MEMORY CONSOLIDATION IN AVOIDANCE-CONDITIONED GOLDFISH:

CHANGES IN BRAIN PROTEIN-SYNTHETIC PATTERNS

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Montgomery, David W., Memory Consolidation in Avoidance-Conditioned Goldfish: Changes in Brain Protein-Synthetic Patterns. Master of Science (Psychology), May, 1971, 83 pp., 3 tables, 6 figures, bibliography, 82 titles.

Three groups of goldfish were prepared; naive, avoidance-conditioned and pseudo-conditioned animals. Five pseudo-conditioned fish were avoidance trained later and found to have no measurable acquisition of the avoidance conditioning paradigm.

Brain proteins synthesized immediately after each treatment were labeled with intracranially injected ^{3}H DL-leucine. Brains were removed for protein extraction and the proteins separated by gel electrophoresis. Following slicing, the amount of radioactivity present in each gel fraction was determined by liquid scintillation photometry.

Several protein fractions were found to have significantly different rates of synthesis when compared across groups. The possible involvement of these proteins in the memory storage process was discussed.

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THESIS

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

by

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Denton, Texas

May, 1971

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CHAPTER I

INTRODUCTION

Molecular biology was recognized as a distinct discipline within the biological sciences during the early 1940's, characterized by its approach to biological problems on an intracellular, or biochemical, level. Since that time there have been numerous attempts to apply the concepts and methodology of molecular biology to an explication of the cellular mechanisms involved in the process of learning and memory formation. Over the last three decades, three basic points of view regarding the involvement of macromolecules in this phenomenon have emerged (1). First, information might be stored within the physical structure of molecules such as proteins, nucleic acids, lipids, or carbohydrates. A precedent for such a view has been set by the explanation of the role of deoxy-ribonucleic acid (DNA) as the cellular repository of genetic information. All information required for the development, maintenance and replication of cells is coded by the sequence of base (deoxy-ribonucleotides) pairs within the molecule, with each three pairs consisting a "bit" of information. A further example of the recording of information

within the primary structure of a molecule is the case of messenger ribonucleic acid (mRNA). This molecule is formed using DNA as a template, and functions in translation, via the protein synthetic apparatus. Although mRNA is a single-stranded molecule, it too contains information in the sequence of the ribo-nucleotides linked together to form the compound.

There are also cases of information storage (recognitional, catalytic) within the tertiary structure or conformation of proteins. Enzymes, that is, proteins with catalytic properties, contain within their active sites the "information" needed to recognize and react with their specific substrates only. Antibodies have the capacity to recognize and react with invading substances foreign to the organism.

Another, more complex approach has been proposed by Linus Pauling (2) in his paper entitled "A Molecular Theory of General Anesthesia." He suggests that memory storage resides within the pattern of molecular orbital electrons of a certain compound or compounds rather than in the more gross physical structure.

The most widely held viewpoint is that macromolecules may affect or control the participation of various cortical neurons in the formation and firing of neural networks in response to sensory input. This view attempts to incorporate

the concepts of molecular biology into the classical explanation offered by neurophysiology.

E. Roy John (3) has recently proposed a statistical, non-deterministic theory of information storage and retrieval. He describes a model in which sensory input selects at random the neural circuits for reverbration. These are characterized by orderly firing of groups of neural cells as compared to the random basal discharge pattern of non-stimulated networks. During the course of repeated firing of the units in the network, a shift occurs in the cellular concentration of an hypothesized "critical substance." This substance is postulated to be required for the reactions resulting in memory storage. The result of this experience is considered to be alteration in the biochemical properties of the cells involved together in circuits. Consequently, a change occurs in the probability of coherent activity in appropriate neural populations when the input stimulus is re-presented. The argument is developed (3) that "the coherence or signal to noise ratio represents the significance or reliability of the information, while the specific information content is reflected by the average activity of a cellular ensemble through time."

Flexner, Flexner, and Roberts (4) have taken a position including parts of the first two approaches mentioned, as

well as the identification of a compound and system similar to that proposed by John. According to these workers, proteins are formed as a result of learning that have, directly or indirectly, an ability to facilitate the firing characteristics of synapses. The lability of biological macromolecules has always been a difficulty of the view that their structure is the memory storage form. To overcome this problem they (4) suggest that the synthesis of these proteins is a self-inducing system. That is, the proteins or their products function as inducers of the mRNA needed to re-synthesize themselves; as they are depleted they would trigger the process of synthesizing more of their kind. This would result in a self-perpetuating mechanism resistant to loss by degradation or accident. However, were the required mRNA lost, it would lead to loss of essential proteins and permanent loss of memory. Some difficulties are presented by this concept, namely that mRNA is believed at the current time to be merely a transcription of the information already present in the cell's DNA. Evidence will be presented in a later section that RNA base ratio changes may be associated with learning, but unless this is actually the case, then Flexner, Flexner, and Roberts (4) theory implies a change in the genetic material itself. An alternative is existence of consolidation

specific mRNA instead of mRNA specific to each learned behavior.

One further example of this second point of view will be discussed. Castellucci et al., in a series of studies on the habituation and dishabituation of the gill withdrawal reflex of Aplysia (5, 6, 7), report that the withdrawal reflex can be explained by alterations in the efficacy of the excitatory synapses between sensory and motor neurons. They conclude (5) that "a prerequisite for studying behavioral modification is an analysis of the wiring diagram underlying the behavior. We have indeed found that once the wiring diagram of the behavior is known, the analysis of its modifications becomes greatly simplified."

A third approach to the macromolecular involvement suggests that these are not directly related to the learning process but are required to maintain the metabolic machinery of the cell. Without a doubt, cells cannot function unless the appropriate macromolecular species are present. However, only these substances and their synthetic mechanisms appear to offer an explanation for the endurance of consolidated memory traces.

Before beginning a discussion of the specific data implicating proteins and other substances in the memory

storage process, this point should be made: regardless of the viability of the theory that learned information is stored within the structures of molecules, macromolecules are necessarily involved in the process at some stage. Protein synthesis and, concomitantly, mRNA synthesis, is the only known mechanism for the expression of genetic information. Further, almost all biochemical reactions are mediated by enzymes so produced. Whatever the course of the memory consolidation process, it must pass through the protein synthetic system at some point. This being the case, further studies of the correlation of protein synthesis during learning and information storage may provide a tool with which to manipulate and elucidate the mechanisms involved.

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CHAPTER II

LITERATURE REVIEW

During the period from 1900 to World War I, theories of a preserverative neural trace achieved prominence as a means of explaining some of the newly discovered phenomena of learning and forgetting. Although these theories varied in detail, in general a neural fixation or consolidation process was assumed to continue after an organism was no longer confronted with the learning stimulus. Muller and Pilzecker in 1900 (1) were the first to make a clear statement of such a consolidation theory. They postulated the existence of a preserverative neural trace or process, subject to extrenal interference and necessary for the fixation of the memory trace for recently acquired information.

Although it was known before this time that severe cerebral trauma was capable of producing memory loss of events immediately preceeding the trauma, it remained for Burnham (2) in 1904 to relate this phenomenon of "retrograde amnesia" to the consolidation process. In an analysis of two cases of retrograde amnesia due to cerebral trauma, he suggested that fixation depends on an ongoing physiological

process, that this process requires time for an event to become sufficiently fixed to be reproduced or remembered, and that interference with cortical processes during this period can prevent consolidation. Since then a large number of physiological agents have been found to produce retrograde amnesia. Glickman in his 1961 review of this area (3) reports that electroconvulsive shock, anoxia, extremes of temperature, anesthesia, and electrical stimulation of the brain will also produce this phenomenon. More recently, Albert (4, 5) demonstrated that spreading cortical depression resulting from the direct application of potassium chloride to the cortex results in memory loss. Sachs (6), and John, Tschigi and Wentzel (7) found that various other electrolytes will also disrupt cortical activity sufficiently to produce amnesia. Lee-Teng (8, 9, 10) has shown similar effects in chicks receiving subconvulsive shock. A more interesting case is the finding that intra-ventricular injections of a ribonucleic acid degradative enzyme, ribonuclease, will inhibit trace consolidation (11). This is one of the few examples of a substance with known, specific action that will produce retrograde amnesia. This enzyme selectively degrades RNA but would also be expected in vivo to prevent protein synthesis by degrading the mRNA needed. Agranoff and his group (12) have shown that

puromycin, an inhibitor of protein synthesis, will prevent the consolidation of memory without affecting the learning process. Muller and Pilzecker's hypothesis that the consolidation of memory is susceptible to external interference has been substantiated.

The degree of susceptibility to interfering agents appears to decrease with the passage of time since the last training trial. The work of Agranoff et al. (12, 13, 14) demonstrated that injections of puromycin immediately after the last avoidance training trial prevented the formation of a long lasting memory trace of the event. When the injections were made thirty minutes after the last trial, only a moderate decrease in memory measurable on the third day occurred. When injected one hour after the last trial, goldfish demonstrated no loss on day three. Injections before training affected neither learning or memory. Even though the consolidation of the memory trace was prevented, the animals show a decreasing recall gradient for several days afterwards. Similar results have been shown to occur in mice treated with puromycin (15, 16) and with cyclohexamide (17, 18). McGaugh (19) has also shown the presence of such a susceptibility timeline in mice treated with electroshock.

The above data suggests that there is another, short-

lived form. This form is not affected by any of the previously mentioned agents and will continue to endure for a period of hours to days, even when the formation of a long-lasting memory has been blocked. Once memory consolidation has occurred, it is well protected against external interference. It is now generally accepted that at least two forms of memory exist simultaneously, but it is not yet clear whether short-term memory is converted to long-term by the process of consolidation or if these are parallel processes. E. Roy John (20) has hypothesized the presence of a third form of memory which he calls a "holding mechanism." According to this point of view, short-term memory is a process parallel but different to long-term with the holding mechanism functioning to hold the trace for the consolidation process. This is supported by evidence presented by Albert (4, 5) showing that consolidation could be prevented by cathodal stimulation of the cortex, but could also be restarted by subsequent anodal polarization. Albert demonstrated with cats that spreading cortical depression would block the recall of information, but that if cathodal polarization of the medial cortex was used to block consolidation, recall was not impaired. This suggests two holding mechanisms with somewhat different functions. One,

affected by cathodal stimulation seems to be involved with the actual consolidation process, perhaps with the function of a template for the synthesis of a permanent storage mechanism. The other holding mechanism, affected by spreading depression but not cathodal polarization, appears to mediate recall during the consolidation period. The evidence presented suggests the life term of the recall holding mechanism to be about three hours, while the time required for consolidation to be about five hours.

Evidence Relating Proteins And Memory Consolidation

Puromycin, shown by several workers to selectively affect the consolidation of memory, (12, 18) inhibits protein synthesis almost immediately after injection and lasts up to 16 hours, depending on the dosage used. Yarmolinsky and De La Haba (21) and Nathans (22) report that this antibiotic inhibits protein synthesis by competing with amino acids for the amion-acyl terminus of transfer ribonucleic acid (tRNA). Once attached to the tRNA it becomes incorporated into the growing polypeptide chain at the ribosomal level. This compound does not have the structure needed for an amino acid to attach to its free end, so the polypeptide is released prematurely as peptidal puromycin. Injections of the two moieties comprising the

puromycin molecule, puromycin aminonucleoside and O-methyl-tyrosine, produced no memory deficits. In addition, no behavioral side effects are attributable to this drug.

Acetoxycyclohexamide and cyclohexanide, drugs that function to prevent memory trace fixation, are also inhibitors of protein synthesis at a different level of cell function (22, 23). These drugs inhibit the transfer of amino acids to transfer RNA, thus, unlike puromycin, preventing the formation of peptide bonds.

This data tends to indicate that continued protein synthesis is affected by these drugs and that it is essential to the consolidation process that protein synthesis occur. Results presented by Lim and Agranoff (24) show that in the goldfish, turnover-time for proteins was much longer for brain tissue than for any of the other organs studied. The theory that information might be coded by the structure of macromolecules would seem more plausible in the light of this data.

Complications in interpreting the above results are presented by the studies showing that intercranial injections of saline two months after puromycin treatment (but not acetoxycyclohexamide) restored the memory lost (25). Puromycin dihydrochloride neutralized with the bases of potassium, lithium, calcium, or magnesium failed to block

the expression of maze learning in mice. This is opposite to the effect of puromycin neutralized with sodium (26). It was suggested by these investigators that it may be due to cationic binding at anionic membrane sites, with resulting exclusion of sufficient peptidal puromycin to make it effective in blocking memory. In any event, it is difficult to understand how small doses of saline could have the capacity to restore memory unless puromycin serves to prevent expression rather than consolidation. This latter approach appears to be incorrect because injections of the drug immediately before recall of a previously learned task have no effect on the efficiency of recall (13). Adequate explanation will have to await more data on this point.

To further complicate the issue, Barondes and Cohen (27) and Flexner and Flexner (28) reported that when both puromycin and acetoxycyclohexamide were injected simultaneously in mice trained to a criterion of 9 out of 10 in a "Y" maze, no effect on the consolidation of memory occurred. A potential explanation for this is that in the case of overlearning, memory consolidation might begin before the training session is complete. Davis (13), however, showed that in goldfish memory consolidation did not begin until the animal was removed from the training tank. A study to

see if this is also the case for mice might clarify the issue. Glassman (29) suggested that the protective effect of acetoxycyclohexamide on memory, when given with puromycin, might be a result of prevention of the ribosomal disaggregation caused by release of peptidal puromycin. This would occur because binding of amino acids and puromycin to tRNA would be prevented by acetoxycyclohexamide.

The relationship between inhibitors of protein synthesis and memory trace formation is by no means conclusive, for other effects of puromycin and the cyclohexamides have been demonstrated. Bondeson, Bevitz, and Edstrom (30) reported that puromycin reduced neural action potentials in the carp fish by 25%. Furthermore, it affected the activation of of phosphorylase by inhibiting 3'5' cyclic AMP (29).

Adair et al. (31) carried out a complex series of studies on the rate of tritiated uridine incorporation into the polysomes of mice trained in various ways. They found that a great increase occurred in the rate of incorporation in mice that had been avoidance-conditioned. However, no increase occurred in animals that were classically conditioned, even though they were as active as those avoidance-trained. Further, no increase occurred in animals that had been previously trained and required to perform the task again for 15 minutes. Since these animals were submitted to the

same degree of stress as the trained groups, stress is ruled out as an explanation. The data also indicated an increased synthesis of either messenger RNA or ribosomal RNA. They suggested that this seemed to "signal the beginning of the synthesis of either a new protein or proteins or an increased rate of synthesis of proteins that are continuously made" (31). It is possible as well that specific proteins might be needed to facilitate synaptic transmission of neurons involved in memory networks.

It is conceivable that this might be the case, for newly synthesized proteins were not inhibited in the process of reaching nerve endings (33) by injections of acetoxycyclohexamide. The protein that appeared at the nerve endings was synthesized before injection, rather than being formed later. Droz and Barondes (34), using electron microscopic radioautographs of nerve ending fractions from mouse cerebrum, showed that radioactive protein is associated with nerve endings 15 and 90 minutes after intracerebral injections of tritiated leucine and lysine. This was felt to be consistent with synthesis of proteins at the nerve endings or rapid transport of particulate protein to nerve endings. Such a synthesis would have to be ribosomal in nature, because acetoxycyclohexamide has been shown not to inhibit nuclear protein synthesis (Edstrom 35, 36).

Barondes and Cohen (37) have reported a case of retreival of "lost" memory similar to that discussed. Animals were brought to a state of arousal by either electric shock or administration of amphetamines or steroids before the short-term memory of training disappeared. Under these conditions, acetoxycyclohexamide-produced loss of memory was reversed. A similar effect on learning was shown by injection of magnesium pemoline, a mild stimulant (38). This drug appeared to enhance avoidance conditioning in rats as well as decreasing the rates of RNA and protein synthesis. Unfortunately, it could not be determined from the data whether this was a performance improvement or an increase in the rate of learning. Gaito et al. (39, 40, 41) studied water maze learning in rats and its effects on DNA, RNA and protein synthesis in the medial ventral cortex. They found that during learning the rate of RNA and of protein synthesis was less than that of the untrained controls. This suggests that increased rates of synthesis are not required for acquisition and is in agreement with the failure of puromycin to affect learning. It was also found that the level of shock used affected the rate of RNA and protein manufacture, with higher values decreasing the rate. Further, motor areas of the cortex demonstrated considerably greater turnover rates than did the areas

actually involved in the acquisition of the response. This is consistent with the data presented by Hyden (42) and Hyden and Lange (43) that during learning of reversal of handedness the rate of protein synthesis was greatly increased. Such an increase in rate would seem to be a logical consequence of increased neuronal activity.

There is other evidence correlating protein systhesis and the information storage phenomenon. Ungar and Ocequerra-Navarro (44), Ungar (45), and Ungar and Irwin (46) demonstrated that habituation to a startle response may be transferred to another mouse by injections of a brain extract from trained animals. The active ingredient appears to be a short chain polypeptide. Based on the preference shown by rats for dark rather than light enclosures, Ungar, Calvan and Clark (47) were able to transfer an acquired fear of dark to naive animals by means of a brain extract. The degree of transfer was found to depend on three factors: the amount of extract administered, the length of training the donors received, and the interval between training and brain removal. The last is particularly interesting, for best results were obtained when the donors were not killed until several hours after training. This suggested that the initial synthesis of the transfer factor began at the end of training and lasted for several hours afterward.

Characterization of the active fraction by enzymatic degradation indicated that the compound was a short-chain peptide of six to ten amino acid residues. This may have been the active region of a hormone, such as adreno-corticotrophic hormone, perhaps exerting some effect other than transfer of information. Bohus and De Weid (48) found that the polypeptide chain comprising the first ten amino acids of the ACTH molecule inhibited extinction of a shuttle box avoidance response in rats. However, when the phenylalanine molecule in the seventh position is replaced by its dextrorotatory form, extinction is facilitated. Whether these are similar compounds is not know, yet both are proteins affecting the memory process.

Before leaving the topic of Ungar's transfer factor, it must be said that some difficulty has been had on the part of other investigators in attempts to replicate this work (49). However a recent study (50) offers an explanation on the basis of the length of intertrial interval used. When a moderately long interval was used between each trial, reliable transfer of training was effected by means of a brain extract. When no interval was used, no transfer occurred.

Involvement of RNA And Other Macromolecules

Although the prime concern of this review is the role played by proteins in the memory consolidation process, it is appropriate to discuss briefly some evidence concerning other macromolecules as well. Two other species appear to be involved here. Ribonucleic acid, whose role in protein synthesis has been discussed, has received the most attention. More recently, the brain mucoids are emerging as a candidate for study.

The synthesis rate of RNA in neurons appears to be affected by stimulation and subsequent firing of the neuron. An increased concentration of neuronal RNA has been shown by Hyden (51) to accompany vestibular stimulation in the rat and by Rappoport and Daginawalla (52) to be increased by olfactory stimulation in the catfish. Wilson, Boggon, Zemp and Glassman (53) found similar effects to occur during learning. These results would appear to be merely a function of increased neural activity. However, Appel-white and Gardner (54) in a study of protozoan habituation reported synthesis of RNA that appeared to be a function of the habituation, rather than related to the contractile response elicited.

In a complex qualitative study, Machlus and Gaito (55, 56) performed hybridization between DNA and RNA from

avoidance-trained rats. The learning task emphasized the differences between the nonbehaving rats and the trained rats, by including a large amount of motor activity. This was to determine if unique species of RNA were produced during a gross behavioral event. Each animal was injected with intracranially tritiated orotic acid, an RNA precursor, 60 minutes before the beginning of training. Following the 90-minute incorporation period, the brains were removed and DNA extracted from one hemisphere and RNA from the other. The results of the hybridization studies suggested that an additional species of RNA appeared in the brains of the learning animals. More importantly, these species were different from those present in the brains of the non-behaving rats. When control was made for the motor activity (56) the same results were obtained. Unfortunately, the RNA extraction procedure used did not permit determination of the type RNA involved. Were it messenger RNA that was uniquely synthesized, its probable role would be the synthesis of new proteins.

Although these are the only reports to date suggesting a synthesis of unique RNA species during learning, there are several investigators reporting changes in the composition or structure of brain RNA synthesized during various behavioral tasks. Rappoport and Daginawala (52)

studied the effects of various chemical agents on the RNA produced by the olfactory brain areas of catfish. Free-swimming fish were subjected to various chemical odorants added to their water. The fish were sacrificed and the RNA extracted from the appropriate brain areas was subjected to base-ratio analysis. The same process was performed on fish to whom irritants were applied to the nares epithelium. The results of the base-ratio analysis indicated that in free swimming fish there was an increase in the amount of RNA extractable as well as a change in the RNA base-ratios. Further, this base-ratio change was specific to the odorant used. The fish to whom olfactory irritants were directly applied demonstrated an increase in the amount of RNA extractable but not in the base-ratios.

Shashoua (57) studied the base composition of RNA from the brains of goldfish subjected to various behavioral tasks. He reported that the RNA synthesized during the acquisition of new swimming skills has a uridine-to-cytidine ratio 20 to 80 percent higher than that of fish not subjected to learning. It was concluded that the RNA changes that took place were not specific to the particular information content being stored but were involved in the consolidation step of new information storage.

Similar base ratio changes have also been reported (58) to occur in planaria conditioned by shock to make a flexion response to a light stimulus. They used three shock- and light-pseudo-conditioned control groups and one conditioned group. The RNA in the head and tail ganglia of the animals was extracted and subjected to base ratio determination. It was reported that the conditioned group had significantly decreased A:C and A:U ratios in the RNA extracted from the head ganglia but not in that from the tail ganglia. No such changes occurred after light or shock alone. Although several possible interpretations for these data are possible, it seems that the synthesis of a different composition RNA occurred during conditioning.

Questions arise concerning the meaning of these base ratio changes. Since it is not likely that preformed RNA is being changed, the learning situations discussed must be serving as inducers of mRNA synthesis in some manner. As mRNA is synthesized from a DNA template, two alternative explanations are possible: first, the RNA is specific to each learned behavior. This would require that changes be made in the DNA itself by the learned behavior in order that mRNA could be formed. This does not appear to be the case; rather, mRNA produced probably has the function of

synthesizing protein necessary for the consolidation step. In such a situation, the mRNA would have a general consolidation function instead of being specific for each learning event. Such an interpretation does not elucidate the cause of the base-ratio changes observed by Rappoport and Daginawala (52) to be specific to the olfactory stimulant used. It appears that a better understanding of basic regulatory mechanisms must be reached before all these data can be satisfactorily explained.

These studies related RNA species and synthesis to the learning-consolidation process. However, in studies using inhibitors of RNA synthesis in an attempt to block the consolidation process, no decremental effects have been seen for the most part. Davis et al. (14) reported no effects of actinomycin-D on memory fixation in goldfish. Unfortunately this drug produces undesirable side effects as well as being highly toxic. The animals had to be tested within several hours after training for memory defects because the drug was fatal after this time. Since short-term memory lasts for this length of time or longer, the results should not be interpreted as an indication of no effect. In mice, Barondes and Jarvik (59) report no effect on inhibiting RNA synthesis by as much as 90% on the ability of the animals to learn an avoidance response.

Oshima, Gorbman and Shimada (60) found that injections of actinomycin-D in homing salmon blocked olfactory recognition of home waters for 4 to 7 hours after administration. The blockage was a temporary phenomenon because memory was recovered less than 48 hours after injection. This indicates that long-term memory is a continuous process rather than a long-lived RNA or protein. It appears from their work that a residual basis for olfactory memory function lasted longer than the interruption in the synthesis of RNA and protein. This brings to mind the holding mechanisms suggested by John (20) and Alberts (4, 5).

A number of studies report transfer of training to naive animals by means of RNA extracted from the brains of trained donors. The earliest reports of this nature were those on Planaria. Jacobson (61) and McConnell (62) report that learning in the true sense occurs in this species. McConnell relates in a subsequent review of this work (63) that transfer of training occurs when naive animals are fed trained ones, and that RNA extracted from trained donors could transfer this learning to naive ones when injected into them. Also, when trained animals were transected into two halves, the anterior would regenerate and the animal could then perform the previously trained task. However, when they regenerated in a solution containing

ribonuclease, no savings were retained. He postulated that the RNase had degraded the RNA essential to maintenance of the memory trace. Many attempts to replicate these experiments by other investigators have failed, but McConnell (64) suggested that these difficulties were due to a lack of environmental conditions required for learning in planaria.

There is sufficient evidence to indicate that this transfer of training phenomenon is genuine, yet the explanation for it is obscure. If memory storage is a process of continuing synthesis of mRNA and protein, then transfer of training by RNA and protein may represent an initiation of the mechanism with these compounds including their own synthesis. A commonly used indication that transfer has occurred is a decrease in response acquisition time, rather than the appearance of the full-blown response. It is possible that the RNA and protein transfer factors may merely facilitate the biochemical reactions necessary for learning to occur, or initiate at an earlier time than would normally be the case. This would serve to speed up the acquisition time for the response to be learned.

Before proceeding on to the role of brain mucoids in the memory process, a recent development in the area

of molecular biology should be discussed because of its implications for research in this area in the future. One of the basic precepts of molecular biology since the elucidation of the structure of DNA has been that RNA synthesis is a DNA dependent process, but that DNA synthesis or replication is self-dependent. This rule was considered to be inviolate; no new information could be incorporated into the cell's genetic information. Errors of replication could occur but these would cause misreading of the code, not addition to it. However in June, 1970, David Baltimore (66), Temin and Mitzutani (67), and Sol Speigelman (68) upset this cardinal belief by demonstrating that certain of the oncogenic viruses have an enzyme capable of synthesizing DNA on an RNA template.

The single DNA strand so produced then replicates its complement and becomes incorporated into the rest of the cell's genetic information. This revives the possibility that DNA might be the residence of memory. Just what the possible ramifications of this new evidence will be for the study of the molecular basis of memory and learning remains to be seen. Certainly it opens a new set of alternatives to be investigated, and possibly a new means of explanation.

Recently the brain mucoids have been proposed by Bogoch (69) to be involved in the process of memory storage and formation. Relating to the work previously mentioned, the incorporation of carbohydrate precursors into the brain gangliosides is also inhibited by puromycin. Further, he reported that certain brain proteins associated with membranes show quantitative changes in pigeons during learning. These are proteins rich in found carbohydrate moieties. During learning, not only do the amino acids compositions of these proteins vary quantitatively, but the number and kind of bound carbohydrate moieties vary to an even greater extent. According to Bogoch (69, p. 298), "The brain mucoids demonstrate sufficient heterogeneity, fixed location, appropriate development, change with pathology, recognition functions, biosynthesis, change with behavior, and change with learning, all consistent with the notion that they are directly involved in the biochemistry of learning and memory."

As indirect support of this possibility, Barondes (70) reported that there is extensive incorporation of carbohydrates into macromolecules at the nerve endings. Whereas protein may be transported from the perykarion, he suggested that this protein could be modified at the nerve ending by the addition of carbohydrate moieties. He proposed that

these carbohydrate-containing proteins could influence nerve-ending function very rapidly after the addition of the carbohydrates, since this reaction occurs at the nerve ending instead of the perykarion.

The previous review suggests that memory is consolidated or fixed in a long-term storate form after the completion of learning by a process involving protein synthesis. It has been demonstrated that the consolidation process takes place within a time period beginning immediately after the end of training. The susceptibility of the process to the effects of the inhibition of protein synthesis is limited to 30 minutes to 1 hour after cessation of training. It is suggested that immediately after learning is over, there commences a process dependent of the synthesis of certain proteins. Once a sufficient concentration of these is reached, the process is no longer dependent on continued synthesis to complete information storage. Were these proteins previously existing in sufficient concentrations and removed by consolidation from the cellular pool, blockage of protein synthesis should not produce its inhibitiory effect. The existence of these proteins, it is proposed, should be demonstrable by a

differential rate or novel synthesis during the period right after training ends.

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CHAPTER III

METHOD

Subjects

The Goldfish (Crassius auratus) was chosen as the experimental animal primarily because Agranoff and his research group (1) have established the time relationship between the end of training and the susceptibility of the memory consolidation process to inhibition of protein synthesis. Further, a reasonably simple apparatus and conditioning procedure has been designed by Bitterman (2) for use with goldfish.

Fish were obtained from Reeder's Fish Farm, Seagoville, Texas, and maintained in a 25-gallon aquarium filled with distilled water. The aquarium received constant aeration, and fish were fed daily with the commercial fish food used by Reeder's. Only fish weighing between 4 and 6 grams ($\bar{x}$ = 5.37 grams) were used.

Training

All animals were deprived of food for a period of 48 hours before use. The subjects were divided into three groups: 1. 21 fish were normal controls and received no

training, but remained in the acquarium until ^{3}H DL leucine incorporation, 2. the pseudo-conditioned control group (26 fish), which received the same sensory stimulation as the experimental group, but no avoidance learning, and, 3. the avoidance-conditioned group (30 fish).

Avoidance training and pseudo-conditioning of groups 2 and 3 was administered in a Bitterman-type shuttle box (Aquatic A-660) obtained from the Lafayette Co., Lafayette, Indiana. Cycle timing was programmed by a cam type timer and programmer (Model 5500) supplied with the shuttle box. The shuttle box was 4" X 8" X 11" clear plastic divided into two equal size compartments by an opaque partition with a doorway to permit movement from one side to the other. The top of the apparatus was stainless steel and had attached to it independent voltage grids for each side and a light source at each end. During conditioning a 3-volt, 0.5-milliampere shock was used as the unconditioned stimulus and a 15-watt light bulb at each end served as the conditioned stimulus. Switching of the cycle from one side to the other during training was performed manually by turning a selector to the appropriate side.

An avoidance training trial consisted of a one-minute cycle commencing with turning on the light at the fish's end of the box. Twenty seconds after the onset of the light,

constant current was applied to the voltage grids at the same end of the apparatus and remained on concomitantly with the light for an additional 20 seconds. At the end of this period (40 seconds after onset of CS light), both light and shock were terminated, followed by a 20-second rest period in the dark before beginning the next trial.

Each avoidance-trained fish received a minimum of 25 trials, divided into blocks of 5 trials, with a 2-minute rest period between each block. Training was terminated after 25 trials if a criterion of 8 correct responses in 10 consecutive trials was reached, or continued until this criterion was achieved. An avoidance (correct) response was scored if the animal avoided the shock by leaving the CS end of the box before onset of the shock. An escape response was scored if the animal left after the onset of the shock. Each response and its latency time was recorded. During training 5 fish were set aside for recall testing 4 days later and 5 were reserved for synthesis rate determination.

A pseudo-conditioning trial was designed to preclude learning of an avoidance response. Both shock and light began at the same time, with the light lasting for 40 seconds and the shock lasting for 4 seconds. This was

followed by a 20-second rest period in darkness before the next trial. During each trial the cycle was manually switched from side to side to follow the fish and prevent the possibility of escape from the stimuli. Each fish was given 25 trials as outlined above, with 2-minute rest periods every 5 trials. After the 25 trials were completed, the shock power was turned off and the animal given 5 more trials with the light and dark phases only. This arrangement was made because no avoidance-trained animal received shock during the last 5 trials. The purpose of this group was to control for the protein synthesis that would be expected as a result of the sensory stimulation alone. Each animal received the mean amount of shock (95 seconds) and light (1200 seconds) received by the avoidance-trained group. During training 5 fish were set aside at random for later avoidance training and 5 were reserved for total rate of sythesis determination.

Radioactive Labeling

Immediately after the last training trial each fish was removed from the training box and injected intracranially with 10 microcuries of ^{3}H DL leucine (New England Nuclear Corp., Boston, Mass., specific activity 5.0 curies per millimole) diluted to 25 microliters with isotonic saline.

Injections were made with a Hamilton 100-microliter syringe at a shallow angle of entrance to prevent damaging the brain with the needle tip, and the solution expelled over the brain rather than into it. The animals were returned immediately to an enclosed portion of their aquarium to permit the consolidation process to occur (3) and watched closely for indication of brain damage. A period of 30 minutes was allowed for incorporation of the labeled leucine into proteins synthesized. This interval was based on the evidence (1) indicating that in the goldfish, the dependence of memory consolidation on protein synthesis largely disappears within 30 minutes after the cessation of training.

At the end of the incorporation period the animals were sacrificed by spinal cord transection just posterior to the brain case. The brain was removed by cutting forward on each side from the posterior margin of the brain case to the optic orbits, peeling off the cartilage and slipping a pair of forceps under the brain. Care was taken to ensure removal of the complete, intact brain. This procedure took approximately 30 seconds per animal. Each organ was placed in a separate vial in 0.5 mls. of cold isotonic saline and frozen at -20^{o}C until needed.

Preparation of Brain Homogenates

A very mild procedure was chosen to prevent denaturing brain protein, a common occurrence when trichloroacetic acid methods are used. Two brains at a time were thawed and pooled in 1 ml of cold 0.14 M saline, 0.01 M Tris, pH 7.2. Triton X-100 (Rhom and Haas) was added to the buffer in a concentration of 0.5% volume to volume to aid in dissolving the membrane-bound protein. Each sample was homogenized in a 5 ml capacity Potter-Eljeham tissue grinder with a motor-driven teflon pedstal for a total of 60 strokes. Homogenates were cooled in an ice bath for several minutes every 20 strokes to prevent heat buildup. Following homogenization, the samples were centrifuged for 10 minutes at 5000 X G in a refrigerated Sorvall RC2-B centrifuge to remove cell fragments and organelles, then stored at 2-5°C.

Determination of Protein Concentration

The protein concentration of each homogenate in milligrams per ml. was determined by the method of Lowry, Rosebourough, Farr, and Randall (4). Due to the sensitivity and narrow range of this quantification method, each sample was diluted by a factor of 100 with glass distilled water in order to reduce the protein concentration to a measurable

level. Each homogenate was run in duplicate in conjunction with standards of recrystallized bovine albumin at concentrations of 50 and 100 micrograms per ml. These standards served as a means of monitoring any deviation from standard in the reagents prepared each time. The absorbance of each sample in optical density units at a wave length of 750 millimicrons was measured with a Coleman-Hitachi Model 124 double-beam spectrophotometer. Prior to each protein determination, glass distilled water was placed in both sample and reference curettes (1cm quartz, 4ml capacity) and the instrument zeroed and set on 100% transmittance. A standard curve of optical density plotted against concentration in micrograms per milliliter was prepared by this method using recrystallized bovine albumin dissolved in glass distilled water. The mean of the two optical density measurements for each brain homogenate was compared to this standard curve and concentration read in terms of micrograms per milliliter. The obtained value was multiplied by the dilution factor of 100 to obtain the protein concentration of the sample.

Polyacrylamide Gel Disc Electrophoresis

Each brain homogenate was electrophoresed in duplicate using a Canalco Model 200 constant rate source and model

6 bath chamber. A tris-glycine buffer system, ph 8.3 was used in conjunction with a separating gel with a concentration of 7.5% acrylamide, ph 8.9. Bromophenol blue at .01% was used as a tracking dye, and amido schwartz, 1% in 7% acetic acid was used as a protein stain. This system was modified in a number of particulars from that devised by Davis (5).

Much longer separating gels than usual were prepared to permit a run length of 4.5 centimeters. In preliminary test runs, the standard 3.0 separating gel for serum proteins was used. A large number of protein bands were visible in the stained gels; however, they were too close together to permit adequate discrimination of individual bands. As these gels were to be sliced into sections for radioactivity determination, it was felt that greater separation between bands would be desirable. Over a number of trials a run length of 4.5 mm was found to give optimum separation between protein components without decreasing the sharpness of resolution.

On the basis of test runs, 750 micrograms of protein was determined to be the maximum amoutn that could be separated with good resolution. However, because of the relatively low protein concentration of the brain homogenates, 750 µg comprised a large fluid volume. The sample gel as

normally prepared will not polymerize when more than 1 part protein solution is added to 30 parts gel. To permit the separation of the desired quantity of protein, the volume of the sample gel layered in the tubes was increased from the standard of 0.1 mls. to 0.4 mls. To further increase the amount of sample volume that could be added, while maintaining polymerization, the concentration of the sucrose used was increased from 40% to 47%. However, the amount of sucrose solution used was decreased from 4 parts in 8 to 3.4 parts, with the remaining volume of fluid being made up by the addition of the homogenates to the gel. These changes allowed mixing as much as 0.080 mls. of homogenate to 0.320 mls. of sample gel (1 part in 5) without adversely affecting its ability to polymerize.

As a result of the increase in volume, hence length, of the sample gel, the volume of the stacking gel had to be increased to 0.40 mls. to maintain the stacking of proteins into a thin disc at the beginning of separation (6). To accommodate a separating distance of 4.5 cm. while leaving sufficient separating gel to prevent over-running the tube, this gel was increased to 5.0 cm. The gel column as finally standardized had a total length of 9.0 cm.

During the trial electrophoresis runs the cleanliness of the glass tubes appeared to be the major determinant of the ease with which the gels could be removed without damage. After each run the tubes were washed thoroughly in warm, soapy water by running a cotton-tipped applicator through them. After rinsing, they were left to soak overnight in a solution of potassium dichromate-sulfuric acid to remove any remaining particulate matter and soap film. The tubes were then rinsed well with distilled water, then with a 1% solution of Canalco column coat, and oven-dried.

Gel Column Preparation

All gels were prepared immediately before use to insure optimum performance. The volumn of homogenate needed for two gel sample layers was calculated from its protein concentration and placed in a vial. Gel solution was prepared and added to the vials in the amount needed to bring the total volumn to 0.80 mls and mixed by shaking.

Six clean glass tubes were inserted sample end down into tube caps and placed in the preparation rack, care being taken to position them vertically. The tubes were marked with the appropriate code number for each of the three samples to be separated, and 0.4 ml. of the sample solution was added to the tubes. Each sample was run in

duplicate. Following this, 0.10 ml. of water was allowed to gently run down the tube wall and layer over the gel solution. This was accomplished with a 1.0 ml. tuberculin syringe fitted with a piece of capillary tubing over the needle. The end of the tubing was placed about 1 mm. above the surface of the gel and layering performed. A flat, smooth interface resulted, as required for the formation of sharp, non-distorted protein bands. The tubes so prepared were then photopolymerized for 30 minutes within one cm. of a fluorescent light source. When polymerization was complete, the water was drained from the tubes and washed with gel solution to remove all traces of water.

Stacking gel solution was then layered over the sample gel layer until the bottom of the meniscus rested on the first file mark, about 0.40 ml. This gel was water-layered as before, to ensure formation of even-sized pores; photo-polymerization was carried out slowly at a distance of 30 cm. from the light source for 15 minutes then moved to within 1 cm. of the light for an additional 15 minutes.

After this step, the water was drained off, the gel surface rinsed twice with separating gel solution, and the tube filled, leaving a bead of excess gel on top. A small square of saran wrap was used to cover the top of the tube, allowing the excess gel to run down the side.

This prevented the formation of air bubbles at the end, as well as preventing gel shrinkage from water evaporation. The tubes were then placed in the dark and allowed to chemically polymerize for 45 minutes.

Electrophoresis Running Procedure

Immediately upon final polymerization the tubes were carefully removed from the tube caps and sample gel was inserted into the upper bath grommets. The lower bath reservoir, with electrode connected, was placed in an ice bath and filled with cold Tris-glycine buffer. The lower ends of the tubes were wetted with buffer to prevent the trapping of air bubbles and the tubes lowered into the lower reservoir. The top of each tube was covered with a drop of buffer and 250 mls. of buffer containing 0.01% bromophenol blue tracking dye and was poured into the upper reservoir. The upper electrode was connected and the power turned on to a current level of 1 milliampere per tube. Electrophoresis was continued at this level until the tracking dye band migrated well into the stacking gel, whereupon the current was increased to 3.0 milliamperes per tube for the duration of the run. The lower initial current level was chosen to prevent loss of protein into the upper buffer by ohmic-heating-induced convection

currents. The run was terminated when the tracking dye reached a file mark on the tube, indicating that the 4.5 cm. separating distance had been achieved.

Band diffusion began as soon as the current was turned off, making it imperative to remove the gels and fix them as quickly as possible. This was achieved within a total elapsed time of 1 to 2 minutes for all six gels by rimming the inside of the tube at the separating gel end with a long 22 gauge needle attached to a water line. By forcing a stream of water around the gel, they were then removed and the separating gel was placed in fixative stain of 7% acetic acid with 1.0% amino black. All gels were stained for a period of 2 hours, then removed and stored in 7% acetic acid until destained.

De-Staining

De-staining tubes (7 cm. X 7 mm, I.D.) were cut from soft glass tubing and one end was partially closed by flaming. Six of these tubes were capped at the constricted end and inserted into the preparation rack. They were filled to a height of 1 cm. with stacking gel solution and photo-polymerized for 35 minutes at a distance of 1 cm. from the light source.

Following polymerization the tubes were filled with cold acetic acid (7%) and the gels slipped into them, tracking-dye-end down. These were inserted into the bath as before, and 7% acetic acid used as the electrolyte. The power supply was placed in the destain mode and the amperage adjusted to 12 milliamperes per tube. Completion of destaining was determined visually and the tubes were removed for storage in acetic acid needed for radioactivity determination.

Radiation Counting

Each gel was placed in an egg-slicer type gel cutter obtained from Canalco and cut into 29 sections of 1.5 mm. thickness. Care was taken during this step to ensure that each gel was sliced in the same way so that any given protein band would correspond to the same slice number every time after background radiation was subtracted.

The gel sections were placed in numbered scintillation vials and prepared for counting by the method of Tishler and Epstein (7). In this procedure, each gel slice was covered with 0.20 ml. of 30% hydrogen peroxide and heated at 55°C in a water bath until completely dissolved. During the process, the dye used for staining was oxidized to a colorless product, preventing quenching of emitted photons by the dye.

Following digestion, 10 mls. liquid scintillation "cocktail" was added to each vial. The "cocktail" was prepared by dissolving in 1 liter of spectroanalyzed toluene 3.0 gms of PPO (2, 5 diphenyloxazole) and 0.01 gm. of a secondary fluor, POPOP (1, 4-di-- 2-(5-diphenyloxazolyl) benzene) plus 15% by volume of Beckman BBL-3 solublizer (Beckman Instruments, Fullerton, Cal.).

The samples were placed in a Beckman Model LLS-100 Liquid Scintillation Counter with direct data readout module. The machine was programmed to count tritium using the whole-spectrum ^{3}Hisoset in the auto-quench mode. No low sample reject was used and each sample was counted for 50 minutes to a pre-set counting error of 2%.

After subtracting background radiation, the number of counts per minute per sample was recorded.

Determination of Rate of Total Protein Synthesis

Three groups of 5 fish each were trained as previously described and their newly synthesized protein tritium was labeled. The brains were removed and pooled within groups. Total brain protein was extracted by method B of Lim and Agranoff (8). This involved homogenizing in 10% trichloroacetic acid (TCA), followed by centrifugation for 10 minutes. The

sediment was heated at 80°C in the presence of 10 mls. of TCA for 30 minutes and re-centrifuged. This pellet was washed twice each with 95% ethanol and ether, then resuspended in distilled water.

The obtained protein solutions were quantified in terms of micrograms of protein per milliliter of solution. One milligram of protein was spotted on each of 5 fiberglass filter disks (Reeve Angel, Clifton, N.J.; No. 934AH, 2.4 cm.), with 5 disks used for each group. These disks were oven-dried for 2 hours, then placed in vials with 10 mls. of "cocktail" (1 liter toluene, 6.0 gms. PPO, 0.010 gm. POPOP) and counted in the LS-100 for 50 minutes at a pre-set error of 0.2% in the auto-quench mode.

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CHAPTER IV

RESULTS AND DISCUSSION

Subjects

All fish were weighed prior to use and those weighing less than 4 grams or more than 6 grams were rejected, with the mean weight of animals used being 5.37 grams (Sd.=0.66gm.). In order to determine the consistency with which the brains were removed, each brain was weighed separately following extraction. For all animals used (N=56) the mean organ weight was 60.1 milligrams with a standard deviation of 5.4 milligrams, while none of the brains weighed less than 53mg. nor more than 66 mg. These data indicate that consistent removal of the brain was achieved.

Training

Avoidance conditioning was performed on 30 fish, as previously described. During training 10 fish were set aside at random for use in the recall study and the determination of rate of total protein synthesis. In addition, 16 fish were sacrificed for the gel electophoresis studies. Of the remaining animals, 3 were rejected for failure to

obtain criterion within 50 trials, and one died from brain damage produced by the intra-cranial injection procedure.

The data presented in Figure 1 shows that a typical acquisition pattern was followed, with an initial period of rapid improvement followed by a leveling off of the curve. During the last 10 trials a mean value of 87.7% correct responses was obtained, corresponding well with the values reported by Agranoff et al. (1, 2). The experimental criterion of 8 out of 10 correct responses was reached in 31 trials with a standard deviation of 7.4 trials.

It has been reported in the literature that subconvulsive shock is capable of blocking the consolidation of newly acquired behaviors (3, 4). As electric shock was employed in the avoidance conditioning procedures, it was felt necessary to determine if recall of the avoidance training had been affected. The 5 animals chosen for this purpose during conditioning were tested for response saving 4 days later. The data in Figure 1 shows an initial baseline or savings of 40% correct responses during the first 5-trial block, with criterion reached in 18 trials, ±2. This is lower than the 50% savings reported by Agranoff on day 3 (1, 2), probably a result of the extra day between training and recall in this study. It is sufficient however, to

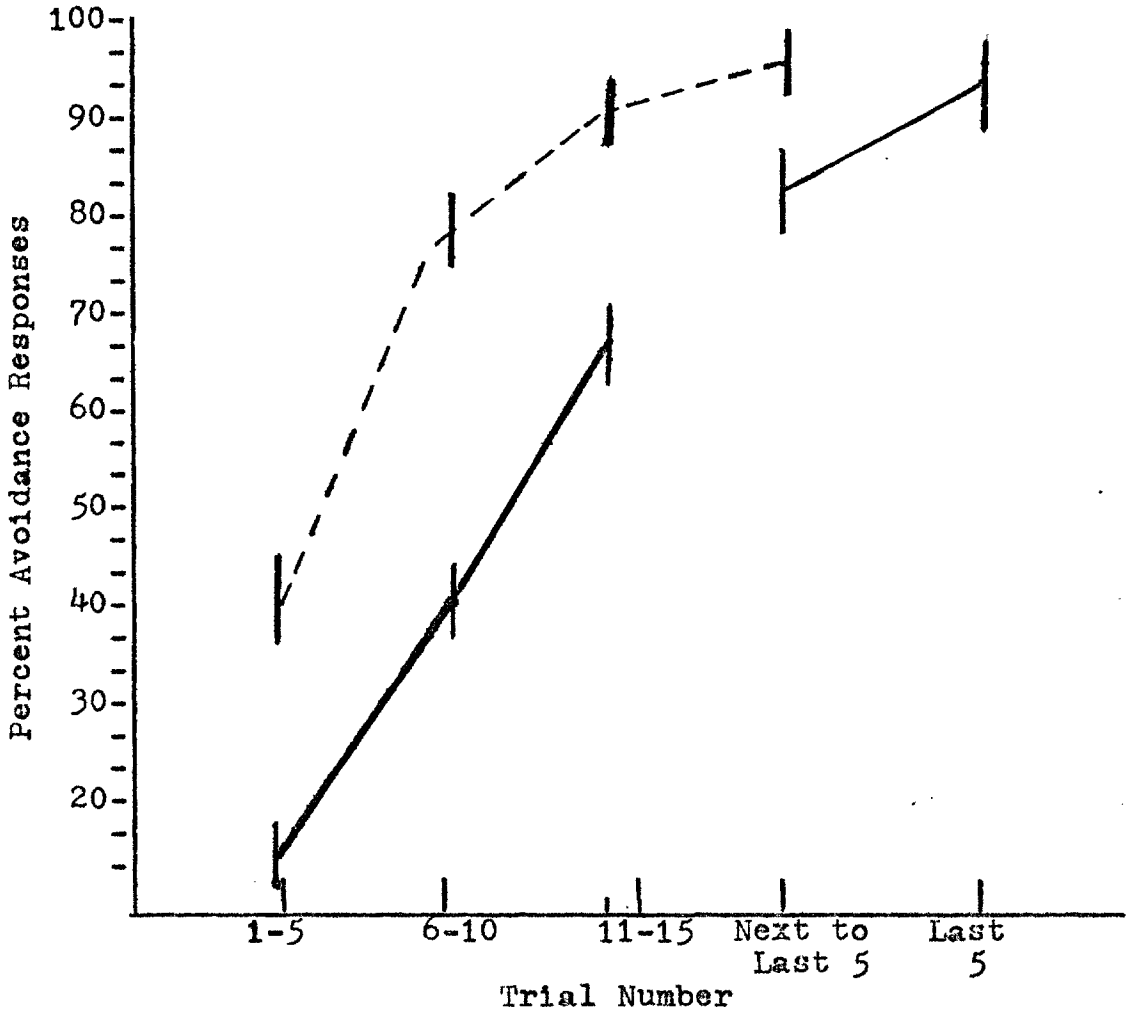

Fig. 1- Total number of avoidance responses made by all subjects on each trial block expressed as the percentage of the maximum possible number of avoidance (80 avoidance responses possible per 5-trial block) responses. (----) represents responses made during recall, (____) responses during conditioning.

demonstrate that the consolidation process had not been prevented by the experimental stimuli.

Twenty-six animals were pseudo-conditioned, 16 of which were sacrificed for electrophoresis of brain protein. An additional 10 were set aside for rate of protein synthesis determination and for avoidance training. Since the pseudo-conditioned group was to serve as the major experimental control it was necessary to ensure that no learning of the avoidance conditioning paradigm had occurred. Five animals were trained the 5th day after pseudo-conditioning (Figure 2). This group achieved 18% correct responses on the first 5-trial block and reached criterion within a mean of 28 trials(Sd.=6.3), as compared to 31 trials for the group avoidance conditioned initially. The mean differences between these two groups were subjected to a two-tailed t test and found to be non-significant ($t=.94$, p .20). On this basis it was concluded that the pseudo-conditioned group learned neither the avoidance response nor sufficient related responses to affect their use as controls. It is not possible to state from this data that no learning occurred to be consolidated after pseudo-conditioning, but only that it was not the same learned by the avoidance conditioned group.

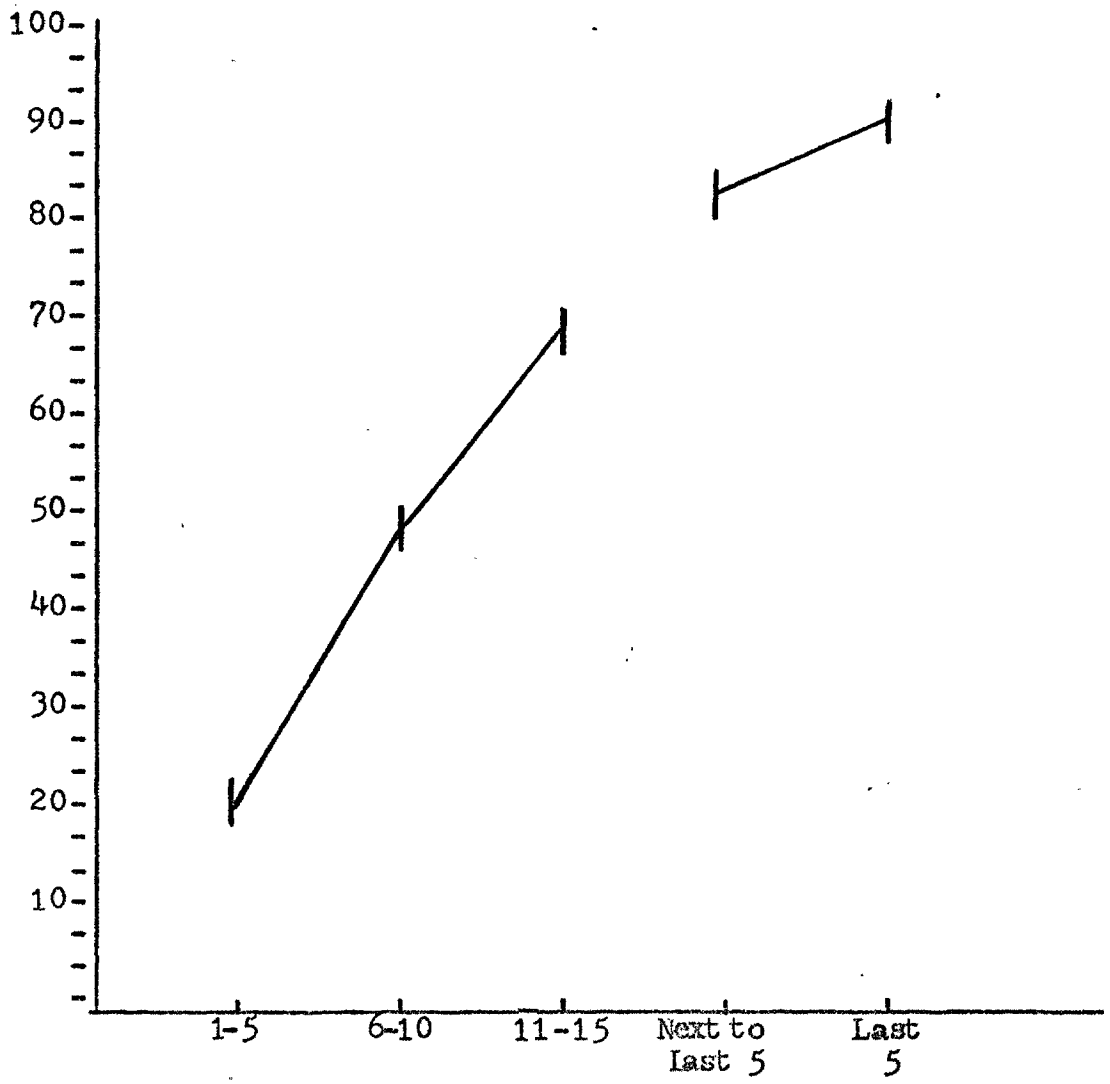

Fig.2- Total number of avoidance responses made by all subjects on each 5-trial block expressed as percent of maximum possible. Avoidance conditioning of 5 pseudo-conditioned fish.

Rates of Total Protein Synthesis

The brains of 5 animals from each experimental condition were removed following incorporation of tritiated DL leucine and pooled. These brains were homogenized and the total brain protein extracted and purified by the method of Lim and Agranoff (5) described in Materials and Methods. A comparison of the total protein yield per brain (Table 1) shows that the yield was virtually the same across groups. The protein extracted comprised 9.1% of the wet brain weight, which agrees well with the values reported for the procedures (5).

Table 1.

Protein Yield Per Brain Milligrams Pure Protein

Optical Density at 750 mu	Avoid. Cond.	Pseudo-Cond.	Non-Trained
ug. per ml. at 1:200	133 ug.	138 ug.	135 ug.
Total Yield in mg.	26.5 mg.	27.6 mg.	26.9 mg.
Yield per Brain in mg.	5.90 mg.	5.52 mg.	5.39 mg.

The purified protein from each group was diluted to a concentration of 1 milligram per milliliter with distilled

water and spotted on fiber glass filter discs. These were oven-dried for two hours and the amount of radioactivity present was determined in a Beckman LS-100 liquid scintillation system for 50 minutes each at 2% pre-set error. The number of counts per minute (minus background) per milligram of protein was calculated and reported in Figure 3.

The non-trained group exhibited the highest rate of synthesis, with the avoidance-conditioned animals and the pseudo-conditioned groups demonstrating a lower rate of synthesis. This represents a depression of protein synthesis of 18% and 20% respectively, but the results of the recall training indicated no adverse effects on memory consolidation.

There appears to be some disparity between these results and those published by other workers. Hyden and Lange (6) report an increase in the rate of protein synthesis in pyramidal neurons of the hippocampus during reversal of handedness experiments in rats. Adair (7) found an increased rate of messenger and ribosomal RNA synthesis following avoidance conditioning in rats, and suggested that this was preparatory to increased protein synthesis.

However, total protein synthesis has been shown to be sensitive to many physical stimuli in goldfish (8), precluding and attempt to ascribe the depressed synthesis to the training

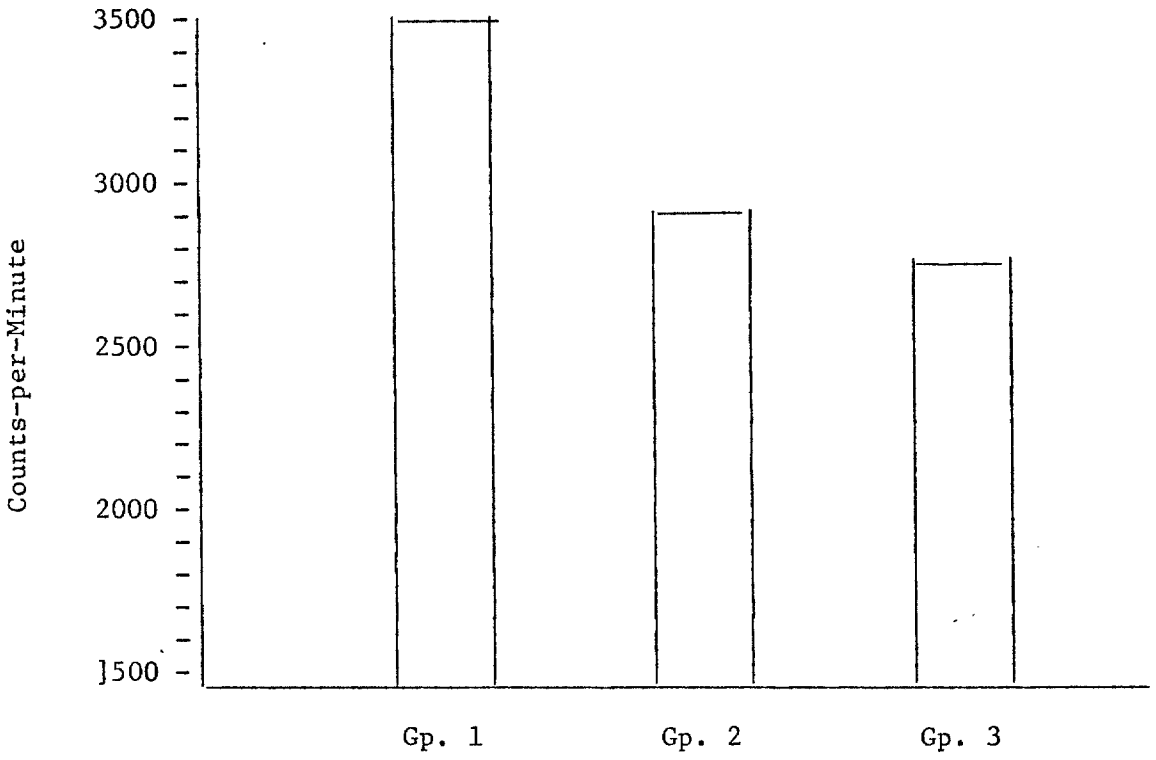

Fig. 3- Mean counts-per-minute per milligram of brain protein from non-conditioned fish (Gp. 1), avoid--ance conditioned fish (Gp. 2) and from pseudo-conditioned fish (Gp. 3).

procedures or to the consolidation process itself. Studies of the effects of protein synthesis inhibitors (1, 8, 9) on the formation of a long-term memory trace indicate that this process is not significantly affected by degrees of inhibition less than 80%. This suggests that any proteins required for consolidation to occur only need to be produced in catalytic quantities.

Gel Electrophoresis

The brains of 48 fish were used in this aspect of the study. Following training and the 3H DL-leucine incorporation period, brains were removed, weighed, and paired to produce 8 brain-pairs per experimental group. Each sample was homogenized and cleaned of cellular debris by centrifugation as described in Materials and Methods. The protein concentration was determined by the method of Lowry, Rosebrough, Farr and Randall (10) and two gels prepared from each sample containing 750 micrograms of brain protein. These gels were electrophoresed, stained and destained, then set aside until use.

The major difficulty presented in this study was ensuring that each gel slice, upon cutting, would contain the same protein fraction. In order to do this it was essential that all gels used had the same separation distance.

Each gel was measured to determine the separation distance achieved. This was performed by measuring with a millimeter ruler the distance between the center of the tracking dye band and the interface of stacking and separating gel layers. Any gel in which the total length of separation was more than 45.5mm. or less than 44.5mm. was rejected. In those cases in which neither of the duplicate gels fell within this range, the sample was run again. A further requirement was made that the protein bands be level, flat, and non-diffuse. Where this criterion was not met by either gel the sample was also re-run.

Slicing the gels was done with a wire slicer designed like an egg cutter. Great care was taken to align the gels in the same manner every time. Ink marks were made on the top of the cutter at three positions, corresponding to the point where the interface, a well separated band in the middle of the gel, and the tracking dye band were to be placed prior to cutting. Scintillation counting of radioactivity was carried out as already outlined.

An attempt was made to quantify the amount of protein present in each band by means of a Canalco Model "E" Microdensitometer (Canalco, Rockville, Md.). The large number of bands present in close proximity, as well as a lack of sufficient instrument sensitivity, made it difficult

to reliably correlate densitometric data obtained with the band or bands to which it belonged. For these reasons, no further efforts were made to quantify in this manner.

Visual inspection of the stained gels permitted counting of 18 separate bands of protein. These bands appeared in each gel regardless of experimental group of origin, and no bands were visible in one group that were not present in the others. Between-group differences in the amount of protein present in each band were not detectable, but only gross variations would be apparent by visual methods.

The number of counts-per-minute in each gel slice was calculated by subtracting background radiation from the obtained radiation and compiled. No attempt was made to convert this to disintegrations per minute, nor to calculate molar incorporation ratios because the quantity of protein in each band was not known. It was felt that since the purpose of this study was to compare the amount of radioactivity in each slice across groups, quantitation in these terms was not necessary.

The mean count-per-minute values for each slice are presented in Figures 4, 5, and 6. It can be seen that very similar profiles are presented for each group, with the peaks and troughs corresponding in most cases. In general,

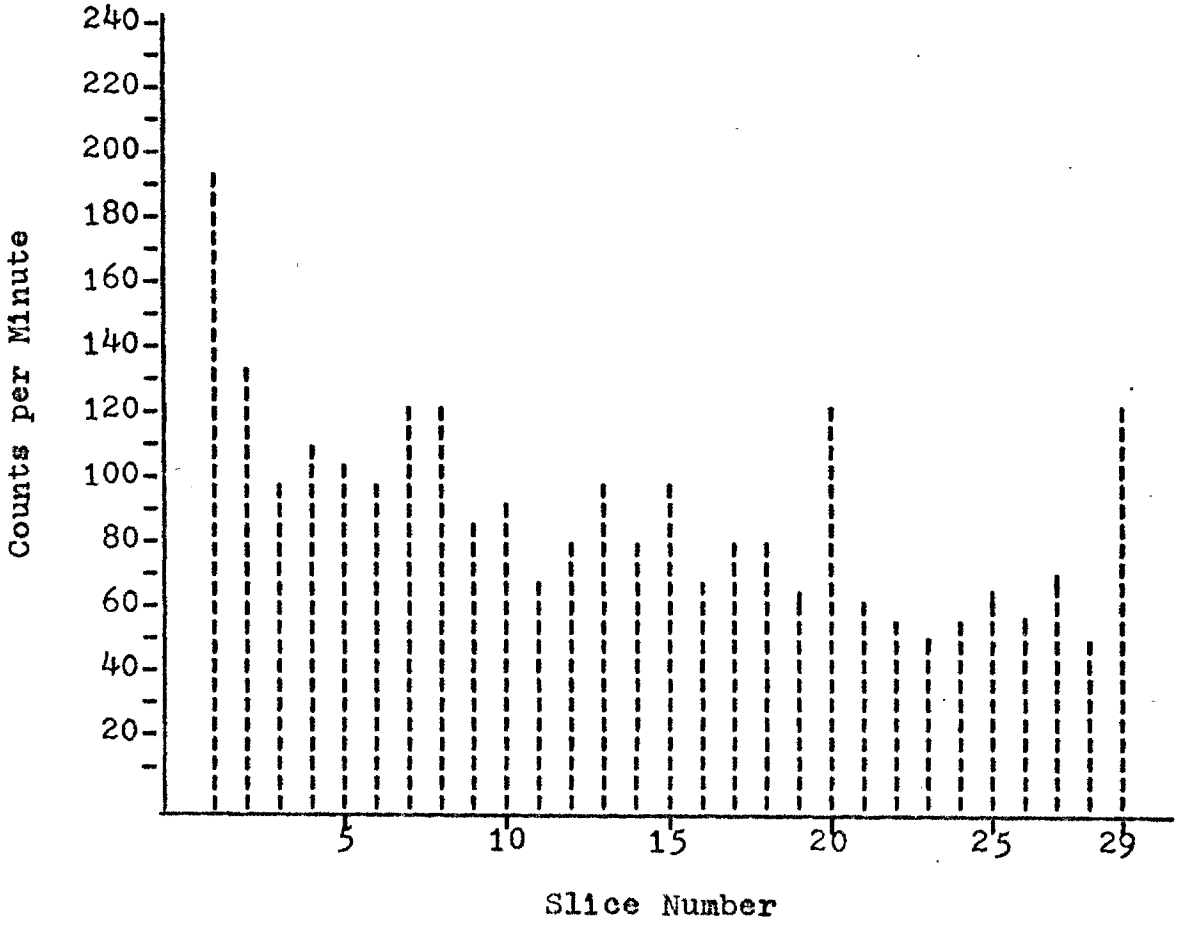

Fig. 4- Mean counts per minute in each gel slice obtained by cutting gel columns containing separated brain protein from non-conditioned fish.

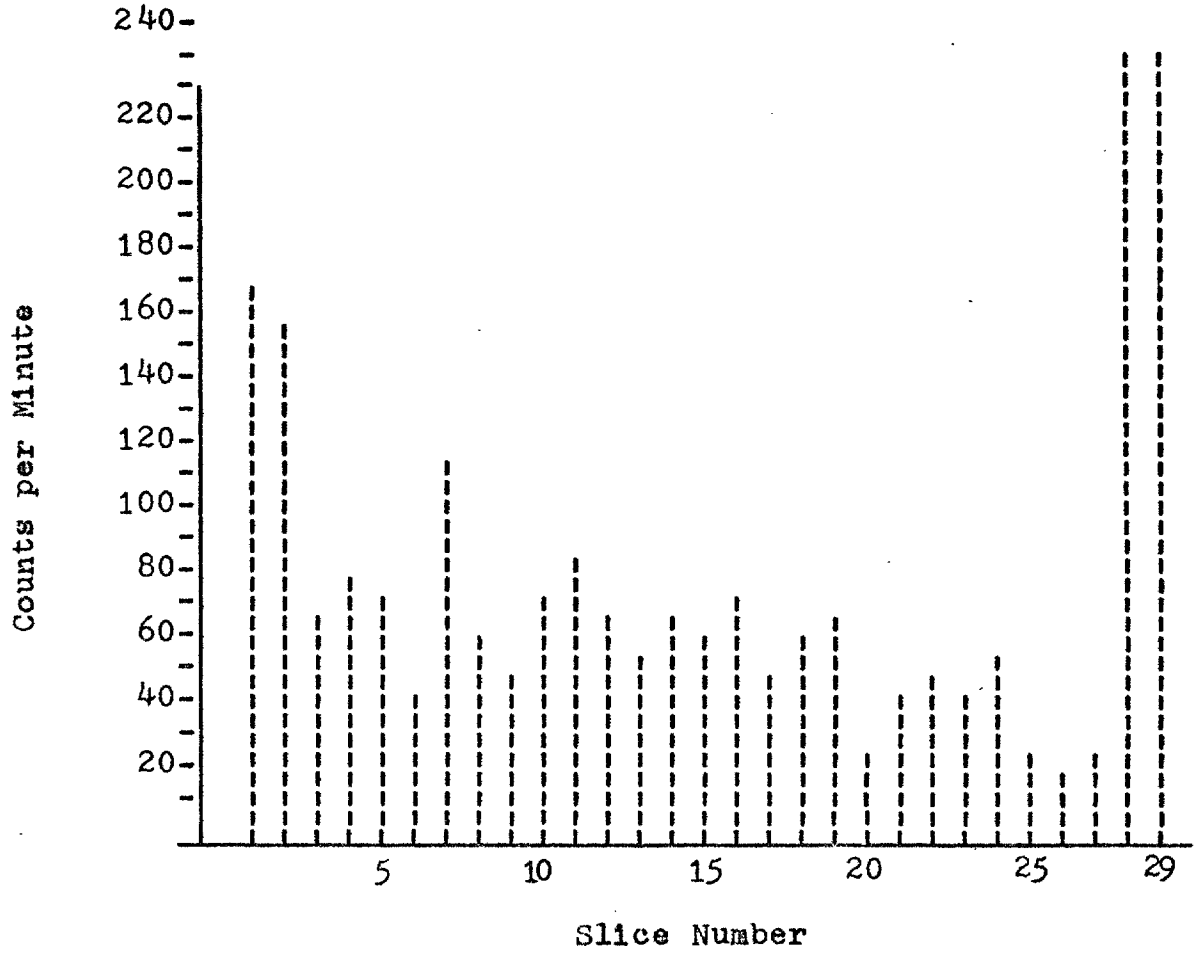

Fig. 5- Mean counts per minute in each gel slice obtained by cutting gel columns containing separated brain protein from avoidance conditioned fish.

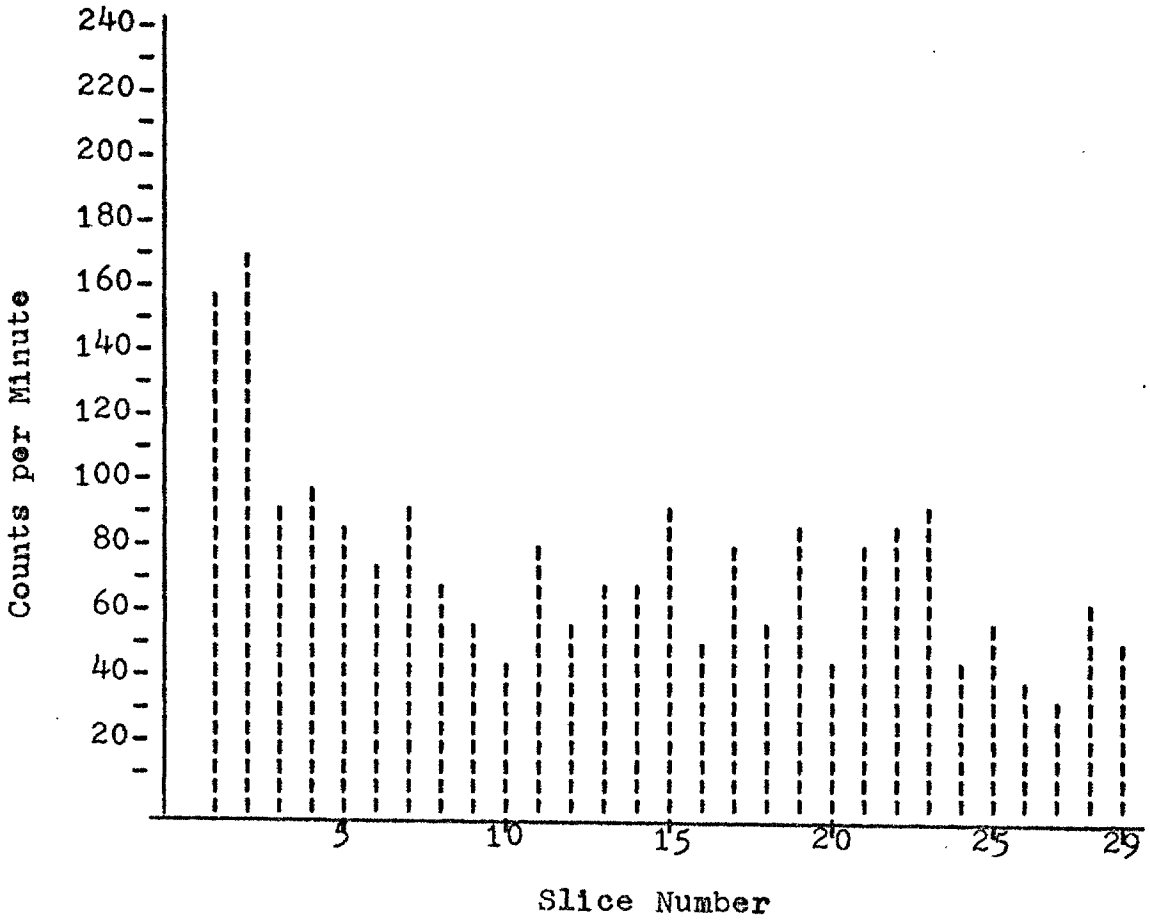

Fig. 6- Mean counts per minute in each gel slice obtained by cutting gel columns containing separated brain protein from pseudo-conditioned fish.

the highest radiation values were obtained in the first two slices below the interface, with a declining trend toward the end of the gel. The range of molecular weight of proteins entering the gel ranges from 150,000 daltons for those large proteins barely penetrating the gel, to small polypeptides found at the end of the gel (11). Any large structures such as membrane fragments or ribosomes not sedimented by the centrifugation steps would be stopped at the interface. The same is true of most RNA species and all but the small DNA fragments.

The probable explanation for this trend is that most proteins are rather large in nature, about 50,000 to 60,000 daltons molecular weight. Proteins of this size or larger would remain close to the point of origin. The remaining smaller proteins and polypeptides would be found in smaller amounts throughout the lower end of the gel. Visual information tends to confirm this, for the greatest protein density occurred from the top to the middle of the gel, with the lower end being much less dense.

Analysis of variance, one-way basis of classification, was run for each of the 29 gels as well as t tests between each group mean. The values for those slices found to contain significant differences are reported in Table 2.

Table 2

Compilation of Statistical Analysis of Radiation Emitted by Each Gel Slice Across Experimental Gps.

	Analysis of Variance		Two-Tailed t test Between Group Means					
Slice No.	F	p	$t_{1,2}$	p	$t_{1,3}$	p	$t_{2,3}$	p
1	1.25	.05	10.3	.001	11.6	.001	1.59	.05
7	17.24	.01	0.3	.05	.83	.05	.55	.05
8	3.52	.05	2.34	.05	2.34	.05	.0	.05
15	2.70	.05	1.84	.05	.32	.05	2.16	.05
17	5.25	.05	3.09	.01	.71	.05	2.38	.05
20	5.73	.01	2.38	.05	1.40	.05	1.00	.05
28	38.60	.001	7.83	.001	.43	.05	7.40	.001

* F= 4.32 required for significance at p .05 level with df.=2 and 21
t= 2.08 required for significance at p .05 level with df.=21

The statistical analyses used revealed significantly different rates of synthesis in the proteins located in 7 different slices. In three cases significant mean differences occurred between the avoidance conditioned group and the pseudo-conditioned group, slices 15, 17, 28. In two cases, 15 and 17, the avoidance-conditioned group had a lower rate of synthesis than the other two groups, which differed very

little from each other. Slice 28, however, exhibited a much higher rate of synthesis in the avoidance-conditioned group than in the normals or the pseudo-conditioned animals.

In the two other instances of significant mean differences, slices 1 and 8, both trained groups had mean count-per-minute values lower than that of the normal, non-trained controls.

In summation, several proteins were found to have diminished rates of synthesis for the avoidance-conditioned group alone, whereas two were found to be lower for both trained groups than for the non-trained animals. In addition, one protein fraction had a significantly higher rate of synthesis in the avoidance-conditioned animals than in either other group. Interestingly, slice 29 in avoidance-conditioned animals showed a just less than significant ($t=1.9$ with $t=2.08 = p\ .05$) increase over that found for the pseudo-conditioned group.

Discussion

Changes in the synthesis of several protein fractions have been reported. The differences in the effects of different experimental treatments suggests a relationship between rates of protein synthesis and the memory consolidation process.

Two protein fractions were found to have a decreased synthesis in the avoidance-conditioned and the psuedo-conditioned groups only. That no effect was seen in these fractions from the non-trained animals indicates some commonality between the affected groups not shared by the normals. As all animals were maintained and treated the same with the exception of the stimuli related to the training paradigm, it is logical to assume the differences to be related to the shock and light stimuli received or the motor responses exhibited by both groups. Hyden (12) reports an increase in brain protein synthesis during high levels of motor activity, tending to rule out this explanation. Visual stimuli are also reported (13) to increase production in visual regions of the brain. Shock, however, tends to diminish the amount of protein, RNA and DNA synthesis in proportion to the intensity and duration of the shock used (7). On this basis, it is suggested that the decreased rate of synthesis in these fractions in both trained groups is a result of the shock received during training. It appears unlikely that this may be explained as a unique susceptibility of these proteins to such inhibition of synthesis; rather, it may represent a more complicated metabolic regulatory response to a disruption of biochemical equilibrium.

The issue is further complicated by the depression in synthesis of two other fractions from the avoidance conditioned group. Recalling that consolidation of memory occurred during the period in which these proteins were radioactively labeled but was repressed until removal from the training environment, these proteins might be those regulating the synthesis of enzymes required for the consolidation process. Although there is no direct corroboration of this interpretation, much evidence has been cited indicating that these proteins are synthesized immediately after training (1, 2) and that this is a regulated mechanism. The data obtained concerning the depression of synthesis of these fractions is believed to represent the first report of protein synthesis inhibition during memory consolidation.

One fraction was found to be synthesized at a significantly greater rate in the avoidance conditioned animals than in either of the other groups. This is not the first case in which a specific protein associated with learning or memory storage has been isolated. From its position at the lower end of the gel a molecular weight on the order of 1,000 to 3,000 daltons is indicated. This corresponds, in size at least, to the ten amino acid residue peptide found by Bohus and DeWeid (14) to prevent

the extinction of shuttle box avoidance responses in rats. Also, Ungar, Calvin and Clark (15) have reported the isolation of a 6 to 10 amino acid polypeptide capable of transferring an acquired fear of the dark to naive animals. The brain protein fraction separated in this study appears too small to serve as a storage site for a memory trace. However, the criterion set by John (16) for his "critical substance" would fit this protein if it can be shown that it must reach a concentration threshold before memory consolidation can occur. In conclusion, it must be said that the evidence implicating the proteins found in this study with memory consolidation processes is strictly correlational. However, it is felt that the synthesis pattern shown by brain proteins in the avoidance-conditioned group indicates the presence of a complex mechanism regulating long-term memory formation. The absence of this pattern in the non-trained or psuedo-conditioned groups during the period in which consolidation occurs further implicates these proteins in that process.

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