2.0 μg 5-HT	0.90 (0.10)	0.95 (0.06)	0.82 (0.10)
10.0 μg 5-HT	1.85 (0.15)	--	1.36 (0.08)

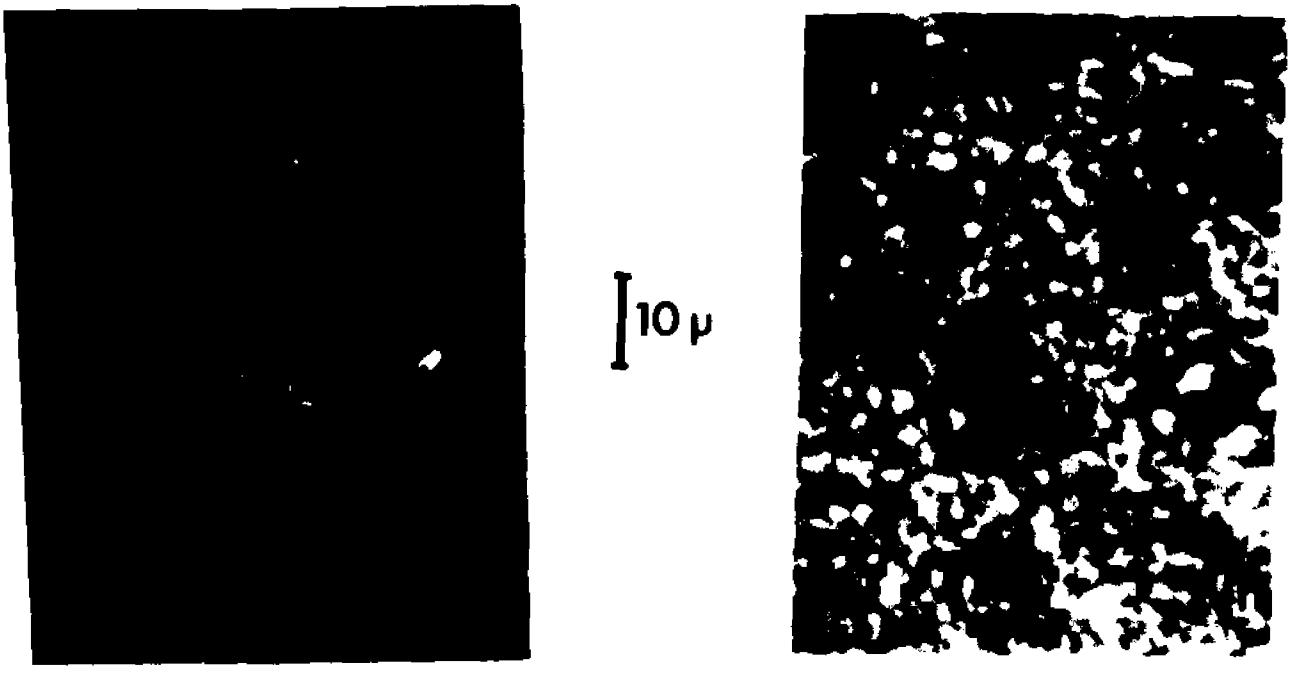
Table 12: Whole brain 5-HT levels after intracranial injection of 5-HT.

Several doses of 5-HT were injected intracranially. At different periods after the injection, as indicated in the Table, animals were sacrificed and homogenates from the whole brain were prepared as quickly as possible in 0.01 M HCl. Brain 5-HT levels were then determined as described in the method section. Each value represents the mean obtained from five animals (tested individually) ± S.D. (S.D. is given in parentheses.)

The increased 5-HT level lasted about 45 minutes after the intracranial injection. The distribution of the 5-HT which was intracranially injected was determined by autoradiographic techniques and shown in Figure 13. Autoradiography of brains injected with labeled 5-HT at various times after the injection revealed a rapid distribution throughout the brain. When animals were killed immediately after

the injection, most of the label did not diffuse from the ventricles. At 2.5 minutes after the injection, the midbrain area produced the largest number of silver grains. Fewer silver grains were produced by the cerebral cortex and none by the cerebellum. At 5 minutes after the injection, the number of silver grains produced by the cerebral cortex were comparable to those found in the midbrain area. At 10 minutes after the injection, the cerebral cortex contained more label than the midbrain. At this time, silver grains were also produced by the cerebellum.

The effect of intracranial injection of 5-HT on the incorporation of C^{14}-leucine into the whole brain proteins as a function of the interval between 5-HT administration and amino acid injection and as a function of dose is shown in Table 13. A small amount of inhibition was found for 2 μg 5-HT and a slightly larger amount of inhibition was found for 10 μg 5-HT. Although the brain serotonin levels obtained by both intracranial injection and ECS were similar, the amount of the inhibition was smaller in the case of intracranial injection than that observed when ECS was administered. The brain level of 5-HT was also increased by MAO inhibitors, but these inhibitors (e.g., pargyline), although not able alone to induce inhibition of protein synthesis, potentiated the effect of intra-



A B

Figure 13: Autoradiograph of various regions of the brain after intracranial injection of H^3-5-HT.

H^3-5-HT was injected intracranially. At varying periods after the intracranial injection animals were sacrificed and the brains were prepared for auto radiography (see method section). For other procedures and legend see Figure 2.

A: Midbrain at 0 minutes after the IC injection
B: Cerebral cortex at 0 minutes after the IC injection
C: Midbrain at 2.5 minutes after the IC injection
D: Cerebral cortex at 2.5 minutes after the IC injection
E: Midbrain at 5 minutes after the IC injection
F: Cerebral cortex at 5 minutes after the IC injection
G: Cerebellar cortex at 5 minutes after the IC injection
H: Midbrain at 10 minutes after the IC injection
I: Cerebral cortex at 10 minutes after the IC injection
J: Cerebellar cortex at 10 minutes after the IC injection

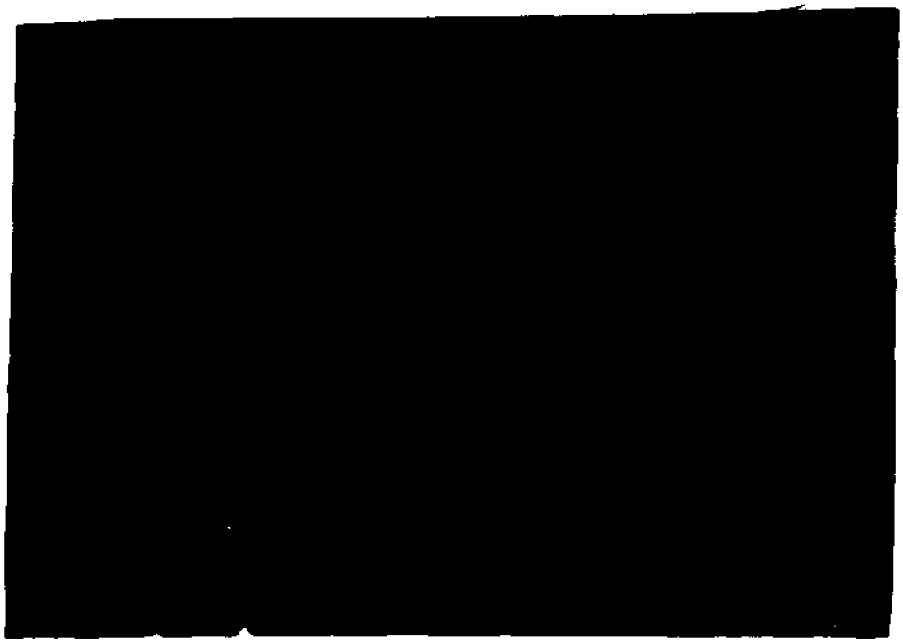


FIG. 13 C

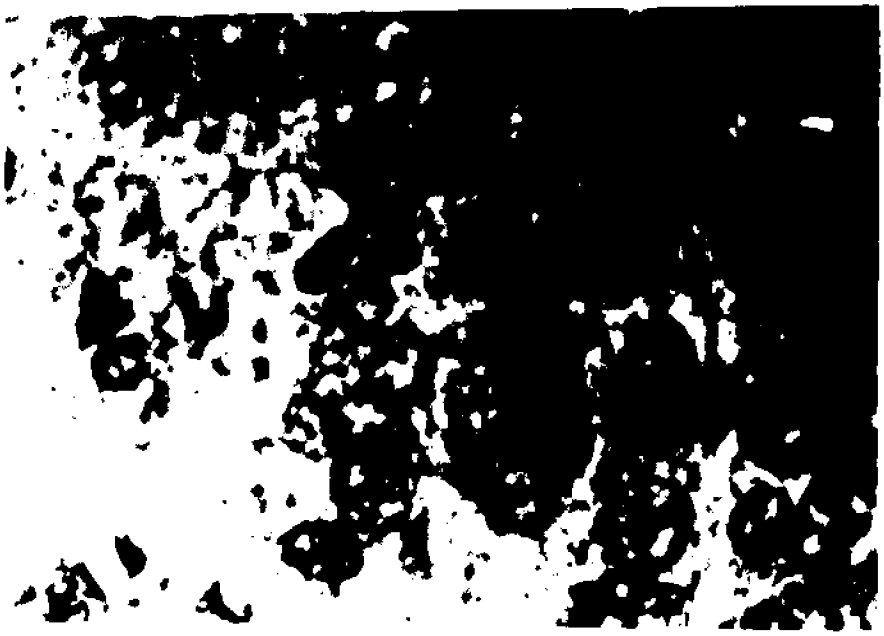


FIG. 13 D

FIG. 13 E

FIG. 13 F

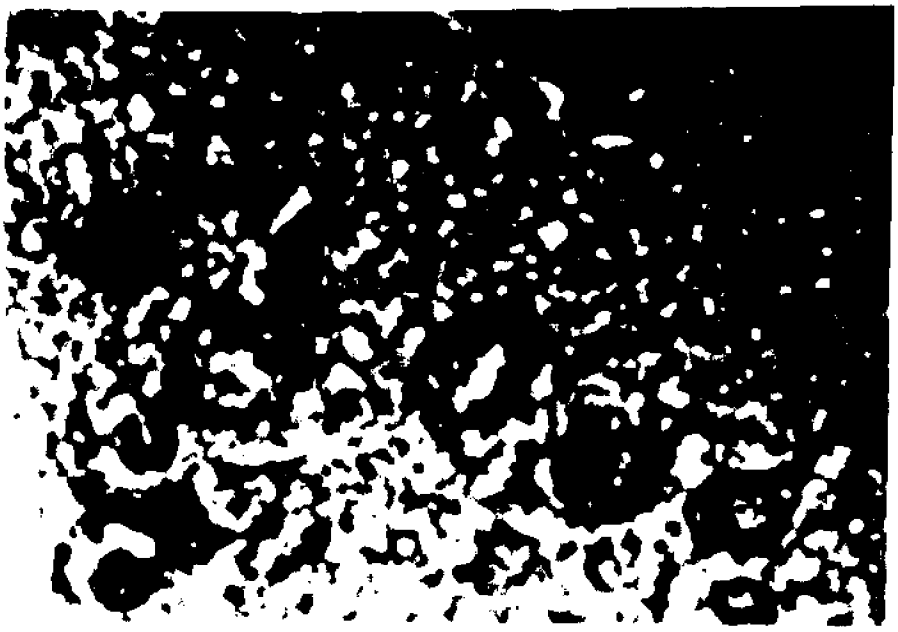


FIG. 13 G

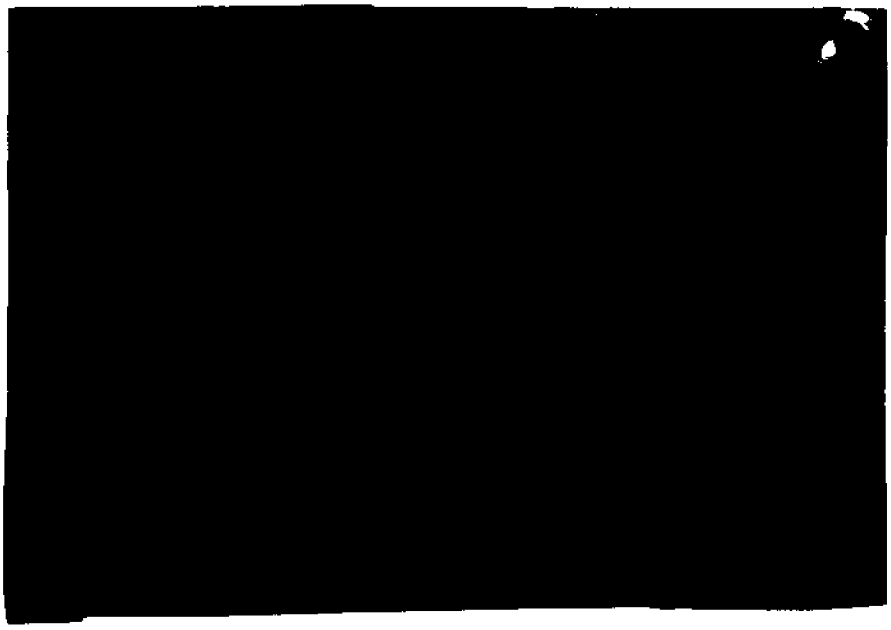


FIG. 13 H

FIG. 13 I

FIG. 14 J

Interval (minutes) before injection of C^{14}-leucine	Incorporation of C^{14}-leucine into the whole brain protein						
	Saline	1 μg 5-HT		2 μg 5-HT		10 μg 5-HT	
	CPM mg prot.	CPM mg prot.	% inhibition	CPM mg prot.	% inhibition	CPM mg prot.	% inhibition
0	141(6)	--	--	135(8)	4	--	--
10	174(7)	--	--	146(3)	16	137(4)	21
30	173(3)	165(4)	5	148(4)	15	139(3)	20

Table 13: The effect of 5-HT on the in vivo incorporation of C^{14}-leucine into whole brain proteins.

Doses of 5-HT were injected intracranially. At three different periods after the injection, C^{14}-leucine was also injected intracranially. Five minutes after the second injection the animals were sacrificed. Three animals received the same treatment and brains from these three animals were pooled, homogenized and analyzed for incorporated radioactivity. Thus three animals resulted in one observation for each treatment. Three observations were made for each experimental variable within this experiment. The values represent the mean obtained from the three observations of each treatment. The numbers in parentheses are the maximum variabilities found within triplicate. It should be noted that at least two additional replications with each experimental variable were performed during the present work, yielding results in the same directions as those above.

cranially injected 5-HT (Table 14). As can be seen from Table 14 the amount of the inhibition was not appreciably increased but smaller quantities of 5-HT were necessary to produce comparable inhibition in the presence of the MAO inhibitor.

Regional studies reveal that the effect of intracranially injected 5-HT on protein synthesis in various regions is similar to that observed after ECS. The similarity is qualitative but not quantitative. As can be seen from Table 15, the largest amount of inhibition was found for the cerebral cortex and the diencephalon. The lowest effect, on the other hand, was observed in the cerebellum.

The labeling of the subcellular fractions extracted from the cerebral cortex following intracranial injection of 5-HT is shown in Table 16. Again, there is a similarity between the pattern of the distribution of the inhibitory effect of 5-HT among the subcellular fractions to that observed with ECS. The magnitude of the effect is different when these two treatments are compared (see also Table 5). A significantly greater amount of inhibition occurred in the synaptosomal fraction as compared with the microsomal fraction.

The similarity of the pattern between the inhibitory effect of 5-HT and ECS gave rise to two major hypotheses: 1) 5-HT is a mediator in the inhibitory effect of ECS and 2) 5-HT produces electrical changes in the CNS which affect protein synthesis in a similar manner to that of ECS.

Pretreatment before IC injection of 5-HT	Incorporation of H^3-leucine into the whole brain protein						
	Saline	2.5 µg 5-HT		5 µg 5-HT		10 µg 5-HT	
	CPM / g tissue	CPM / g tissue	% inhibition	CPM / g tissue	% inhibition	CPM / g tissue	% inhibition
control	5624(171)	5092(241)	10	4617(190)	18	4191(231)	25
pargyline	5850(220)	4320(124)	26	4248(246)	27	4272(130)	27

Table 14: The effect of intracranial injection of 5-HT on the incorporation of H^3-leucine into the whole brain protein extracted from animals pretreated with pargyline.

Animals were divided into two main groups of 20 animals each. One of these groups was injected twice (IP) with 25 mg/Kg pargyline, 18 hours and again two hours, before the intracranial injection of either saline (5 animals) or three different doses of 5-HT (5 animals per dose). The other main group was treated with saline in a similar manner to the pargyline treated group. Intracranial injection of either saline or 5-HT was subsequently given as described for the first group. Ten minutes after the intracranial injection, H^3-leucine was also injected intracranially. The animals were sacrificed five minutes later and the incorporated radioactivity was then determined individually for each animal. Each value represents the mean for 5 animals which received the same treatment. The numbers in parentheses are the standard deviations (S.D.)

Brain region	Incorporation of C^{14}-leucine into protein		
	control	5-Hydroxytryptamine	
	CPM/mg protein	CPM/mg protein	% inhibition
cerebral cortex	144(3)	116(5)	20
diencephalon	321(6)	256(9)	20
cerebellum	214(2)	216(5)	0

Table 15: The effect of 5-HT on the incorporation of C^{14}-leucine into different regions of a mouse brain.

Animals were injected with 2 μg 5-HT intracranially and 10 minutes later with C^{14}-leucine. Five minutes after the last injection animals were sacrificed and brains from five animals were pooled and developed for radioactive determination. Each value represents the mean of the two groups which received the same treatment. Each group consisted of five animals. Numbers in parentheses represent the variability between the two groups.

Subcellular fraction	Incorporation of C^{14}-leucine into protein		
	control CPM mg prot.	5-HT CPM mg prot.	% inhibition by 5-HT
whole homog.	144(4)	116(2)	19
mic. (P_3)	264(6)	203(5)	23
mit. (C)	68(1)	53(2)	22
synapt. (B)	38(1)	25(1)	34
soluble fraction(S_3)	151(2)	121(3)	20

Table 16: The effect of 5-HT on the incorporation of C^{14}-leucine into the brain proteins of different subcellular fractions extracted from cerebral cortex of a mouse brain.

For procedure and legend, see footnote to Table 15.

In order to test the second hypothesis, electrophysiological studies were done. These studies are shown in Figure 14. Bilateral electrodes (for description of the apparatus used see Figure 15 and the method section) with a cannula were placed into the mouse brain. Saline or 5-HT were injected through the cannula to the left hemisphere and gross EEG was then taken simultaneously from each hemisphere. As can be seen from Figure 14, considerably reduced amplitude compared to pre-injection electrical activity was noted for the hemisphere into which 5-HT was injected. Slightly higher amplitude was noted for the other hemisphere especially at 15 minutes after the injection. Injection of saline did not cause such changes in electrical activity. A flattening of the EEG was observed by Lenox et al [103] for a monkey immediately after the convulsion period produced by ECS. Based on the assumption, following general practice, that the reference electrode was"indifferent", no indication for seizure activity in either hemisphere can be seen. The mechanisms underlying the foregoing effects remain to be elaborated. The EEG changes last about 30-45 minutes after tne injection. The inhibition of protein synthesis in the hemisphere into which 5-HT was injected was not greater than the inhibition observed in the other hemisphere. On the other hand, a correlation was found between the duration of the electrical changes and the period in which protein synthesis was inhibited after the injection of 5-HT. In order to test possible attenuation of the inhibition produced by 5-HT, mianserine, a

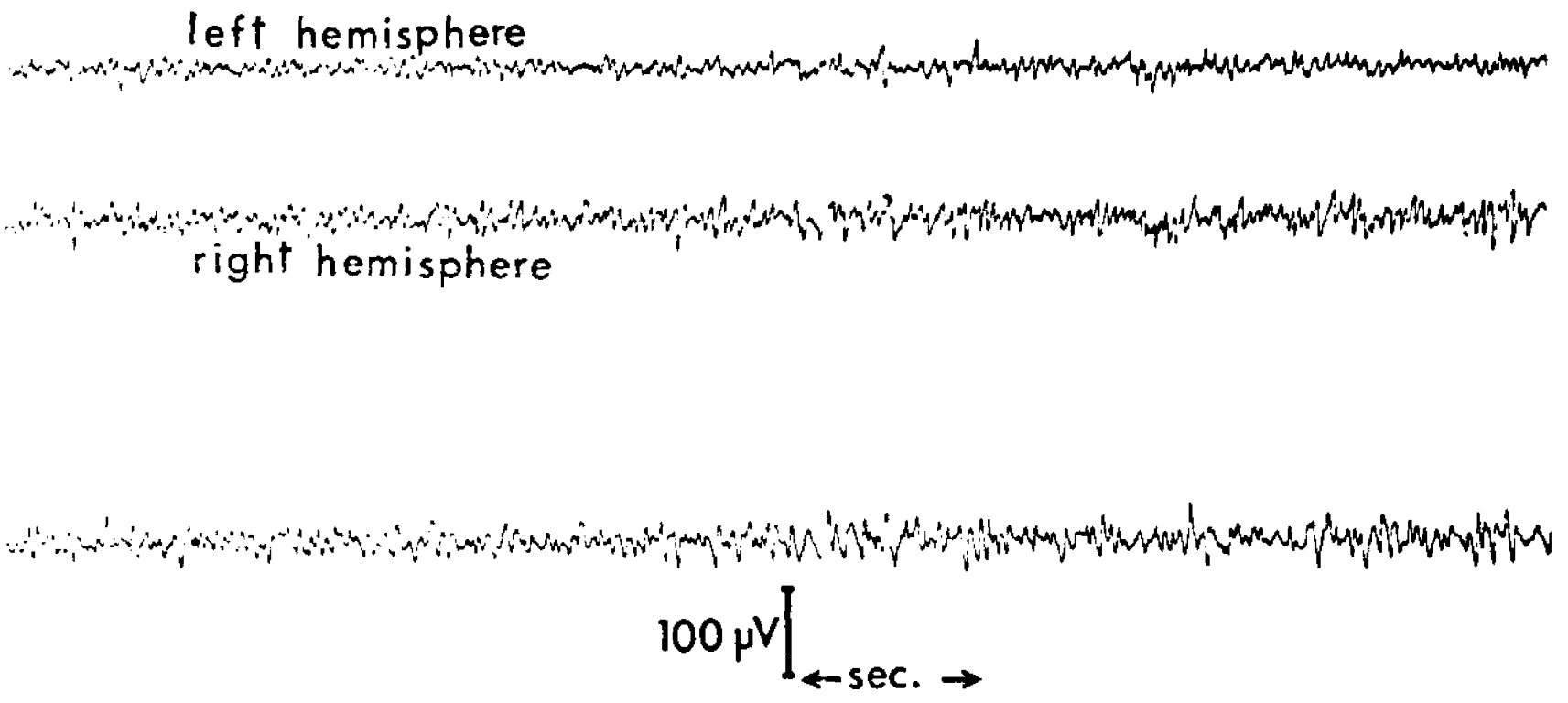
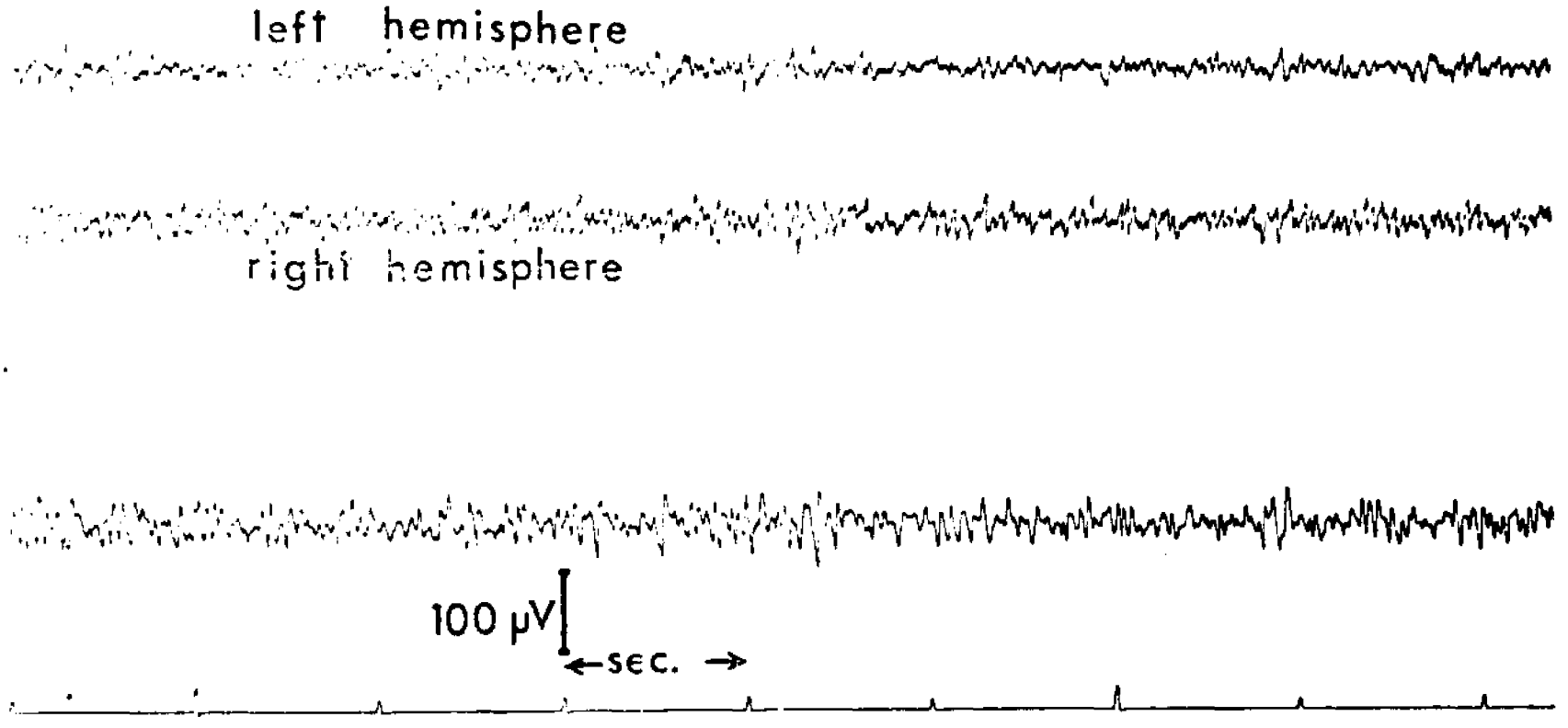


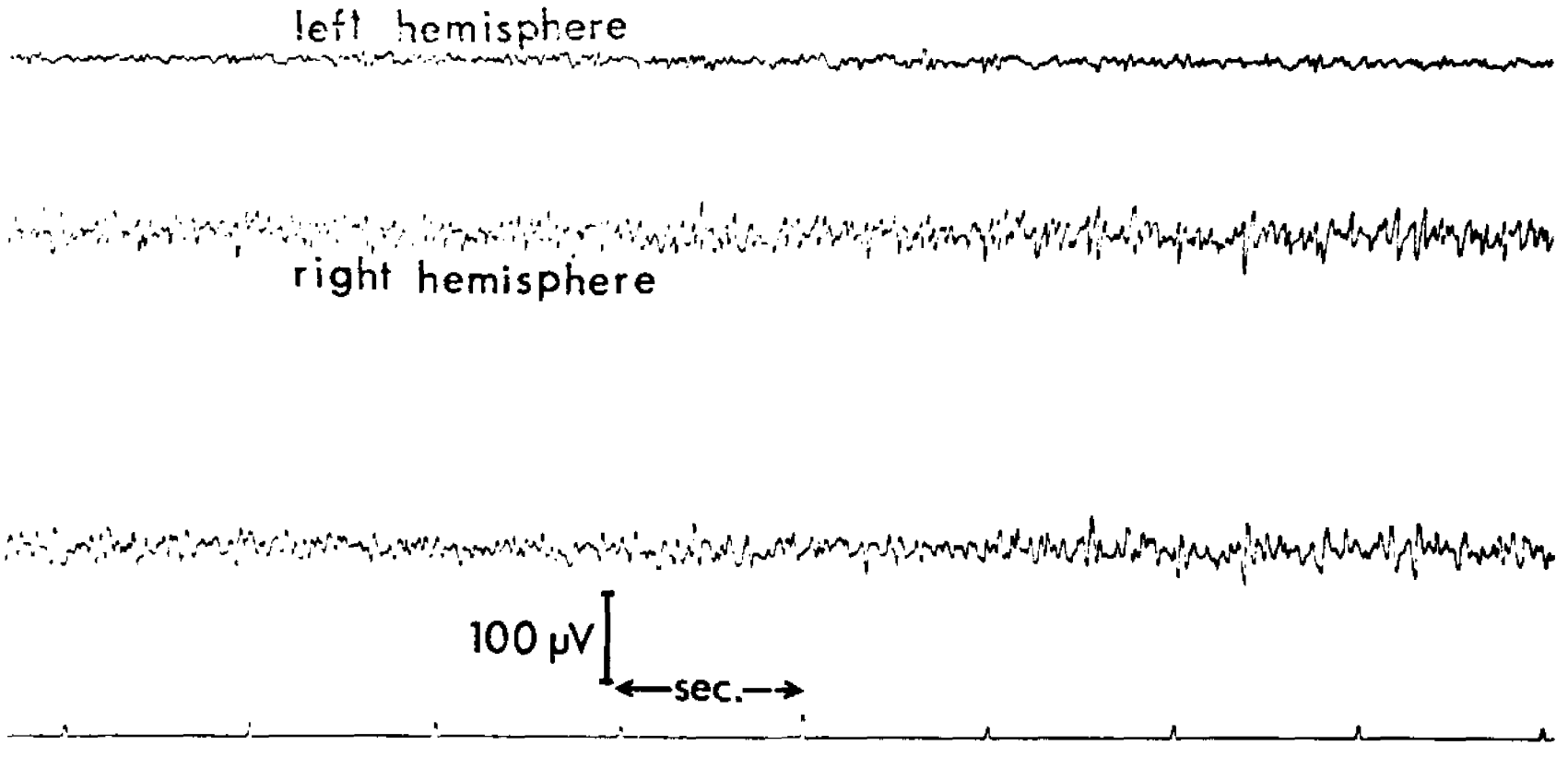
Figure 14: Effect of intracranial injection of 5-HT on the EEG recorded from the cerebral cortex of a mouse brain.

(A) EEG before injections

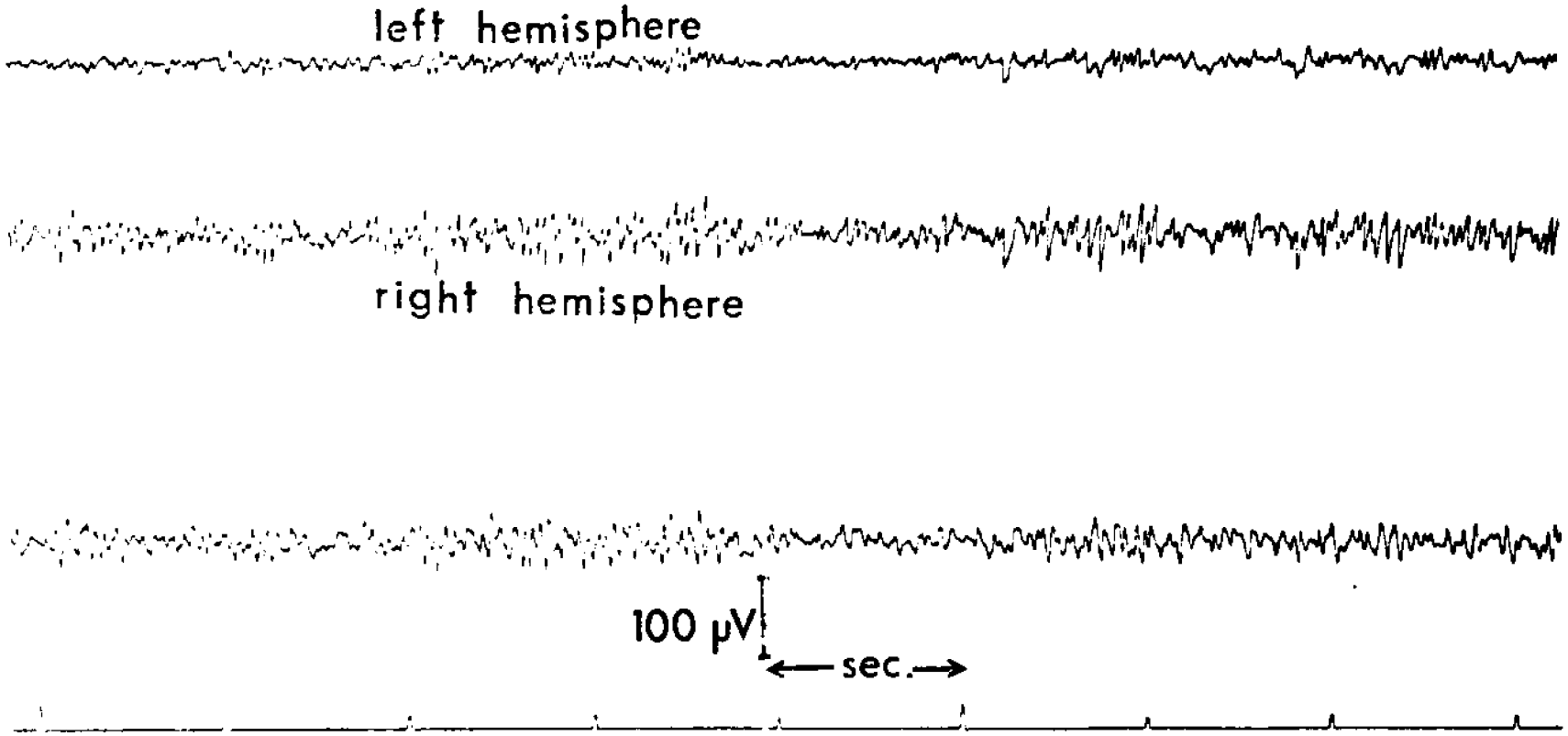
EEG was recorded using bilateral electrodes (see method section for detailed procedure). 10 λ of saline or 5-HT dissolved in saline (10 μg/animal) was injected through the cannula connected to the electrodes rack (See Figure 15). Saline was always injected before 5-HT. An interval of at least 45 minutes was kept between injections.



(B) EEG 5-13 seconds after the injection of saline



(C) EEG 5-13 seconds after the injection of 5-HT



(D) EEG 15 minutes after the injection of 5-HT

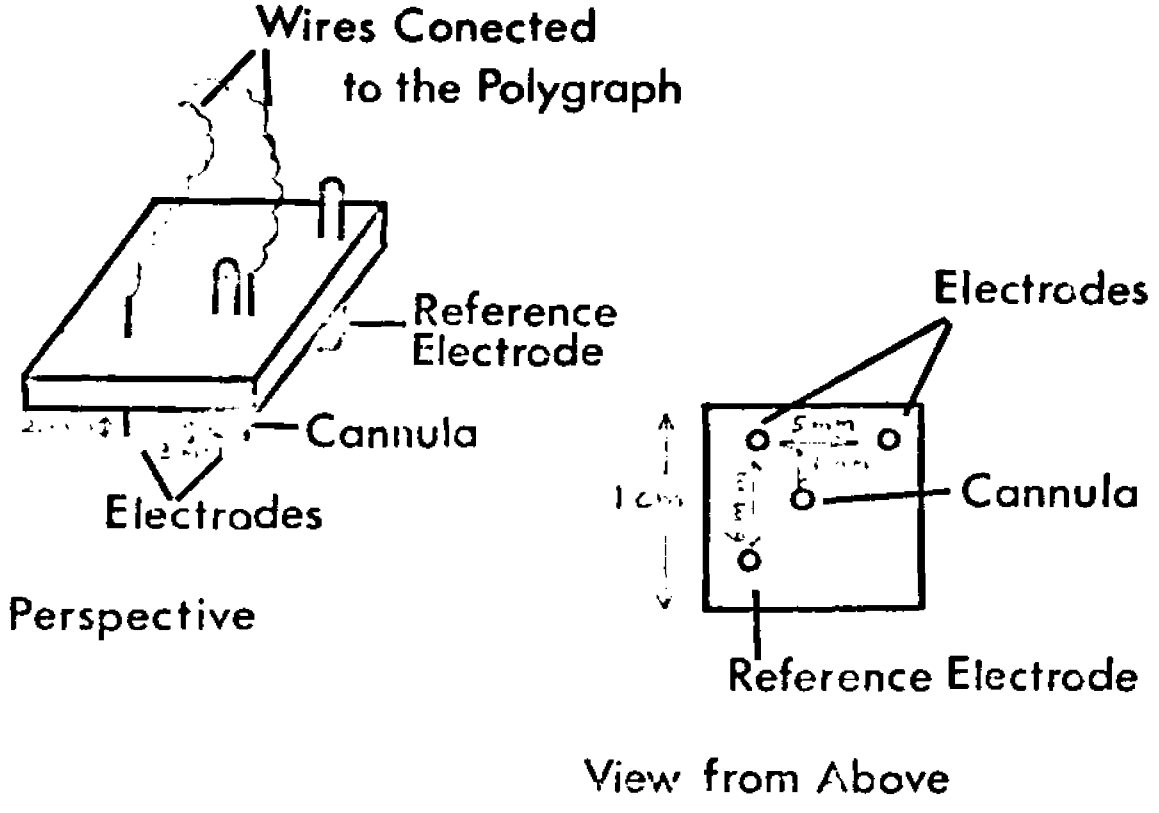


Figure 15: Apparatus for cortical EEG recording and intracranial injections.

central antagonist of serotonin, was injected IP and the incorporation of labeled leucine was then examined following the injection of 5-HT. As can be seen from Table 17 there is no evidence for such attenuation. However, the ability of mianserine to bind serotoninergic receptors, or to attenuate electrical changes produced by 5-HT, is still questionable [101].

Treatment	Incorporation of H^3-leucine into whole brain protein	
	CPM/g tissue	% inhibition
control	7840±825	--
control and mianserine	7320±950	7
5-HT	6320±680	19
5-HT and mianserine	5780±840	21

Table 17: The effect of 5-HT on the incorporation of H^3-leucine into the whole brain protein extracted from animals pretreated with mianserine.

For procedure and legend, see footnote to Table 10.

In vitro studies on protein synthesis in the brain:

1. Characterization of the in vitro system

The requirements of the brain microsomes for various components of the reaction mixture is described in Table 18. In agreement with previous study [13] the system showed an absolute requirement for a source of ribosomes (microsomes), and soluble enzymes. On the other hand, in contrast to this same study, the data showed no dependency on poly U.

Conditions of incubation	Incorporation of C^{14}-phenylalanine into brain microsomes CPM
complete system	891
without microsomes	12
without S_{100}	52
without Magnesium ions	104
complete + poly U	941
without ATP	75
without creatine phosphate	112
without GTP	845

Table 18: The dependency of microsomal protein synthesis in vitro on various components of the reaction mixture.

Incubation of microsomes was carried out for 15 minutes under standard conditions as described in the method section. Values represent the mean of the duplicates performed routinely for each experimental variable. Variability between duplicates never exceeded 6%.

Moreover, no consistent requirement was shown for GTP. The discrepancies can be explained on the basis of the washing procedure that was used in the present experiment. It is possible that some endogenous nucleotides were left in the microsomal preparation after the washing procedure. The poly U dependency was increased by 10 fold (see Table 19) if microsomes were preincubated without radioactive amino acid, thus indicating that most of the microsomes were in aggregate form upon isolation. Small quantities of Mg^{++} were added with the medium in which the microsomes were prepared in order to maintain the integrity of the polysomes. Therefore, no absolute requirement for

Mg^{++} was evident. The kinetics of the incorporation with postmitochondrial supernatant (S_{30}) is described in Figure 16. The incorporation is linear up to 6 minutes and then the curve is gradually flattened. The pH optimum curve and the Mg^{++} optimum curve are shown in Figures 17 and 18. In agreement with previous studies which had been done with liver microsomes, the pH optimum was found to be in a range between 7.2-7.8. The optimum Mg^{++} was found to be 6 mM for the native RNA dependent polymerization and 8 mM for the poly U dependent polymerization. A comparison between liver and brain microsomes and ribosomes is shown in Figure 19. Liver microsomes showed more linearity than brain microsomes when the incorporation was measured as a function of increasing amount of microsomes. When liver ribosomes and brain ribosomes were compared, full linearity was shown for all the amounts tested. The results could be explained by the presence of an inhibitor in brain microsomes and/or on the basis of deficient factors in the soluble fraction extracted from the brain. The soluble fraction extracted from liver was more efficient than that extracted from brain (Figure 20). Titration of the brain microsomes with the soluble fraction from either liver or brain showed that for 5 mg of microsomal protein, 0.25 ml of the soluble fraction (prepared as described in the method section was the smallest saturating amount. Further consideration and interpretation of these data will be presented in the discussion section.

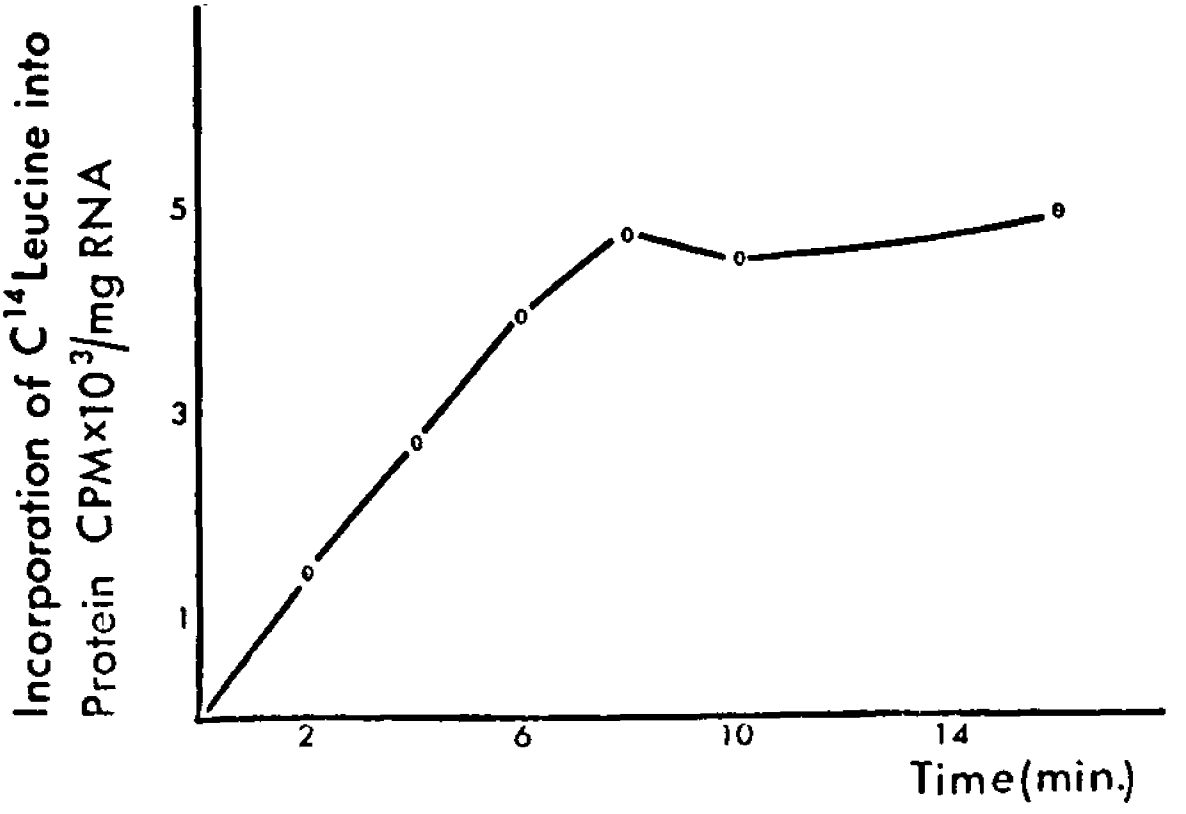


Figure 16: The kinetics of the incorporation of C^{14}-leucine into microsomal protein in vitro for the S_{30} system obtained from brain tissue.

S_{30} was incubated with a standard incubation mixture (see method section) and two aliquots of 0.5 ml each were taken at each duration indicated on the graph. TCA 5% was added to the aliquots and the incorporated radioactivity was determined. The reaction mixture was pre-incubated at 37°C. for two minutes before the microsomes were added.

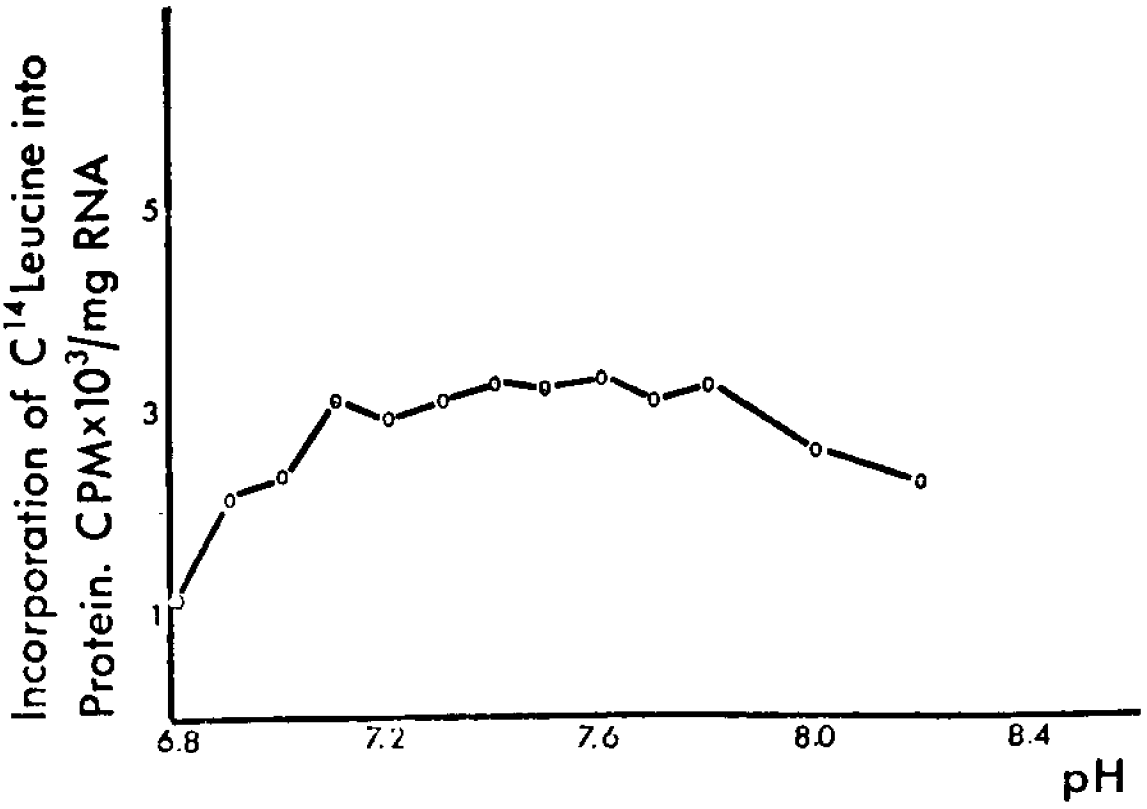


Figure 17: The pH dependency of the incorporation of C^{14}-leucine into microsomal protein in vitro.

Postmitochondrial supernatant (S_{30}) was incubated in the presence of a standard reaction mixture with tris buffers of different pH values. All determinations were done in duplicate and values represent the mean of the duplicates.

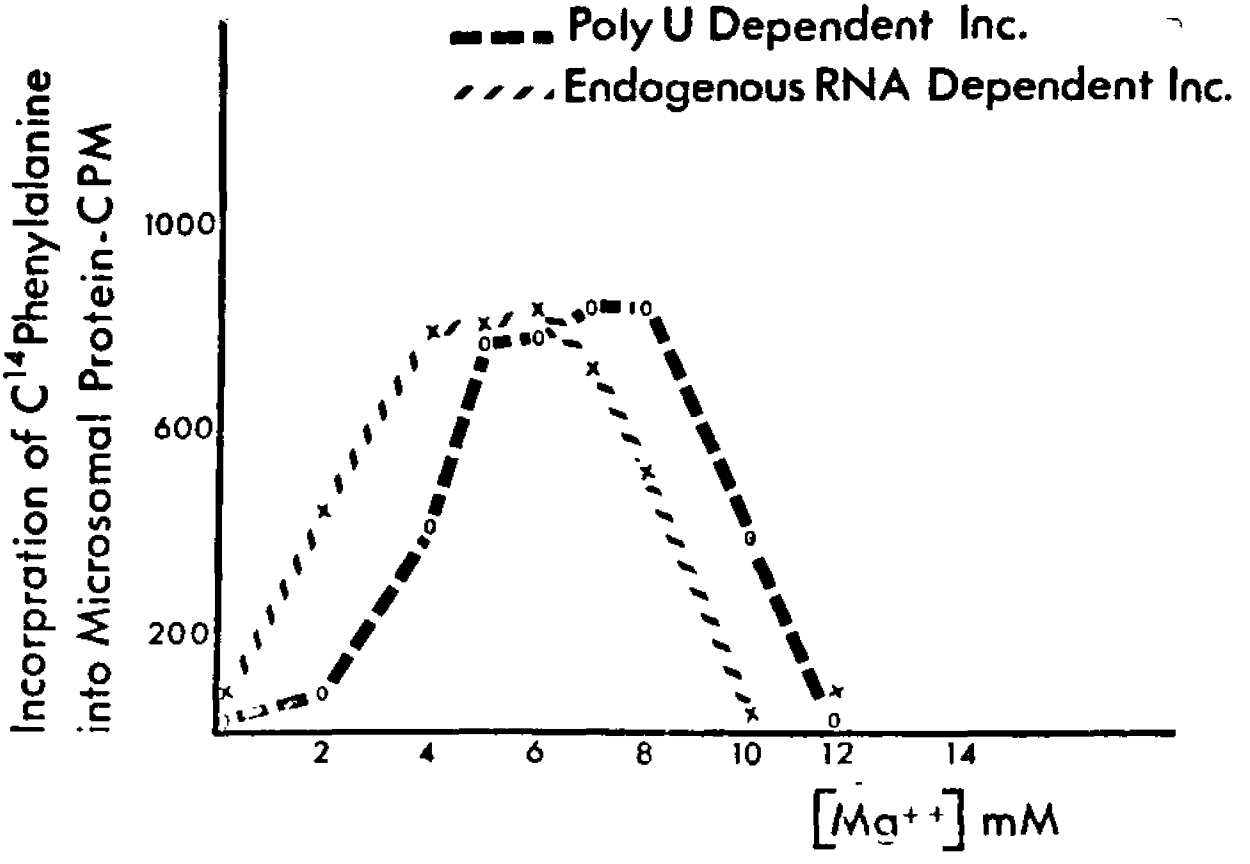


Figure 18: Optimum $[Mg^{++}]$ for the incorporation of C^{14}-phenylalanine into microsomal protein in vitro for endogenous RNA dependent incorporation and poly U dependent incorporation.

Postmitochondrial supernatant (f_{20}) was incubated in the presence of standard reaction mixture with various concentrations of Mg^{++}, with and without poly U. When poly U was to be added the f_{20} was preincubated for 10 minutes in the absence of labeled amino acid in order to disaggregate the polysomes to monosomes. The C^{14}-phenylalanine was added after the preincubation along with the poly U and 10mM creatine phosphate.

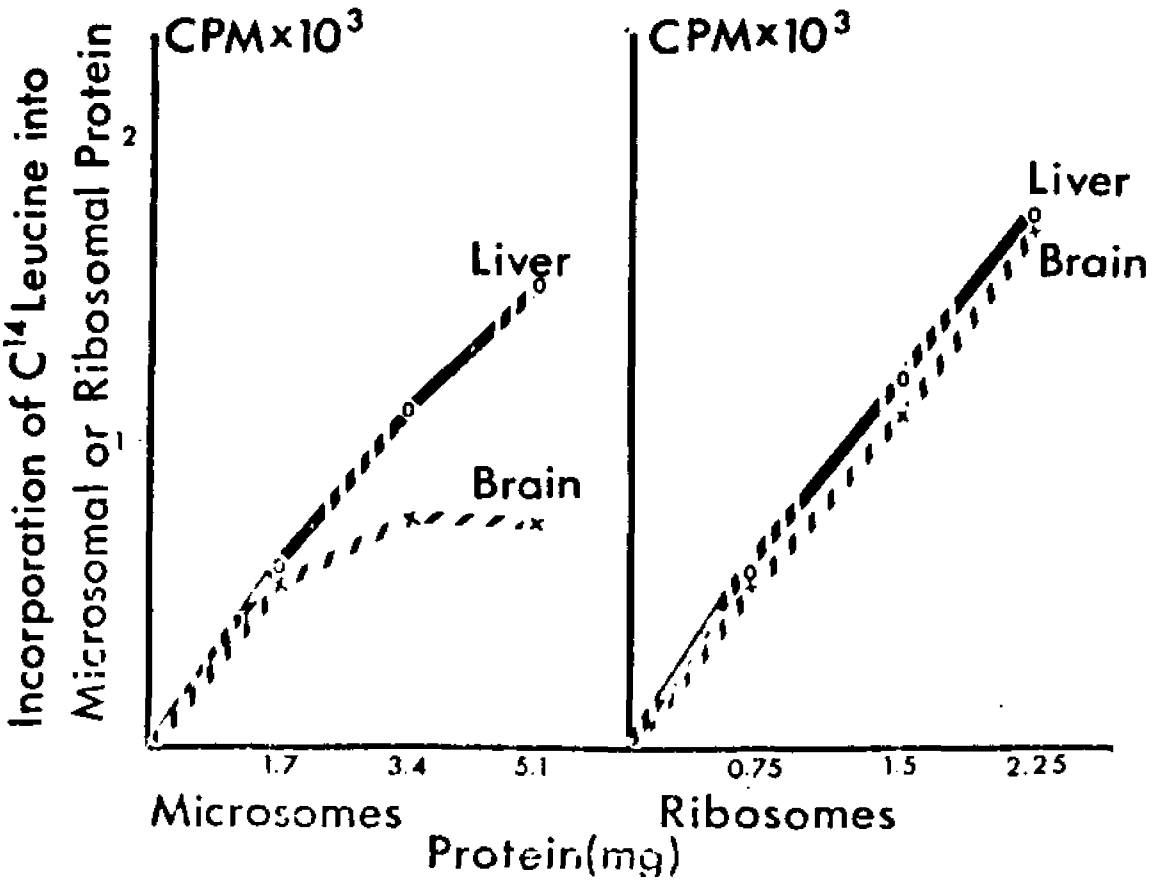


Figure 19: The incorporation of C^{14}-leucine into brain and liver microsomes and ribosomes; titration of microsomal and ribosomal protein.

Varying amounts of ribosomes and microsomes in the presence of 0.25 ml S_{100} from brain tissue were incubated with a standard reaction mixture for 15 minutes. The reaction was terminated with 5% TCA and the incorporated radioactivity was determined as described in the method section.

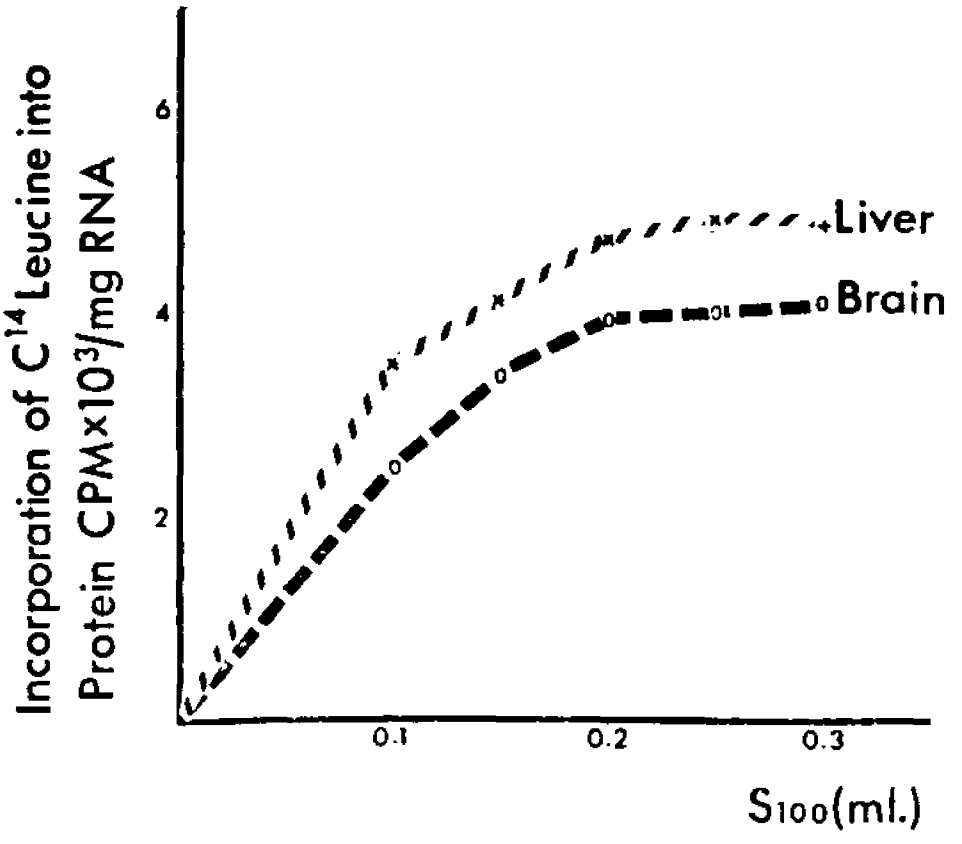


Figure 20: The effect of varying amounts of soluble enzymes (S_{100}) on the incorporation of C^{14}-leucine into microsomal protein in vitro with brain microsomes.

Varying amounts of S_{100} fraction (see method section) from liver or brain as indicated on the graph were added to a standard incubation mixture containing brain microsomes (5 mg of microsomal protein). The incorporated radioactivity was determined as described in the method section.

2. The effect of ECS on the incorporation of C^{14}-phenylalanine into microsomal protein in vitro

Multiple ECS has been shown to produce disaggregation of the polysomal aggregates into monosomes [75]. If a single ECS were to cause such disaggregation, one would expect to find a decreased native RNA dependent incorporation of labeled amino acid into protein with microsomes extracted from animals pretreated with ECS, and an increased poly U dependent incorporation of labeled amino acid into protein with these same

Interval (minutes) between treatment and sacrifice	Incorporation of C^{14}-phenylalanine into protein CPM/mg protein					
	Without preincubation			After preincubation		
	A	B	A/B	A	B	A/B
$\overline{ECS}$ 0'	170	18	0.106	10	183	18.0
ECS 0'	168	20	0.119	12	180	15.0
ECS 5'	173	15	0.087	--	--	--
ECS 10'	163	14	0.085	--	--	--
ECS 30'	155	34	0.219	--	--	--
$\overline{ECS}$ 30'	181	13	0.072	--	--	--

Table 19: The endogenous RNA dependent and poly U dependent incorporation of C^{14}-phenylalanine into microsomal protein of S_{30} extracted from animals previously treated with ECS.

Animals treated with ECS as described in the method section (5 animals per group). At varying periods after the treatment animals were sacrificed and microsomes were prepared from the brains as described in the method section. Conditions of incubation were the same as those described in Table 18. Preincubation of the microsomes was carried out for 10 minutes with a standard reaction mixture without labeled amino acid. Poly U, C^{14}-phenylalanine and additional Cr.P. were added to the system after the preincubation. Incubation for 15 min. was carried out and incorporated radioactivity then determined. A represents endogenous RNA dependent incorporation and B represents poly U dependent incorporation (incorporation detected in presence of poly U minus incorporation detected in absence of poly U.) $\overline{ECS}$ represents sham treatment and ECS represents actual treatment.

microsomes. As can be seen from Table 19 microsomes extracted from ECS pretreated animals did not show such responses. Moreover, preincubated microsomes, which were disaggregated during the preincubation showed large response to poly U, without differences between microsomes extracted from animals pretreated with ECS compared to control microsomes.

Since changes in ATP levels were observed after ECS, microsomal ATPase was measured for microsomes extracted from animals pretreated with ECS. As shown in Table 20, no significant changes in microsomal ATPase occurred as a consequence of ECS previously given to the animals.

Treatment	μmoles Pi released/min/mg
$\overline{\text{ECS}}$	0.184 (0.014)
ECS	0.199 (0.014)

Table 20: ATPase activity in microsomes extracted from animals previously treated with ECS.

The ATPase activity was determined as described in the method section, using microsomes containing 5 mg microsomal protein. Each value represents the mean obtained from five animals; each was determined individually. Numbers in parentheses are standard deviations. $\overline{\text{ECS}}$ represents sham treatment and ECS represents actual treatment.

A loss of the inhibitory effect from the *in vitro* system can be attributed to a dilution or removal of an inhibitor resulting from ECS administration. In order to determine

whether 5-HT is related to such inhibitory effect, or serves as an inhibitor per se, the effect of this biogenic amine was examined using the in vitro system.

3. The effect of 5-HT on the incorporation of C^{14}-amino acids into microsomal protein in vitro

The effect of increasing concentrations of 5-HT on the incorporation of C^{14}-leucine into microsomal protein of brain microsomes as compared with liver microsomes is described in Table 21. In agreement with a recent study

Addition to incubation mixture	Incorporation of C^{14}-leucine into protein			
	Brain postmitochondrial supernatant (S_{30})		Liver postmitochondrial supernatant (S_{30})	
	CPM/mg RNA	% inhib.	CPM/mg RNA	% inhib.
none	5686	--	4574	--
0.006 μ mole 5-HT	5680	0	4500	2
0.060 μ mole 5-HT	5640	1	4370	4
0.600 μ mole 5-HT	5540	2	4420	3
1.500 μ mole 5-HT	5364	6	4521	1
3.000 μ mole 5-HT	4164	27	4485	2

Table 21: The effect of increasing amounts of 5-HT on the incorporation of C^{14}-leucine into liver and brain microsomes in vitro.

Postmitochondrial supernatant was incubated with the standard reaction mixture. Various concentrations of 5-HT as indicated were added to the incubation medium. Other conditions as described in the method section.

[104], high concentrations of 5-HT are able to inhibit about 30% of the incorporation of the labeled amino acid into protein. There is a slight quantitative discrepancy between the present study and the study mentioned above [104]. In this study, 3 mM of 5-HT were required in order to produce 30-35% inhibition, whereas Hemminki [104] reported that 1 mM 5-HT is able to produce the same amount of inhibition. The dif-

ferences in preparation and species may explain this discrepancy. As can be seen in Table 21, there is differential sensitivity of brain and liver microsomes. Brain microsomes are sensitive to 5-HT, whereas liver microsomes are insensitive. Liver and brain are known to possess high MAO activity. Therefore, the possibility that 5-HT was oxidized during the incubation period by contaminant mitochondrial MAO was considered. MAO inhibitors such as pargyline or iproniazide inhibit protein synthesis on their own, but when 5-HT was added together with the inhibitors a synergestic effect between the 5-HT and the MAO inhibitor was observed (Table 22).

Exp. #	Addition to the incubation mixture	Incorporation of C^{14}-leucine into protein		
		Without 5-HT	With 5-HT	
		CPM/mg RNA	CPM/mg RNA	% inhibition
1	none	5020	3405	32
	+ pargyline	3780	1361	57
2	none	5300	4060	23
	+ iproniazide	2642	908	66

Table 22: The effect of 5-HT and MAO inhibitors on the incorporation of C^{14}-leucine into protein in vitro.

Postmitochondrial supernatant (S_{30}) from brain was incubated with the standard reaction mixture. 5-HT (0.003 M) and pargyline (.01 M) or iproniazide (phosphate sale 0.01 M) were added to the incubation medium before the reaction had been started.

During the course of the present work, liver and brain microsomes were compared. Liver microsomes showed less sensitivity to 5-HT even in the presence of the MAO inhibitor. When the oxidation rate of 5-HT was measured, it was found that 5-HT was not oxidized or degraded during the incubation period,

even when the MAO inhibitor was absent. The synergistic effect between the MAO inhibitors and the 5-HT must therefore be attributed to a different mechanism rather than being related to the MAO inhibition *per se.*

Recent studies suggested that 5-HT is capable of binding to macromolecules such as DNA and RNA, and that various derivatives of 5-HT showed varying capabilities of such binding [70]. The equilibrium constant of the binding reaction as well as the number of binding sites per macromolecule were reported [105]. The bound 5-HT may or may not inhibit protein synthesis. In the event that the bound 5-HT does not inhibit protein synthesis an increased RNA concentration would bring about a decrease in the inhibitory effect of the 5-HT. To test this possibility, an excess of soluble yeast RNA was added with and without 5-HT in the presence and absence of MAO inhibitor. The results are summarized in Table 23. While excess RNA was able to eliminate the inhibitory effect of 5-HT in the absence of MAO inhibitor, inhibition was still observed when MAO inhibitor was added to the system.

Previous studies [11,104] have indicated that among GABA, NE, epinephrine, DA, and serotonin, the latter was the only one which was capable of inhibiting protein synthesis *in vitro*. In order to obtain more information about the specificity of the inhibitory effect of 5-HT and the relation of this effect to the binding of 5-HT to RNA, the effect of various derivatives of 5-HT was examined. In the event that the bound molecules inactivate the tRNA or the ribosomal RNA, one would expect to observe a decrease in the incorporated amino acid

in each case that a 5-HT derivative, which is capable of binding RNA, is present in the system. The results of this experiment are described in Table 24. It is clear that serotonin is highly specific and the free 5-hydroxyl as well as the free amino group must be present in order for

Addition to the incubation mixture	Incorporation of C^{14}-leucine into protein % of control
+5-HT	76
+5-HT+pargyline	55
+5-HT+RNA	106
+5-HT+pargyline+RNA	56

Table 23: Attenuation of the inhibitory effect of 5-HT on protein synthesis in vitro with soluble RNA.

Each experimental variable was performed in duplicate. The percent incorporation was calculated from the mean of the duplicate where the control mean was considered as 100%. For each case described in the Table the control contained all the subsidiary substances except the 5-HT (See Appendix I-d). The final concentrations of the 5-HT, pargyline and RNA in the incubation mixture were 0.003 M, 0.01 M, and 12.5 mg/ml, respectively.

the inhibitory effect to occur. There was no correlation between the potential inhibitory effect of serotonin derivatives and its binding equilibrium constant, the number of sites into which the derivative binds, or the product of the last two parameters.

In order to find the stage at which protein synthesis is inhibited by 5-HT, the amine was added to two different systems incorporating amino acids in vitro: 1) Soluble enzymes without microsomes with free amino acids as a precursor. 2) Complete system with amino acyl-tRNA's as a

Addition to the incubation mixture	Incorporation of C^{14}-leucine into protein % of control	
	without pargyline	with pargyline
none	100	100
+5-HT (oxalate salt)	73	56
+5-HT (creatinine salt)	71	54
5-methoxy tryptamine	100	97
N acetyl serotonin	100	93
melatonin	100	94
5-methoxy,1-methyl tryptamine	100	92

Table 24: The effect of 5-HT and its derivatives on the incorporation of C^{14}-leucine into protein by brain microsomes in vitro.

Values are the percents of the control and were calculated as described in Appendix I-d. The 5-HT and its derivatives were added to yield final concentrations of 0.003 M each. Pargyline was added to yield final concentrations of .01 M.

precursor. As indicated in Table 25, amino acylation was inhibited by 5-HT in the absence of pargyline. The incorporation of the amino acid from amino acyl-tRNA into protein was not affected by 5-HT in the absence of pargyline. On the contrary, when pargyline was added to the system, amino acylation was not affected by 5-HT while the transfer of the amino acid from the amino acyl-tRNA into the polypeptide was greatly inhibited.

Serotonin was not found to inhibit amino acylation by competetive inhibition for endogenous tryptophane which chemically resembles serotonin. As can be seen from Table 26, the inhibitory effect of 5-HT on amino acylation of tryptophane is similar to that on leucine. Moreover, the poly U dependent incorporation of labeled phenylalanine into protein was inhibited by 5-HT to the same extent to which the native RNA

Addition to the incubation mixture	Incorporation of C^{14}-leucine into t-RNA		Incorporation of C^{14}-leucine from aa-tRNA into protein	
	CPM	% inhibition	CPM	% inhibition
none	559	--	314	--
+5-HT	383	31.5	296	5.7
+pargyline	556	--	243	--
+pargyline+5-HT	527	5.2	133	45.3

Table 25: The effect of 5-HT on the incorporation of C^{14}-leucine into t-RNA and on the transfer of the labeled amino acid from C^{14}-leucyl-tRNA into protein.

Soluble fractions (S_{100}) from a brain were incubated under standard conditions with and without 0.003 M 5-HT. Each experimental variable was done in quadruplicates; a pair of replicates was then incubated for an additional 10 minutes with 50 µg RNAase and the other pair with an equivalent volume of H_2O. The incorporated radioactivity was then determined. The mean difference between the two pairs of replicates was considered as the amount of radioactivity incorporated into tRNA.

The incorporation of C^{14}-leucine from aa-tRNA into protein was determined by using the labeled preincubated S_{100} as a precursor. 0.04 M cold l-leucine was added in order to preclude any apparent incorporation from free amino acid into protein. The 5-HT and the pargyline were added to yield final concentrations of 0.003 M and .01 M, respectively.

dependent incorporation was affected (Table 27). Based on these findings, interaction of 5-HT with aminoacyl synthetase may be given as a mechanism causing the inhibitory effect of 5-HT. The inhibition of protein synthesis by MAO inhibitors operates through a different mechanism and may be accounted for by the non-specific action of the MAO inhibitors on enzymes other than MAO [102].

4. The activation of the amino acid within the synaptosomes; in vitro studies

Many of the previous studies on the synaptosomal protein synthesis in vitro were controversial because of the possible occurrence of microsomal or bacterial contamination which

Addition to the incubation mixture	Incorporation of C^{14}-leucine into tRNA		Incorporation of C^{14}-tryptophane into tRNA	
	CPM	% inhibition	CPM	% inhibition
none	862	--	567	--
5-HT	421	51.2	379	33.2

Table 26: The effect of 5-HT on the incorporation of C^{14}-leucine and C^{14}-tryptophane into tRNA.

The incorporation of the labeled amino acids into tRNA was determined and calculated as described in Table 25.

Addition to the incubation mixture	Incorporation of C^{14}-phenylalanine into protein			
	endogenous RNA dependent incorporation		poly U dependent incorporation	
	CPM	% inhibition	CPM	% inhibition
none	529	--	1720	--
5-HT	368	30.5	1155	32.8
pargyline	407	--	809	--
pargyline+5-HT	243	40.3	374	53.8

Table 27: The effect of 5-HT on the incorporation of C^{14}-phenylalanine into protein with and without poly U.

The incubation procedure was similar to that described for Table 19. The poly U dependent incorporation was determined on preincubated microsomes. The preincubation procedure and the additions to the incubation mixture are similar to those described in Table 19.

might incorporate labeled amino acids during the incubation period. A different approach to the problem would be to isolate the amino acyl synthetase system from the synaptosomal fraction. An attempt was made to isolate this system. Table 28 shows that amino acyl synthetase activity was detected in synaptosomal fraction. The incorporated

Source of the amino acyl synthetase	Incorporation of C^{14}-leucine into tRNA CPM			
	tRNA from S_{100}		tRNA from SS	
	-RNAase	+RNAase	-RNAase	+RNAase
S_{100}	804	36	111	39
SS	382	41	195	32

Table 28: The incorporation of C^{14}-leucine into synaptosomal and cytoplasmic tRNA's by homologous and heterologous amino acyl synthetases.

Guinea pig brains were used for the preparation of the fractions. The pH5 fractions from the soluble fractions of the cell body (S_{100}) and of the synaptosomes (SS) were prepared according to Moldave [85] and were used as the source of amino acyl synthetases and for the extraction of tRNA according to the procedure described in the method section. The amino acyl synthetases were incubated with various types of tRNA. The incubation was performed in quadruplicate; a pair of replicates was then taken and incubated for an additional 10 minutes with 50 µg RNAase. The other pair was incubated with equal volume of H_2O for the same period. The incorporated radioactivity was then determined.

amino acid is released by RNAase and therefore cannot be attributed to bacterial or microsomal contaminations. An attempt to purify amino acyl synthetase from synaptosomes using DEAE cellulose column chromatography was not successful. However, amino acyl synthetase from soluble fraction (S_{100}) of brain homogenate was easily isolated using the above technique. The failure to isolate the synaptosomal enzyme may be attributed to the small quantity of this enzyme in the synaptosomal fraction. In crossover experiments, tRNA's which were isolated from a soluble fraction of synaptosomes and from a soluble fraction (S_{100}) of brain homogenate were examined for their ability to serve as substrates for the enzymes from these two sources. The results, summarized in Table 28, indicate that some specificity was found for the enzymes from these two sources. The activity of the enzyme from the synaptosomal source was greater than that from the S_{100} source when tRNA from the synaptosomal source was used. The activity of the enzyme extracted from S_{100} was greater than that from the synaptosomal source when tRNA from S_{100} was used as acceptor of amino acid. These results indicate the possibility of limited local aminoacylation and hence protein synthesis within the synaptosomes.

Behavioral studies:

The hypothesis developed by Essman [106] that 5-HT mediates the amnesic effect produced by ECS suggested a test

of the effect of an elevated amount of 5-HT on passive and active avoidance responses. In addition, a correlation between retrograde amnesia produced by ECS or by 5-HT and the inhibition of protein synthesis produced by such treatments would provide information about the possible relationships between the two processes.

In a previous study it has been suggested that MAO activity in the brain may be reduced as a consequence of bilateral nephrectomy. In these animals, impaired acquisition of passive avoidance was indicated [107]. An experiment was performed to determine whether the injection of MAO inhibitors would produce such an impairment of acquisition of passive avoidance. The results of this experiment clearly showed that injection of pargyline in doses capable of elevating the brain 5-HT levels did not cause impaired acquisition of passive avoidance (Figure 21). On the other hand, intracranial injection of 5-HT or administration of ECS produced marked amnesic effect (Figure 21). The time course of such amnesic effect revealed typical retrograde amnesia (Figure 22). A comparison between the amnesic effect produced by ECS to that produced by 5-HT is also presented in Figure 22. As can be seen, there is qualitative similarity, but differences in the recovery time are pronounced. A recent investigation indicated that the effect of 5-HT is specific and analogues of 5-HT did not produce amnesia [108]. From Figure 22 it is clear that 5-HT had proactive as well as retroactive effect. During the course of the present work, the effect

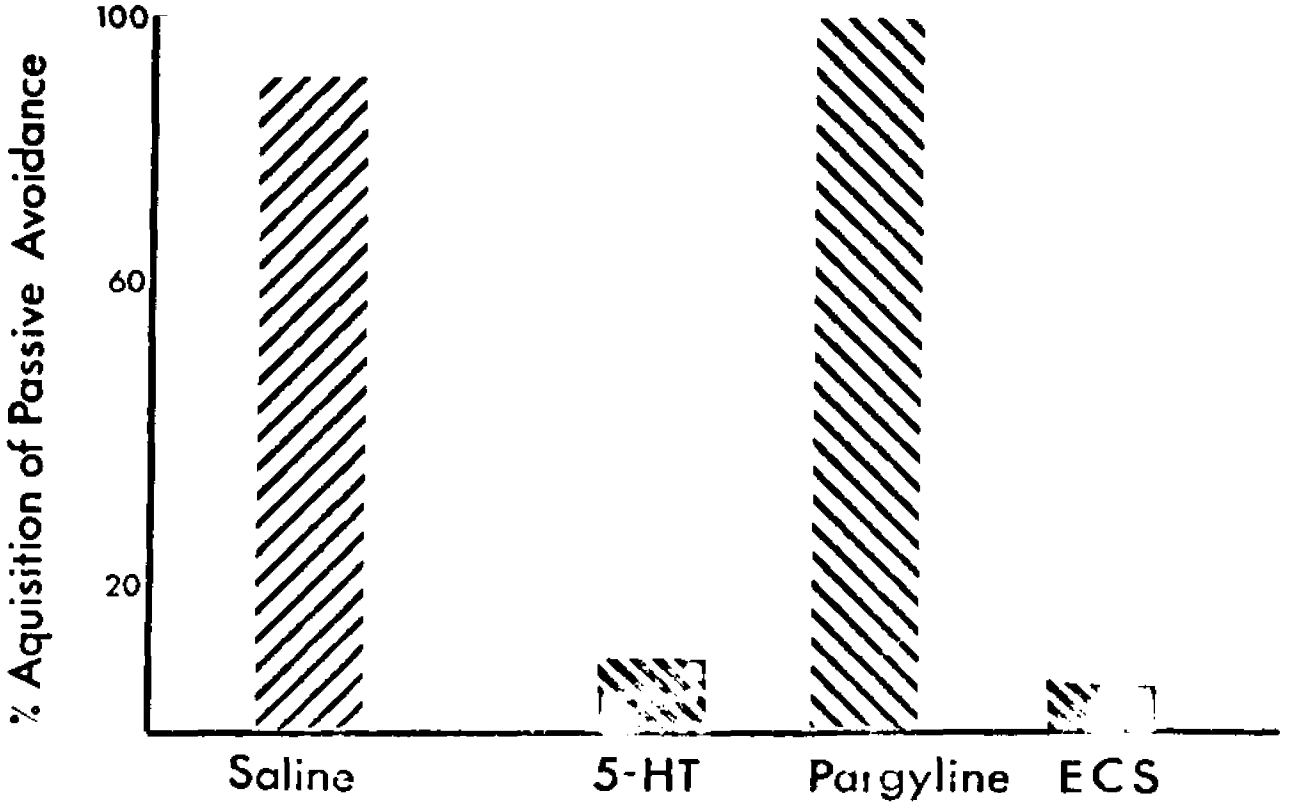


Figure 21: The effects of MAO inhibitors, intracranial injection of 5-HT, and ECS on passive avoidance acquisition in mice.

Pargyline (25mg/Kg) was administered twice, once immediately after the training trial (see method section) and again two hours before the testing trial. ECS or 5-HT was administered immediately after the training trial. The time interval between the training trial and the testing trial was 24 hours. Animals that failed to enter into the larger chamber (see method section) within two minutes were considered to be trained, and acquired the passive avoidance response. Results expressed as a percent of animals acquiring passive avoidance out of the total number of animals within the experimental group (10 animals per group).

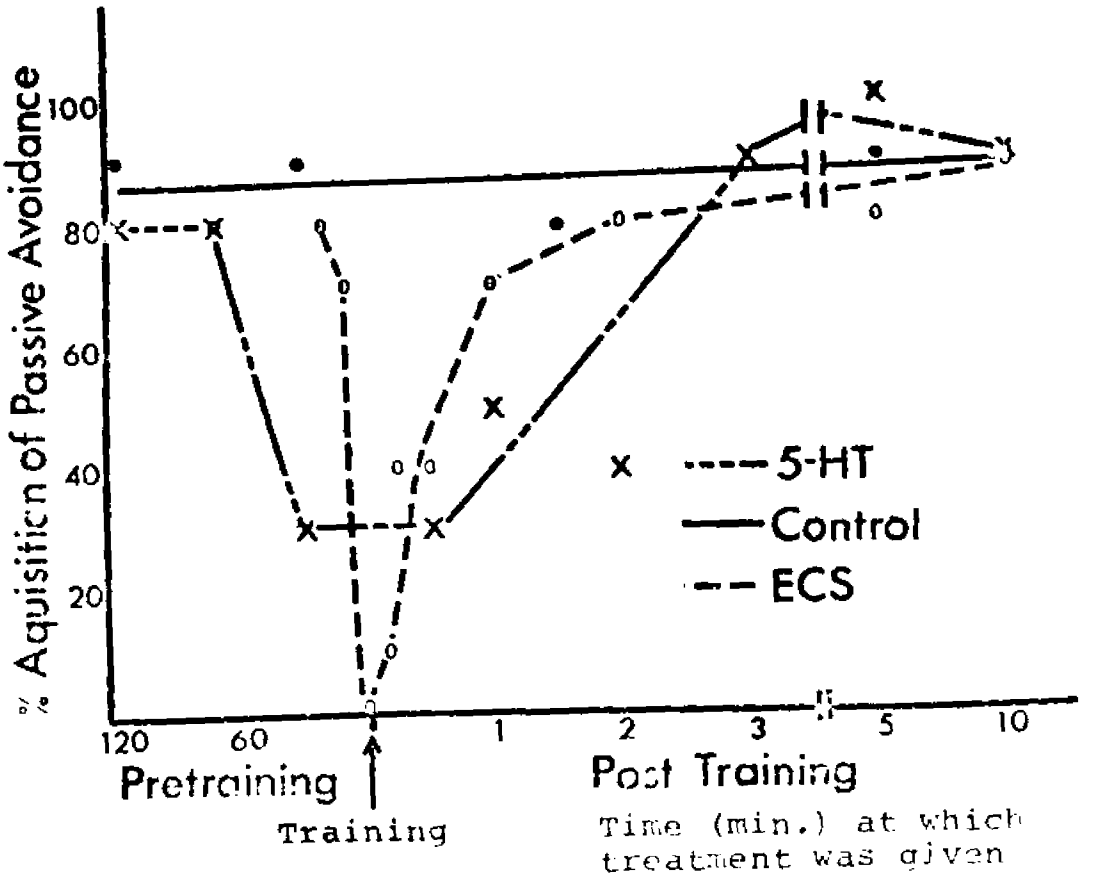


Figure 22: Proactive and retroactive effects of intracranial injection of 5-HT and of ECS administration on passive avoidance acquisition.

For procedure and legend see Figure 21. ECS and 5-HT were administered at different intervals before and after training, as indicated in the graph.

of 5-HT on active avoidance acquisition was also tested. A proactive effect was shown also for the active avoidance acquisiton, but unlike the ECS, 5-HT increased the latencies in the training trials in both the active avoidance and the passive avoidance experiments. On the second day of the active avoidance experiment, the 5-HT treated animals showed a similar learning curve to that of the control animals. This finding indicates the possibility that the proactive effect of 5-HT is related to performance rather than acquisition. This possibility should be considered in further experiments.

DISCUSSION

Two primary questions were of concern: 1) Does the incorporation of the labeled leucine into brain proteins truly represent protein synthesis? 2) Did the decreased incorporation of the amino acid into protein observed after ECS or intracranial injection of 5-HT originate from changes in protein synthesis? The procedure of washing following hydrolysis and a consecutive extraction of the lipids which was used in the present experiments (see method section), ensures that only proteins will remain in the precipitate in which the radioactivity is measured. The observed linearity of the incorporation precluded non-specific adsorption of the labeled amino acid on the brain proteins. Moreover, the requirements for a source of energy, Mg^{++} ions, and the pH optimum shown in the in vitro system, strongly support enzyme-dependent reaction. The different rates of incorporation observed when different routes were used for the administration of the amino acid may easily be explained when the blood brain barrier is considered. Under conditions of IP injection, the blood brain barrier mechanism restricted passage of the labeled leucine from the circulatory system into the central nervous tissue [109]. The short period of linearity which was shown for the IC injection is consistent with the fast

disappearance of the precursor from the brain after intracranial injection [110]. This fast disappearance was described and as discussed cannot be explained only by rapid metabolism [111]. Moreover, the long period of linearity observed when labeled leucine was injected IP suggests that the bodily metabolism of this precursor is relatively slow.

Administration of ECS or intracranial injection of 5-HT produces a decreased incorporation of labeled amino acids into the whole brain protein. A decreased rate of incorporation of amino acids may be explained by six alternative mechanisms:

1. A decreased permeability of the labeled amino acid from the peripheral circulatory system into the cerebral fluid may determine this site as the rate limiting step in the transport of the amino acid from the blood to the brain. A decreased availability of the precursor at the site where protein synthesis takes place will be the result of these consequences. Two recent independent studies dealing with the general problem [78,79] suggested that inhibition of protein synthesis rather than the above had occurred, but the procedures used in the aforementioned studies (the labeled amino acid was injected IP) did not eliminate a possible effect of ECS on this site. In the present study a procedure to confirm or deny this mechanism was utilized. The labeled precursor was injected intra-

cranially and therefore the availability of the labeled precursor was not limited by the blood brain barrier. Changes in the disappearance rate of the labeled precursor from the brain as a consequence of ECS can also be ruled out. Increase in cerebrovascular permeability (considering this site as the rate limiting step) would result in enhanced incorporation of the labeled amino acid into the brain proteins when IP injection was used. This was not found by Cottman et al [78] and Dunn et al [79]. Further, in case of increased disappearance rate, the degree of the inhibition would be expected to increase with time when intracranial injection was used. (The labeled amino acid will be diluted isotopically with the endogenous amino acid in the circulatory system.) The present studies showed that the inhibition is decreased as a function of time, a finding which is not consistent with increased disappearance rate. It was therefore concluded that the cerebrovascular permeability does not become the rate limiting step after the administration of ECS or IC injection of 5-HT.

2. A decrease in the spreading of the labeled amino acid as a consequence of ECS result in smaller amounts of the labeled amino acid at the site of protein synthesis. This mechanism may be ruled out when data from the present autoradiographic studies are considered. There were no differences in the distribution of the silver grains throughout the brain before and after ECS. Moreover, it was found that the concentration of free leucine within the brain

increases by 10-27% following ECS [78]. If slower spreading had resulted, this increase would not be expected. In addition, if a decreased spreading would occur, regions which are far removed from the site of the injection would be more affected than regions near the site of the injection. As was demonstrated in the present work, the cerebellum presented the smallest amount of inhibition, whereas the cerebral cortex presented the largest. Topographically, the cerebellum is located at a larger distance than the cerebral cortex from the site of the injection. The spreading of the amino acid intraventricularly injected is probably determined by the flow of the cerebrospinal fluid. This flow is passive and probably not affected by ECS.

3. Decreased cellular permeability following ECS would result in a decrease in the availability of the precursor in the intracellular sites. It has been reported that transport of amino acid into and out of the brain cells is an energy-requiring process [112]. Inhibitors of respiration or of oxidative phosphorylation inhibit transport. In general, a correlation has been made between ATP levels and transport activity in brain slices [112]. It was observed in the present study that ECS produces a decrease in ATP level. The decrease is of short duration and recovery to normal level occurred at one minute after the administration of the shock. On the other hand, inhibition of the incorporation of the labeled amino acid into protein was

still pronounced at 10 minutes after the administration of the shock. It is not likely, therefore, that decreased transport was the principal factor which determined the slower incorporation rate observed 10 minutes after the administration of the shock. Moreover, it seems that transport rates are a major factor in determining the levels of amino acids inside the cell [112]. Therefore, the results reported by Cottman et al [78] on elevated concentration of free leucine following ECS appear to rule out a decrease in transport. In addition, the autoradiographic studies described in the present work did not indicate a decrease in the number of silver grains which were visualized in the cellular area in the cerebral cortex following ECS. However, the effect of ECS upon protein synthesis at the cerebral cortex was most pronounced. On the other hand, changes in permeability were observed in the cerebellum where inhibition of protein synthesis was minimal. Considering these findings, it appears that although changes in cellular permeability may play some role in determining the decrease in the rate of the incorporation, especially at the first minute after the administration of the shock, this role is minor and the major part of the inhibition cannot be attributed to changes in the cellular permeability.

4. Increased rate of the amino acid metabolism following ECS would result in decreased concentrations of this precursor for the protein synthesis. It was observed in

the present work (Table 1) that the rate of the incorporation is not highly concentration dependent and a slight decrease in the precursor concentration would not be expected to be reflected in a decreased incorporation rate. In addition, the short duration of the incubation limited significant metabolism, the rate of which was shown to be slow in itself as argued above. Changes in metabolic rate cannot therefore be considered a determining factor in the decreased rate of the incorporation observed after ECS.

5. Decreased rate of incorporation could be observed as a consequence of isotopic dilution. As was indicated before, ten to twenty-seven percent increase in the free leucine concentration was observed four minutes following ECS [78]. The labeled amino acid could be therefore isotopically diluted. This kind of artifact can be excluded given the following considerations: a) Increased amounts of the amino acid injected up to concentrations in which isotopic dilution was effective did not change the amount of the inhibition. b) The amount of the inhibition was much greater (around 50%) than the one expected from isotopic dilution alone (maximum 27%). c) With corrected values of the specific activity of the amino acid within the polypeptide chain and the specific activity of the free leucine, significant inhibition was found immediately after ECS [78].

6. Changes in the rate of protein synthesis per se may bring about decreased rate of the incorporation of the

amino acids into protein. Changes in the rate of protein synthesis may be a result of a) presence of inhibitors produced, or increased in concentration, as a result of ECS; b) decreased concentrations of one or more factors required for protein synthesis; c) destruction of part of the protein synthesis machinery, such as degradation of RNA or disaggregation of polysomes to monosomes; d) conformational changes which caused a decrease in the affinity of the protein synthesis apparatus to its substrate.

Changes in the rate of protein synthesis and the factors which may play a role in determining these changes were further considered during the course of the present work.

An account of the mechanism of inhibition should include consideration of two sites of protein synthesis: the microsomal sites located in the perikaryon and the synaptosomal site located at the nerve terminal. Not much is known about the mechanism of protein synthesis at the latter site, but some information is available concerning the mechanism at the microsomal sites [35]. The amount of incorporation of labeled amino acid into microsomal proteins is much greater than the incorporation into the synaptosomal or mitochondrial proteins (see Table 7). Therefore, when the whole brain protein synthesis is measured by the incorporation of the labeled amino acid into the brain protein, the microsomal protein synthesis is primarily what is dealt with. The incorporation of C^{14}-leucine into the whole brain protein was inhibited by ECS. As was mentioned above, one way to explain the inhibitory effect is that an inhibitor of protein

synthesis, which was produced or increased in amount after the ECS, inhibits the microsomal protein synthesis. Evidence suggesting presence of a microsomal inhibitor bound to microsomes extracted from the brain, is presented in Figure 19, where brain and liver microsomes are compared. The decreased linearity which was shown for brain microsomes may be explained by the presence of such inhibitor in the microsomal fraction. However, the concentration of such a hypothetical inhibitor is not changed as a consequence of ECS. This conclusion is based upon the finding that microsomes extracted from animals pretreated with ECS were not inhibited when compared to control microsomes. Nevertheless, these findings do not preclude the possibility that a soluble inhibitor was produced as a consequence of ECS because a soluble inhibitor may be removed or diluted through the preparation procedure and thus not be detected in the in vitro studies. This possibility will be discussed at a subsequent point.

Decreased concentrations of a factor required for protein synthesis as a consequence of ECS was mentioned above as another alternative mechanism mediating the inhibitory effect. The finding of Orrego and Lipman [99] may have bearing upon this point. In their study they showed that protein synthesis is inhibited in brain slices after electrical stimulation. An interpretation for the inhibitory effect involved the drop in ATP levels which was observed during the stimulation period [113]. The present study showed that ECS is followed by a dramatic drop of the brain ATP levels. However,

there was no correlation between the recovery of ATP to normal level and the recovery of protein synthesis to normal rate. The drop in ATP could produce long-lasting conformational or aggregational change in the brain ribosomes, but the lack of inhibition in the microsomes extracted from animals pretreated with ECS excluded such interpretation. A correlation was shown for the recovery time of the ratio ATP/ADP+AMP to pretreatment level and the recovery time of the protein synthesis to normal rate. These two adenine nucleotides have been shown to be inhibitors of protein synthesis in reticulocytes [114], and in cell-free systems extracted from rat liver [82]. The ratio of these nucleotides to ATP level is an important factor determining the inhibitory effect. It appears that ADP and AMP and especially the change of the ATP/ADP+AMP ratio as a consequence of ECS may mediate the inhibitory effect of the shock treatment.

A comparison of the delayed recovery of the elevated levels of ADP and AMP with the brief recovery period of the decreased ATP to a normal level shows that ECS was followed by an increase in the total adenine nucleotides pool. This could be explained by the degradation of RNA observed after ECS [68], by increased conversion of guanine nucleotides to adenine nucleotides, or by increased do novo biosynthesis.

The drop in ATP level and the initial elevation in ADP and AMP concentrations suggest changes in ATPase activity.

An increase of the ATPase activity which is located in the vicinity of the protein synthesis machinery in both the microsomal and synaptosomal sites [115], would supply increased concentrations of ADP and AMP, protein synthesis inhibitors, at the site of the protein synthesis. An activation of the ATPase, especially Na^+ and K^+ stimulated ATPase could occur following the delocalization of K^+ and Na^+ which has been shown to take place during neuronal activity [116,117] and after ECS [60]. An increased Na^+ and K^+ stimulated ATPase activity in animals treated with ECS was recently reported [118]. This increase was observed only in the particulate fraction extracted from brains that had been frozen in liquid N_2 prior to the preparation. In the present study microsomes extracted from animals pretreated with ECS did not show significant increase in ATPase activity in vitro. However, the microsomes were prepared from fresh tissue which was merely chilled prior to preparation. It is suggested that the inhibitory effect of ECS on the in vitro system would be visible in a system in which the latent Na^+ and K^+ stimulated ATPase was preactivated.

Disaggregation of brain polysomes following multiple ECS was shown by Vesco and Giuditta [75]. In the present study there is no evidence to support such disaggregation after a single ECS. Microsomes extracted from animals pretreated with ECS did not show a greater response to poly U. The greater response to poly U which was demonstrable after preincubation of microsomes was also the same in microsomes extracted from ECS pretreated animals as well as in the

control microsomes. This experiment indicated that the ribosomes were in the polysomal aggregation state after a single ECS and therefore disaggregation cannot be accounted for by the protein synthesis inhibition observed after a single ECS.

The electrophoresis studies with the postmitochondrial supernatant (S_2) provided some evidence for the stage at which protein synthesis was inhibited by ECS. It appears that the labeling of low molecular weight bound proteins was greatly inhibited by ECS. Thus examination of S_2 without treatment of SDS showed that most of the inhibition was located at the starting point where non-migrating particulate proteins were located. After treatment with SDS, a detergent which solubilizes the microsomal proteins, most of the inhibition was located in the fast migrating fraction which consisted of low molecular weight proteins. Inhibition of the initiation of the polypeptidation would be consistent with this finding. Inhibition of the elongation as well as aminoacylation would result in more homogenous distribution of the inhibition along the gel.

As was mentioned above, consideration should also be given to the inhibitory effect at the synaptic site. In the present work it was shown that the incorporation of the labeled amino acid into synaptosomal proteins was inhibited to a greater extent than the incorporation into microsomal proteins. Similar results were described independently by Dunn et al [79]. The latter group distinguished between

two sites at which labeled proteins appear: fractions which are storage sites of the newly synthesized proteins and fractions which are sites of synthesis. Based on this distinction Dunn et al [79] suggested that increasing rate of synthesis during the pulse will result in a higher relative labeling of synthesizing to receiving fractions. Thus, if there is an initial inhibition of protein synthesis after ECS, followed by a recovery, inhibition in the synaptosomes which may be considered as the receiving fraction, will be more pronounced than that in microsomes which are the principal site of protein synthesis within the cell. The rate of the appearance of labeled proteins in the synaptic site (see Figure 5) and the differential inhibition observed in the present work are consistent with such an interpretation. However, the observed decrease in the differential inhibition as a function of time is not consistent with the above hypothesis. The recovery from the inhibition in the synaptosomal site was faster than the recovery observed in the microsomal site. This finding leads to two new possible interpretations for the differential effect. a) ECS produces inhibition in ribosomal protein synthesis as well as inhibition of axoplasmic flow. Both processes require energy. Further, the short period of inhibition observed in the synaptosomal fraction may be correlated with the short period of decrease in ATP after ECS. b) The inhibition of ribosomal protein synthesis as well as synaptosomal protein as a consequence of ECS, are independent of each other.

In vivo [27,28] as well as in vitro experiments [31,32,33] have yielded support for the idea that synaptosomes are an independent site of protein synthesis. This hypothesis is further supported by results reported here. After a relatively short pulse (5 minutes) a significant amount of labeling was found in the synaptosomal proteins (see Table 7). This brief duration makes unlikely the possibility that these labeled proteins were supplied by axoplasmic flow. However, microsomal contamination was observed in the synaptosomal fraction. Nevertheless, this contamination is insufficient to explain the total labeling observed within the synaptosomal fraction (see Table 7). The highest specific activity of proteins within the synaptosomal site was observed in the fraction enriched with outer membranes (see Tables 8 and 9). This finding obviates the intrasynaptosomal mitochondria as the principal site for protein synthesis within the synaptosomal fraction. The electrophoresis studies clearly showed significant differences in the distribution of the labeling when the synaptosomal fraction is compared with the microsomal fraction. The highly labeled and slowly migrating band which was shown for the synaptosomal membrane fraction did not appear in the postmitochondrial fraction when both were treated with SDS and electrophoretically separated. It can be argued that the ribosomes do not contribute incorporated radioactivity to the newly synthesized proteins of this band. Although some labeled bands from both sources, microsomal and synaptosomal, showed similar migratory

properties which might be an indication for some microsomal contamination in the synaptosomal fraction, it is clear that this contamination does not contribute all of the labeled proteins to the synaptosomal fraction. In addition, the presence of tRNA and aminoacyl synthetase activity with partial specificity within the synaptosomal fraction strongly suggests that local protein synthesis may take place within the nerve ending. The failure to purify aminoacyl synthetase from this site may be attributed to the small quantity of the enzyme available from the synaptic site.

The biphasic curve shown for the kinetics of the labeling rate of the proteins at the nerve terminal was an interesting finding. This type of curve would be interpreted as containing two components, the synthesizing phase and the receiving phase, i.e., the deposit at the nerve ending of the labeled proteins previously synthesized at the perikaryon. It is evident that only the first phase is inhibited by ECS, which may indicate independent inhibition of protein synthesis at the synaptosomal site. Nevertheless, the alternative explanation cannot be completely ruled out. That is to say, it is still possible that the rate of the axoplasmic flow, within a short period after ECS, is decreased as a result of the shock treatment.

The present study also bears on the following issues: the effect of an elevated brain level of 5-HT upon inhibition of protein synthesis and the possible relationships between ECS, 5-HT, and the inhibitory effect upon protein synthesis

produced by ECS. It has been suggested by Essman [106] that 5-HT may mediate many of the effects produced by ECS such as changes in RNA content and retrograde amnesia. In addition, Essman has shown that 5-HT in very low concentrations (3×10^{-7}M) is able to significantly inhibit protein synthesis in synaptosomes in vitro [80]. This finding raised the hypothesis that 5-HT may modulate protein synthesis in the synaptic site. As was shown in the present work, an elevation of the whole brain 5-HT levels per se did not affect the incorporation of labeled amino acid into the whole brain proteins. An elevation of 5-HT produced by MAO inhibitors was not accompanied by inhibition of protein synthesis. On the other hand, intracranial injection of 5-HT produced small but significant inhibition of the incorporation of labeled amino acid into the whole brain protein. The intracranial injection was in most cases intraventricular. This was shown in histological and anatomical studies in which stain was injected intracranially and the site into which the stain was injected was identified as the lateral ventricles. That the labeling originated from the 5-HT, intracranially injected, was taken up by the brain tissue and rapidly distributed throughout the brain, was shown in the autoradiographic studies. The spreading of the labeling is probably controlled by the flow of the cerebral fluid. Although relevant to the issue, no differentiation could be made in respect to the cellular sites into which the 5-HT was taken up. However, the labeling was generally

not restricted exclusively to areas which are known to be rich in endogenous serotonin. The midbrain which is rich in endogenous serotonin showed greatest labeling only at 2.5 minutes after the intracranial injection. The cerebral cortex which does not contain high endogenous serotonin showed a comparable labeling to the midbrain 5 minutes after the injection and greater labeling at 10 minutes after the injection. This finding is consistent with the previous finding for brain slices suggested that 5-HT can be taken up by both the catecholamine transport system and the 5-HT transport system and that the transport by the latter system is faster [119]. The serotonin which was taken up is probably stored at synaptic sites. This was suggested by the finding that 5-HT which was accumulated in brain slices was released by depolarizing procedures such as electrical stimulation or high external K^+ concentrations [120]. In fact, the elevated 5-HT levels observed after the injection of MAO inhibitors occur at the synaptic site. Some of the 5-HT is transferred to the ventricles and can be found in an effluent collected from the perfused ventricles after the injection of MAO inhibitors [121]. Hence, there is a similarity between the intracranial injection and the effects of MAO injection. It was therefore surprising to find that, whereas the injection of MAO inhibitor does not alter cerebral protein synthesis, intracranial injection of 5-HT does inhibit cerebral protein synthesis. That changes in 5-HT concentrations at the cell body versus synaptic sites resulted from

the injection of MAO inhibitors differ from those produced by IC injection of 5-HT can be given as a partial explanation. However, in the present work it was not possible to determine whether these two treatments differentially changed the concentration of the 5-HT at these two sites. A consideration of the mechanism by which intracranial injection of 5-HT inhibits protein synthesis leads to two hypotheses: 1) 5-HT *per se* inhibits cerebral protein synthesis. 2) Intracranial injection of 5-HT produces electrical changes similar to those produced by ECS and as a consequence of this electrical activity protein synthesis is inhibited. In regard to the first hypothesis, it was shown by *in vitro* studies that 5-HT is able to inhibit the incorporation of labeled amino acid into microsomal protein. The effective concentrations of the 5-HT were relatively high and the question whether they fit the range of a compartmental endogenous concentration remains to be determined. A possibility that artifacts in the *in vitro* system changed the sensitivity of the brain microsomes, or that the effectiveness of the 5-HT in this system is reduced may exist. An increase in the density or in concentrations of the ribosomes in the *in vitro* system compared with the *in vivo* situation would result in an increased binding of 5-HT and a reduction of the effectiveness of the 5-HT as an inhibitor. That high concentrations of RNA attenuate the inhibitory effect of 5-HT was shown by the addition of soluble RNA to the *in vitro* system. This kind of artifact should be

further considered. However, there is evidence in the present work to support the view that the effect of 5-HT in vitro does not represent its in vivo effect or its mediatory effect in the case of ECS. This evidence came from the observation that the regional effect of 5-HT upon protein synthesis inhibition was not correlated with the distribution of the 5-HT at different regions of the brain. In addition, the stage of the synthesis which was inhibited by ECS (initiation of polypeptidation) is different than that shown for 5-HT in vitro.

As was observed in the present study, intracranial injection of 5-HT produced changes in the EEG continuously recorded after the injection. These changes cannot be attributed to artifacts produced by changes in volume of the cerebral fluid or to changes in osmolarity, because saline which was injected as a control did not produce these kinds of changes. It is therefore suggested that electrical changes took place within the brain after the intracranial injection of 5-HT. The nature of these changes remains to be established. It is suggested that intracranial injection of 5-HT induces protein synthesis inhibition at the ribosomal site via the electrical changes produced by this treatment. Delocalization of ions such as K^+ and Na^+ as a consequence of the electrical changes can be given as a mechanism connecting the electrical changes and the protein synthesis inhibition. The similarity between the regional

effects of 5-HT and ECS, and the quantitative differences shown for these treatments are both consistent with such a view. The absence of the inhibitory effect upon protein synthesis after the injection of MAO inhibitors is also consistent with this view. Depression of the electrical activity in the midbrain raphe has been observed after injection of MAO inhibitors [122]. Moreover, it was indicated that pargyline impairs ganglionic transmission and this impairment does not correlate with the MAO inhibition [102]. It seems, therefore, that pargyline could prevent the electrical activity produced by elevated amount of 5-HT. The failure of mianserine to attenuate the inhibitory effect of 5-HT may not be consistent with the above hypothesis. Mianserine has been shown to be a serotonin antagonist by several behavioral and physiological tests [101]. However, these tests may not be enough to determine its action upon the electrical activity produced by 5-HT. More direct experiments with this drug are required. Finally, the intracranially injected 5-HT was not concentrated near the ribosomes, the principal site of protein synthesis. This was shown in the autoradiographic studies described in the present work. It is therefore concluded that the ribosomes are not the direct site which is affected by 5-HT in vivo.

The 5-HT seems to be concentrated principally at the nerve ending and this site is therefore most likely to be affected by 5-HT per se. The ability of 5-HT to inhibit

protein synthesis in the synaptosomes in vitro as shown by Essman [80], is consistent with such a view. The synaptosomal protein synthesis system in addition to its exposure to high concentration of 5-HT, may also be more sensitive to this substance than microsomes. The possibility of differential sensitivity to 5-HT of the protein synthesis apparatus from different sources was shown in the present work for liver and brain microsomes. The differential sensitivity, together with the site of localization of 5-HT suggest two modes of action of 5-HT upon protein synthesis; one in the synaptic site and one in an unknown site which affects the ribosomal protein synthesis. While in the synaptosomal site the action of 5-HT is directly upon the protein synthesis apparatus, the effect of this substance at the ribosomal site is indirect and appears to operate through the induction of electrical activity. According to this hypothesis, the mediatory effect of ECS through 5-HT would operate only at the synaptic site.

The relationship between the inhibition of protein synthesis and the development of retrograde amnesia is the final problem given consideration here. In the present work an attempt was made to demonstrate the ability of intracranial injection of 5-HT to produce a retrograde amnesia and to compare this effect to the retrograde amnesia produced by ECS. As indicated in the behavioral studies, the effect of 5-HT was more prolonged. This can be explained by the relatively long duration of the electrical changes produced by intracranial injection of 5-HT.

Several studies have suggested that protein synthesis must play a role in the consolidation process [37,40]. In the present work an elevated 5-HT level produced by intracranial injection of 5-HT was accompanied by retrograde amnesia. In conditions under which 5-HT level was elevated but protein synthesis inhibition was not noted (injection of MAO inhibitors), retrograde amnesia was not seen. It is suggested that the inhibition of protein synthesis may play a role in producing the amnesic effect resulting from ECS or 5-HT. Additional support for such a view came from a recent work, which shows a coincidence between the extent of the protein synthesis inhibition induced by ECS or 5-HT and the amnesia induced by these treatments [108]. The effects were age dependent. Animals that were resistant to the inhibitory effect on protein synthesis (17 day old animals) did not show retrograde amnesia.

A relevant issue is the proactive effect suggested for the electroconvulsive shock. ECS given one hour prior to the training trial, significantly reduced the latencies observed in the subsequent training trial [125]. However, when ECS was given 15 minutes before the training trial and compared with ECS which was given immediately after the training trial, it was found that the effect of the post-training ECS was significantly more pronounced [126]. While the proactive effect can be explained by the hyperactivity or by the fear induced by ECS, the retroactive effect of ECS is probably related to impaired acquisition.

In the present study no significant differences were found between sham ECS treated animals and animals treated 10 or 20 minutes prior to the training trial. The possibility that ECS induces retrograde amnesia through the inhibition of protein synthesis was considered [127]. Taking this into account and recalling the fact that impaired acquisition of passive avoidance is more pronounced for post-training ECS than for pre-training ECS, one may suggest that only a short lasting inhibition of protein synthesis may play a role in the induction of the amnesic effect. As was indicated in the present work such a short lasting inhibition exists for the nerve endings. In this case the membrane protein should play some role in the memory consolidation process. This concept is not new. In 1964 it had been suggested by Bodian [123] and later by Best and Noel [124] that synaptic transmission coupling to protein syntheses might have occurred and its possible role in memory consolidation was given consideration. Synthesis of synaptic proteins could lead to the formation of new functional interneuronal relationships by forming new synaptic connections either by increased synthesis of enzymes involved in the synaptic function or by synthesis of structural proteins such as the receptor proteins which is an important factor in the synaptic function. Such new functional interneuronal relationships may lead to a recording of new information. The inhibition of protein synthesis in the

synaptic site may interfere with the formation of new interneuronal relationships and thus inhibit memory consolidation. It is apparent that more understanding of the protein synthesis at the nerve terminals is required to clarify the relationship between protein synthesis in this site and the formation of new synaptic connections. The use of ECS to produce inhibition of protein synthesis at the nerve terminal may serve in the future as a tool to interfere with the formation of these new intersynaptic connections and thus to clarify the role of these connections in the memory consolidation process.

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Appendix I

a) Calculations of the percent inhibition of protein synthesis induced by ECS or 5-HT:

When an experimental variable was repeated within a single experiment, an average between the observations which were related to the same treatment was calculated. The percent inhibition in all cases was calculated from the means. The control was always considered as 100%. The following equation was used for the calculations:

$$100 - \frac{\text{mean of experimental group(ECS or 5-HT)}}{\text{mean CPM of control}} \times 100 = \% \text{ inhib.}$$

when the effect of ECS or 5-HT was examined in the presence of an auxiliary treatment (see, for example, Table 10 concerning the effect of ECS after the injection of mianserine), the group receiving only the auxiliary treatment (mianserine according to the example indicated above) was considered as the control group.

b) Calculation of percent radioactivity in various subfractions:

During the preparation the total volume of each fraction was determined and aliquots were taken for radioactive determination. The total amount of radioactivity in each fraction could be calculated in this way. For the percent radioactivity in each fraction the radioactivity of the whole synaptosome was considered as 100%. The calculations

were done as follows:

$$\frac{\text{amount of radioactivity in fraction X}}{\text{amount of radioactivity in whole synapt.}} \times 100 = \text{\% radioactivity in fraction X}$$

c) Calculation of the ratio ATP/ADP+AMP:

Adenine nucleotide concentrations were determined after chromatographic separation (see method section) by measuring the absorbance at 260 nm. The ratio of ATP/ADP+AMP was calculated as follows:

$$\frac{\mu \text{ moles ATP found at time T after treatment}}{\mu \text{ moles ADP+ moles AMP found at time T after treatment}} = \frac{\text{ATP}}{\text{ADP+AMP}} \text{ ratio at time T after treatment}$$

d) Percent incorporation of control - calculations for Tables 23 and 24:

The effect of 5-HT or its derivatives on the incorporation of C^{14}-leucine was measured. Therefore, when additional substance was added to the incubation system to measure its effect upon the inhibitory effect of 5-HT <u>in vitro</u>, the samples containing the auxiliary substance but not the 5-HT were considered as the control for those cases in which the effect of the auxiliary substance was measured. Percent of control was calculated according to the following equation:

$$100 \times \frac{\text{CPM found in the group treated with 5-HT}}{\text{CPM found in the control group}} = \text{\% incorporation of control}$$