AGE RELATED CHANGES IN MEMORY, OXIDATIVE METABOLISM, COENZYME-A, AND ACETYLCOENZYME-A IN THE RAT FOREBRAIN

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PREFACE

The aging process has received a great deal of attention in the last twenty years. The reasons for a heightened interest in aging are evident when one examines trends in the population of the United States. Simply put, our society is growing older. That is, an increasingly larger portion of the population is comprised of older adults. In 1960 only 9.2% of the nation's population was over age 65. However, present figures indicate that number having increased to approximately 12.7%. Additionally, it is projected that by the year 2030, 21.2% of the United States'population will be 65 years old or older (Special Committee on Aging, United States Senate, 1986).

Because our society is "aging", it has become important for researchers to address the issue of aging and its implications for society as well as for the individual. Topics addressed have ranged from providing extended care for a growing elderly population to new medical treatment for a growing number of special problems specific to the aged.

Not only has a great deal of applied aging research been carried out, but basic research has also been on the increase. Such research has been carried out by the biological, as well as the behavioral, sciences. These studies generally are aimed at providing insight into the changes

that affect the individual organism at a number of levels. For instance, biological scientists have researched changes in basic cell processes and physiological processes that result from normal aging.

Behavioral scientists have also been interested in changes as a function of aging. One particular area of interest to behavioral scientists is that of memory and cognition. Cognitive psychologists have generated a great deal of data over the past thirty years concerning thought processes of the aged.

Although the topic of aging has been studied extensively for about thirty years, it has been only recently that the behavioral and biological sciences have begun to view each other's research as relevant to their own efforts. In fact, when one looks at the literature, it has only been within the past twenty years that these two disciplines have begun to collaborate in the study of aging. Nowhere has this collaboration been more evident than in the study of memory and its changes in the aging process.

The following discussion will present evidence generated in the study of aging and memory. This review of the literature naturally follows the path of the study of aging and memory. That is, it begins by discussing evidence generated by behavioral scientists and biologists separately, and culminates in a synthesis of the two disciplines as they relate to memory and aging. Finally, as a result of the presented evidence, hypotheses are presented which are

tested by investigating behavioral changes in memory and associating these with changes in basic biological processes.

I wish to express my thanks to a number of people who assisted me in this project and throughout my coursework at Oklahoma State University. I wish to express my sincere gratitude to Dr. Robert Stanners, my major advisor, for his guidance, thoughtful insights, and invaluable assistance. I would also like to thank the other members of my committee; Dr. James Price for his expert quantitative advice, and Dr. David Thomas for his helpful input.

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I wish to extend my thanks to Dr. James Thomas who introduced me to the subject of Psychology many years ago. Additionally, I wish to express my thanks to David and Leora Simmons for their faith in me, their support of my goals, and their encouragement.

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CHAPTER I

LITERATURE REVIEW

Aging and Memory

Within the past thirty years psychology has increasingly concerned itself with aging and its consequences for the individual. Specifically, the cognitive sciences have conducted a number of studies aimed at discovering those changes in basic thought processes that change as a result of aging. Such studies have resulted in a considerable data base that indicates specific changes in cognition as a function of age. Although all of the findings are not unequivocal, some age-related changes have become quite well established.

One area of cognition that has received a great deal of attention, with regard to age-related change, is memory. Both popular and scientific literature are replete with anecdotal evidence that memory declines as people get older. However, empirical investigation of memory and aging is a fairly recent undertaking. In order to effectively understand the results of previous investigations, it is important to understand 1) what is meant by the term "memory", and 2) which aspects of memory do, in fact, change as a result of aging.

The most popular model of memory in use is an information processing model. This approach views memory as a system in which incoming information is processed via discrete stages or processes. The information is acted upon in each stage and this "action" then determines the extent to which the information is stored in memory for future use. Generally, memory is viewed as being composed of three components, a sensory store, a brief store, and a permanent store.

Such a model has proven to be useful for scientific investigation of memory, but it has also spawned some confusion. A particular problem one encounters is in defining the stages in the processing system. A number of investigators have used a number of different terms to define them. For example, the brief storage of incoming information has been referred to as "short term memory", "working memory", "immediate memory", and "primary memory". Such terminology makes it difficult at times to synthesize findings from different studies.

For reasons cited above, the present study adopts one model of memory. This model is that of Waugh and Norman (1965) and proposes three types of memory; a sensory memory store, a primary memory store (PM), and a secondary memory store (SM). Sensory memory is modality-specific storage component with a very brief duration of 300-1000 ms for visual information (Haber, 1970; Neisser, 1967), and about 2000 ms for auditory information (Neisser, 1967). PM as

defined by Waugh and Norman (1965) is a temporary memory store consisting of those items that are being attended to at a given moment, or are presently activated. Finally, SM is a memory store that holds information that is not being immediately attended to and is more stable and permanent than the other memory components.

The area of memory research where the most consistent age differences have been observed is SM. As previously mentioned, this memory store is a more permanent and stable store of information and consists of information that is outside of immediate attention. Indeed, this component of memory is often referred to as long-term memory by most investigators.

There exists an enormous literature on age differences in SM. For more thorough coverage of the topic, the reader is directed to reviews by Craik (1977), Hartley, Harker, & Walsh (1980), Erber (1982), Botwinick (1984), and Poon (1985). The present review is intended to clearly establish an argument for the existence of age differences in SM.

When one examines the empirical findings, a clear pattern emerges. That is, older humans appear to show a consistent memory deficit in SM, but not in sensory memory, or PM. A number of studies have established an age-related decline in recall and cued recall of words (Schonefield and Robertson, 1966; Hultsch, 1975; Craik & Rabinowitz, 1985; Drachman & Leavitt, 1972; Treat, Poon, & Fozard, 1981; Smith, 1977). Additionally, age-related deficits have been

established for long-term memory of prose (Byrd, 1985; Taub, 1979), performed activities (Kausler & Lichty 1985), recognition of faces (Smith and Winograd, 1978), and spatial memory (Thomas, 1985).

Given the previous discussion of the literature, it becomes quite evident that SM functioning declines with advanced age in humans. This effect does not appear to be ameliorated by providing encoding instructions. Bäckmän, Mäntylä, and Erngrund, (1984) found that providing subjects with encoding instructions equally benefited both young and old subjects in recall performance. However, young subjects still exhibited superior recall. Similar results were reported by Smith and Winograd (1978) for facial recognition. Thus, memory deficits do not seem to be corrected by acquisition of new, or restructuring of old, cognitive processes insofar as comparison with younger controls is concerned. This suggests the possibility of more basic physiological processes being involved in SM decline in the aged. One possibility is that of biological changes in the aged brain.

Aging, Memory, and Acetylcholine

Within the last fifteen years, a great deal of research has been directed toward studying the neurological changes associated with aging of the central nervous system. These studies include investigation of changes in basic cell processes, neural physiology, and neurochemistry. One line of

research that is of interest to the cognitive sciences as well as the biological sciences deals with neurological changes associated with age-related decline of memory function. Although these studies are at a basic level, they have begun to provide important information concerning the underlying neurochemical mechanisms involved in memory and the aging process.

Much of the neurochemical research of aging and memory is based on the cholinergic hypothesis of memory (Deutsch, 1971). Although simplistic, this theory is currently the most developed and tested theory regarding neurochemistry, memory, and aging. Briefly stated, the theory proposes that memory is regulated at the synapse of neurons that utilize acetylcholine (ACh) as a neurotransmitter. During learning, postsynaptic sensitivity to the neurotransmitter is increased. As more trials occur, this sensitivity is increased even more. Following training, postsynaptic sensitivity decreases. If sensitivity declines to a certain point, then forgetting is said to have occurred. According to Deutsch (1971), decline in sensitivity depends upon neurophysiological conditions at the time of learning. That is, if at time of learning the physiology of the synapse is altered (i.e. reuptake inhibition, receptor blocking) or its efficiency is diminished (i.e. a reduction in neurotransmitter) then the formation of the memory trace is altered or does not occur at all.

Given Deutsch's (1971) theory, it is reasonable to expect age-related changes in the cholinergic system to be somehow correlated with changes in memory. Investigators have, in fact, examined the role of ACh in memory and age-associated declines. These studies have followed three main courses and have provided significant data linking the cholinergic system to geriatric memory. The three major courses include 1) pharmacological manipulation of the cholinergic system, 2) mechanical or chemical lesions of cholinergic pathways, and 3) direct measurement of Ach and its chemical precursors.

Pharmacological Studies

Pharmacological studies have been conducted using both human and animal populations. These studies utilize drugs that have specific pharmacological action upon the cholinergic synapse. Scopolamine is one such pharmacological agent. Scopolamine binds to the cholinergic synapse at sites where ACh normally binds, thus blocking ACh from binding to the postsynaptic receptor. Scopolamine, however, does not create a synaptic potential.

When scopolamine is introduced into the synapse, normal levels of ACh are released at the pre-synaptic site, however, this ACh does not bind to the postsynaptic receptor. Instead, the ACh in the synaptic cleft is degraded by Acetylcholinesterase (ACHE). The result is choline and acetate. Choline is taken up into the pre-synaptic cell via

a high affinity transport mechanism while the acetate is removed from the synaptic cleft. This prevents the occurrence of a potential at the receptor. Thus, scopolamine alters the cholinergic system by blocking central neural activity at synapses selectively stimulated by ACh.

Another drug commonly used in the study of memory and the cholinergic system is physostigmine. The action of physostigmine is to increase the acculmulation of ACh in the synaptic cleft. It has been shown to reverse the effects of scopolamine. Presumably, this is done by increasing the concentration of ACh in the synaptic cleft by inhibiting the production of ACHE, thus increasing the duration in which ACh is present in the cleft. Because scopolamine and ACh bind at, and thus compete for, the same post-synaptic receptor sites, an increase in ACh allows more ACh to bind in place of scopolamine.

When scopolamine is administered to healthy young human subjects, PM is not impaired (Ostfeld & Auruguete, 1962; Safer & Allen, 1971), but SM is significantly impaired on a verbal cued-recall task (Crow & Grove-White, 1973; Drachman & Leavitt, 1974). Also, scopolamine has been found to decrease performance in free recall as well (Crow & Grove-White, 1971).

Drachman and Leavitt (1974) used scopolamine to compare the performance of young subjects to that of old subjects using a delayed free recall task. Under no-drug conditions, young subjects performed significantly better than old sub-

jects. However, when young subjects were administered scopolamine, their performance declined to the level of older subjects given a placebo. Finally, following scopolamine administration, performance of older subjects was diminished significantly more than young subjects.

To demonstrate that the effect of scopolamine was on the central nervous system, Drachman and Leavitt (1974) injected another group of subjects with methyl scopolamine. Methyl scopolamine acts in the same manner as scopolamine, but is unable to cross the blood-brain barrier. Its effect then is to block binding of ACh at the postsynaptic receptors in the peripheral nervous system. Thus, when healthy young subjects were injected with scopolamine, and another group was injected with methyl scopolamine, the scopolamine group showed significantly poorer memory function on SM tasks, but not PM tasks.

Further evidence of cholinergic involvement in memory function is provided by Drachman (1977). In this study, the deleterious effect of scopolamine upon memory of young subjects was reversed by the administration of physostigmine.

Thus, given the pharmacological data from studies using human subjects, it is apparent that the central cholinergic system has a large degree of influence in memory function. These data are supported in the findings of pharmacological studies using non-human subjects as well.

Deutsch (1971) administered scopolamine to rodents prior to an appetative avoidance learning task. Retention of the

task was significantly reduced under scopolamine. A different approach was used by Glick, Mittag, & Green (1973) who chemically decreased choline acetyltransferase (the catalyzing enzyme for Ach synthesis) in laboratory rodents. The effect of this treatment was to decrease retention of a previously learned passive avoidance response. Finally, improvement on a spatial memory task has been achieved in aged animals administered the Ach releasing stimulant 3,4-dyaminopyridine (Davis, Idow, & Gibson, 1983).

Given the above evidence, it has become increasingly evident that the central cholinergic system plays an important role in memory function. Age-like effects of certain pharmacological agents appear to indicate a prominent role of Ach in both human and non-human memory. However, such evidence does not, by itself, provide conclusive proof. It is possible that these pharmacological agents have more widespread effects on the central nervous system. Thus, additional data are needed to corroborate these findings. One group of data that fulfills this task concerns lesions of neurological pathways.

Lesion Studies

A number of researchers have studied the role of ACh and memory by destroying known cholinergic pathways and studying the subsequent effects on memory function. These studies typically are conducted with the use of chemical or mechanical lesions. It is now well established that the origin of

ACh afferent neurons is located at the nucleus basilis magnocellularis (Fibiger, 1982: Fibiger & Lehmann, 1982). Hence, lesions to this area would be expected to produce a memory deficit if the cholinergic hypothesis of memory were valid. Such lesions have been shown to cause a decrease in the retention of a passive avoidance response (Friedeman, Lere, & Kustner, 1983; Sanberg, Sanberg, Hanin, Fisher, & Coyle, 1984) and an active avoidance response (LoConte, Bartolini, Casameti, Marconcini-Pepeu, & Pepeu, 1982) in laboratory animals. The findings further support a cholinergic involvement in memorial functioning.

ACh and ACh Precursors

A third approach to studying the role of ACh in memory and aging is to measure the level of ACh or its precursors. ACh is synthesized in the nerve endings of cholinergic neurons. Three chemical substances are involved in this process. These are Choline (Ch), acetyle-coenzyme A (ACoA), and choline-acetyltransferase (CAT). Ch is pumped in the cholinergic neuron by a high affinity mechanism. ACoA is produced by the mitochondria of the cholinergic neuron. These two substances combine and their reaction is catalyzed by CAT. The result is ACh and coenzyme A (CoA) (Cotman & McGaugh, 1980). This reaction is summarized in the following equation.

$$Ch + ACoA \rightleftharpoons ACh + CoA$$

Research has been conducted to determine age-related changes in the production of ACh, availability of chemical precursors to ACh, and the relationship of these to memory. Two important studies indicate a relationship between ACh levels and memory in aged animals. Gibson and Jenden (1981), using two strains of mice, showed that whole-brain ACh synthesis declines with age. This decline in ACh was significantly correlated with memory performance. Bartus (1979) indicates a strong correlation between memory function and ACh synthesis in old rats. In this study, the decline in ACh synthesis between young and old rats was on the order of approximately 50%-60% and corresponded with an approximate 50% decline in memory performance. These two studies provide strong and direct support for the role of ACh in age-related memory decline.

Because the amount of ACh is known to decrease in older animals, inquiries have been undertaken to determine if this decrease is due to a shortage of any of its synthetic precursors. Most of these studies have dealt with the catalyzing enzyme CAT. In their review of the literature, Bartus, Beer, Dean, & Lippa (1982) point out that the majority of these studies do not support an age-related decrease in CAT for human or rodent populations. Where such a decline does exist is in the brains of humans suffering from senile dementia of the Alzheimer type. Consequently, when considering the role of the cholinergic system in normal aging, the role of CAT does not provide a causal link.

The ACh precursor, Ch, has been studied, but not to the extent of CAT. Sherman, Keuster, Dean, & Bartus (1981) report a 22% decrease in the uptake of Ch in the hippocampus of aged rats. Furthermore, Gibson & Jenden (1981) found that ACh synthesis from choline decreased by 50.4% in ten month old mice and by 75.9% in thirty month old mice. However, they also report that total choline concentration did not decrease. Finally, administration of lecithin (a precursor to Ch) increases serum content of Ch but does not improve memory function in aged humans (Drachman, Glosser, Fleming, & Longnecker, 1982). Hence, it appears that the age-associated decline in ACh synthesis cannot be due to a lack of available choline. Rather, this decline must be due to functions within the cholinergic neuron itself that are responsible for the utilization or reuptake of Ch in ACh synthesis.

The final possibile explanation of the decrease in ACh synthesis is a decrease in ACoA. Although ACh, CAT, and Ch have been studied, no study has directly investigated the association of ACoA in memory function, aging, and ACh. The lack of information concerning aging and ACoA leaves an important gap in the literature concerning ACh, aging, and memory. A number of studies, however, provide strong evidence that ACoA may play an important role in cholinergically mediated memory function in old age.

The first group of studies concerns mitochondria. As previously mentioned, ACoA is produced in the mitochondria

of the neuron. It is now well established that oxidative metabolism of brain mitochondria decreases with age (Hansford, 1983; Sylvia, Harik, Lamanna, Wilkerson, & Rosenthal, 1983; Harmon, Nank, & Floyd, 1987). Additionally, Blass & Gibson (1979) have shown that the synthesis of Ach is dependent on adequate supplies of oxygen. They found that ACh production in the mouse brain was significantly reduced by subjecting the animals to mild hypoxia. Because ACoA is the result of oxidative metabolism in the synaptic mitochondria, it is possible that ACoA is rate limiting in the production of ACh under conditions of decreased oxidative metabolism.

This hypothesis has been supported indirectly by a number of researchers using the drug piracetam, a drug chemically similar to gamma-aminobutyric acid that increases oxidative metabolism in the brain. Wolthius (1971) showed that rats administered piracetam exhibited significantly better learning and long-term retention of a maze task. Bartus, Dean, Sherman, Friedman, & Beer (1981) drastically increased the retention of aged rats on a passive avoidance task by administering a combination of the drug piracetum and choline. These authors argued that by increasing oxidative metabolism, an increase in ACoA was effected which then allowed for the acetylation of choline. However, these authors never directly measured ACoA content of the rodents' brains.

Findings similar to those of Bartus et al (1981) and Wolthius (1971) have been reported by Dimond and Brouwers (1976) and Mindus (1979) using humans as subjects. Dimond and Brouwers (1976) used young adults as subjects in their research. Subjects given doses of piracetam performed significantly better than placebo-treated subjects on a delayed-recall memory test. Mindus (1979) administered piracetam to elderly adult subjects. He reports that the memory of aged subjects with slight memory loss improved after the administration of piracetam.

Given the evidence generated by the use of piracetam, it is likely that ACoA is involved in age-related declines in memory. Thus, it is possible that by increasing oxidative metabolism via piracetam, ACoA concentrations are increased. This would then lead to increased ACh concentrations, thus leading to increased memory according to the cholinergic hypothesis of memory.

A second course of examination also points to a possible decline in ACoA as a function of age and a primary mechanism in the age-related memorial function. It is now well established that ACoA in the brain is synthesized from pyruvate by synaptic mitochondria (Cotman & McGaugh, 1980; Tucek, 1982). Deshmuk, Owen, & Patel (1980) have demonstrated an age-related decline in pyruvate-driven respiration in synaptosomes isolated from rat forebrain. Finally, Gibson, Jope, and Blass (1975) have demonstrated that ACh production can be limited by pyruvate utilization in brain slices. Thus,

it appears from these studies that pyruvate driven metabolism in mitochondria can be rate limiting in the production of ACh. It is highly likely, then, that one of the bases of the reduction of ACh is a decreased availability of ACoA as a result of decreased pyruvate-driven metabolism.

A final group of studies supporting a possible decline in ACoA have been presented earlier in this paper. These are the studies of Ch utilization. As previously mentioned, Ch concentration in the brain does not appear to decline as a result of advanced age. Rather, the uptake of Ch declines. Such a decline might be associated with the amount of available ACoA. Support for this argument is provided by the work of Jope & Jenden (1980). They report that while ACoA production in cholinergic neurons was impaired, Ch was accumulated and was only acetylated subsequent to the resumption of ACoA production. This led these investigators to conclude that the transport of Ch is kinetically coupled with its acetylation. Since ACoA is the compound providing the acetyl group for the acetylation of Ch, it is reasonable to infer that a decrease in Ch uptake could be the result of an age-related decrease in ACoA production. This, then, would likely lead to a decrease in ACh production.

This argument agrees with the findings of Bartus et al (1981). They found that animals given piracetam alone showed superior memory over animals given saline or choline only. However, when piracetum and choline were administered

together, performance was superior to any other treatment. Thus it appears that when the animals were administered choline and piracetam together, acetylation of choline was increased. The only possible mechanism is an increase in ACoA due to increased oxidative metabolism.

Given the previous arguments that link ACh to memorial function in man and laboratory animals, it then becomes important to determine if any link exists between concentrations of ACoA and memorial function. The following research was developed to investigate if such a connection exists. Of specific interest were the following questions; 1) Is there an age-dependent decrease in ACoA concentrations in the CNS? 2) Is the amount of ACoA associated with oxidative metabolism? 3) Are changes in memorial function associated with changes in ACoA and/or oxidative metabolism?

The research presented here is an attempt to address these questions. Specifically, the following hypotheses were tested.

Hypothesis 1: An age-related deficit in long-term passive avoidance retention exists, with retention performance decreasing at older age (this would be a replication of previously cited studies).

Hypothesis 2: An age-related difference in oxidative metabolism exists, with metabolic activity decreasing as age increases. Furthermore, this decrease will be apparent only in the synaptic fraction of brain mitochondria (this would be a replication of Harmon, Nank, & Floyd (1987)).

Hypothesis 3: An age-related difference in concentrations of ACoA exists, with concentrations decreasing as

a function of advancing age. It is expected that a decrease in ACoA will be found only in synaptic mitochondria and not in the nonsynaptic fraction.

Hypothesis 4: Oxygen-driven metabolism will be positively associated with levels of ACoA in synaptic and nonsynaptic mitochondria.

Hypothesis 5: Concentration of synaptic ACoA will be positively correlated with memorial function.

CHAPTER II

EXPERIMENTAL METHODS

Behavioral Testing

The purpose of the behavioral testing was to determine the existence of an age-related difference in memorial function of the rodent subjects. A single-trial step-through passive avoidance task was used for this purpose. The choice of this task was prompted by a number of factors. First, numerous researchers have shown that it reliably reflects memorial function in laboratory animals. Second, the task has been used to generate a large amount of data against which the current research can be compared. Finally, this task does not affect normal levels of ACh (Jope, 1978).

Subjects

Subjects for the experiments were female Fisher 344 rats obtained from the National Institute of Aging colonies (Harlan, Sprague, Dawley, Inc. - contractors). Seventy-two animals were used, and were received in three shipments of 24 animals nimals were in three age groups; young (approx-

imately 3 months), middle age (approximately 12 months), and old (approximately 30 months).

Upon delivery, all animals were kept in quarantine for approximately two weeks. In quarantine, the animals were housed four to a cage with same-age animals, were kept on a twelve-hour light/dark cycle, and were given food and water *ad libitum*. After quarantine, the animals were moved to another facility and were housed under the same food, water, light/dark, and cage-mate conditions as in quarantine.

Prior to any testing, the animals were visually inspected by the experimenter for locomotor ability, general physical condition, and lesions or tumors. No significant physical limitations were found for any of the animals.

Apparatus

The testing apparatus consisted of a 36 cm x 36 cm x 36 cm chamber with a grid floor and an open top. Chamber walls consisted of black Plexiglas. The floor of the chamber was constructed of parallel metal rods approximately 1.5 cm apart. The rods were connected in series to a Lafayette Instruments model 82400 power source capable of delivering a shock of 0-5 mA. Shock duration was controlled by a Lafayette Instruments model 58010 electronic timer connected to the power source.

The chamber front was situated flush with the edge of a table approximately 1 m high. A 6.5 cm x 6.5 cm opening was located on the center of the front wall of the chamber. A 7

cm x 26 cm wire-mesh runway extended from the opening and beyond the edge of the table. An experimenter-controlled guillotine door closed off the opening. A 60-watt incandescent lamp was located directly above the opening.

Procedure

All animals were tested for memory using a single-trial step-through passive avoidance task. During testing, only the animal being tested was allowed in the experimental room. All other animals were left in an adjacent room. All animals from the same cage were tested during the same session. Finally, both learning and retention trials were carried out between 2:00 p.m. and 5:00 p.m.

The learning portion of the task was carried out over two days. On the first day, an animal was removed from its cage and brought into the experimental room. The room was totally dark except for the light above the runway. The animal was placed in the chamber and allowed to roam freely for two minutes to become habituated to the chamber. The animal was then removed from the chamber and placed on the end of the runway farthest from the chamber with its head facing away from the chamber. The animal was released by the experimenter and a timer was started. Upon entry into the chamber (all four feet on the grid floor) the door was lowered and the latency to enter was recorded. The animal remained in the chamber for 20 s and was then returned to its cage.

A second day of training was carried out twenty-four hours after the habituation trial. This training consisted of two trials. On the first trial an animal was placed on the runway as on day one. The animal was released and latency to enter the chamber was recorded. The second of these trials was identical to the first trial, but upon entering the chamber the animal was presented with a 0.2 mA shock lasting 0.4 s. The animal remained in the chamber for 20 seconds and was then returned to its cage.

Immediately following the second day of training, the animals were taken to their housing place and left for one week. After one week, retention of the passive avoidance response was tested. The animal was place on the runway as before and latency to enter the chamber was recorded. If the animal did not enter the chamber after five minutes, it was assigned a latency of five minutes. The primary measure of interest was retention of the conditioned avoidance response after the one-week period. This was reflected by the latency to enter the chamber. To determine the existence of an age difference in retention, the conditioned avoidance responses were analyzed using a two-way repeated-measures analysis of variance (ANOVA). Factors were trials (pre-shock, post-shock) and age (3, 12, and 30 months). Although individual retention scores were used in this analysis, in subsequent analyses the mean retention score for each group of four animals was used. This was necessary

to correlate neurochemical measures that required the brain tissue of four same-age rats.

Neurochemical Measures

Neurochemical and neurophysiological measures were carried out on synaptic and nonsynaptic fractions of mitochondria. The mitochondrial fractions were isolated from the forebrains of four rats. Following isolation, the mitochondria were measured for oxygen driven metabolism using malate + glutamate and succinate as substrates. Concentrations of CoA and ACoA were also measured in each fraction.

Mitochondrial Isolation

Of all oxygen used by the brain, approximately 90% is utilized by mitochondria. Additionally, essentially all of the ACoA used in the synthesis of ACh is produced as a result of oxidative metabolism in the mitochondria. Thus, in order to measure both oxidative metabolism and concentration of CoA and ACoA, it is necessary to isolate mitochondria of the brain.

Two distinct populations of mitochondria exist in neural tissue. Synaptic mitochondria are found in the synaptosomes of neural tissue. Nonsynaptic mitochondria are found in the soma of the neuron and in nonsynaptic neurons (i.e. glial cells). When studying age-related changes in mitochondrial function, it is important to distinguish between these two distinct populations of mitochondria

(Neidele, van den Berg, & Grynbaum, 1969; Lai & Clark, 1975; Harmon, Nank, & Floyd, 1987). The experiments presented here utilize both types of mitochondria.

Synaptic (S) and non-synaptic (NS) fractions of brain mitochondria were isolated using a modified version of the procedure of Lai & Clark (1979). All centrifugation was carried out in a Sorvall RC-5B refrigerated centrifuge using a Sorvall SA-600 rotor. Centrifuge temperature was held constant at 4° C.

To obtain a sufficient amount of mitochondria, brain tissue of four same-age animals was pooled. Each group of four animals consisted of cage-mates that were tested for passive avoidance during the same session. All animals were sacrificed by decapitation approximately eighteen hours after final retention testing. Immediately after decapitation, an incision was made directly in front of the cerebellum. All brain tissue, from the frontal cortex to the incision, was lifted out of the cranium and placed into ice-cold isolation medium (IM) containing 0.32 mM Sucrose, 1 mM K^+-EDTA, and 10 mM Tris-Cl buffer (pH 7.4).

After tissue was removed from all four animals, the tissue was chopped into a slurry and excess IM was poured off. The tissue was homogenized fourteen times up and down in 15 ml IM using a glass "C" homogenizer (all homogenization utilized a glass homogenizer and Teflon pestle). The homogenate was centrifuged at 1302 X g for three minutes. Following centrifugation, the supernatant was poured off and

retained; the pellet was resuspended in 15 ml IM, and centrifuged again. This procedure was repeated three times.

The pooled supernatants were then centrifuged for ten minutes at 17,000 X g. The pellet from this step was removed and homogenized in 5 ml of 3% Ficoll solution [in 0.24 M mannitol, 0.06 M sucrose, 50 μM K-EDTA, and 10 mM Tris Cl (pH 7.4)] using a "B" homogenizer. The homogenate was layered on top of 15 ml of 6% Ficoll solution (using the same buffer as 3% Ficoll solution) and centrifuged at 32,570 X g for thirty minutes. The supernatant containing myelin was carefully aspirated off and discarded while the pellet was homogenized in 10 ml of 6% Ficoll in a "B" homogenizer. The homogenate was layered on top of 20 ml of 12% ficoll and centrifuged for 30 minutes at 32,750 X g. This step produced a pellet of NS at the bottom of the centrifuge tube and a layer of synaptosomes floating at the interface of the 6% and 12% Ficoll layers.

The supernatant above the synaptosomes was aspirated off and discarded. The synaptosomes were then aspirated off, suspended in 20 ml of 6mM Tris Cl (pH 8.1), and homogenized five times up and down using a "B" homogenizer. The homogenate was then centrifuged at 14,500 X g for ten minutes. The pellet was then resuspended in 20 ml IM and poured into 40 ml of ice-cold 6 mM Tris CL (pH 8.1) and allowed to sit for forty minutes at approximately 0° C. This step lyses the synaptosomal vesicles.

While the synaptosomes were lysing, the pellet of NS was homogenized 3 times up and down in 10 ml IM using a "B" homogenizer. The homogenate was centrifuged at 14,500 X g for ten minutes. This step was repeated a second time after which the NS were suspended in 1 ml IM.

After lysing was completed, synaptosomes were homogenized eight times up and down using a "C" homogenizer. The suspension was then centrifuged for ten minutes at 14,500 X g. The pellet from this step was resuspended in 10 ml of 6 mM Tris Cl (pH 8.1) using a "B" homogenizer, and was then centrifuged for ten minutes at 8,000 X g. Following this centrifugation, the supernatant was aspirated off and the pellet was resuspended in 5 ml of 3% Ficoll using a "B" homogenizer. The resuspended pellet was layered on top of 10 ml of 6% Ficoll and centrifuged at 11,700 X g for thirty minutes.

The supernatant was then aspirated off and the pellet was resuspended in 10 ml IM, three times up and down, using a "B" homogenizer. This suspension was centrifuged for ten minutes at 14,500 X g. This step was repeated and the resulting pellet was suspended in 1 ml IM.

Oxygen Consumption

Oxygen consumption was measured using a water-jacketed chamber fitted with a Clark oxygen electrode. The electrode was connected to an amplifier. Output was recorded by a Linear model 255 strip chart recorder. Measurements were

made at 37° C. Both succinate and malate plus glutamate-driven activities were measured. Succinate (9.5 mM), and 4.74 mM glutamate plus 4.75 mM malate were used as substrates. These were expressed as rotenone-sensitive and thenoyltriflouroacetone (TTFA)-sensitive activities, respectively.

Oxygen activity was measured immediately after mitochondria were isolated. Fifty microliters of mitochondria were added to 1.9 ml of medium containing 225 mM mannitol, 75 mM sucrose, 15 mM Tris-SO_4, and 50 mM K-EDTA (pH 7.4). Oxidative metabolism was recorded for approximately 5-7 minutes. Oxidative metabolism was then inhibited by the addition of 5 µl of 47.6 (final concentration) µM TTFA or 10 µM rotenone for succinate and malate + glutamate, respectively. The rate of oxygen activity was calculated as the rate of activity due to substrate minus the rate of activity after the addition of TTFA or rotenone. Oxygen activity was calculated on the basis of nmol O_2/min/nmol heme *a*.

Statistical analysis of results from the oxidative metabolism was carried out using four one-way ANOVAs. Each ANOVA was calculated for oxidative metabolism of each mitochondrial fraction using a particular substrate. The classification variable of interest in these analyses was age. This variable consisted of three levels; 3, 12, & 30 months of age.

Two additional analyses were carried out using data derived from measuring oxidative metabolism. The purpose of

these analyses was to determine the correlation between oxidative metabolism and response latency, oxidative metabolism and CoA concentrations, and oxidative metabolism and ACoA concentrations. Rates of oxidative metabolism for each combination of mitochondrial fraction and substrate were used as predictors to calculate all possible regressions on a number of criterion variables. Criterion variables used were 1) mean group response latency, 2) synaptic CoA concentrations, 3) nonsynaptic CoA concentrations, 4) synaptic ACoA concentrations, and 5) nonsynaptic ACoA concentrations. Each criterion variable was used in a separate single-criterion multiple regression equation.

Optimum regression equations were determined using Mallow's C(p) statistic. Using the results from analysis of all possible regressions, the model with optimum R^2 was analyzed further. This analysis was a single or multiple regression (as indicated by C(p)) analysis. The purpose of this analysis was to test the significance of R^2 and the regression parameters.

Cytochrome Calculations

Cytochrome content of the mitochondrial samples were calculated from dithionite-reduced MINUS ferricyanide-oxidized difference spectra measured with a DBS-3 Johnson Research Foundation (University of Pennsylvania) scanning dual-wavelength spectrophotometer. Millimolar extinction coefficients of 28, 19.5, and 24 (1 cm light path) were used

for the absorbance differences at the specified wavelength pairs of cytochrome *b* (562-575 nm), cytochromes *c* + c_1 (554-538 nm), and cytochrome aa_3, respectively.

To obtain spectra, mitochondria and a sucrose-phosphate buffer containing 0.25 M sucrose and 50 mM sodium phosphate buffer (pH 7.4) were added to a quartz cuvette (total volume 0.34 ml). Dithionite was added to the cuvette and the spectrum of the sample was recorded. Ferricyanide was then added to the cuvette and the spectrum recorded. The computed difference spectrum was then plotted.

These data were not analyzed for age differences. Rather, they were used to compute heme *a* content per ml of tissue. Heme *a* content was then used as standard reference for determining oxidative metabolism and concentrations of CoA and ACoA (i.e. nmol ACoA per heme *a*.).

Coenzyme A and Acetyl-coenzyme A Assays

Concentration of coenzyme A (CoA) and acetyl-coenzyme A (A-CoA) were determined using the procedure of Allred and Guy (1969). CoA and A-CoA were measured using a Varian DMS-100 ultraviolet-visible wavelength spectrophotometer. Absorbance was measured at 340 nm using a double-beam ultraviolet light source with band width of 1.0 nm. Rate of absorbance was measured using a Linear model 585 strip chart recorder. All samples were measured in 1.0 ml quartz cuvettes against an H_2O blank.

In this assay, CoA and A-CoA are recycled through a cycle of three enzyme-catalyzed reactions with each cycle generating NADH. Each cycle involves the following reactions.

1. acetyl phosphate + CoASH ⟶ A-CoA + phosphate
2. A-CoA + oxalacetate ⟶ citrate + CoASH
3. malate + NAD ⟶ oxalacetate + NADH

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malate + acetyle phosphate + NAD ⟶ citrate + phosphate + NADH

The rate of NADH formation is limited by the amount of CoA and A-CoA present. Thus, CoA and A-CoA concentrations were measured as the rate of NADH production.

To calculate actual concentrations of CoA and A-CoA, it was necessary first to calculate a primary standard curve. The curve was calculated by adding to a cuvette increasingly larger amounts (0 µl - 5 µl) of 1.007 mM CoASH to 0.45 ml of premix. The premix contained 0.25 ml of Tris buffer (1 M, pH 7.2), 50 µl of 1 M KCL, 50 µl of 0.2 M malate, 50 µl of 80 mM acetyle phosphate, 50 µl of 2 mM β-NAD. Added to this was 1.5 µl of malate dehydrogenase, 3.5 µl of citrate synthase, 50 µl of 20 mM dithiothreitol, and enough water to make the total volume 1.0 ml.

This mixture was incubated in a water bath for two minutes at 30° C. Following incubation, 50 µl of phosphotransacetylase, which catalyzes the above reaction, was added to the cuvette to start the reaction. The cuvette was then placed in the spectrophotometer and the rate of NADH production was recorded on the chart recorder. A linear

regression equation was calculated using Co-ASH as a predictor and the rate of NADH production as the criterion variable. CoASH concentration accounted for 99.1% of the variance in the model. Thus, a linear relation was established and the standard curve then used in subsequent calculations of CoA and A-CoA. A plot of the standard curve can be found in Figure 1. Figures may be found in Appendix A.

Concentrations of CoA and A-Coa of tissue samples were calculated using the following procedures. Equal amounts of NS or S and 6% perchloric acid were combined and allowed to sit on ice for 30 minutes. The mixture was then homogenized five times up and down using a glass "A" homogenizer and Teflon pestle. The homogenate was centrifuged at 8000 X g in a tabletop centrifuge. Following centrifugation, the supernatant was poured off and neutralized to pH 4-5 with KOH.

Neutralized supernatant was then added to a cuvette containing 0.45 ml premix (same as was used for the standard curve). Added to this was 1.5 µl of malate dehydrogenase, 3.5 µl of citrate synthase, 50 µl of 20 mM dithiothreitol, and enough water to make the total volume 1.0 ml.

Following incubation at 30° C, the cuvette was placed into the spectrophotometer and a background rate was obtained. The cuvette was then removed and 50 µl of phosphotransacetylase (PTA) was added to start the reaction. The cuvette was then placed in the spectrophotometer and the rate of NADH production was recorded on the chart recorder.

This rate was calculated as the rate before PTA was added minus the rate after PTA was added. This rate reflected the amount of CoA + A-CoA. To obtain a measurement of A-CoA only, neutralized supernatant was mixed with 50 µl of 20 mM N-ethylmaleimide and incubated for five minutes at 30° C. Following incubation, 0.1 ml of 20 mM dithiothreitol was added and incubation was continued for another five minutes. After this incubation, 0.45 ml of the premix, 3.5 µl of citrate synthase, 1.5 µl of malate dehydrogenase, and enough water to make the total volume 1.0 ml were added and incubated for two minutes.

Following the final incubation, the cuvette was placed in the spectrophotometer and the background rate was recorded. The cuvette was removed and 50 µl of PTA was added to start the reaction. This rate of NADH formation was recorded and the cuvette removed. The rate of NADH produced from A-CoA was calculated as the rate with PTA minus the rate without PTA.

CoA and A-CoA were calculated as follows. ACoA was calculated by inserting the rate of NADH formation into the linear function obtained from the standard curve. Concentration of CoA was calculated by subtracting the rate of NADH production in the ACoA run from the rate of NADH production in the CoA + ACoA determination assay. This value was then inserted into the standard curve regression equation to produce a measure of CoA concentration. All concen-

trations of ACoA and CoA were expressed as nmol enzyme/min/nmol heme *a*.

As with the oxidative metabolism data, these data were analyzed using ANOVA. Two one-way ANOVAs were calculated for concentration of CoA. Each ANOVA was calculated for a separate mitochondrial fraction. The classification variable of interest in these analyses was age. This variable consisted of three levels; 3, 12, & 30 months of age.

Likewise, two one-way ANOVAs were calculated for concentration of ACoA. Each ANOVA was calculated for a separate mitochondrial fraction. The classification variable of interest in these analyses was, again, age, which consisted of three levels; 3, 12, & 30 months.

An additional regression analysis was carried out using CoA and ACoA data. Nanomol concentrations of CoA and ACoA from each mitochodrial fraction were used as predictors and response latency was used as the criterion variable. All possible regressions were calculated to determine the correlation between CoA and ACoA, derived from synaptic and non-synaptic fractions, and mean group response latency.

Optimum regression equations were determined using Mallow's C(p) statistic. Using the results from analysis of all possible regressions, the model with optimum R^2 was analyzed further. This analysis was a single or multiple regression (as indicated by C(p)) analysis. The purpose of this analysis was to test the significance of R^2 and the regression parameters.

As can be seen, regression equations were not calculated using combinations of oxidative metabolism and enzyme data as predictors when mean group response latency was used as the criterion. Due to missing values, these regression analyses had a small number of cases (n=12). To investigate combinations of oxidative metabolism and enzyme concentration would have resulted in eight predictors. A number of authors (Draper & Smith, 1981; Tabachnick & Fidell, 1983) indicate that for better interpretability, the number of predictors should be 3 - 5 times greater than the number of cases. Thus, given the small number of cases in this study, it was necessary to split the predictors.

The same reasoning was used in regression analyses using synaptic and nonsynaptic CoA or ACoA as criterion variables. Because of a small number of cases, it was necessary to limit the number of predictors. This was done by using as predictors only enzyme and oxidative metabolism measures from the same fraction of mitochondria. Thus, only synaptic measures of CoA/ACoA and oxidative metabolism were used as predictors in regression analyses having synaptic ACoA/Coa as predictors. Likewise, only nonsynaptic measures of CoA/ACoA and oxidative metabolism were used as predictors in regression analyses having nonsynaptic ACoA/CoA as predictors.

CHAPTER III

RESULTS AND DISCUSSION

Passive Avoidance Retention

Results from the passive avoidance retention testing supported Hypothesis 1. The ANOVA indicated both main effects to be significant. The main effect for age was significant, $F_{(2,68)} = 9.65$, $p < .0002$, as was that for trials $F_{(1,68)} = 144.43$, $p < .0001$. The interaction of age and trials was also significant, $F_{(2,68)} = 4.40$, $p < .016$.

An analysis of simple main effects indicated the effect of trials to be highly significant ($p < .0001$) at all levels of age. Statistics were $F_{(1,68)} = 77.55$, $F_{(1,68)} = 56.30$, and $F_{(1,68)} = 20.73$ for 3, 12, and 30 month animals, respectively. Thus, all groups exhibited significantly greater post-shock latencies.

The age by trial interaction can be explained by examining the results of a simple main effects test for age computed for both pre-shock and post-shock trials. This analysis indicated no significant age effect in the pre-shock trials, $F_{(2,138)} = 0.90$, $p > .05$. However, in post-shock trials, a significant effect of age was found, $F_{(2,138)} = 13.82$,

$p < .001$. An ANOVA summary table of these results may be found in Table 1. Tables may be found in Appendix B.

Mean group retention latencies for the pre-shock trial were 56.28, 31.33, and 22.96 s for 3, 12, and 30-month old animals, respectively. Retention latencies for post-shock testing were 273.1, 216.08, and 137.49 s for 3, 12, and 30-month old animals, respectively. These results support the hypothesis that a memory deficit exists in aged rodents (Hypothesis 1). That is, there were no age differences in performance prior to learning. However, retention of the shock-induced avoidance response did show a significant age-related decline.

Oxidative Metabolism

Malate + Glutamate. Analysis of variance for malate + glutamate-driven respiration showed no significant age differences for either mitochondrial fraction. For the nonsynaptic fraction, respiration rates were 60.46, 45.13, and 66.92 nmol for 3,12, and 30 month old animals. These means were not significantly different, $F_{(2,13)} = 2.01$, $p < 0.17$.

Measurements of the synaptic fraction gave rates of 47.39, 53.05, and 49.78 nmol for 3, 12, and 30 months of age, respectively. Again, these means did not differ significantly, $F_{(2,11)} = 0.13$, $p < 0.87$. ANOVA summary tables for oxidative metabolism using malate + glutamate as sub-

strate with nonsynaptic and synaptic mitochondria may be found in Tables 2 and 3, respectively.

Succinate. For respiration measurements when succinate was used as substrate, ANOVA results show no significant effect for age, $F_{(2,13)} = 3.49$, $p < 0.06$ in the nonsynaptic fraction. Mean rates were 44.83, 27.53, and 61.0 for 3, 12, and 30 month-old animals, respectively. For the synaptic fraction, no significant effect of age was found, $F_{(2,11)} = 2.11$, $p < 0.17$. Mean oxidative metabolism rates for the synaptic fraction were 54.40, 69.29, and 82.65 nmol for 3, 12, and 30 month-old rats, respectively. ANOVA summary tables for oxidative metabolism using succinate as substrate with nonsynaptic and synaptic mitochondria may be found in Tables 4 and 5, respectively.

Regression results were generated by calculating all possible regression equations for succinate-driven and malate-driven respiration of synaptic and nonsynaptic origin each (four predictors). Mean sub-group response latency was the criterion variable. As indicated by Mallow's C(p) statistic (see Appendix C for a discussion of this statistic), the optimum regression equation was found to be a two-predictor equation. This equation had as its predictors 1) synaptic succinate metabolism and 2) synaptic malate + glutamate metabolism, C(p) = 1.21, p = 2 (see Table 6). This equation rendered an $R^2 = 0.504$, $F_{(2,9)} = 4.58$, $p < 0.0425$ (see Table 7).

Although these regression results appear to indicate a significant multiple correlation between synaptic respiration and ACoA, an examination of simple correlations indicates otherwise. The simple correlations between sub-group response latency and succinate and malate + glutamate respiration are $r = -0.46$ and $r = 0.045$ $(n=12)$, respectively. Neither correlation is significant at the 0.05 level. The significant multiple correlation, when both are used as predictors, is explained by examining the simple correlation between the two predictors themselves. This correlation yields $r = 0.715$, $p < .009$. Thus, it appears that malate + glutamate acted as a suppressor variable in the equation. Therefore, the significant multiple correlation was not considered any further.

The final regression analysis carried out with respiration data investigated the correlation between CoA and ACoA concentrations and respiration. As with retention data, all possible regression equations were calculated using succinate-driven and malate + glutamate-driven respiration of synaptic and nonsynaptic origin as predictors, with synaptic and nonsynaptic CoA and ACoA, each, used as criterion variables.

In these regression equations, CoA or ACoA were also used as predictors depending on what the criterion was. That is, if ACoA were being used as a criterion, then CoA would be a predictor in the model. Conversely, if CoA were

used as the criterion, then ACoA would be used as a predictor in the model.

Because synaptic and nonsynaptic mitochondria are two distinct fractions, regressions were calculated in the following manner. All regression equations were computed for the same mitochodrial fraction. That is, only oxidative metabolism, CoA, and ACoA from synaptic mitochondria were used as predictors for criteria from synaptic mitochondria. Likewise, only nonsynaptic predictors were used in equations with nonsynaptic criterion variables.

When synaptic ACoA was used as the criterion variable, Mallow's C(p) indicated the optimum equation to be a two-predictor equation having synaptic CoA and succinate metabolism as the predictors, C(p) = 2.38, p = 2 (see Table 8). As can be seen in Table 9, the model was not significant, $R^2 = 0.25$, $F_{(2,9)} = 1.53$, $\underline{p} < .27$.

For nonsynaptic ACoA, a one-predictor model, having nonsynaptic CoA as the predictor, was indicated, C(p) = 1.21, p = 1 (see Table 10). This model was not statistically significant. As shown in Table 11, $R^2 = 0.003$, $F_{(1,10)} = 0.834$, $\underline{p} < 0.38$. Thus, the concentration of ACoA was not associated with respiration using either succinate or malate + glutamate as substrates. Given the above results, there exists no support for hypothesis 4.

Discussion. The results from measurement of respiration rates do not support hypothesis 2. Also, they are not consistent with those of investigators who have previously mea-

sured oxidative metabolism of nonsynaptic and synaptic mitochondria. Harmon, Nank, & Floyd (1987) report a moderate age-dependent decline of respiration in nonsynaptic mitochondria (86, 74, and 60 nmol at 3, 12, and 30 months) and a very large decline in synaptic mitochondria (86, 51, and 24 nmol at 3, 12, and 30 months) when malate + glutamate was used as substrate. There is no discernible reason why the results of the current research should deviate from those of previous researchers; however, two possible explanations exist.

As concerns the regression data, it is not surprising that oxidative metabolism did not significantly correlate with retention. The reasons for this are many. First, given the lack of an age effect in the ANOVAs for respiration, and the significant age effect in the ANOVA for retention, it would be expected that respiration and retention would not be associated. A final reason concerns sample size. As can be seen, the sample sizes for all analyses were small (n = 12). With such small sample sizes, correlations would have to be quite large to attain significance.

One other explanation of the regression data can be posed, and is much more parsimonious than those previously given. That is, that succinate and malate + glutamate-driven oxidative metabolism are not correlated with either memory or ACoA levels. Given the statistical results, it is this explanation that must be accepted.

Although the metabolic chains that utilize succinate and malate + glutamate as substrates produce CoA and ACoA, they are not the only ones. As previously mentioned, Pyruvate-driven metabolism also produces ACoA. It is argued by Tucek (1983) that the ACoA used in acetylation of choline is derived from Pyruvate metabolism. Thus, it is possible that functioning of this metabolic chain is more associated with memory and ACoA concentrations. Future research would need to be conducted to investigate this hypothesis.

Although not significant, these findings pose interesting questions for research dealing with ACh, aging, and memory. As previously mentioned, other researchers have established a correlation between oxygen utilization of the brain and production of ACh. Specifically, the work of Blass and Gibson (1979) demonstrates that mild hypoxia in animals severely compromises the cholinergic system. This effect is such that an increase in choline does not result in any further increase in ACh concentrations.

The results of this research, those of Blass and Gibson (1979), and the previously mentioned results of Bartus, et al (1981) bring up some interesting questions. First, if, as has been demonstrated, ACh concentrations decrease as a result of decreased oxygen utilization, what is the mechanism responsible? The present results shed little light on the matter.

As was argued previously, a possible candidate for this mechanism is ACoA. However, the present research provides

little support for this argument. That is, there is no evidence that concentrations of ACoA are correlated with oxidative metabolism, nor is there an indication that oxidative metabolism is correlated with memorial function.

A second question to be asked concerns oxidative metabolism and aging. Specifically, does oxidative metabolism decrease with advanced age? The previously cited findings of Harmon et al (1987) seem to indicate that oxidative metabolism does decrease as a function of age. However, the previous findings do not agree with their results in that the present data show no decline in oxidative metabolism as a function of age.

Although results differ between the current study and that of Harmon et al (1987), it is likely that their findings are more accurate. The reason for this argument is two-fold. First, their results were obtained with eight animals in each experimental cell. The present experiment was conducted with as few as four and as many as six animals in each cell. Thus, the larger number of subjects in the Harmon et al (1987) study could have provided more statistically reliable data.

A second difference between the Harmon et al (1987) study and the present one concerns procedures used for measuring oxidative metabolism. In measuring oxidative metabolism, the accepted method is to measure as many separate oxidative metabolism rates as necessary, using the same tissue sample, until the rates of consecutive runs are

within ±10%. To attain such accuracy requires a large number of runs per sample as well as a large amount of tissue. This was the procedure used by Harmon et al (1987).

The research presented here was unable to follow this procedure due to scarcity of tissue. The maximum number of individual rates generated was reduced to approximately three per tissue sample per substrate. Thus, the variance within each sample was increased due to the inability to obtain a sufficient number of rates to meet the ±10% criterion. It is highly likely that this within groups variance contributed to the lack of significant age differences in the ANOVAs.

Another possible explanation of differences found concerns the age of the older rodents. Through personal communication with one of the authors, the mortality rate for Harmon, et al (1987) was at the level of approximately 40% - 50%. According to Campbell, Krautner, & Wallace (1980), it is at this mortality rate where an animal would be considered truly "senescent". This mortality rate occurs in the Fisher 344 rat at approximately 30 months of age (Campbell et al, 1980). In the present research, only one of the older animals died. This was the only death among all of the animals. Thus, the animals used in this research may have been selected from a population that is not truly senescent.

One final explanation of the discrepancies between Harmon et al (1987) and the present study concerns the mito-

chondrial isolation technique used. As previously mentioned, this technique was a modification of that used by Lai & Clark (1979) and had not been used prior to this study. Thus, it may be that this isolation technique did not fully separate out the mitochondrial fractions. The result, if this occurred, would be that the synaptic fraction would be "contaminated" with nonsynaptic mitochondria.

After isolation, the ratio of nonsynaptic to synaptic mitochondria is approximately 10:1. Thus, if only 10% of the nonsynaptic fraction were to be present with the synaptic fraction, the resulting preparation would be only 50% synaptic. Obviously, this contamination would eliminate any measured differences between the two mitochondrial fractions.

CoA and ACoA

ACoA. Analysis of variance revealed no significant differences between age groups for concentrations of ACoA in either the synaptic or nonsynaptic fractions. For the synaptic fraction, mean concentrations were 0.34, 0.39, and 0.30 nmol for 3, 12, and 30 month-old animals, respectively. These means were not significantly different, $F_{(2,13)} = 0.25$, $p < .78$ (see Table 12). Mean concentrations of ACoA for the nonsynaptic fraction were 0.28, 0.30, and 0.34 nmol for 3, 12, and 30 month-old animals, respectively. Again, these means did not differ significantly, $F_{(2,13)} = 0.38$, $p < .69$ (see Table 13). Thus, Hypothesis 3 was not supported.

That is, the data indicate that concentrations of ACoA do not decrease with advanced age in either synaptic or nonsynaptic mitochondria.

CoA. In synaptic mitochondria, ANOVA indicated no significant difference between age groups for CoA concentrations, $F_{(2,13)} = 1.59$, $p < 0.24$ (see Table 14). Mean concentrations were 0.30, 0.46, and 0.21 nmol for 3, 12, and 30 month-old animals, respectively. For the nonsynaptic mitochondria, mean CoA concentrations were 0.15, 0.33, and 0.40 nmol for 3, 12, and 30 month-old animals, respectively. These means did not differ significantly, $F_{(2,13)} = 3.13$, $p < .078$ (see Table 15). Thus, CoA concentrations do not differ significantly in either mitochondrial fraction as a function of increased age.

Regression results were generated by calculating all possible regression equations using CoA and ACoA concentrations in synaptic and nonsynaptic mitochondrial fractions as predictors. As with respiration equations, mean sub-group response latency was the dependent variable. The optimum R^2 was reached with a two-predictor model. The predictors were 1) synaptic CoA and 2) synaptic ACoA, $C(p) = 2.25$, $p = 2$ (see Table 16). For this model, $R^2 = 0.462$ was not significant, $F_{(2,9)} = 1.53$, $p < .062$ (see Table 17).

Discussion. The ANOVA for enzyme concentration clearly shows no significant effect for age, mitochondrial fraction, enzyme type, or any interaction of these. Thus, Hypothesis

3 was not supported. However, by examining Figures 2 and 3, the data do show a partial trend in the direction predicted by the hypotheses. One interesting aspect of these results is the change in enzyme concentration levels at different ages for different mitochondrial fractions. For synaptic mitochondria, Figure 2 shows that CoA and ACoA concentration increases from 3 to 12 months but at 30 months decrease to a level below that of 3 months. In contrast, both CoA and ACoA increase at older ages in nonsynaptic mitochondria as seen in Figure 3.

This differential behavior of these enzymes should be investigated further. Although not significant, these results do indicate the possibility of differential age-related changes in enzyme activity of synaptic and nonsynaptic mitochondria.

As can be seen from the above discussion, an association between enzyme concentration and memory was not proven and thus, hypothesis 5 was not supported. However, the R^2 of the regression equation was close to being significant at the .05 level, $F_{(2,9)} = 3.858$, $p < .062$. Figure 4 shows the regression lines generated by this equation.

It is interesting to note that although this multiple correlation was not significant, the two predictors in the equation were 1) of synaptic origin and 2) were enzymes. Also of interest is the comparison of ANOVA results with multiple regression results. That is, ANOVA indicated no age differences in CoA or ACoA concentrations. However,

multiple regression indicated that these two approach significance as predictors of memory. What this would seem to indicate is that memory may be linked to CoA and ACoA concentrations in synaptic mitochondria, regardless of age. Due to the lack of a truly significant multiple R^2, however, this argument is only speculation.

As in the measurement of oxidative metabolism, the collection of these data was hindered by a small number of subjects in each experimental cell. Again, cell sizes ranged from three to six. Again, the small number of subjects hinders interpretation of the data. Although the data appear to, at least partially, behave in the predicted manner, they are nonetheless statistically not significant. Thus, to attempt a generalization of the trends discussed would provide little useful information.

Another difficulty in collection of these data concerns the ability to obtain multiple rates for each synaptic fraction. As with the oxidative metabolism measures, the standard procedure for measuring CoA and ACoA rates is to collect multiple rates for the same sample until they are within ±10%. Due to the small amount of tissue available, this was not possible. The maximum number of runs possible, per sample, was one for synaptic mitochondria and two for nonsynaptic mitochondria. Thus, the problem of within cell variance was again encountered and, quite possibly contributed to the nonsignificant results.

General Discussion

To summarize the findings, a significant age difference was found for long-term retention of a single-trial step-through passive avoidance task. Older animals demonstrated a greater decline in retention of the response than either the young or middle aged animals. However, performance on the memory task was not correlated with oxidative metabolism of mitochondria, nor was it associated with concentrations of CoA or ACoA in the mitochondria. Furthermore, no difference in enzyme concentration was found between animals of different ages.

Thus, no support can be given to the hypothesis that ACoA is a causal factor in memory decline in the aged. Indeed, from these results, it must be stated that there is no decline, as a function of age, in ACoA in the forebrain of the rat. Finally, succinate-driven and malate + glutamate-driven respiration do not appear to be associated with either memory function or levels of ACoA.

Although these results provide no support for the previously stated hypotheses, except hypothesis 1, this does not mean that more research is unnecessary. In fact, the contrary could be argued.

As previously mentioned, the sample sizes for this research were quite small. It is possible that more reliable results could be obtained by doubling, or even tripling, the number of animals in each age group. The effect of increasing cell size could have two effects. First, within-

groups variance could be reduced, thus providing more reliable statistical analysis. Second, increased cell size would provide more tissue for metabolism and enzyme analysis, thus providing more stable measurements of metabolism and enzyme concentrations.

Additional benefit could be obtained by using more sensitive techniques to measure enzyme levels. Although the current technique is sensitive to the picomol level (Allred & Guy, 1969), it requires a large amount of enzyme. For example, to obtain a single reading, 0.40 ml of synaptic mitochondria must be used. Given a total of 1 ml of tissue obtained per group, this allows for only one measurement of each enzyme. Thus, a more sensitive technique requiring only microliter volumes of tissue (i.e. high performance liquid chromatography) would be useful. Such a method would allow multiple runs and drastically reduce the variance found within experimental cells.

Finally, more generalizable results might be obtained by using older animals that are in the 50% survival range. It is highly likely that such animals are truly senescent and would provide more reliable data than the current aged group.

One final aspect of the present data should be addressed. As previously mentioned, metabolism and enzyme measurements were expressed as amount/nmol heme *a*. This measurement standard reflects data relative to the amount of

mitochondrial cytochrome present in the isolated brain mitochondria.

It is well established that as the brain ages, neural tissue is lost. Thus, a decrease in enzyme levels could be a result of this loss. That is, the production of ACoa from each mitochondria has not changed. Rather, the total amount of ACoA in the brain has decreased simply because there are fewer mitochondria, due to fewer neurons, to produce it. Thus, the total ACoA produced will decrease with age and correlate positively with learning data. However, since these neurochemical data were not calculated on a total brain standard (i.e. brain weight, brain volume), and a measure of the mitochondrial content in the brain was not obtained, we can only speculate on what might be.

Summary

This research has been an attempt to investigate previously untested hypotheses. Although little support was generated for the hypotheses, future research is urged. Although not statistically significant, some findings exhibited trends that should be investigated further. Additionally, future research with larger sample sizes and more sensitive assay methods should clarify some of the questions raised by this research.

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APPENDIX A

FIGURES

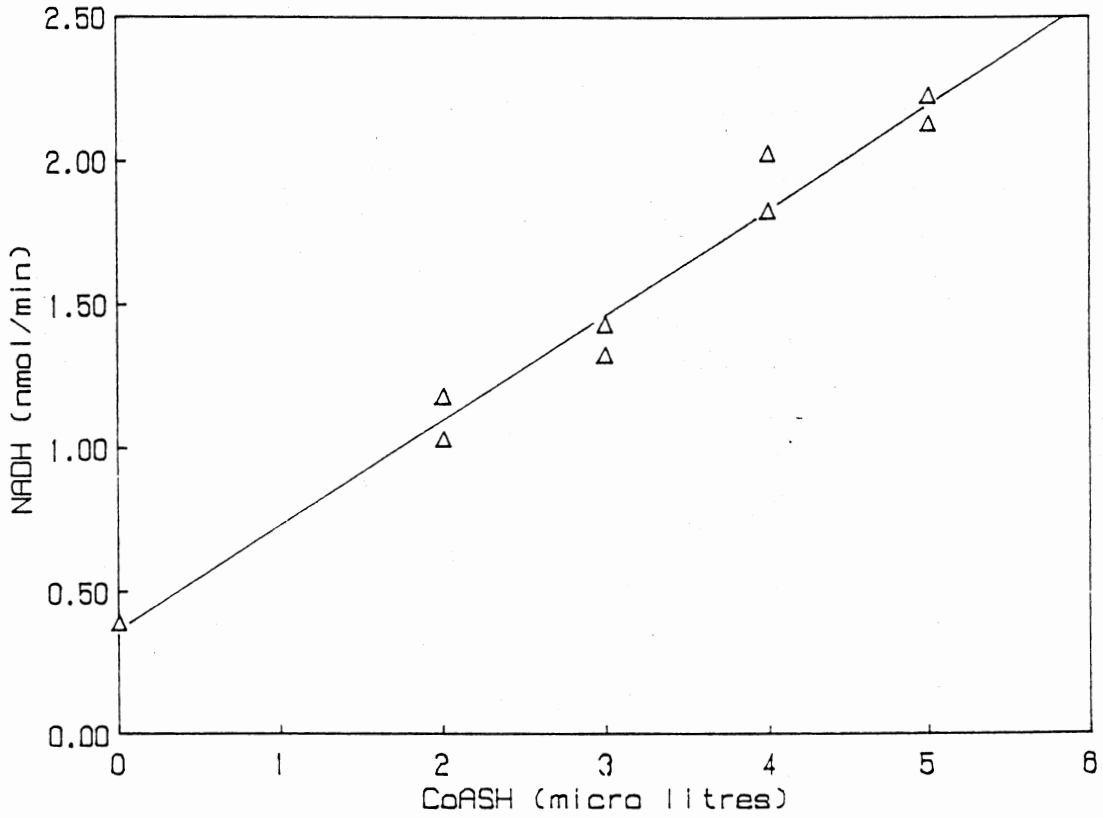

Figure 1. Standard Curve for CoA and ACoA Assays

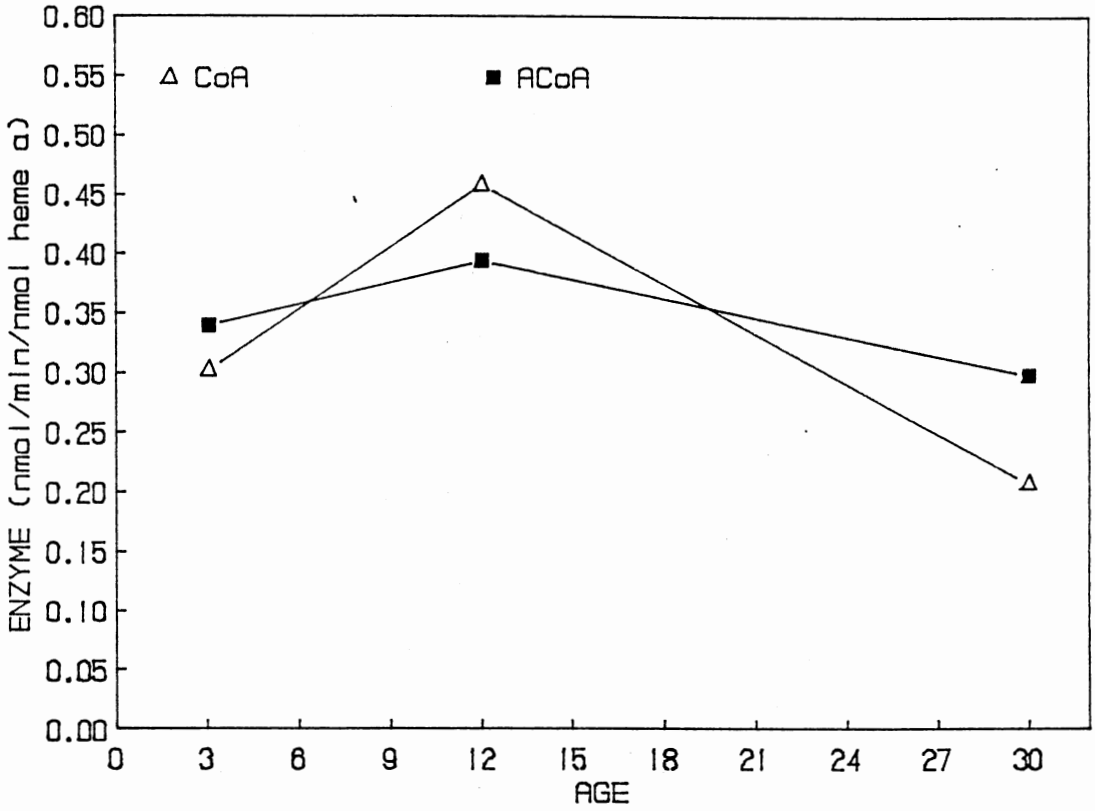

Figure 2. Comparison of Means of Synaptic CoA and ACoA for Each Age Group

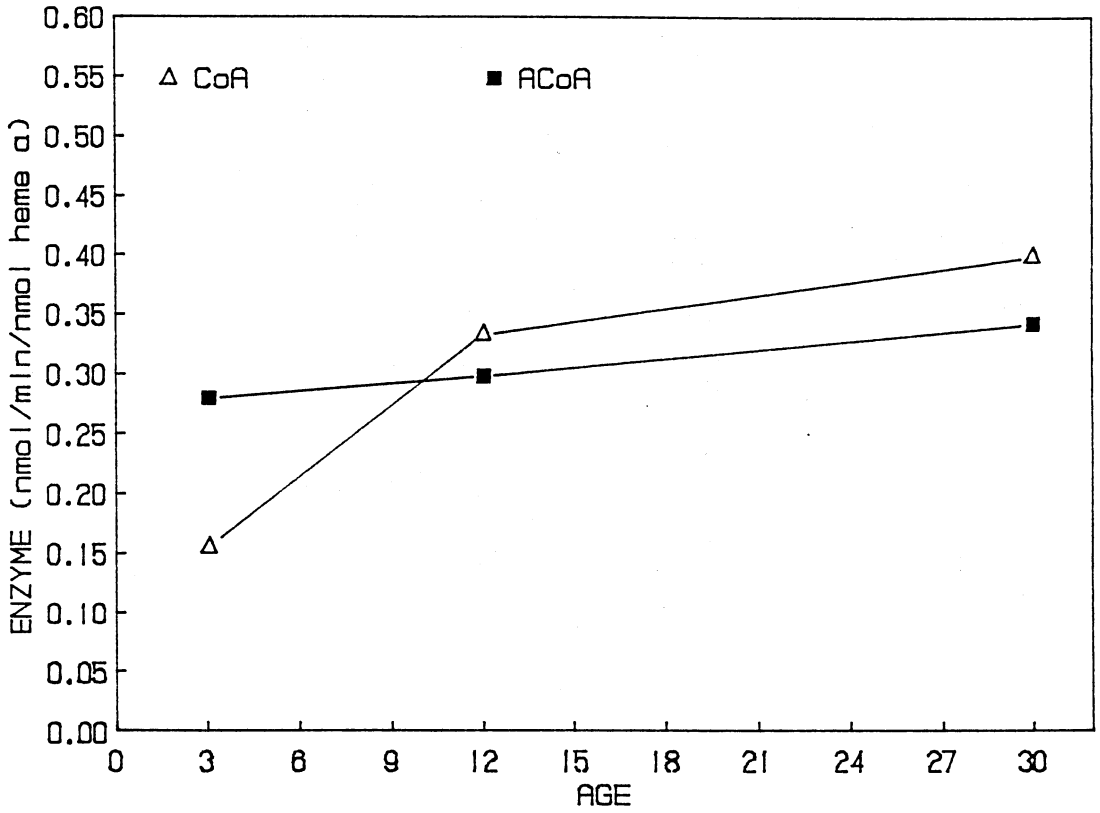

Figure 3. Comparison of Means of Nonsynaptic CoA and ACoA for Each Age Group

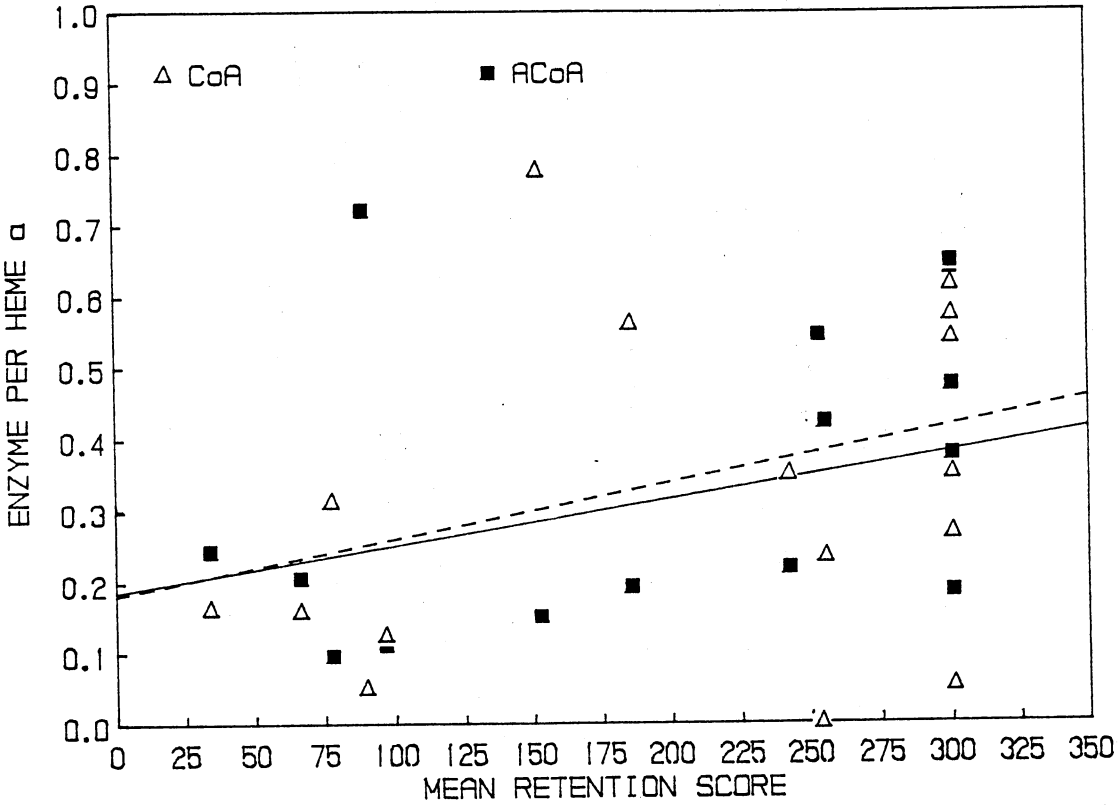

Figure 4. Regression Lines of Synaptic CoA and ACoA Using Passive Avoidance Retention as the Dependent Variable

APPENDIX B

TABLES

TABLE 1

ANOVA SUMMARY TABLE FOR PASSIVE AVOIDANCE RESULTS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Age	2	167621.17	83810.58	9.65	.0002
Subj. in Age	68	590453.47	8683.14		
Trials	1	1060475.38	1060475.38	144.43	.0001
Trial X Age	2	64024.12	32012.06	4.40	.0156
Trial X Subj. in Age	68	494636.68	7274.07		
Trials @ 3 months	1	564124.44	564124.44	77.55	.0001
Trials @ 12 months	1	409555.65	409555.65	56.30	.0001
Trials @ 30 months	1	150819.40	150819.40	20.73	.0001
Trial X Subj. in Age	68	494636.68	7274.07		
Age @ Pre-Shock	2	14228.63	7114.32	.90	n.s.
Age @ Post-Shock	2	217417.15	108708.57	13.82	.001
Within cells	138	1085090.15	7862.97		

TABLE 2

ANOVA SUMMARY TABLE FOR MALATE + GLUTAMATE-DRIVEN OXIDATIVE METABOLISM OF NONSYNAPTIC MITOCHONDRIA.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Age	2	1261.212254	630.606127	2.01	0.1730
Error	13	4070.525344	313.117334		
Total	15	5331.737599			

TABLE 3

ANOVA SUMMARY TABLE FOR MALATE + GLUTAMATE-DRIVEN OXIDATIVE METABOLISM OF SYNAPTIC MITOCHONDRIA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Age	2	72.77540757	36.38770378	0.13	0.8757
Error	11	2980.22875655	270.92988696		
Total	13	3053.00416412			

TABLE 4

ANOVA SUMMARY TABLE FOR SUCCINATE-DRIVEN OXIDATIVE METABOLISM OF NONSYNAPTIC MITOCHONDRIA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Age	2	2801.829829	1400.914915	3.49	0.0612
Error	13	5219.556056	401.504312		
Total	15	8021.385885			

TABLE 5

ANOVA SUMMARY TABLE FOR SUCCINATE-DRIVEN OXIDATIVE METABOLISM OF SYNAPTIC MITOCHONDRIA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Age	2	1791.647686	895.823843	2.11	0.1677
Error	11	4672.057330	424.732485		
Total	13	6463.705016			

TABLE 6

REGRESSION MODELS FOR RETENTION TIME USING SYNAPTIC AND NONSYNAPTIC MEASURES OF OXYDATIVE METABOLISM AS PREDICTORS

Predictors in Model	R^2	C(p)	Variables in Model
1	0.21463863	3.43263	SSR[a]
1	0.00676885	6.45862	NSSR[b]
1	0.00199138	6.52816	SMR[c]
1	0.00003540	6.55664	NSMR[d]
2	0.50438473	1.21475	SSR SMR
2	0.22101917	5.33974	SSR NSMR
2	0.21876665	5.37253	SSR NSSR
2	0.03427175	8.05825	NSSR NSMR
2	0.00816189	8.43834	SMR NSSR
2	0.00201173	8.52787	SMR NSMR
3	0.50514279	3.20371	SSR SMR NSSR
3	0.50455873	3.21221	SSR SMR NSMR
3	0.30633909	6.09773	SSR NSSR NSMR
3	0.04357525	9.92282	SMR NSSR NSMR
4	0.51913675	5.00000	SSR SMR NSSR NSMR

[a] Synaptic mitochondria, succinate used.

[b] Nonssynaptic mitochondria, succinate used.

[c] Synaptic mitochondria, malate + glutamate used.

[d] Nonssynaptic mitochondria, malate + glutamate used.

TABLE 7

MULTIPLE REGRESSION SUMMARY TABLE USING RESPONSE TIME AS THE CRITERION AND OXYDATIVE METABOLISM AS PREDICTORS

Source	DF	Sum of Squares	Mean Square	R-square	F Value	Prob>F
Model	2	52990.30417	26495.15208	0.5044	4.580	0.0425
Error	9	52068.99020	5785.44336			
Total	11	105059.29437				

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	303.911487	85.15606494	3.569	0.0060
SSR[a]	1	-4.829688	1.59899905	-3.020	0.0145
SMR[b]	1	4.595039	2.00323304	2.294	0.0475

[a] Synaptic mitochondria, succinate used.

[b] Synaptic mitochondria, malate + glutamate used.

TABLE 8

REGRESSION MODELS USING SYNAPTIC ACOA AS CRITERION AND SYNAPTIC MEASURES OF OXYDATIVE METABOLISM AND CoA AS PREDICTORS

Predictors in Model	R^2	C(p)	Variables in Model
1	0.1229	1.84713	SSR[a]
1	0.0340	2.84560	SMR[b]
1	0.0176	3.02991	SCO[c]
2	0.2538	2.37831	SCO SSR
2	0.1320	3.74564	SSR SMR
2	0.0660	4.48635	SCO SMR
3	0.2875	4.00000	SCO SSR SMR

[a] Synaptic mitochondria, succinate used.

[b] Synaptic mitochondria, malate + glutamate used.

[c] Synaptic CoA

TABLE 9

MULTIPLE REGRESSION SUMMARY TABLE FOR THE EQUATION USING SYNAPTIC ACoA AS THE CRITERION AND OXIDATIVE METABOLISM AND SYANPTIC CoA AS PREDICTORS

Source	DF	Sum of Squares	Mean Square	R-square	F Value	Prob>F
Model	2	0.14359	0.07180	0.2538	1.530	0.2679
Error	9	0.42224	0.04692			
Total	11	0.56583				

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	0.945593	0.33873656	2.792	0.0210
SCO[a]	1	-0.378924	0.30166454	-1.256	0.2407
SSR[b]	1	-0.006239	0.00369684	-1.688	0.1257

[a] Synaptic CoA

[b] Synaptic succinate-driven oxidative metabolism

TABLE 10

REGRESSION MODELS USING NONSYNAPTIC ACOA AS THE CRITERION AND NONSYNAPTIC MEASURES OF OXYDATIVE METABOLISM AND CoA AS PREDICTORS

Predictors in Model	R^2	C(p)	Variables in Model
1	0.0777	0.52609	NSCO[a]
1	0.0298	0.96905	NSMR[b]
1	0.0034	1.21360	NSSR[c]
2	0.0827	2.47971	NSCO NSMR
2	0.0808	2.49781	NSCO NSSR
2	0.0694	2.60286	NSSR NSMR
3	0.1346	4.00000	NSCO NSSR NSMR

[a]Nonsynaptic CoA

[b]Nonsynaptic mitochondria, malate + glutamate used.

[c]Nonsynaptic mitochondria, succinate used.

TABLE 11

MULTIPLE REGRESSION SUMMARY TABLE FOR THE EQUATION USING NONSYNAPTIC ACoA AS THE CRITERION AND OXIDATIVE METABOLISM AND NONSYANPTIC CoA AS PREDICTORS

Source	DF	Sum of Squares	Mean Square	R-square	F Value	Prob>F
Model	1	0.01344	0.01344	0.0777	0.843	0.3802
Error	10	0.15946	0.01595			
Total	11	0.17290				

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	0.243242	0.09279262	2.621	0.0255
NSCO[a]	1	0.269157	0.29317744	0.918	0.3802

[a] Nonsynaptic CoA

TABLE 12

ANOVA SUMMARY TABLE FOR ACoA CONCENTRATIONS
OF SYNAPTIC MITOCHONDRIA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Age	2	0.02337225	0.01168613	0.25	0.7847
Error	13	0.61498815	0.04730678		
Total	15	0.63836040			

TABLE 13

ANOVA SUMMARY TABLE FOR ACoA CONCENTRATIONS
OF NONSYNAPTIC MITOCHONDRIA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Age	2	0.01199306	0.00599653	0.38	0.6915
Error	13	0.20535210	0.01579632		
Total	15	0.21734516			

TABLE 14

ANOVA SUMMARY TABLE FOR CoA CONCENTRATIONS
OF SYNAPTIC MITOCHONDRIA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Age	2	0.16021714	0.08010857	1.59	0.2409
Error	13	0.65436262	0.05033559		
Total	15	0.81457976			

TABLE 15

ANOVA SUMMARY TABLE FOR CoA CONCENTRATIONS
OF NONSYNAPTIC MITOCHONDRIA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Age	2	0.16870326	0.08435163	3.13	0.0777
Error	13	0.35038678	0.02695283		
Total	15	0.51909004			

TABLE 16

REGRESSION MODELS USING RETENTION TIME AS THE CRITERION AND SYNAPTIC AND NONSYNAPTIC MEASURES OF ENZYME CONCENTRATION AS PREDICTORS

Predictors in Model	R^2	C(p)	Variables in Model
1	0.22670496	3.85559	SCOA[a]
1	0.17405836	4.66272	SCO[b]
1	0.05913053	6.42471	NSCO[c]
1	0.01060670	7.16864	NSCOA[d]
2	0.46159412	2.25444	SCO SCOA
2	0.28920838	4.89733	SCOA NSCO
2	0.26402621	5.28340	SCOA NSCOA
2	0.26117086	5.32718	SCO NSCOA
2	0.19497477	6.34205	SCO NSCO
2	0.06047340	8.40412	NSCO NSCOA
3	0.51407999	3.44977	SCO SCOA NSCOA
3	0.47964395	3.97771	SCO SCOA NSCO
3	0.30562016	6.64572	SCOA NSCO NSCOA
3	0.29752028	6.76990	SCO NSCO NSCOA
4	0.54341646	5.00000	SCO SCOA NSCO NSCOA

[a] Synaptic mitochondria, ACoA.

[b] Synaptic mitochondria, CoA.

[c] Nonsynaptic mitochondria, CoA.

[d] Nonssynaptic mitochondria, ACoA.

TABLE 17

MULTIPLE REGRESSION SUMMARY TABLE FOR THE EQUATION USING NONSYNAPTIC ACoA AS THE CRITERION AND OXIDATIVE METABOLISM AND NONSYANPTIC CoA AS PREDICTORS

Source	DF	Sum of Squares	Mean Square	R-square	F Value	Prob>F
Model	2	48494.75266	24247.37633	0.4616	3.858	0.0617
Error	9	56564.54171	6284.94908			
Total	11	105059.29437				

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	37.564866	59.54105450	0.631	0.5438
SCO[a]	1	189.955609	95.86371888	1.982	0.0789
SCOA[b]	1	233.117626	106.33171565	2.192	0.0560

[a] Synaptic CoA

[b] Synaptic ACoA

APPENDIX C

MALLOWS C(p) STATISTIC

Mallows' C(p) statistic is one of many methods used to determine an optimum multiple regression equation. The statistic has the form

$$C(p) = RSS_p / s^2 - (n-2p)$$

where RSS_p is the residual sum of squares for a multiple regression model having p parameters, including the intercept. s^2 is the residual mean square for the model containing all predictors, including the intercept. s^2 has the form

$$s^2 = RSS_A / (n-p)$$

where RSS_A is the residual sums of squares for the model containing all predictors.

When deciding whether a model having p predictors is an adequate predictor of the larger model, it is necessary to examine the value of C(p) relative to p. That is, a model with p parameters that is a good predictor of the larger model will have $C(p) \approx p$. This can be seen in the following equations. as previously stated,

$$C(p) = RSS_p / s^2 - (n-2p) \text{ and}$$

$$s^2 = RSS_A / (n-p).$$

Thus,

$$C(p) = [RSS_p(n-p) / RSS_A] - (n-2p).$$

Hence, as RSS_p approaches RSS_A, C(p) approaches p.

Given this description of C(p), its use can be easily explained. For a given multiple regression equation having P predictors, C(p) is calculated for all equations having *p* predictors, where *p* = 1 ... P. For each equation, C(p) is plotted, or compared, against *p*. Given the definition of C(p), it follows that the "best" model will be that one where C(p) is plotted closest to the line representing *p*, or in the case of comparison, where the difference abs[C(p)-*p*] is minimized.

VITA

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Candidate for the Degree of

Doctor of Philosophy

Dissertation: AGE RELATED CHANGES IN MEMORY, OXIDATIVE METABOLISM, COENZYME-A, AND ACETYLCOENZYME-A IN THE RAT FOREBRAIN

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