THE EFFECT OF PERIPHERAL STIMULATION UPON THE PROTEIN METABOLISM OF THE CORTEX

by

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This paper is dedicated to my wife Leslie who administered crucial encouragement during despairing moments.

R. L. S.

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TABLE OF CONTENTS

page

I. Introduction.............................. 1

II. Methods and Materials.................... 21

III. Results.................................. 27

IV. Discussion............................... 40

V. Summary.................................. 51

Bibliography.............................. 53

Appendix.................................. 58

I

INTRODUCTION

(1)

In recent years there has been a proliferation of hypotheses concerning the nature of the biochemical changes in the brain which underlie learning. Two rather specific hypotheses have received major interest: (1) Learning is transmuted in such a way that the incorporated information is "stored" in the macromolecules of brain nucleic acids. (For reviews see references 7, 9, 21, and 22). (2) Learning affects the activity of brain cholinesterase presumably because learning affects the biochemistry of synaptic transmission. (For reviews see references 5, 49, and 50). A complete review of the many studies bearing on these two hypotheses would not be pertinent to the experiment to be presented. However, one major point deserves recognition because it is common to both lines of experimentation. Simple activation or stimulation of the experimental subject as a variable distinct and clearly separate from learning has been a point of difficulty in the designs of experiments in both approaches.

This difficulty can be demonstrated with examples from some of the reference experiments done to elicit evidence on the hypotheses mentioned above. Hyden and Egyhazi [31, 32] trained rats to walk a tight wire for food reward, a task

which presumably involved changes in the vestibular system attributable to learning. When the animals' performance reached criterion, they sacrificed them, and after isolating nerve cells of Deiter's nucleus (a relay nucleus of the vestibular system) and freeing the cells of attached glial cells by microdissection, they extracted the ribonucleic acid (RNA) from the nuclei of these cells and measured the concentration of RNA per cell, as well as the base ratios of the RNA extracted. Animals kept in their home cages during the course of the experiment served as controls. However, to control for vestibular activation _per se_ an additional control group was subjected to vestibular stimulation by being spun in a low speed centrifuge each day the experimental animals were receiving training. The results showed an increase in concentration of RNA per Deiter cell nucleus in animals which were trained and in animals which were passively stimulated as compared to the home cage controls. However, the trained animals, in addition, were found to have an altered nucleic acid base ratio relative to the spun controls. The adenine fraction was increased and uracil fraction was decreased.

Having described the essentials of the experiment a criticism will be made: Although the "trained" animals and the "stimulated controls" differed with respect to learning, they also differed with respect to the stimulus conditions to which they were subjected. Perhaps, in addition, they differed with respect to their emotional state as well as their nutritional state (since the acquisition of food was dependent on the performance of the task in the case of the "trained" group). So, although the problem of "activation" vs "learning" is acknowledged in this experiment, the controls are inadequate.

Later Hyden and Eghazi [33] did an experiment in which they forced right-handed rats to obtain food only with their left hands. Since the contralateral, right cortex controls the left hand, it was presumed that the contralateral cortex mediated this reversal of handedness. It was therefore assayed for RNA in a manner similar to that described in the above experiment. An homologous area in the ipsilateral cortex served as a control. A further control involved right-handed rats who received no training in reversal of handedness. Again, it was found that training resulted in an increase in the amount of RNA per nerve cell nucleus and that training altered the ratio of bases in the RNA. The comparison in this case was between right ("trained") cortex and the left ("untrained") cortex.

One of the important difficulties in the described experiment lies in the fact that a small number of animals (N=3) were used to compare the opposite cortices of animals which had received no training, whereas a larger number (N=8) were used to show differences in the experimental group. Thus we are left with the question of whether the differences would have occured regardless of the training. However, this experiment demonstrates the advantages of using an animal as its own control because in this experiment such questions as motivational level, nutrition, and equality of stimulus conditions were not at issue. However, the question can still be raised– did the changes occur because of increased activation of the contralateral cortex involved in controlling the trained limb, or were the changes reflective of the learning process itself?

Krech, Rosenzweig, Diamond, and Bennett [5,49,50] in a long series of experiments investigating the effects of learning on cholinesterase (hypothesis number 2 above) have found that no simple relationship exists. They have found,

however, that "enriched" environments, as opposed to "impoverished" environments will increase the cholinesterase activity in the brains of developing rats. Again the question may be asked: Does environmental complexity affect cholinesterase activity because it affects learning or because it affects the general activity level of the animals?

In summary, experiments in the field of learning and biochemistry, although provocative at times, are plagued by the inability to clearly distinguish the effects of learning from the effects of simple activation or stimulation.

(2)

Having discussed the issue of "activation" vs. "learning", attention will now be paid briefly to the various biochemical substances which have been examined in hope of finding changes underlying the learning process.

It is unlikely that any single molecular species is the reservoir of learned information. In the broad sense, a very large number of substances are undoubtedly necessary for adequate functioning of the memory mechanism, as attested to by the fact that inadequacies of a large variety of substances may result in mental retardation in humans.[18]

However, in order to intersect the process at some point, various likely candidates have been examined. RNA and cholinesterase, as mentioned previously, are but two of these. As pointed out by Eccles,[17] changes in RNA may be basic to a variety of changes going on in the cell including the production of a protein of which cholinesterase is one kind. Thus the RNA and the cholinesterase hypotheses are not at all mutually exclusive.

It is the assumption of the experiment to be described in this thesis that the examination of protein metabolism would be a strategic point at which to intersect the complex events of active nerve tissue. This is purely arbitrary but presumes that if changes can be shown to occur in the general class of substances identified as protein, then more specific molecular entities might be profitably studied.

To summarize, (1) although the biochemical changes accompanying learning are of great interest, a more basic question may well be: What are the changes which accompany the simple activation of nervous tissue?, and (2) although a variety of substances have been examined in an attempt to find biochemical changes reflective of the plastic changes which occur in the brain, at this time it seems quite arbitrary which one is selected; furthermore, (3) brain protein seems to be a substance which is likely to be involved in the neural plastic processes regardless of what they might be.

With these considerations in mind, the focus for attention becomes the following: Will simple activation or stimulation of the organism result in changes in the metabolism of brain protein? The answer to this more restricted question may indirectly shed light on the problem of the biochemistry of learning. In reviewing the literature which pertains to this question, the metabolism of brain protein will be discussed first, followed by a review of studies dealing with the question of the effects of stimulation on brain protein metabolism directly.

(3)

Protein accounts for about forty per cent of the dry weight of the brain.[51]

For some time it was thought that the proteins of the nervous system were relatively inert.[20] This conclusion was based on the fact that intravenous infusions of amino acid hydrolysates resulted in little change in the concentration of amino acids within the brain although this same procedure resulted in increases in all other tissues assayed. When radioactive amino acids became available, it was found that little uptake of labeled amino acids occurred when they were administered intravenously.[48] However, when the "blood-brain barrier" was bypassed and the labeled amino acids introduced directly into the cerebrospinal fluid, active incorporation into proteins was found.[23] From such studies, it has been concluded that the overall rate of synthesis of brain protein is not unusually slow and in the adult animal the rate of incorporation of amino acids in isolated brain tissue is intermediate between that in heart muscle and liver.[48]

Estimates of the mean half-life of brain proteins have been reported as fourteen days[23] using C^{14}-methionine as a precursor and as 10.7 days[58] using C^{14}-lysine as a precursor. Both of these studies utilized the infusion of a single dose of precursor and then measured the activity at various points in time after the dose was given.

When the rats were administered C^{14}-lysine in their diets both prenatally (by feeding their mothers) and postnatally, followed by a switch to an unlabeled diet at various points in time, it was possible to calculate the half-life of their brain proteins in a different way.[37] A figure comparable to that obtained by the single infusion technique was obtained, i.e., a mean half-life of fourteen days. However, this study produced an additional point of information. Since it was assured that all of the

proteins in the brains of these animals had been labeled with radioactive amino acids (since they were exposed to radioactive label from conception) an estimate could be made of how much of the protein was absolutely stable. It was found that after 150 days on a regular diet, only a miniscule portion of radioactivity was left in the brain. These authors concluded that only a very small faction of protein labeled with lysine was stable throughout a lifetime. They suggested that this may have ramifications for biochemical theories of learning.

Although the mean half-life of proteins as measured in the previous experiments is moderately short, it is well known that the brain proteins are a heterogeneous array of molecular species so that the mean half-life does not give any information as to the synthesis and breakdown of individual proteins. Brain proteins have been fractionated and classified in a variety of ways[48] and of the various types, the saline soluble faction, accounting for about twenty per cent of the total, has the highest rate of turnover.[45] In fact, one study found that some water soluble proteins labeled with S^{35}-methionine may have a half-life as short as four hours.[43]

It may be concluded that the proteins of the brain are not inert, as was once thought, but are synthesized and broken down with moderate speed. Furthermore, some proteins in the brain may be very active. Although some proteins are more stable than others, almost none remain stable throughout the lifetime of the organism.

Having established that the brain protein is relatively metabolically active, two questions arise: What is the general metabolism of brain protein, and are there any features of its metabolism which make it unique? In general, it may be concluded

that synthesis of protein in the brain occurs through steps which are similar to synthesis of protein elsewhere in the body. In other words, there is evidence that amino acids are polimerized in association with microsomes by interaction with nucleic acids.[14, 11] However, recently there has been evidence accumulated that protein synthesizing systems may occur in association with a fraction of subcellular particles rich in mitochondria as well as a subcellular fraction of ribosomes.[11, 34] This is an interesting finding in light of the knowledge that synaptic endings are known to be rich in mitochondria.

In conclusion, it may be said that the brain has a moderately rapid steady-state breakdown and synthesis of protein. Moreover, studies have shown that this synthesis and breakdown is, for the most part, not qualitatively different from that in the rest of the body.

(4)

Since the brain has a moderately rapid steady-state breakdown and synthesis of protein, one could reasonably ask if this rate is altered by changes in brain function. The answer to this question is as yet unclear. Reports from previous studies are conflicting. However, part of the problem arises from the fact that the term "stimulation" has been used to denote a variety of operations. In addition, a variety of indices for detection of protein metabolism have been employed. I will discuss the previous experiments by categorizing them along the dimension of the stimulation employed. Three general categories will be subsumed: The effects of convulsive stimulation, the effects of discrete stimulation of known neural pathways, and the effects of more general behavioral activation.

Convulsive Stimulation

Gaitonde and Richter[23] injected S^{35}-methionine into the cisterna magna of a group of rats who were subsequently given electroconvulsive shock. These workers were among the first to use the intracisternal route of injection for studying protein metabolism. They found that this route of injection would result in a much higher saturation of labeled proteins in the brain compared to the intraperitoneal route. However, they found no differences in the incorporation of S^{35}-methionine when comparing animals given electroconvulsive shock with untreated controls.

Dingman, Sporn, and Davis[16] injected C^{14}-proline intracisternally. Electroconvulsive shock was administered and the protein from the rat's brain was fractionated into seven different sub-cellular fractions, each of which was analyzed separately for C^{14}-proline uptake. There were no differences between the animals who had received the electroconvulsive shock and those who had not. However, proline is a rather special amino acid since in most tissues it is incorporated mainly into collagen, a protein that is virtually absent from the brain.

Krawczynski, Wiszniowski, Drewnowska, and Kujawa[36] combined S^{35}-methionine with either serotonin or LSD-25 or both and injected the mixture into the cranium. The report does not say whether this was an intracisternal or an intra-cerebral injection. The dose of LSD-25 or serotonin was sufficiently great to induce convulsions within an hour after the injection. The brain proteins were analyzed for uptake of S35-methionine. Treated animals showed significantly increased uptake compared to the untreated controls.

Geiger, Horwath and Kawakita[26] measured the incorporation of C^{14}

derived from labeled glucose into the proteins of the cat cortex. They employed a controlled perfusion technique in which the brain circulation was taken over and perfused with an artificially constituted, simplified blood. They took successive samples from the same animal while the experiment was in progress, justifying this on the basis of a pilot experiment which showed that variations in uptake in separated areas of the cortex are small in the unstimulated cat. After the perfusion was going well and the brain electrically normal, Metrazol convulsions were induced. These convulsions resulted in a marked increase in the incorporation of glucose into brain protein. For approximately fifteen minutes after the convulsion, the incorporation dropped to normal after which it began at a high rate again.

A somewhat different experiment was done by Baily and Heald.[4] Instead of measuring uptake of a labeled amino acid, they measured total protein content in whole rat brain following either treatment with ether, Nembutal, electroconvulsive shock, or Metrazol. They found no significant differences between the experimental group and the untreated controls. However, the Metrazol or electrically induced convulsions were short of duration, ranging from one to two minutes. Also, they used small groups of animals.

In summary, experiments employing convulsive activity to activate the brain have been about equally divided on the question of whether or not activation of the brain results in a higher incorporation of amino acids into protein.

Discrete Stimulation of Known Pathways

Brattgard[8] has shown that rabbits raised in the dark have decreased

amounts of RNA and protein in their retinal ganglion cells. Apparently light stimulation is necessary for the development of normal amounts of protein in the retina. However, Gomiato and Boggio[28] found that the effects of stimulus deprivation on the retina could be blocked by narcosis. Evidently there are more complex factors involved than just the presence or absence of light.

Hyden[29, 30] has measured the protein and RNA content of individual cells and has found changes as a result of physiological stimulation. He found that vestibular stimulation resulted in an increase in protein and RNA in the cells of the first and second order in the vestibular pathway. Acoustic stimulation, however, caused a decrease in the nucleoproteins involved in the acoustic pathway. Hyden interprets both increases and decreases in nucleoproteins as reflecting a lively protein production resulting from stimulation.

Geiger[25] did an experiment in which he activated the cortical somatic sensory area by stimulation of the brachial plexus. The opposite sensory area was frozen three seconds before the stimulation was begun and served as a control. Stimulation lasted for five to twenty seconds. Samples were taken immediately after the stimulation ceased. Geiger found that nonprotein nitrogen increased by an average of 161 microequivalents per milligram in the somatic sensory area on the side opposite the stimulation (i.e., the receiving area for the brachial plexus stimulation). The chemical changes were proportional to the number of stimuli and were reversible with rest.

Altman, Das and Chang,[3] in a recent study, trained rats for a long period on a series of visual discrimination tasks. They then injected them with H^3-leucine

intracisternally and later sacrificed the animals and studied histological sections of the brains with autoradiographic techniques. An objective measure of the amount of radioactivity in single nerve cells can be accomplished with this technique combined with "microdensitometry", a scanning method for measuring the density of reduced silver granules on a photographic plate. No differences were found between the group that had a long history of visual learning tasks and the group that did not. This study included cells of the visual pathways as well as cells concerned with other functions.

Another study which employed autoradiographic techniques was done by Watson.[59] Radiolabeled uridine, a precursor of DNA, was injected into rats and they were given vestibular stimulation. He found that areas of the brainstem known to be involved in transmission of vestibular input showed a rise in the number of "uridine granules" per nucleus over a period of days following the stimulation as compared to quiescent controls.

Talwar, Chopra, Goel and D'Monte[54] measured protein biosynthesis in developing rabbits before and after opening of the eyes and also in adult animals kept in the dark or exposed to light stimulation. To measure protein metabolism, labeled valine or glucose was injected intracisternally and samples of occipital cortex were analyzed. In both conditions light stimulation was reported to increase the incorporations of C^{14} -glucose and C^{14} -valine.

Singh and Talwar[52] reported the findings of a study which investigated the parameters of light stimulation which affected protein synthesis in the occipital cortex. The design of the experiment was similar to the previous one by Talwar, et al., except that parameters of stimulation were varied and a different animal,

the rhesus monkey, was used. Increases in light intensity were found to increase protein metabolism up to a point of 150 candle-power; a further increase caused depressed protein metabolism. Flicker frequency was also investigated and it was found that continuous light stimulation has little stimulatory affect, whereas flickering light at a frequency of seven per second was shown to optimally stimulate protein metabolism in the occipital cortex.

However, Metzger[42] also measured protein metabolism in the occipital cortex as a function of light stimulation but was unable to show any effects. In his experiment, a surgical section of the corpus callosum, anterior commisure, and optic chiasm was done on several Macaque monkeys after which they were allowed to recover. Later, the monkeys were restrained, and one eye of each monkey was covered; then strong visual stimulation was given to the other eye. Tritiated water was used as the radioactivity label. In some instances, shock was paired with the light. The stimulation was of variable flicker frequency and lasted for three hours after which the animals were decapitated. As was stated, no differences were found in any of the cortical areas measured.

Sokoloff [53] found highly discrete changes in blood flow of the brain following visual stimulation. The changes were so dramatic that investigators could assign a particular histological slide to the stimulated or unstimulated group without any other information. However, the areas affected were so discreet and so small a portion of the structure containing them that average blood flows in each of the structures as a whole were not significantly different in the control and experimental animals. Sokoloff argues that cerebral circulation is regulated chiefly by chemical means which adjust the blood flow to the tissue metabolism.

The work of Lorenzo, Fernandez and Roth[38] has shown that changes in radiosulfate permiability occur in discreet functional areas of the brain in response to auditory or visual stimulation. The changes were studied by means of autoradiography. Lorenzo, et. al. interpret the findings as changes in the blood brain barrier permiability to sulfate. It is unknown whether these changes occur as a function of blood flow or of localized expansion of the extracellular fluid space.

General Behavioral Activation of the Brain

Vrba and Folbergova[57] produced compulsory swimming in large groups of rats (N=24) and measured the TCA insoluble substances (nucleic acids and proteins) as well as the TCA soluble glutamine. Swimming was required of the animals for a period of 4.5 hours in one group, and 6.5 hours in another. The animals were sacrificied immediately after the forced swimming period. The exercised animals were found to have a decrease in the TCA insoluble nitrogenous substances and an increase in the TCA soluble glutamine.

Glasky[27] made the observation that chlorpromazine decreased the incorporation of radiolabeled leucine into brain areas but swimming overcame the effect.

Altman[1], in 1963, reported that forced activity in an activity wheel at rates of 7 to 12 r.p.m. increased the incorporation of H^3-leucine into all twelve areas of the brain and spinal cord that were studied. Non-exercised animals served as controls. The method of measurement of incorporation was autoradiography combined with microdensitometry. The largest increase was noted in the motor area of the cortex. However, Altman suspected that the result might be due to non-specific arousal effects.

To test for this possibility, he did a later experiment in which he controlled for arousal.[2] In the later experiment, no differences were found between the exercised and the non-exercised rats.

Furthermore, to test the arousal hypothesis, Das and Altman did an additional experiment.[15] They raised one group of animals in a "restricted" environment and another in an "enriched" environment. Again the uptake of H^3-leucine was measured and an increased incorporation in animals raised in the "impoverished" environment was found. This finding was attributed to the greater stress reaction on the part of the restricted rats to the handling and injection procedures.

Ninoiya, Buxton and Michaelis[44] forced rats to do exhaustive exercise for a period of two hours after which evidence of clinical shock was manifest. Under these circumstances they found an increase in total nitrogen as measured by the Kjeldahl method. If the animals were tranquilized prior to the treatment, no differences were noted.

The experiments of Bennett, Diamond, Krech and Rosenzweig,[5, 49, 50] referred to earlier in this chapter, have studied the effects of general activation of the organism by comparing the effects of "enriched" versus "impoverished" environments on the biochemistry and anatomy of developing rats. The rats raised in an "enriched" environment were found to have increased brain volume, increased brain weight, and increased total brain cholinestrase activity as compared to their littermates raised in "impoverished" environments. Drawing a conclusion which bears on the hypothesis being explored here, one could say that total protein in the brains of the "enriched" group was greater than the total protein in the "impoverished" group,

assuming that the difference in brain weight and volume between the groups reflected equally increased amounts of all the molecular species in the brain. Or more simply, general activation of the rats during their developmental period through use of an "enriched" environment resulted in increased amounts of brain protein as well as other constituents.

Perhaps the behavioral converse of general activation of the organism is sleep. One might predict that during sleep, metabolic processes would be slowed because of the inactivity of the of the organism. Studies thus far have failed to confirm this prediction, and furthermore, a recent study by Reich, Driver, and Karnovsky[47] has shown in rat brain a surprising increased uptake of inorganic phosphate into an, as yet, unidentified compound in the brain during sleep. Preliminary extraction and identification procedures indicate that this compound is most likely a lipoprotein of some kind. The increase in uptake of phosphate into this substance is dramatic -- two to three-fold with a sleep period of only thirty minutes.

In summary, the effects of general behavioral activation on protein metabolism of the brain remains ambiguous, as do the effects of convulsive stimulation and discreet stimulation of known neural pathways reviewed previously.

(5)

In review, the following points have been made thus far:

1. In search for biochemical events underlying the learning process, it has been difficult to design experiments in which changes in brain biochemistry accompanying learning could not be attributable to simple neural activation instead.

2. Thus one might more profitably ask what are the changes which accompany simple neural activation apart from learning.

3. Protein seems to be a reasonable candidate for a substance which might undergo changes during neural activation.

4. However, as yet, the literature is inconclusive as to whether or not protein metabolism is actually affected by neural activation.

(6)

The Experiment

The experiment to be described is designed to render judgment on the following hypothesis. Discrete electrical stimulation of the forepaw of the cat will differentially increase protein metabolism in the cortical areas which are known to be the projection sites for the stimulation employed.

A general outline of the operations involved is as follows: An anesthetized cat is injected intracisternally with C^{14}-valine. Valine is chosen for several reasons. It is regularly found in most proteins. It is normally found in the cerebrospinal fluid. It is an essential amino acid and enters into no specialized metabolism as does glutamic acid, for example. However, other amino acids could have been chosen using similar criteria. Immediately after the injection, electrical stimulation to one or the other forepaw is begun, by way of a Grass stimulator connected to electrodes which are placed into the skin pad of the forepaw.

After the period of stimulation, the animal is sacrificed, the skull cap removed, and small blocks of tissue are excised from mirror image sites on both sides of the cortex which are known to be areas of evoked potential response for the

stimulation point employed. In other words, the forepaw region of S-I (the primary sensory cortex) then entire S-II region (the auxilliary sensory cortex), and the forepaw region of M-I (the primary motor cortex) are sampled. In addition, other areas of the cortex are sampled which are assumed to have no activation or bilaterally equal activation from forepaw stimulation (see Figure 1). These samples are from the hindpaw sensory, the hindpaw motor, the auditory and the suprasylvian "association" areas.

After the sample blocks of tissue are removed, they are quickly weighed and then homogenized. The protein is precipitated with trichloroacetic acid (TCA) and then purified by a number of extraction procedures. The concentration of the protein is determined and the radioactivity is measured by assay in a liquid scintilation spectrometer. By knowing the concentration (in mg. of protein per cc.) and the radioactivity (in counts per minute per mg.), one can then calculate the specific activity of the protein by dividing the radioactivity by the concentration. The units for specific activity are counts per minute per milligram of protein.

Specific activities can then by compared between the two sides of the cortex. If the specific activity of one side is greater than the other, one can conclude with reasonable confidence, that this difference reflects a difference in rates of metabolism.

FIGURE I

Cortical Sample Areas.

A. Primary forepaw sensory area (S-I)

B. Primary hindpaw sensory area (S-I)

C. Primary forepaw motor area (M-I)

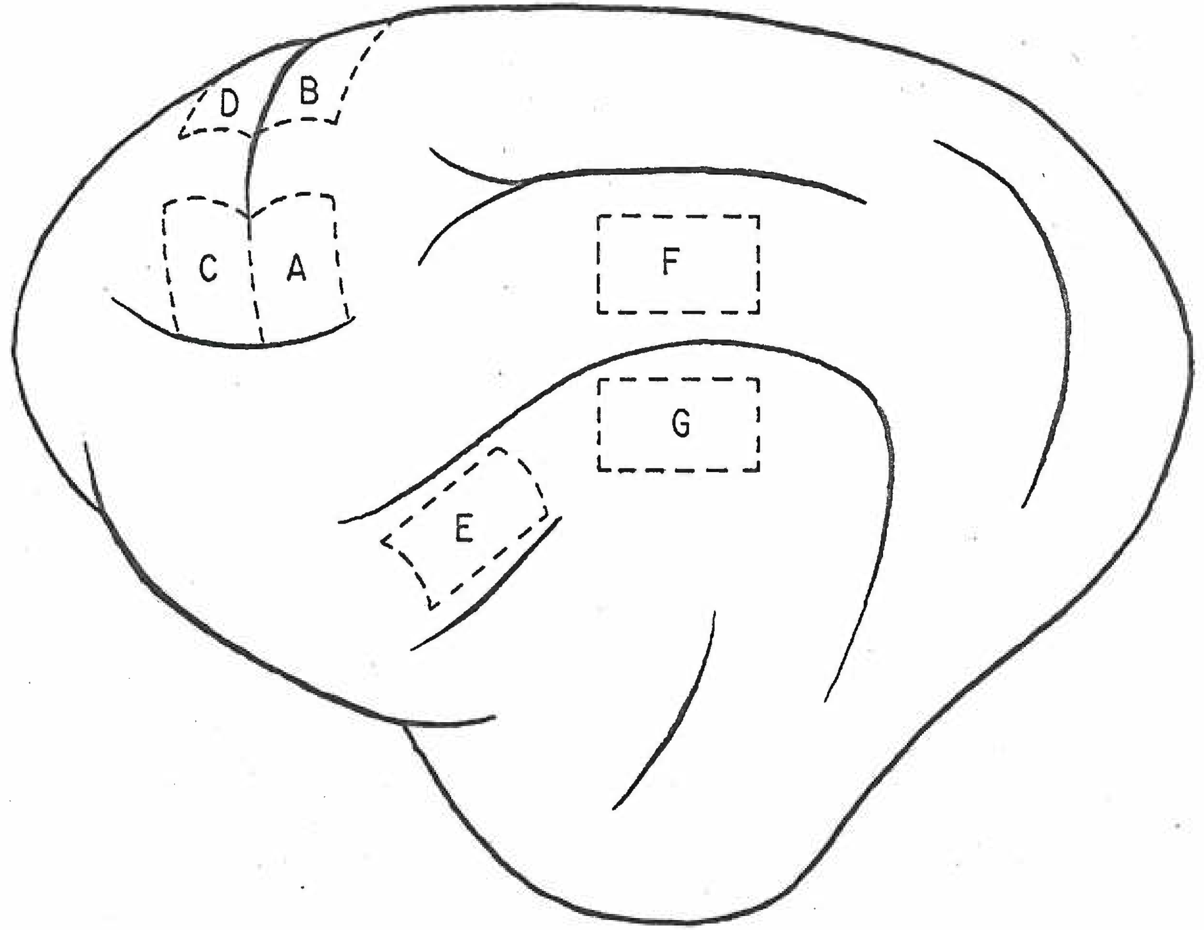
D. Primary hindpaw motor area (M-I)

E. Auxillary sensory area (S-II)

F. Syprasylvian "association" area

G. Primary auditory area

CORTICAL SAMPLE AREAS



The prediction is made that the contralateral specific activity will be greater than the ipsilateral specific activity for the forepaw sensory region of S-I since it is known that sensory impulses are transmitted almost exclusively to the contralateral side, thus activating it differentially. In addition, it is predicted that the forepaw region of M-I and the region S-II also might show contralateral-ipsilateral differences in specific activity because they too have projections from the contralateral forepaw.

On the other hand, it is predicted that no ipsilateral-contralateral differences will be found for the hindpaw sensory, the hindpaw motor, and the auditory cortical projection areas since they are not receiving areas for the stimulation employed. In addition, it is predicted that no ipsilateral-contralateral differences will be found for the suprasylvian "association" area since forepaw stimulation is known to be projected bilaterally to this area in equal amounts.

In addition, data on the time course of C^{14}-valine uptake into the cortex as well as its disappearance from the cerebrospinal fluid after injection are obtained.

II

METHODS AND MATERIALS

Animals

Twelve mongrel cats averaging 3.6 Kg. (range 2.2 to 5.0 Kg.) were obtained from the University of Oregon Animal Care Department. Immature cats were excluded on the presumption that their protein metabolism might be different from adult cats.

The first nine cats were anesthetized with 40 mg. per Kg. of Nembutal; the last three with 70 mg. per Kg. of Chloralose. Anesthesia was administered intraperitoneally. Depth of anesthesia was maintained at the point at which no retraction resulted when the hindpaw was pinched briefly. To maintain this level, small doses of anesthetic were occasionally administered during the course of the experiment. In addition, the first three cats received 0.1 cc. of atropine sulfate to reduce trachobronchial secretions, but this was discontinued because of possible central effects.

Surgery

As soon as the animal was unresponsive, an endotracheal tube was inserted by way of a midline anterior neck exposure. A midline incision was then made over the posterior cranium and posterior aspect of the neck and the muscle attachments

of the occipital bone were severed. A small hole was then drilled in the lip of the occipital bone just above the attachment of the tectorial membrane, thus providing access to the cisterna magna. Upon completion of this drilling operation, cerebrospinal fluid (C. S. F.) was seen to flow freely from this hole. Immediately a small polyethylene tube, the same diameter as the hole, was inserted and attached to a syringe, whereby samples of C. S. F. could be withdrawn and isotope injected. The junction between the tube and the bone was then sealed with dental cement. Blood loss from these procedures was slight.

Isotope Injection

Uniformly labeled C^{14}-valine with a specific activity of 190 microcuries per millimole was obtained from International Chemical and Nuclear Corporation on one occasion and from New England Nuclear on another. The dilution was such that 1.0 cc. contained 100 microcuries. The first nine animals received 10 microcuries each and the last three, 50 microcuries each.

The injection procedure involved first withdrawing about one cc. of C. S. F. from the cisterna, of which 0.2 cc. was set aside for use as a blank in the determination of C. S. F. specific activity. The remaining C. S. F. was mixed with the volume of isotope to be injected so that after the injection of isotope was accomplished, the volume of C. S. F. within the cranium was essentially unchanged by the procedure. The polyethylene catheter was occluded but left in place so that samples of C. S. F. could be withdrawn at intervals during the experiment.

Directly after the injection of isotope was accomplished, the stimulation began.

Stimulation Procedures

Electrical stimulation was varied between right and left forepaw for each experiment so that sidedness effects would be randomized. The stimulating electrodes were placed in the footpads of the paw to be stimulated.

A variety of stimulation equipment was used in the first nine experiments in search of the combination that would be the most effective. These stimulation parameters are included in the appendix. For the last three experiments, a Grass stimulator was employed, using the following stimulation parameters: 100 msec. pulse width, five pulses per second, and voltage varying from 10 to 50 volts. The selected pulse frequency of five pulses per second was based on the fact that at this frequency evoked potentials in primary sensory areas show almost full amplitude during long trains of pulses. Higher frequencies result in rather sharp diminution of evoked potential amplitude.[56] A grossly evident muscle twitch was noted on each pulse with the stated level of stimulation.

Sampling Procedures

During the course of stimulation, samples of C. S. F. were obtained from some of the animals by withdrawing a small aliquot (about 0. 3 cc.) through the plastic catheter indwelling in the cisterna magna, at intervals of 30, 60, 120 and 240 minutes after the injection of the isotope. From these samples, measured volumes, usually 0. 1 cc., were transferred to scintillation vials for radioactivity assay.

After the stimulation period had ended, each animal was sacrificed by an injection of 1. 0 cc. of Nembutal into the brain stem. Then the overlying skin and

fascia were removed from the cranial vault and it was opened with trephine and rongeurs, exposing the brain as widely as possible. After the dura mater was removed, samples of cortex were carefully dissected from the areas previously mentioned (see Figure 1) by the use of No. 11 surgical blade and a small dental spatula. Care was taken to make sure that the depth of the excised sample was at the level of the junction of the gray and white matter and no deeper. In addition, care was taken to make sure that samples from mirror image sites were as analogous as possible with regard to amount of tissue excised and with regard to the conformation of the excised area.

After the excised samples were quickly cleaned of visible clots and adherent pia mater they were weighed on a Roller-Smith balance to within a tenth of a milligram accuracy and were then transferred to a Potter-Elvehjem tube where they were homogenized with 1.0 cc. of distilled water.

Purification of Protein

After the cortical samples were thoroughly homogenized, they were transferred to other tubes where purification procedures were carried out. First, one cc. of 20% TCA (trichloroacetic acid) was added to each sample of homogenate. This resulted in a dense precipitate. About ten minutes were allowed for complete precipitation to occur. The tubes were then centrifuged for ten minutes at 2,000 r.p.m. The supernatant was then decanted.

The TCA insoluble precipitate (containing the protein fraction) was then washed twice with 2.0 cc. of ethyl ether to remove any lipids. The precipitate was treated with 6% TCA at 90 degrees centigrade for twenty minutes to remove any

nucleic acids. After centrifugation the precipitate was resuspended in cold 6% TCA, centrifuged again, and the supernatant disgarded. The precipitate was then solubilized in 1.0 cc. of 1.0 normal NaOH. In most cases the solubilization was enhanced by heating the samples to 90 degrees centigrade for one to two hours. This solution of protein in 1.0 N NaOH was used for both the assay of radioactivity and the determination of protein concentration.

Protein Assay Procedure

Protein concentration was determined by a modification of the Lowry technique[39] which is based on developing a color by reaction with the phenolic groups of tyrosine and phenylalinine within the protein.

Reagents:
A. 2% Na_2CO_3 in 0.10 N NaOH.
B. 0.5% $CuSO_4 \cdot 5 H_2O$ in 1% Na and K tartrate.
C. Mix 50 cc. of A with 1.0 cc. of B.
D. Folin-Ciocalteu Reagent (1 part to 2 parts water)

All reagents were analytical grade.

Aliquots of the purified protein solution were diluted, usually 5:1, so that the color developed would be in optimal range for spectophotometric resolution.

A 0.1 cc. aliquot of the diluted protein solution was transferred to a tube and 3.0 cc. of reagent "C" was added. The mixture was allowed to react for ten minutes; then 0.2 cc. of reagent "D" was blown in quickly and the tube shaken immediately. The color was developed for twenty minutes and then read on a Beckman spectrophotometer at 600 millimicrons. The protein concentration was calculated employing a standard curve constructed by applying the same procedures to solutions of purified bovine albumin of known concentration.

Radioactivity Determination

Radioactivity was determined by the method of liquid scintillation. The scintillation solution contained two parts toluene to one part absolute ethanol, 0.4% PPO (2,5-diphenyloxazole) and 0.1% POPOP (1,4-bis-2-(5-phenyloxasoyl)). 0.2 cc. of purified protein solution was transferred to a counting vial and 19.0 cc. of scintillation solution was added. For determination of radioactivity in the C.S.F., 0.1 cc. aliquots were added directly to the scintillation vials, followed by 19.0 cc. of scintillation solution. After the scintillation vials were prepared they were counted on a Packard Tri-Carb spectrometer for as long as it took to count at least 1,000 counts above background. This assured a count-recount variability of no more than 5%.

Control procedures employed in the counting procedure include the use of duplicate samples for all of the last three experiments and for some of the others; the assay of a scintillation blank to control for contamination of the scintillation solution; the assay of a reagent blank to control for contamination of the reagents; and the assay of a standard solution of known radioactivity in order to calculate the efficiency of the counting procedure. Counting efficiency was calculated to be 58% which is in good agreement with the usual efficiency for this procedure.

Quenching effects were calculated by adding a known amount of radioactivity to each vial after it was counted and also to a blank after it was counted. All the vials were then recounted to see if the sample tubes were the same as the blank when the previously counted radioactivity was subtracted. By this means it was found that with the small aliquots of protein solution counted (usually 0.2 cc.), there was virtually no quenching effect.

III

RESULTS

A total of twelve complete experiments were performed. At the end of the first nine experiments, it became clear that there was too much variability in the results and in the technique used to draw any strong conclusions about the proposed hypothesis, although several of the experiments were very suggestive. The variability of these first nine experiments may tentatively be attributed to several factors: (1) Use of experimental techniques which had never been employed by the experimenter previously; for example, cannulation of the cisterna magna, (2) Inexperience on the part of the experimenter with the required chemical techniques, which led to laboratory handling errors, (3) The use of too low a dosage of C^{14}- valine (10 microcuries), resulting in radioactivity measurements which were barely above background, (4) Trial-and-error attempts to find optimal conditions to show the hypothesized effects: for example, attempting on several occasions to record evoked potentials from the cortex during the course of the experiment and on several other occasions, to use various different types of stimulators. These difficulties will be further discussed in Chapter IV. The pertinent data for all the experiments are nevertheless included in tabular form in the appendix; however, the first nine experiments will be thought of as "pilot experiments."

Time Course of Precursor Incorporation into Cortical Proteins

Although the first nine experiments produced data which were ambiguous with regard to the stated hypothesis, they did produce information on the time course of incorporation of C^{14}-valine into cortical proteins. Table 1. shows the pertinent data from six out of the first nine experiments. The decision to select these particular six experiments was made on the basis of the relatively low variability between samples which most of the selected experiments demonstrate. As can be seen from Table 1., there is a steady progressive increase in the amount of radioactivity incorporated into the protein fraction of brain cortex which is roughly proportional to the length of time from injection to sampling.

Figure 2 shows the time course relationship plotted as specific activity (counts per minute per milligram of protein) versus time (in hours). It appears from the graph that the rate of incorporation over the four hour period is almost constant, with a slight upswing at the four hour point. In addition it appears to be a monotonic increasing function, but since the time intervals are rather far apart, one cannot rule out a peak between two of the points.

Time Course of Disappearance of Precursor From C.S.F.

Figure 3 shows the time course of the disappearance of C^{14}-valine from the cerebrospinal fluid as assayed by determination of the concentration

TABLE 1.

SAMPLE POINTS USED TO DEMONSTRATE TIME COURSE OF INCORPORATION OF C^{14}-VALINE INTO CORTICAL PROTEIN

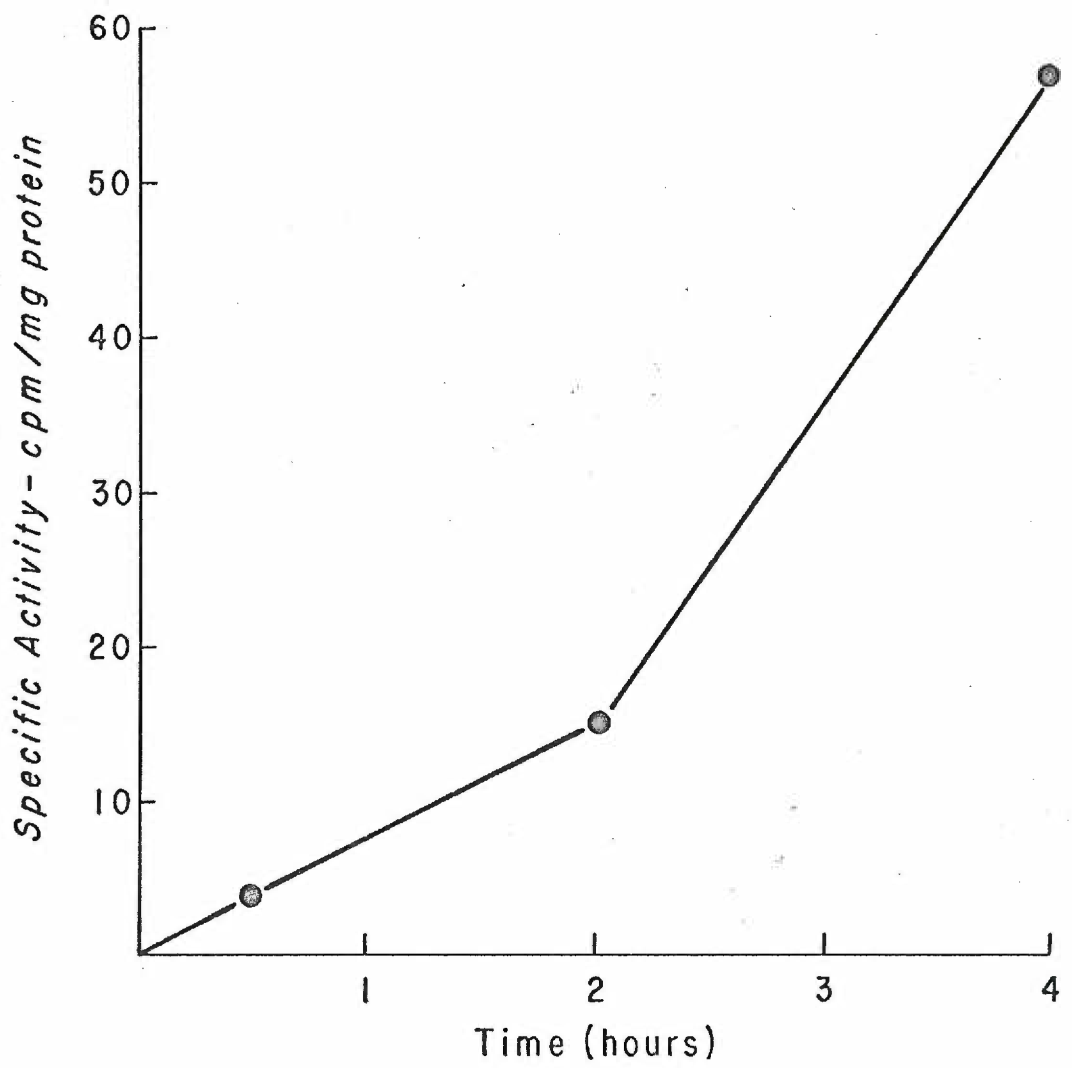
Duration of experiment [1]	Experiment code number	Mean specific activity[2]	Std. Dev.	Group Mean
I. 30 min.	67-96	4.3	2.2	4.3
II. 120 min.	67-58	13.9	2.9	
	67-63	11.0	2.1	
	67-72	25.7	8.2	
	67-83	11.1	4.6	15.4
III. 240 min.	67-105	57.3	4.4	57.3

1 Duration of time from injection of C^{14}-valine to sacrifice of animal.

2 Mean specific activity is calculated as counts per minute per milligram of protein in each sample area. The mean is derived from the fourteen samples taken from each animal.

Figure 2

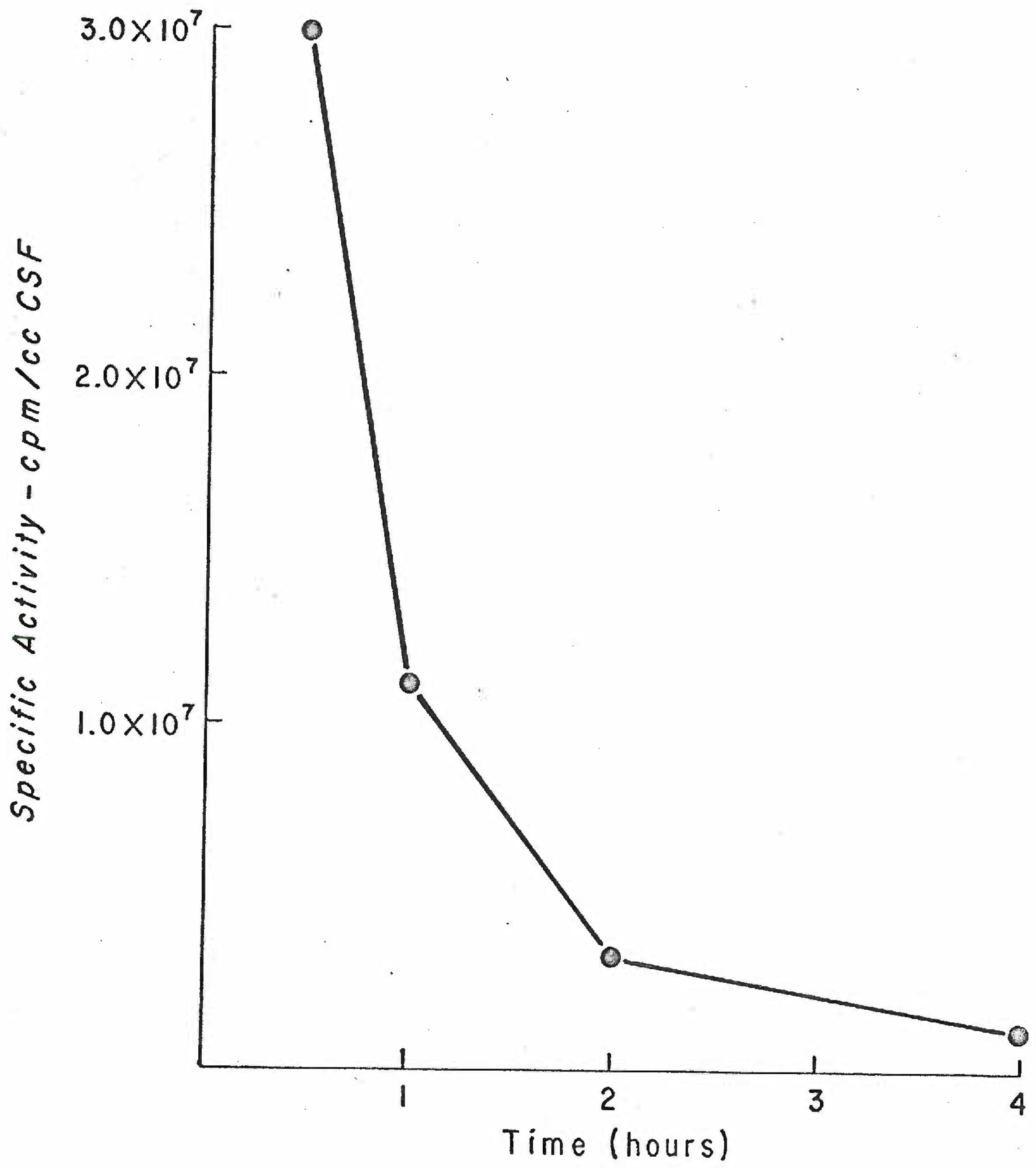
The time course of incorporation of C^{14}-valine into cortical protein is plotted as specific activity (counts per minute per milligram protein) versus time (hours). The data is also presented in Table 1.



Specific Activity - cpm/mg protein
60
50
40
30
20
10
1
2
3
4
Time (hours)

Figure 3

The time course of the disappearance of C^{14}-valine is plotted as the specific activity (counts per minute per cc. of cerebrospinal fluid) versus time (hours). The data was derived by taking samples of cerebrospinal fluid at various time intervals after a single dose of C^{14}-valine was delivered to the cerebrospinal fluid space by way of an indwelling plastic catheter.


3.0X10⁷
2.0X10⁷
1.0X10⁷
Specific Activity - cpm/cc CSF
1
2
3
4
Time (hours)

of radiolabeled substance in aliquots taken from the cisterna magna at various intervals of time, as described in Chapter II. The data presented is from one animal only. Curves from other animals in which this procedure was done had a similar appearance, but did not include sample points for all four hours.

The data shows a rapidly declining concentration of C^{14}-valine in the cerebrospinal fluid indicating its disappearance into other body compartments. By inspection, the curve shown in Figure 3 seems to be exponential; however, plotting it on semi-log or log-log co-ordinates does not result in a linear relationship.

Comparisons of Specific Activity Between Activated and Control Areas of the Cerebral Cortex

As was mentioned previously, the first nine experiments performed were unsatisfactory from a technical standpoint and were therefore inadequate to answer the proposed question developed in Chapter I; namely, will peripheral stimulation result in increased protein metabolism in the cortical areas activated by the stimulation?

The last three experiments performed, as outlined in Chapter II, made use of larger doses of C^{14}-valine, resulting in more reliable radioassays. Furthermore, they were all conducted for a period (from administration of precursor to sacrifice of the animal) of four hours, which resulted in adequate protein labeling. These two factors, plus the experience gained from

performing the first nine experiments, segregate these latter experiments as representing the "best effort" to show differences between activated and control cortical areas. Consequently the subsequent discussion pertains to these final three experiments only.

Table 2 and Figure 4 show results of the final three experiments. Mean specific activity for each contralateral cortical area is compared to mean specific activity for each ipsilateral cortical area and the difference between the two is tested using the one-tailed "t" test for repeated measures.[46] No significant differences between specific activity in the ipsilateral and contra-lateral samples are demonstrated when comparing each sample area individually using the 0.05 level of confidence. In general, the differences are in the direction predicted, for example, mean contralateral specific activity is higher than mean ipsilateral specific activity in the sensory forepaw region of the cortex. However, differences between the two sides are about as great in the auditory area with a "t" of comparable size.

In addition to the primary receiving area for the forepaw, which is located in the somatic sensory cortex, fibers are known to project to the forepaw motor region and to Area S-II. It can be seen that mean contralateral specific activities are greater than mean ipsilateral specific activities for each of these three areas, and in the motor forepaw area this difference approaches statistical significance even with the small "N" involved.

Since sensory fibers are known to project to three of the cortical areas,[12, 13, 24, 35, 41] the data were regrouped as in Table 3 and Figure 5.

TABLE 2

RESULTS OF "t" TESTS FOR DIFFERENCE SCORES BETWEEN CONTRALATERAL AND IPSILATERAL CORTICAL AREAS (FROM EXPERIMENTS I, II and III)

	Mean Specific Activity (Counts per minute per mg. protein)	"t"	Significance
1. Contralateral sensory forepaw	172		
2. Ipsilateral sensory forepaw	157	0.61	N.S.
3. Contralateral sensory hindpaw	164		
4. Ipsilateral sensory hindpaw	162	0.28	N.S.
5. Contralateral motor forepaw	190		
6. Ipsilateral motor forepaw	165	2.83	N.S.
7. Contralateral motor hindpaw	171		
8. Ipsilateral motor hindpaw	179	0.76	N.S.
9. Contralateral Area S-II	201		
10. Ipsilateral Area S-II	168	1.33	N.S.
11. Contralateral Suprasylvian	146		
12. Ipsilateral Suprasylvian	147	0.01	N.S.
13. Contralateral Auditory	177		
14. Ipsilateral Auditory	162	0.54	N.S.

* For "p" of 0.05 (one-tailed test), "t" must be greater than 2.92 (df=2). The "t" is calculated by use of the formula for repeated measures.

Figure 4

Each bar graph represents the mean specific activity of the protein fraction extracted from selected sample areas in the cerebral cortex. The mean specific activity is derived from averaging the specific activities of samples taken from corresponding cortical areas from three different cats. The sample numbers on the graph correspond to the following sample areas:

1. Contralateral sensory forepaw area.
2. Ipsilateral sensory forepaw area.

3. Contralateral sensory hindpaw area.
4. Ipsilateral sensory hindpaw area.

5. Contralateral motor forepaw area.
6. Ipsilateral motor forepaw area.

7. Contralateral motor hindpaw area.
8. Ipsilateral motor hindpaw area.

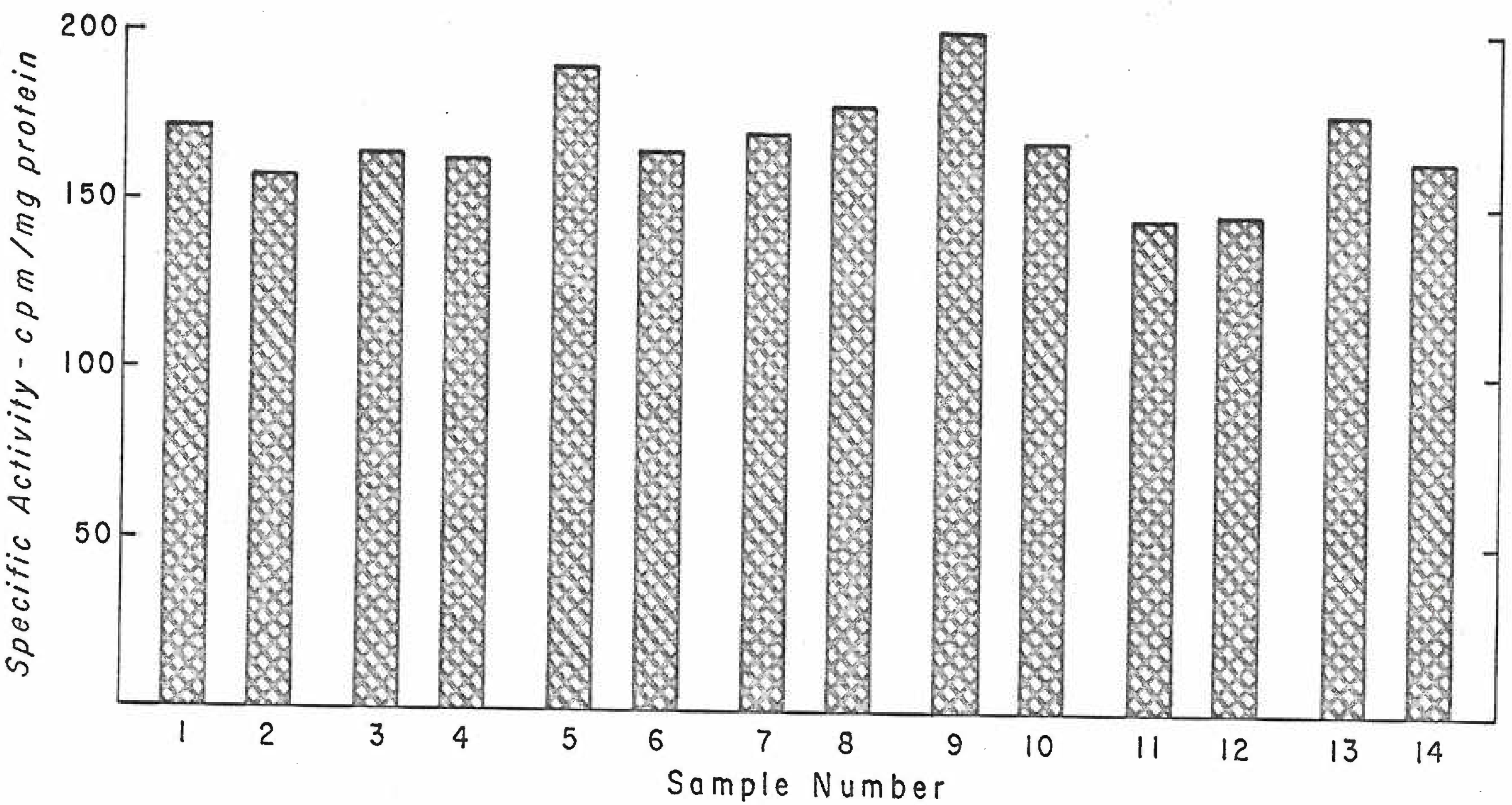
9. Contralateral area S-II.
10. Ipsilateral area S-II.

11. Contralateral suprasylvian area.
12. Ipsilateral suprasylvian area.

13. Contralateral auditory area.
14. Ipsilateral auditory area.

For a pictorial description of the above anatomical areas see Figure 1.

This data is also presented in Table 2, along with tests for statistical significance.



Specific Activity - cpm/mg protein
200
150
100
50
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Sample Number

Figure 5

The bar graphs labeled "forepaw projection" represent mean specific activities derived by grouping together sample areas with known projections from the forepaw and further grouping these as to whether they are contralateral or ipsilateral. The specific groupings are shown in Table 3, Part A.

The bar graphs labeled "no forepaw projection" represent mean specific activities derived by grouping together sample areas which have few, if any, projections from the forepaw, or have bilaterally equal projections from the forepaw, and further grouping these as to whether they are contralateral or ipsilateral. The specific groupings are shown in Table 3, Part B.

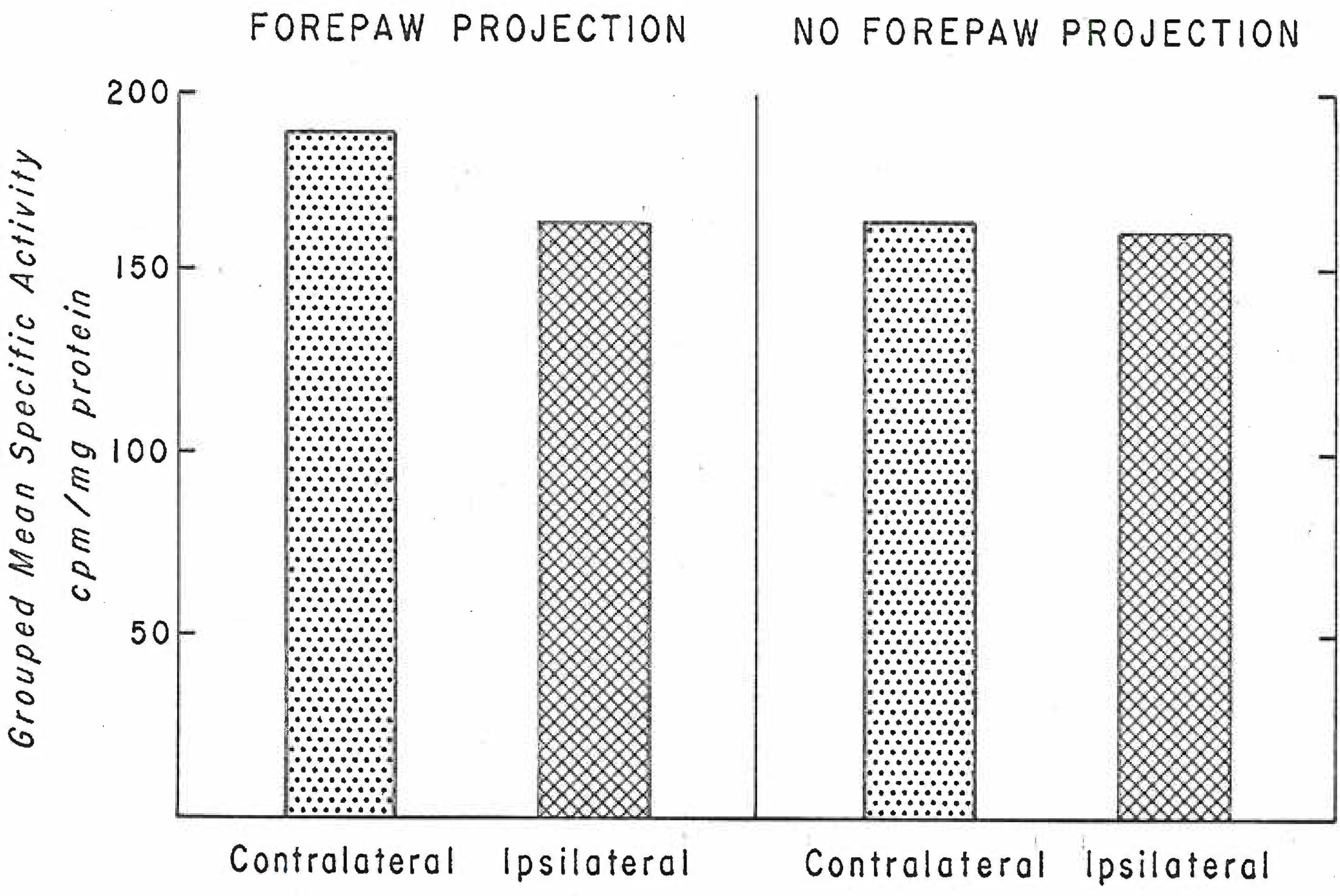

FOREPAW PROJECTION
NO FOREPAW PROJECTION
Grouped Mean Specific Activity
cpm/mg protein
200
150
100
50
Contralateral
Ipsilateral
Contralateral
Ipsilateral

TABLE 3 (Part A)

GROUPED DATA COMPARING DIFFERENTIALLY ACTIVATED SAMPLE GROUPS

Experiment Code Number	Sample Area	Specific Activity in C.P.M./mg. Protein	
		Contralateral	Ipsilateral
I	Sensory forepaw	204	146
	Motor forepaw	210	183
	Area S-II	172	161
		$\overline{X}_1 = 195.3$	$\overline{X}_1 = 163.3$
II	Sensory forepaw	109	124
	Motor forepaw	136	102
	Area S-II	181	76
		$\overline{X}_2 = 142.0$	$\overline{X}_2 = 100.7$
III	Sensory forepaw	202	202
	Motor forepaw	233	210
	Area S-II	251	266
		$\overline{X}_3 = 225.3$	$\overline{X}_3 = 226.0$
Group Mean		187.5	163.3

Different at $p = 0.10$
(one-tailed test; $df = 2$)

TABLE 3 (Part B)

GROUPED DATA COMPARING SAMPLE GROUPS WITH NO DIFFERENTIAL ACTIVATION

Experiment Code Number	Sample Area	Specific Activity in C.P.M./mg. Protein	
		Contralateral	Ipsilateral
I	Sensory hindpaw	145	133
	Motor hindpaw	166	159
	Suprasylvian	149	164
	Auditory	198	156
		$\overline{X}_1 = 164.5$	$\overline{X}_1 = 153.0$
II	Sensory hindpaw	118	123
	Motor hindpaw	111	117
	Suprasylvian	73	71
	Auditory	90	124
		$\overline{X}_2 = 98.0$	$\overline{X}_2 = 108.7$
III	Sensory hindpaw	228	230
	Motor hindpaw	237	260
	Suprasylvian	217	207
	Auditory	242	206
		$\overline{X}_3 = 231.0$	$\overline{X}_3 = 225.7$
Group Mean		164.5	162.4

That is, the contralateral specific activities for the primary sensory forepaw, primary motor forepaw and S-II regions are pooled for each animal and a mean value obtained. The three means are then combined producing a group mean for the three animals. (Table 3, Part A) A parallel grouping procedure is then employed for the corresponding ipsilateral regions (Table 3, Part A). The group means are then tested for a significant difference in specific activity between contralateral ("activated") and ipsilateral ("non-activated") cortical samples employing the "t" test for repeated measures. By regrouping the data as described, errors of measurement are reduced and the overall effect of stimulation is more accurately assessed. The results of this "t" test show the means to be different at approximately the 0.10 level of confidence. (One-tailed test; df = 2) This relatively high alpha level of 0.10 can be attributed tentatively to the small number of subjects employed.

The other areas of the cortex analyzed in this experiment were similarly grouped together but on the basis that they are thought to have few, if any, sensory projections from the forepaw, or, as in the case of the suprasylvian area, bilaterally equal projections from the forepaw. (Table 3, Part B) Means for each animal were obtained for ipsilateral and for contralateral data by pooling the four sampled areas. Group means for the three animals were derived in a fashion exactly like that for the previously discussed cortical areas (Table 3, Part A). This grouping procedure results in contralateral and ipsilateral group means which are 164.5 and 162.4 C.P.M. per mg. protein, respectively. This obviously is not a significant difference.

As demonstrated in Figure 5, one of the striking findings of the data is the similarity between the mean specific activities for all the control groupings, (ipsilateral "forepaw projection;" contra- and ipsilateral "no forepaw projection") each group mean ranging close to 164 CPM/mg. protein. Considering the variability within the groups, the surprising correspondence between these numbers may well be partially attributed to serendipity. However, a degree of correspondence between these three groups would be predicted from the hypothesis.

In summary, the results are as follows:

(1) The proteins of the cat cortex were shown to be metabolically active and to contain, within a short period of time, measurable amounts of radioactivity after an intracisternally administered dose of C^{14}-valine.

(2) When grouped together those cortical areas which are known to receive differentially more neural projections from the contralateral forepaw had a higher level of radioactive label concentration than corresponding areas on the ipsilateral side of the cortex which receive differentially fewer such projections. ($p = 0.10$)

(3) On the other hand, no apparent differences in radioactive label concentration were found between the ipsilateral and contralateral sides when comparisons were made within the group which was presumed to have few, if any, sensory projections from the forepaw, or bilaterally equal projections from the forepaw.

(4) When comparing each pair of homologous anatomical areas separately, differences in specific activity between contralateral and ipsilateral

sides of the cortex are generally in the direction predicted but fail to reach the 5% level of significance.

IV

DISCUSSION

The major hypothesis investigated in this project was: electrical stimulation of the forepaw of the cat will increase protein metabolism in those cortical areas which are known to be the projection sites for the mode and location of stimulation employed.

A total of twelve experiments were performed. In the first nine experiments an effort was made to solve procedural problems and to arrive at optimal experimental parameters to demonstrate the hypothesized relationship. Therefore, from the standpoint of rendering judgment on the stated hypothesis, these first nine experiments must be disqualified. The issues and problems raised in these first nine experiments are worthy of discussion, however, since the experimental conditions for the final three experiments were based on the preliminary findings of these first nine experiments.

I. The Pilot Experiments

1. The problem of optimal time parameters.

Information about the time course of incorporation of valine into the cortical proteins of the cat after injection into the cisterna magna is not

available from the current literature. The only reported investigation employing both valine as a precursor amino acid and the cisterna magna as a route of injection also employed the twenty-day-old rabbit as the experimental animal.[6] Nevertheless, this study of rabbit protein metabolism which found a peak incorporation time (from injection to sampling) of two hours, was utilized at the inception of the investigation presented in this thesis as the only available estimate of the peak incorporation time in the cat, even though the data had been derived from the immature rabbit.

Based on the data derived from the rabbit, time periods equal to or shorter than two hours (from injection to sampling) were selected in the early experiments so that sampling would presumably occur during the ascending slope of the incorporation function. It was desirable that sampling of the cortical proteins should occur before the peak of incorporation because if activation of the cortex did indeed stimulate protein metabolism, it would likely shift the peak of incorporation closer to the time of injection. Consequently, when comparing the two sides of the cortex, the peak of incorporation would occur sooner in the contralateral side of the cortex which was activated than in the ipsilateral side of the cortex which was not activated. Thus, if sampled on the ascending slope of the incorporation function, the contralateral cortex might show <u>more</u> incorporation than the ipsilateral cortex. But in contrast, if sampled on the descending slope of the incorporation function, the contralateral cortex might show <u>less</u> incorporation than the ipsilateral cortex. Clearly an ambiguous interpretation of the data

results unless one arbitrarily employs one portion of the curve. Consequently, an attempt was made to employ only the ascending portion of the incorporation function.

This desire to sample the cortex only during the ascending slope of the incorporation function was one of the factors which led to the selection of rather short time intervals between injection and sampling in the first nine experiments. Unfortunately, these short time periods (two hours or less) resulted in rather scanty labeling of cortical protein which, in turn, led to rather significant measurement errors.

In retrospect, it can be seen that the time course of incorporation of valine into cortical protein is quite different for the cat than for the immature rabbit. (See Figure 2) Instead of peaking at two hours, the curve seems to be rising steadily up to at least four hours although a peak between two and four hours cannot be absolutely ruled out.

In summary, inadequate prior information about the time course of incorporation of valine into cortical protein led to the selection of sub-optimal time parameters for significant labeling of cortical proteins, at least for the first nine experiments. In the last three experiments this was remedied by using a longer time interval from injection to sampling, i.e. four hours.

2. The problem of optimal precursor dosage.

The dose of radiolabeled valine employed in most of the first nine experiments was ten microcuries. This resulted in an average specific activity

in the proteins isolated from the cortex of about twice background. Theoretically less radioactivity than this can be assayed, but for practical routine assay procedures this is a rather small amount. The scanty labeling that occurred with this small dose may have contributed to some of the variability noted in the first nine experiments; consequently, the dose was increased by a factor of five in the final three experiments.

3. The problem of optimal stimulation.

A variety of stimulation techniques were used in the first nine experiments. These included continuous line current (60 cycles per second a.c.) fed through a variable voltage output transformer delivered directly to the paw; pulsed output line current (identical to the first method mentioned but with the addition of a relay which made and broke the circuit at frequencies of from three to five per second); and finally, a Grass physiological stimulator which could electronically vary the stimulation parameters. These various methods were tried in an effort to find optimal stimulus conditions, using as a definition of "optimal," that stimulation technique which resulted in the biggest difference in specific activity between the two sides of the cortex. The results of this trial-and-error resulted in no important differences between the techniques used. Theoretically it might be possible to decide which mode of stimulation activated the most neurons by monitoring the unit discharge "hash" in those cortical areas which were to be sampled; however, this refinement was not attempted.

4. The problem of surgical technique.

Delivery of the dose of C^{14}-valine to the cerebrospinal fluid space was initially a difficult problem. Simply puncturing the overlying membrane with a needle and injecting the valine, as was done in the first few experiments, resulted in leakage from the site of injection. This problem was resolved successfully by the use of a technique which was invented in the course of the subsequent experiments. The technique, described more specifically in Chapter II, involved drilling a tiny hole in the lip of the occipital bone through which a plastic catheter could be passed, thus allowing free access to and from the cerebrospinal fluid space. This assured absolutely quantitative delivery of valine to the cerebrospinal fluid space as well as mixing of the valine with the cerebrospinal fluid.

In excising the samples of cortical tissue, the aim was to sever the cortex precisely at the junction of white and grey matter. It was the subjective judgment of the experimenter that his skill improved in the course of the experiments, but no objective measurements were made as to how close the excisions were to the desired depth.

In summary, in the first nine experiments much effort was invested in trying to find optimal experimental conditions for demonstrating the proposed hypothesis. The specific methods used in each experiment are shown in tabular form in the appendix. The preliminary data and the refined methodology from these experiments were employed in the design and execution of the final three experiments. Thus, the final three experiments are considered the most reliable and are the only experiments used to render judgment on the proposed hypothesis.

II. The Final Three Experiments.

When the specific activities of ipsilateral and contralateral samples are compared for each individual cortical area, no significant differences were found at the 0.05 level of probability. (See Table 2 and Figure 4) This is not too surprising considering that the data is derived from only three animals. A very dramatic difference would be necessary to reach statistical significance with this small number of subjects.

As pointed out in Chapter III, sensory fibers from the forepaw project to at least three of the areas sampled, namely the somatic sensory forepaw area,[13, 41] the somatic motor forepaw area,[24, 35] and the S-II region.[12, 13] These three regions were grouped as described in Chapter III, and the contralateral and ipsilateral specific activites then compared. The means for these groupings are different at the 0.10 level of confidence. (See Table 3 and Figure 5)

It is clear that the validity of this grouping procedure depends not only on the fact that cortical areas mentioned above receive fibers from the forepaw, but also that these projections from the forepaw are differentially distributed to the contralateral side. In the case of the somatic sensory areas, there is little question that the majority of the input is contralateral.[13, 41] Under chloralose anesthesia small ipsilateral evoked responses can be seen, but these are very small compared to the size of the contralateral evoked responses. * As for the somatic motor area, the evoked responses have again been shown to be almost

* Thompson, R.F.: Personal Communication. March 21, 1968

exclusively contralateral.[24, 35] On the other hand, Area S-II has been shown to receive both ipsilateral and contralateral projections when studied by the method of gross evoked potential.[13] However, when single cells were studied, it was found that only a very few responded to ipsilateral stimulation; the majority were responsive to contralateral stimulation only.[12]

In conclusion, there is little doubt that the primary somatic sensory and primary somatic motor areas receive differentially more input from the contralateral side. In addition, there is supporting evidence that sensory projection from the forepaw to S-II is mainly contralateral. However, it is recognized that previous studies are not in agreement on this point. In summary, the three sample areas which receive differentially greater sensory input to the contr-lateral cortex are shown to be different at the 0.10 level of confidence when the grouped contralateral and grouped ipsilateral specific activities from these areas are compared.

Four of the cortical sample areas, the somatic sensory hindpaw area, the somatic motor hindpaw area, the primary auditory area, and the suprasylvian area, were grouped together on the basis that they received few, if any, projections from the contralateral forepaw or in the case of the suprasylvian area, received exactly bilaterally equal input from the forepaw.[55] (Table 3; part B)

For the sensory and motor areas, the validity of this grouping depends on the discreteness of the somatotopic organization of these areas. In other words, it presumes that only the forepaw area was activated while the hindpaw area, only a few millimeters away, remained at baseline activity. Studies on the primary somatic sensory area have shown that the somatotopic organization of evoked

responses is quite discrete and reproducible.[13, 41] When the primary somatic motor area was studied for evoked responses, a "map" of somatotopic organization similar to the "map" for the primary somatic sensory cortex was obtained.[40]

However, when single cells in the somatic motor area were studied, it was found that most of the cells were polysensory, i.e., they responded to visual, auditory, and somatic sensory stimulation.[10] Furthermore, these cells were not found to be place specific but were responsive to tactile stimulation from many points on the skin. A smaller number of cells were modality specific but were not place specific. In fact, no place specific cells could be found anterior to the central suclus. Thus, once again, there is some disparity between the findings of gross evoked potentials and single cell analysis.

Be that as it may, the second group of cortical areas which was not differentially activated by stimulation to the contralateral forepaw was found to have very similar contralateral and ipsilateral grouped mean specific activities. (See Table 3, part B and Figure 5) In addition, the means were very similar in magnitude to the mean for the ipsilateral grouping in Table 3, part A. Thus all three control areas have closely corresponding mean specific activities all ranging around 164 CPM/mg. protein.

In conclusion, the study presented in this thesis lends support to the proposed hypothesis that cortical areas which are activated by peripheral stimulation have a differentially greater uptake of C^{14}-valine, and, by inference, these activated cortical areas contain protein which is metabolically more active than protein in non-activated areas.

III. Comparisons to Previous Studies.

The experiment presented in this thesis has employed a procedure for investigating the effects of peripheral stimulation on cortical metabolism which is different from any of the previous studies reviewed in Chapter I. In general, it can be said that this study adds corroboration to those studies previously mentioned which have found a positive relationship between neutral activity in the cortex and increased protein metabolism.

From the viewpoint of the experimenter, this study has two main advantages:

1. Comparisons were made between cortical areas within the same animal, thus minimizing the spurious effects of differences in anesthesia level, blood pressure, adequacy of respiration, nutritional state or innate idiosyncracies in uptake and metabolism of valine between individual animals. In this respect, the study presented in this thesis is analogous to those studies reported by Hyden and Egyhazi;[33] Metzger, et al.;[42] and Geiger, et al.[26]

2. A second strength of the experiment is that the cortical areas which were assayed were activated neither by electroconvulsive shock nor by convulsive inducing drugs; rather, they were activated by incoming neutral impulses. Consequently, one cannot attribute the changes which occurred to "drug effects" or to nerve discharge that was initiated by stimulation other than synaptic transmission. Thus the experiments of Gaitonde and Richter;[23] Dingman, et al.;[16] Geiger, et al.;[26] and Baily and Heald[4] stand in contrast to the experiment presented in this thesis because of their use of electroconvulsive stimulation and of convulsion inducing drugs.

Speculation

The most inviting explanation for increased uptake of valine into activated cortical areas is to infer that these areas were areas of increased protein metabolism. One might hypothesize that the enzymes involved in transmitter synthesis and release are manufactured at an increased pace consonant with the increased frequency of synaptic transmission in the activated cortex. Perhaps additional enzymes are induced to cope with the increased functional demand for transmitter synthesis.

An alternative explanation for the increased turnover of protein might be that with the increased energy demands of the neurons brought about by the increased frequency of firing, protein is pressed into the service of energy metabolism by way of conversion to sugar, i.e., there is increased gluconeogenesis. However brain tissue is unique in the fact that it meets almost all of its energy demands through oxidation of glucose to the exclusion of other metabolites. Therefore it seems unlikely that gluconeogenesis would account for this change in protein metabolism.[19]

A further alternative explanation for the increased labeling of protein in the activated areas of the cortex is to hypothesize that this process is secondary to changes in blood flow rates, as is suggested by the experiments of Sokoloff,[53] or by changes in blood brain barrier, as is suggested by the experiments of Lorenzo.[38] In other words, one could argue that no particular function can be assigned to the altered uptake of valine; rather, this alteration occurs as a secondary process.

Further Studies

Several experiments are suggested by the results of this study. Having investigated the functional state of proteins in the cortex under the influence of activation, one might ask more specifically whether there are any particular protein species which are especially active metabolically during increased neural activity as measured by the technique of amino acid tracer uptake. In addition, it might be profitable to study the changes in activity of enzymes (such as choline acetylase or cholinesterase) thought to be involved in synaptic transmission, hypothesizing that these enzymes might be significantly altered by cortical activation of the type described in this study.

V

SUMMARY

Hypothesis

An experiment was performed to tender judgment on the following hypothesis: Discreet electrical stimulation of the forepaw of the cat will differentially increase the protein metabolism in the cerebral cortical areas which are known to be the projection sites for the stimulation employed.

Procedures

In each experiment an anesthetized cat was injected intracisternally with C^{14}-valine and electrical stimulation to one of the forepaws was begun immediately thereafter. After a stimulation period of four hours (for the main experimental group), the cat was sacrificed, the skull cap was removed, and small blocks of tissue were excised from mirror image sites on both sides of the cortex, which were known to be areas of sensory projection for stimulation delivered to the contralateral forepaw. In addition, samples were excised from other mirror-image cortical sites assumed to have few, if any, projections from the forepaw, or exactly equal bilateral projections from the forepaw.

After the samples of cortical tissue were excised, the protein fraction from each was isolated, purified, and assayed for specific activity (counts of radioactivity per minute per milligram of protein).

Results and Conclusions

(1) As a group, cortical areas known to receive neural projections preferentially from the contralateral (stimulated) forepaw has a larger specific activity than the group of homologous control areas taken from the opposite side of the cortex. The difference was significant at the 0.10 level of confidence. This difference was interpreted as support for the hypothesis stated above.

(2) No differences in specific activity were found when comparing ipsilateral and contralateral sample groups of those areas known to receive few, if any projections from the forepaw, or bilaterally equal projections from the forepaw.

This finding of "no difference" was attributed to the fact that neither side of the cortex was preferentially activated in the case of these sample groups, and thus, this finding is consistent with the hypothesis.

(3) When each sample area was compared individually the differences were in the direction predicted but did not reach the 0.05 level of probability.

This lack of difference was tentatively attributed to the small number of subjects (N=3) which were used in comparing individual cortical areas.

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APPENDIX

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREAS	C. P. M. PER mg. PROTEIN
67-53	Variac transformer output to right forepaw.	2 Hours	5 C. in 1.0 cc. volume.	1 Contralateral Forepaw Sensory area	37.7
				2 Ipsilateral Forepaw Sensory area	18.1
				3. Contralateral Hindpaw Sensory area	no data
				4 Ipsilateral Hindpaw Sensory area	no data
	Adjusted to maximize forepaw contraction.		Percu-taneous needle delivery to cisterna magna.	5 Contralateral Forepaw Motor area	18.2
				6 Ipsilateral Forepaw Motor area	22.1
				7 Contralateral Sensory head area	18.2
				8 Ipsilateral Sensory head area	24.3
					$\overline{X}$=27.7
					S. D.= 8.38

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREAS	C.P.M. PER mg. PROTEIN
67-58	Variac transformer output to right forepaw.	2 Hours	5 C. in 1.0 cc. volume.	1 Contralateral Forepaw Sensory area	10.4
				2 Ipsilateral Forepaw Sensory area	14.2
				3 Contralateral Hindpaw Sensory area	15.4
				4 Ipsilateral Hindpaw Sensory area	8.1
	Adjusted to maximize forepaw contraction.		Delivery through exposed tectorial membrane	5 Contralateral Forepaw Motor area	15.2
				6 Ipsilateral Forepaw Motor area	16.4
				7 Contralateral Hindpaw Motor area	12.7
				8 Ipsilateral Hindpaw Motor area	14.4
				9 Contralateral Suprasylvian area	20.0
				10 Ipsilateral Suprasylvian area	12.0
				11 Contralateral Visual area	14.2
				12 Ipsilateral Visual area	14.0
					$\overline{X}$=13.9
					S. D.= 2.9

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREAS	C. P. M. PER mg. PROTEIN
67-63	Variac transformer output to right forepaw.	2 Hours	5 C. in 1.0 cc. volume.	1 Contralateral Forepaw Sensory area	10.5
				2 Ipsilateral Forepaw Sensory area	8.9
				3 Contralateral Hindpaw Sensory area	10.1
				4 Ipsilateral Hindpaw Sensory area	10.1
	Adjusted to maximize forepaw contraction.			5 Contralateral Forepaw Motor area	15.0
				6 Ipsilateral Forepaw Motor area	13.1
				7 Contralateral Hindpaw Motor area	11.4
				8 Ipsilateral Hindpaw Motor area	8.2
				9 Contralateral S-II area	13.8
				10 Ipsilateral S-II area	9.4
					$\overline{X}$=11.0
					S. D.= 2.12

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREAS	C. P. M. PER mg. PROTEIN
67-72	Output of	2 Hours	10 C.	1 Contralateral Forepaw Sensory area	19.8
	R. F. T.'s		2.0 cc.	2 Ipsilateral Forepaw Sensory area	22.7
	Pulse Power		volume.	3 Contralateral Hindpaw Sensory area	22.9
	Amplifier.			4 Ipsilateral Hindpaw Sensory area	18.5
			Delivered	5 Contralateral Forepaw Motor area	26.5
	To right		through	6 Ipsilateral Forepaw Motor area	18.8
	forepaw.		plastic	7 Contralateral Hindpaw Motor area	45.4
			catheter.	8 Ipsilateral Hindpaw Motor area	43.2
				9 Contralateral S-II area	23.4
				10 Ipsilateral S-II area	20.1
				11 Contralateral Suprasylvian area	23.6
				12 Ipsilateral Suprasylvian area	24.7
				13 Contralateral Visual area	no data
				14 Ipsilateral Visual area	24.8
					$\bar{X} = 25.7$
					S. D. = 8.2

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREA	C. P. M. PER mg. PROTEIN
67-83	Same as before, but then switched midway to 50 v. output of Tectronix 161 Pulse Generator to right forepaw.	2 Hours	10 C. 2 cc. volume through plastic catheter.	1 Contralateral Forepaw Sensory area	5.3
				2 Ipsilateral Forepaw Sensory area	6.7
				3 Contralateral Hindpaw Sensory area	8.6
				4 Ipsilateral Hindpaw Sensory area	9.1
				5 Contralateral Forepaw Motor area	12.8
				6 Ipsilateral Forepaw Motor area	17.5
				7 Contralateral Hindpaw Motor area	7.5
				8 Ipsilateral Hindpaw Motor area	7.1
				9 Contralateral S-II area	7.6
				10 Ipsilateral S-II area	14.7
				11 Contralateral Suprasylvian area	11.1
				12 Ipsilateral Suprasylvian area	9.4
				13 Contralateral Visual area	20.5
				14 Ipsilateral Visual area	18.0
					$\overline{X} = 11.1$
					S. D. = 4.6

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREA	C. P. M. PER mg. PROTEIN
67-93	Output of Variac Transformer. Frequency of two pulses per second. Pulse duration of 40 msec. to left forepaw.	70 min.	10 C. 0.1 cc. volume through plastic catheter.	1 Contralateral Forepaw Sensory area	10.4
				2 Ipsilateral Forepaw Sensory area	7.2
				3 Contralateral Hindpaw Sensory area	104.9
				4 Ipsilateral Hindpaw Sensory area	44.7
				5 Contralateral Forepaw Motor area	14.8
				6 Ipsilateral Forepaw Motor area	10.6
				7 Contralateral Hindpaw Motor area	28.0
				8 Ipsilateral Hindpaw Motor area	28.6
				9 Contralateral S-II area	568.6
				10 Ipsilateral S-II area	30.0
				11 Contralateral Suprasylvian area	50.7
				12 Ipsilateral Suprasylvian area	24.8
				13 Contralateral Visual area	29.7
				14 Ipsilateral Visual area	220.5
					$\bar{X}$=not calculated S. D.= not calculated

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREA	C. P. M. PER mg. PROTEIN
67-96	Output of Variac Transformer. Frequency of five pulses per second. Pulse duration of 20 msec. to left forepaw.	30 minutes	10 C. 0.1 cc. volume through plastic catheter.	1 Contralateral Forepaw Sensory area	1.5
				2 Ipsilateral Forepaw Sensory area	1.1
				3 Contralateral Hindpaw Sensory area	6.3
				4 Ipsilateral Hindpaw Sensory area	6.5
				5 Contralateral Forepaw Motor area	8.6
				6 Ipsilateral Forepaw Motor area	2.9
				7 Contralateral Hindpaw Motor area	6.0
				8 Ipsilateral Hindpaw Motor area	3.3
				9 Contralateral S-II area	4.3
				10 Ipsilateral S-II area	2.8
				11 Contralateral Suprasylvian area	2.2
				12 Ipsilateral Suprasylvian area	3.0
				13 Contralateral Auditory area	2.1
				14 Ipsilateral Auditory area	1.2
					$\overline{X}=4.3$
					S. D. = 2.2

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREA	C. P. M. PER mg. PROTEIN
67-105	Output of Variac Transformer. Continuous stimulation to right forepaw.	4 Hours	10 C. 0.1 cc. volume through plastic catheter.	1 Contralateral Forepaw Sensory area	59.8
				2 Ipsilateral Forepaw Sensory area	50.3
				3 Contralateral Hindpaw Sensory area	51.2
				4 Ipsilateral Hindpaw Sensory area	56.3
				5 Contralateral Forepaw Motor area	57.8
				6 Ipsilateral Forepaw Motor area	62.8
				7 Contralateral Hindpaw Motor area	53.5
				8 Ipsilateral Hindpaw Motor area	63.0
				9 Contralateral S-II area	52.4
				10 Ipsilateral S-II area	56.3
				11 Contralateral Suprasylvian area	56.5
				12 Ipsilateral Suprasylvian area	55.3
				13 Contralateral Auditory area	63.9
				14 Ipsilateral Auditory area	62.8
					$\overline{X}$= 57.3
					S. D.= 4.4

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREA	C. P. M. PER mg. PROTEIN
67-110	Output of Variac Transformer. Continuous stimulation to right forepaw.	4 Hours	10 ·C. 0.1 cc. volume through plastic catheter	1 Contralateral Forepaw Sensory area	16.1
				2 Ipsilateral Forepaw Sensory area	17.6
				3 Contralateral Hindpaw Sensory area	29.6
				4 Ipsilateral Hindpaw Sensory area	37.5
				5 Contralateral Forepaw Motor area	25.0
				6 Ipsilateral Forepaw Motor area	18.5
				7 Contralateral Hindpaw Motor area	1050.8
				8 Ipsilateral Hindpaw Motor area	24.6
				9 Contralateral S-II area	30.5
				10 Ipsilateral S-II area	72.4
				11 Contralateral Suprasylvian area	lost
				12 Ipsilateral Suprasylvian area	lost
				13 Contralateral Auditory area	lost
				14 Ipsilateral Auditory area	lost
					$\overline{X}$=not calculated S. D.=not calculated

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREA	C. P. M. PER mg. PROTEIN
L. G. E I	Grass Stimulator. Five pulses per second. 100 msec. pulse width to left forepaw.	4 Hours	50 C. 0.5 cc. volume through plastic catheter.	1 Contralateral Forepaw Sensory area	204
				2 Ipsilateral Forepaw Sensory area	146
				3 Contralateral Hindpaw Sensory area	145
				4 Ipsilateral Hindpaw Sensory area	133
				5 Contralateral Forepaw Motor area	210
				6 Ipsilateral Forepaw Motor area	183
				7 Contralateral Hindpaw Motor area	166
				8 Ipsilateral Hindpaw Motor area	159
				9 Contralateral S-II area	172
				10 Ipsilateral S-II area	161
				11 Contralateral Suprasylvian area	149
				12 Ipsilateral Suprasylvian area	164
				13 Contralateral Auditory area	198
				14 Ipsilateral Auditory area	156
					$\bar{X}$=167.6
					S. D.= 22.5

ANIMAL -NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREA	C. P. M. PER mg. PROTEIN
L. G. E. II	Grass stimulator. five pulses per second. 100 msec. pulse width. To right forepaw.	4 Hours	50 C. 0.5 cc. volume through plastic catheter.	1 Contralateral Forepaw Sensory area	109
				2 Ipsilateral Forepaw Sensory area	124
				3 Contralateral Hindpaw Sensory area	118
				4 Ipsilateral Hindpaw Sensory area	123
				5 Contralateral Forepaw Motor area	136
				6 Ipsilateral Forepaw Motor area	102
				7 Contralateral Hindpaw Motor area	111
				8 Ipsilateral Hindpaw Motor area	117
				9 Contralateral S-II area	181
				10 Ipsilateral S-II area	76
				11 Contralateral Suprasylvian area	73
				12 Ipsilateral Suprasylvian area	71
				13 Contralateral Auditory area	90
				14 Ipsilateral Auditory area	124
					$\overline{X}$ = 111.1
					S. D. = 27.8

ANIMAL NUMBER	STIMULATION USED	STIMULATION DURATION	TRACER DOSE	SAMPLE AREA	C. P. M. PER mg. PROTEIN
L. G. E III	Grass stimulator. five pulses per cecond, 100 msec. pulse width to left forepaw.	4 Hours	50 C. 0.5 cc. volume through plastic catheter.	1 Contralateral Forepaw Sensory area	202
				2 Ipsilateral Forepaw Sensory area	202
				3 Contralateral Hindpaw Sensory area	228
				4 Ipsilateral Hindpaw Sensory area	230
				5 Contralateral Forepaw Motor area	223
				6 Ipsilateral Forepaw Motor area	210
				7 Contralaterah Hindpaw Motor area	237
				8 Ipsilateral Hindpaw Motor area	260
				9 Contralateral S-II area	251
				10 Ipsilateral S-II area	266
				11 Contralateral Suprasylvian area	217
				12 Ipsilateral Suprasylvian area	207
				13 Contralateral Auditory area	242
				14 Ipsilateral Auditory area	206
					$\overline{X}$ = 227.2
					S. D. = 20.7