CHANGES IN PROTEIN ELECTROPHORETIC PATTERNS OF RAT BRAIN WHITE AND GREY MATTER AS A RESULT OF AGE AND EXPERIENCE

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Thomas Eugene Hooper CHANGES IN PROTEIN ELECTROPHORETIC PATTERNS OF RAT BRAIN WHITE AND GREY MATTER AS A RESULT OF AGE AND EXPERIENCE. (Under the direction of W.James Smith) Department of Biology, East Carolina University, October 1968.

The purpose of this study was to examine changes in rat brain protein patterns resulting from age and experience, using the method of disc electrophoresis on acrylamide gel. Rats ranging in age from twelve days to one year were used in the experiments. Differences in protein patterns of white and grey cerebral tissue were shown to occur with age. A considerable increase in some protein peaks occurred in the oldest rats and in rats having undergone a learning experience. A preliminary study of trained versus untrained rats indicated that a significant difference in brain protein patterns may exist and that further work is needed. Phosphate and triton extracts from cerebral white and grey matter yielded protein peaks basically similar in size and position. Two additional peaks were present in phosphate extracts that were not present in triton extracts.

Autoradiographs of brain slices incubated in a media containing uniformly labelled C^{14}L-leucine and noradrenalin yielded faint patterns which were unsuitable for determination of individual protein synthesis. In vitro protein synthesis was shown to continue in the brain slices as indicated by the autoradiograph pattern.

Densitometer patterns of protein extracts from incubated brain slices stimulated with noradrenalin yielded patterns similar to control incubated and fresh unincubated tissue.

ACKNOWLEDGMENT PAGE

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INTRODUCTION

The relationship of RNA and protein synthesis to the acquisition of learned behavior has been extensively studied in recent years (1-9). The importance of protein synthesis in the brain and its effect on memory and learning was shown by experiments carried out by Flexner et al (1), who observed that injection of puromycin, an inhibitor of protein synthesis, prevented fixation of learned behavior in mice. Barondes and Cohen (2) also reported the effect of puromycin in preventing memory retention in mice after intracerebral injection. Agranoff et al (3) reported similar effects in goldfish. Hydén and Lange (4) demonstrated a bilateral increase in protein synthesis in the hippocampus of rats during a behavioral test involving transfer of handedness; highest protein was in the learning side. Structural changes in RNA base sequences occuring during learning activity have been shown by Hydén and Egyhazi (5-7). Their observations indicated that new and specific RNA was produced. Shashona (8), in experiments on goldfish, reported similar RNA base sequence changes during learning activity. Zemp et al (9) reported increased incorporation of uridine into RNA during learning experiments, indicating an increased synthesis of RNA during learning. Since the major or only role of RNA is thought to be protein synthesis, all of these studies, therefore, would indicate a relationship between protein synthesis, memory and learning.

Hydén (10) has proposed that permanent changes in the base pattern of the RNA molecule, resulting perhaps from frequency modulation by the first series of incoming stimuli from the sensory cells, may cause the synthesis of specific proteins. When the particular frequency occurs again as incoming stimuli, the specified protein is activated. The protein then effects the transmission of nerve impulse at a synapse by the activation of the transmitter substance. Thus, intraneuronal molecular storage of information may be based on production of specific protein in each neuron following initial change of the RNA base pattern.

Other theories also exist. Neurotransmitters such as noradrenalin or acetylcholine have been proposed as the agents which induce the synthesis of specific proteins that facilitate the response to subsequent stimuli (11). However, little evidence has been reported which supports such a theory.

Even if the proposed relationships between protein synthesis and memory and learning prove incorrect, it is nevertheless pertinent to study protein metabolism in any tissue since proteins play a central part in the metabolic activity of all tissues. Thus, it is logical to assume that further knowledge of protein metabolism in the cell would be helpful in understanding the basic processes of the neural tissue.

The purpose of the studies reported here was two-fold. First, we wished to study the effect of noradrenalin, a neurotransmitter (12), on the pattern of protein synthesis in brain slices <u>in vitro</u>. The goal was to measure the amount of synthesis of as many proteins as

we could isolate from brain slices, and to measure the effect of noradrenalin on the synthetic rate of each protein present. It has been shown that total protein synthesis continues at a linear rate for many hours in vitro (13).

Secondly, an attempt was made to detect any changes that might occur in the overall pattern of brain protein from rats of various ages, since, if learning does involve a change in the kind or amount of protein, differences might be expected to exist between an older experienced animal and a young naive animal. The method of disc electrophoresis on acrylamide gel, as developed by Ornstein (14), was used since this method produces good separation of proteins and permits the use of a small quantity of sample. Earlier disc electrophoretic studies conducted on rat brain tissue indicated that some change in protein pattern does occur with advancing age (15). In this study, the brain tissue was roughly divided into grey matter and white matter in order to compare the pattern of both types of tissue and to determine where any changes in protein pattern might occur with age. Also an attempt was made to evaluate the effectiveness of the method in detecting such small changes in protein as might occur in these studies.

MATERIALS AND METHODS

Preparation and Incubation of Brain Slices for Study of Protein Synthesis

Albino rats of the Holtzman strain were used for all experiments. Animals were lightly anaesthetised with ether and decapitated. The brain was rapidly removed (within two minutes) and placed in ice cold incubation media containing 120 Mm NaCl, 6.7 Mm KCl, 1.25 Mm $CaCl_2$, 1.3 Mm $MgCl_2.6\ H_2O$, 10.0 Mm Na_2HPO_4, and 6.0 Mm Glucose (16). After cooling for approximately one minute, the whole brain was placed with its anterior portion upwards in the barrel of a 10 ml glass syringe, which had been modified by cutting off the end leaving the barrel open at both ends, and which was packed in ice. A 2% agar solution, 35-40°C, was poured around the brain and allowed to solidify for approximately two minutes. This procedure greatly facilitated holding the brain in position for slicing.

Nichrome wire .07 mm thick, 1.0 mm wide, and 6 cm long, held tautly in a U-shaped spring steel holder, was used to slice the brain. Whole brain slices about 0.5 mm thick were obtained by advancing the brain with the plunger of the syringe. The movement of the plunger was regulated by a threaded bolt so that the advancement of the brain in the syringe could be calibrated in terms of the number of turns of the threaded bolt. It was found that by using this method four to five pairs of slices could usually be obtained from each brain.

The brain slices were placed in ice cold incubation media and any agar adhering to the tissue was removed by gently bubbling oxygen into the incubation media. In some cases it was necessary to remove the agar by gently teasing the tissue with a glass rod. Studies of viability of brain slice tissue prepared in this manner indicated the oxygen uptake was constant for three to five hours as determined using the Warburg technique (17).

For incubation, brain slices were transferred to 20 ml glass test tubes containing about 1 ml of incubation media to which had been added 0.05 ml of uniformly labelled L-leucine - C^{14}, (10^6 cpm), and 10^{-3} M noradrenalin in experimental tubes. Controls containing 0.05 ml uniformly labelled L-leucine - C^{14} but no noradrenalin were incubated in the same manner. Oxygen was slowly bubbled through the incubation media during the four hour incubation period. Procedures of protein extraction and electrophoresis are described below.

Isolation of White and Grey Matter

Albino rats of varying ages from twelve days to one year were used in these experiments. In one age group, 250 days, animals had been trained to perform simple tasks as a preliminary investigation of detection of differences in protein between trained and untrained animals. Rats of age 365 days were retired breeders. Animals were killed and their brains removed and cooled in physiological saline. The sections of the brain were identified, the the cerebrum was quickly removed, and the remaining portions of the brain were discarded. The cerebral tissue was roughly separated

into white and grey matter with the aid of a dissecting microscope. White or grey tissue from rats of the same age was pooled if necessary to obtain a sufficient amount of sample. Extracts of the soluble cytoplasmic and membrane bound (insoluble) protein fraction were obtained in the manner described below.

Protein Extraction from Brain Tissue

Tissue was weighed and 0.05 M sodium phosphate buffer, pH 6.8, was added in a one to four weight to volume ratio to extract the soluble cytoplasmic protein fraction. The tissue was homogenized in a cold ground glass tissue grinder and centrifuged at 5°C at 28,000xg for twenty minutes. The supernatant fluid was decanted and all samples were labelled and frozen.

A second extract was made of the pellet by adding a non-ionic detergent, Triton X-100, to the pellet in a one to one weight to volume ratio based on the original weight of the tissue. The solution was again homogenized in a chilled tissue grinder and allowed to stand for about ten minutes to extract the membrane proteins. The solution was then centrifuged at 28,000 xg for twenty minutes, and the resulting supernatant was decanted, labelled, and frozen.

Preparation of Gels and Electrophoresis

Standard 7% gels were prepared according to the method of Ornstein (18) and Davis (19) as modified by Askew (20). Chemical formulations supplied by the Canal Industrial Corporation[1] were

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[1]Canal Industrial Corporation, 5635 Fisher Lane, Rockville, Maryland.

used in all cases. For each gel, 0.08 ml of the protein sample was used. For electrophoresis, gels were placed in a holder which could accommodate six gel tubes and suspended between upper and lower chambers filled with tris glycine buffer, pH 6.8. Two drops of 0.005% bromophenol blue dye were added to the upper chamber to serve as a tracking dye ahead of the protein discs. Electrodes were placed in the upper and lower chambers and a direct current of five milliamps per tube was supplied through a voltage regulated power supply. Electrophoresis was assumed to be completed when the tracking dye reached the end of the separation gel, usually in about one hour. Essentially all of the proteins small enough to enter the gel migrated toward the annode (21).

After electrophoresis the gels were immediately removed from the glass electrophoresis tubes by dislodging them with a thin stream of distilled water, which was directed between the side of the tube and the gel with a hypodermic needle nozzle. Gels were immediately placed in a solution of aniline blue-black in 10% acetic acid for a minimum of one hour for staining. After fixation of the protein bands by the stain, excess dye was removed by placing the gels in glass gel holders which were then suspended between upper and lower chambers filled with a solution of 10% acetic acid. Electrodes were again placed in the upper and lower chambers and a current of twelve milliamps per tube was applied. Any dye which was not attached to a protein molecule migrated into the lower chamber. Destaining was usually completed in approximately one hour. Gels were then scanned using a Canalco Model

F densitometer and recorded using a Texas Instruments Serve Riter.

Tracings of the optical densities of the stained protein bands were examined using a lighted scanning panel so that tracings could be superimposed. Corresponding peaks were identified, and numbered, and the peak heights were measured. Gels of the youngest rats were considered as standard. Each peak in the experimental gels (prepared from rats older than twelve days) was compared to the same peak in the standard gel (prepared from rats of age twelve days), and a ratio obtained by dividing each experimental gel peak height by the peak height of the corresponding peak of the standard gel. The average ratio of peak heights of each experimental gel was obtained. This procedure defined the standard gel as 100%. All gels were then normalized to the standard gel by dividing the average ratio of each gel into the experimental peak height ratio of each peak. The percentage change from the standard for each peak was then plotted versus the age of the animal from which it was obtained.

RESULTS

Autoradiographs

Protein extracts from incubated brain slices stimulated with noradrenalin yielded densitometer patterns similar to control incubated slices and fresh unincubated tissue. No significant differences were noted between stimulated and unstimulated or incubated and unincubated tissue. Autoradiographs of the gels containing protein extracts of brain tissue incubated in uniformly labelled C^{14}L-leucine and stimulated with noradrenalin showed one band at the point of origin of the gel. This band corresponded to the protein that did not enter the gel in electrophoresis. Other bands, faint and unsuitable for densitometry, were observed. Repeated efforts to improve resolution by increasing exposure time failed to provide bands which were suitable to provide data on individual protein bands. Due to lack of equipment and time to pursue alternate procedures, this portion of the experiment was terminated.

Phosphate Extracts

Gel tracings of phosphate extracts from white matter had fourteen peaks in all age groups tested; grey matter had thirteen peaks. All peaks were compared with the corresponding peaks in the youngest age group (twelve days) which was considered the standard. A percentage increase in peak height in age groups beyond the standard twelve day standard indicated a lesser amount of protein in that peak as compared to the twelve day standard. Peaks present in the phosphate extracts with no corresponding peaks in the triton extracts

were numbered according to the number of the peaks between which they occurred. For example, a peak found between peak nine and peak ten was numbered peak nine point five. All peaks in which the range of values for duplicates exceeded ±6% are listed in table 1. All other peaks had values within ±6% of each other and a majority were within ±3%.

White Matter. - Gel tracings of cerebral white matter had fourteen peaks. Peak four point five was present as a shoulder on peak five. Peaks one, three, and twelve showed the largest percentage rises in the older age groups when compared to the standard twelve day group as shown in figure 1. Peak one exhibited the largest increase of 111% in the 365 day rat, with an average rise of 73% in the older groups when compared to the twelve day standard. Peaks three and twelve were up an average of 57% and 23% respectively over the twelve day standard, with the greatest increase occurring between age twelve days and age 100 days. Peaks nine point five decreased 30% from the standard in the 365 day rat with an average decrease of 26% for all age groups beyond the twelve day standard. Peaks eight and eleven decreased an average of 19% and 24% respectively for all age groups beyond the standard twelve day group. All other peaks remained within +10%-16% of the standard twelve day group. Phosphate extracts of the white matter in the trained 250 day rat were not obtained due to contamination of the protein sample.

Grey Matter. - Gel tracings of cerebral grey matter had thirteen peaks except in rats of 100 days in which peak nine point five was

Table 1: Peaks in which range of values exceeded ±6% for duplicates.

	Grey Phosphate Extracts	White Phosphate Extracts
Age	Peak number and range	Peak number and range
12 days	1±10%	none
100 days	7±8%	none
230 days	3±9%	9.5±8% , 1±10%
250 days	*	no values obtained
365 days	1±15% , 12±14%	1±25% , 6±7% , 7±8% , 12±7%

	Grey Triton Extracts	White Triton Extracts
Age	Peak number and range	Peak number and range
12 days	none	none
100 days	1±8% , 12±10%	1±10% , 12±7%
230 days	11±9%	9±8%
250 days	*	*
365 days	12±12%	10±9% , 11±11% , 12±8%

* only one sample used.

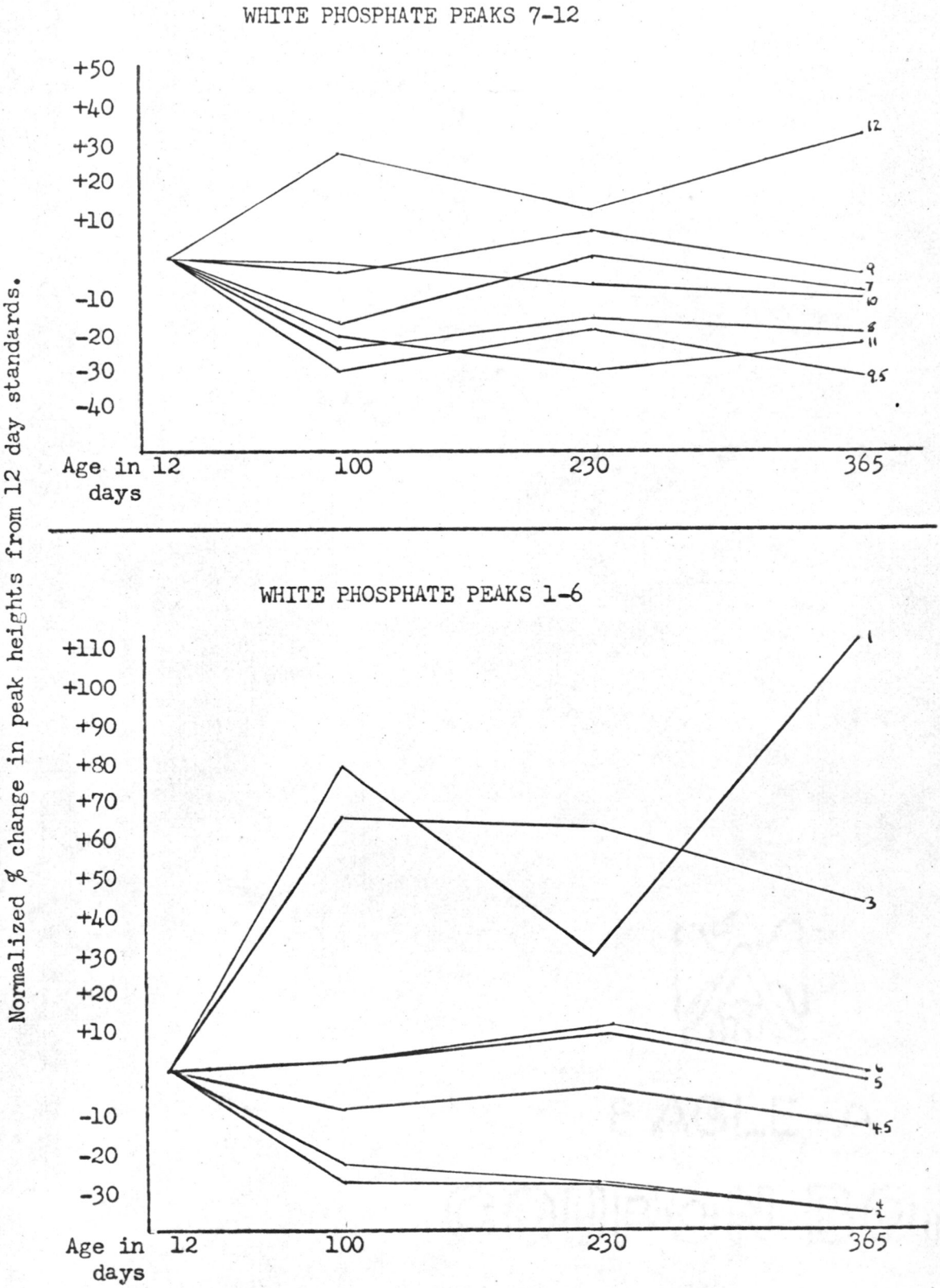

Figure 1: Changes in Peak Heights of Phosphate Extracts from Cerebral White Matter.

missing. Peak four point five which was present in white matter was not present or was indistinct in tracings of grey matter and no attempt was made to measure it. Peaks three and twelve showed the largest increase when compared to the standard twelve day age group, as shown in figure 2. Peak three showed the largest increase over the twelve day standard, with an average increase of 39% and a maximum increase of 56% in the 365 day rat. The largest increase from the standard in peak three again occurred between the twelve and 100 days. Peak twelve showed an average increase of 23% for all ages beyond the twelve day standard. Peaks one and four showed the largest decrease in protein content. Peak one had a large decrease in the 100 and 230 day rats and a much smaller decrease in the 250 and 365 day rats, with an overall average decrease of 27% for groups beyond the twelve day standard. Peak four decreased in all age groups beyond the twelve day standard with a maximum decline of 36% in the 250 day rats and an average decline of 27%. All other peaks remained within +14%-18% of the twelve day group in all age groups beyond the standard, indicating little change in protein content in those peaks. A comparison of typical white and grey phosphate tracings is shown in figure 3.

Triton Extracts

Triton extracts were very similar to phosphate extracts in both position and height of peaks. Twelve peaks were present in both white and grey triton extracts. Two peaks (four point five and nine point five) present in phosphate extracts were not present in Triton extracts. Figure 4 illustrates a typical phosphate and

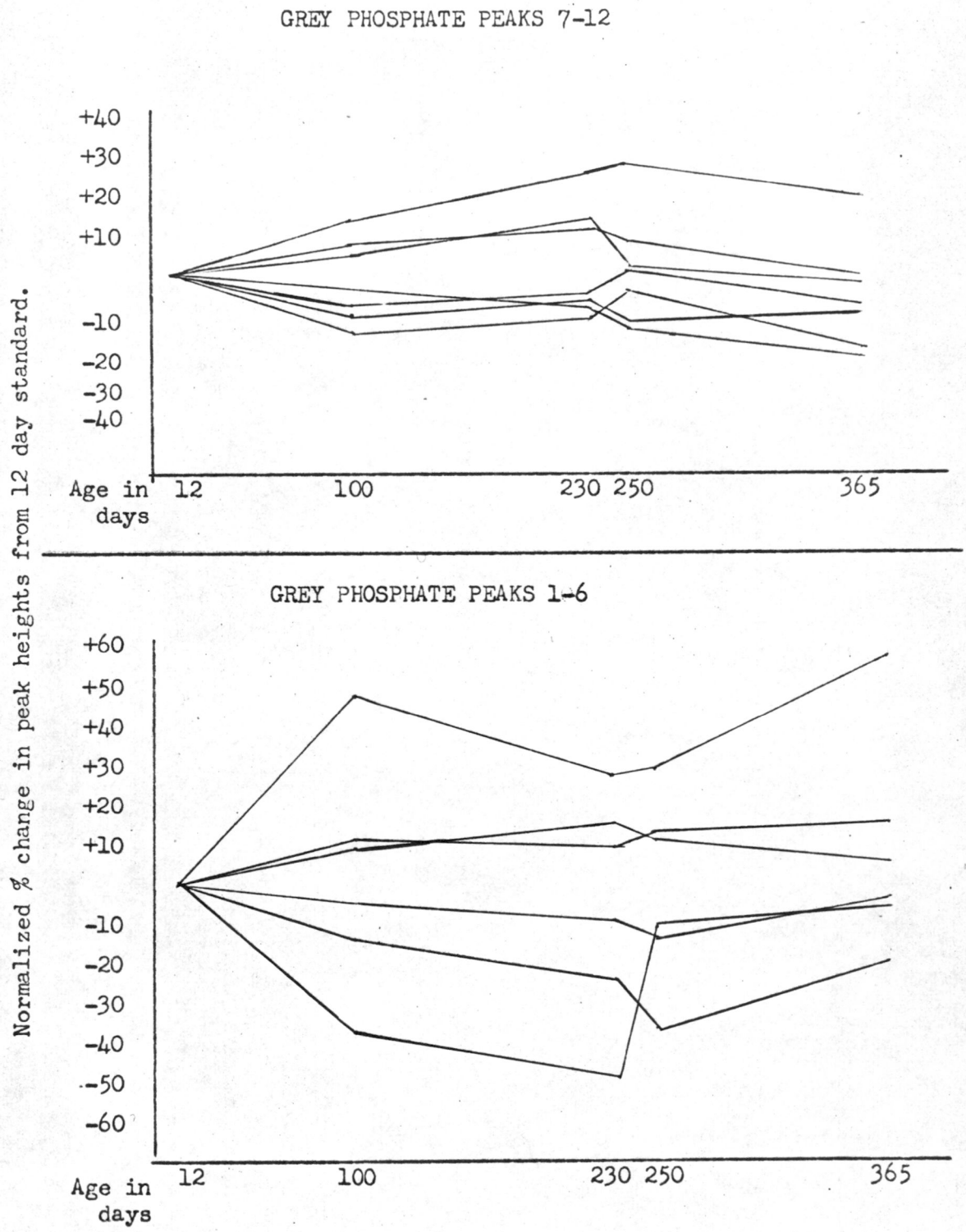

Figure 2: Changes in Peak Heights of Phosphate Extracts from Cerebral Grey Matter.

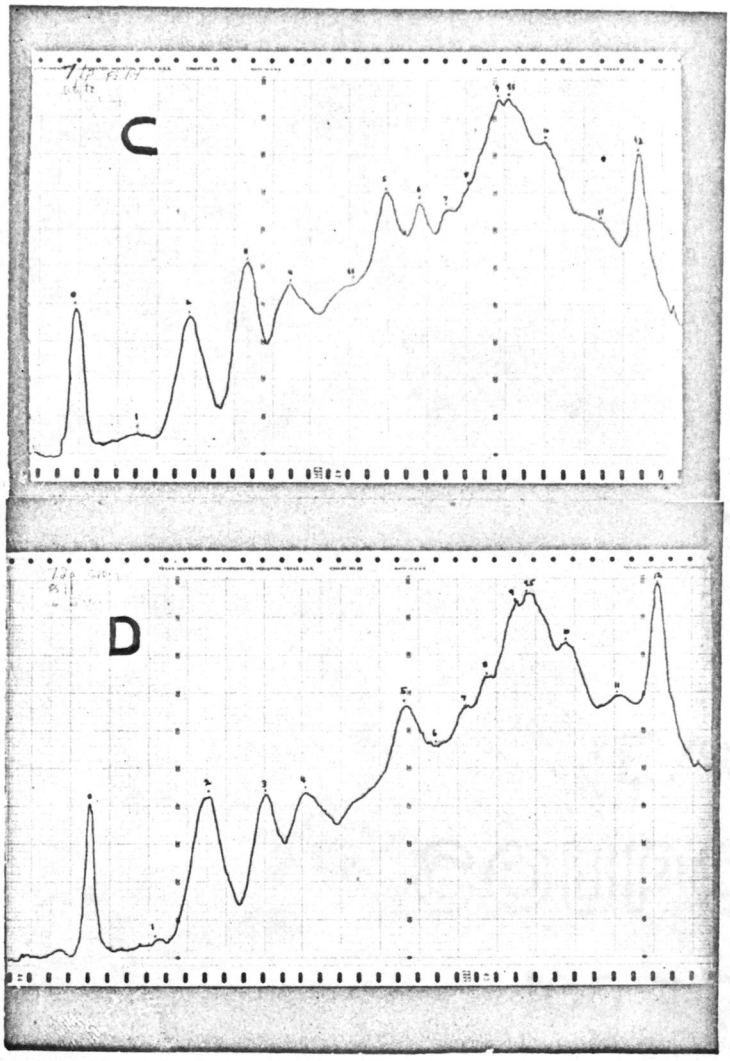

Figure 3: Comparison of tracings of phosphate extracts of white matter (C) and grey matter (D) from a 230 day old rat.

Triton extract from cerebral grey matter.

White Matter. - Peak twelve showed the largest increase from the standard twelve day group with an average rise of 39% for all age groups beyond the standard twelve day group as shown in figure 5. The largest increase was 78% in the trained 250 day group. Peak twelve was well below the other age groups in the 230 day group. Peaks one and three showed the largest decline in protein present, with average declines of 26% and 31% respectively in all groups beyond the twelve day standard. Most of the decrease in peak three occurred between age twelve days and age 100 days, while peak one remained close to the standard in the 100 day groups beyond the twelve day standard. All other peaks remained within +20%-13% of the standard twelve day group.

Grey Matter. - The twelve peaks present in cerebral grey matter corresponded, at least in approximate height and position, to the twelve peaks present in cerebral white matter. This does not necessarily mean, however, that they are composed of the same protein. Peak two showed a large increase between age twelve days and age 100 days and continued to show a large increase in all older age groups compared to the twelve day standard as shown in figure 6. Peak twelve remained close to the standard twelve day group in both the 100 and 250 day age groups, but showed a large increase in the 250 day trained rat and the 350 day age group. Peak eleven showed the largest percentage drop with an average decrease of 21% for all groups beyond the standard twelve day group. All other peaks were within +8%-15% of the standard twelve

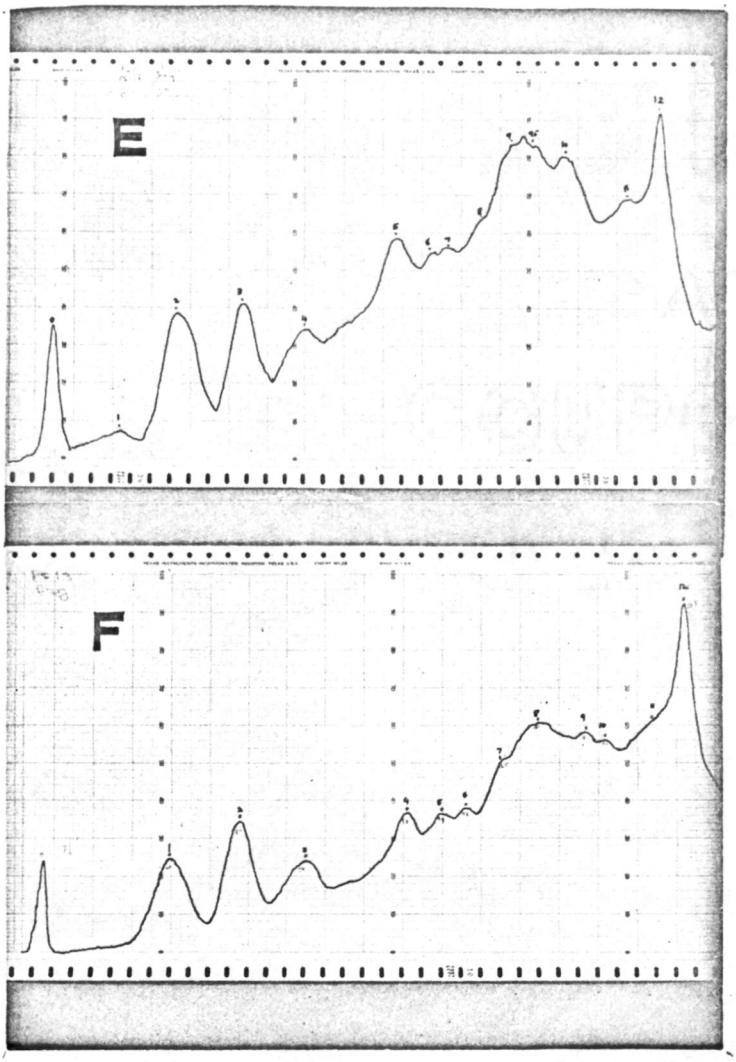

Figure 4: Comparison of tracings of phosphate (E) and triton (F) extracts of grey matter from a 250 day old rat.

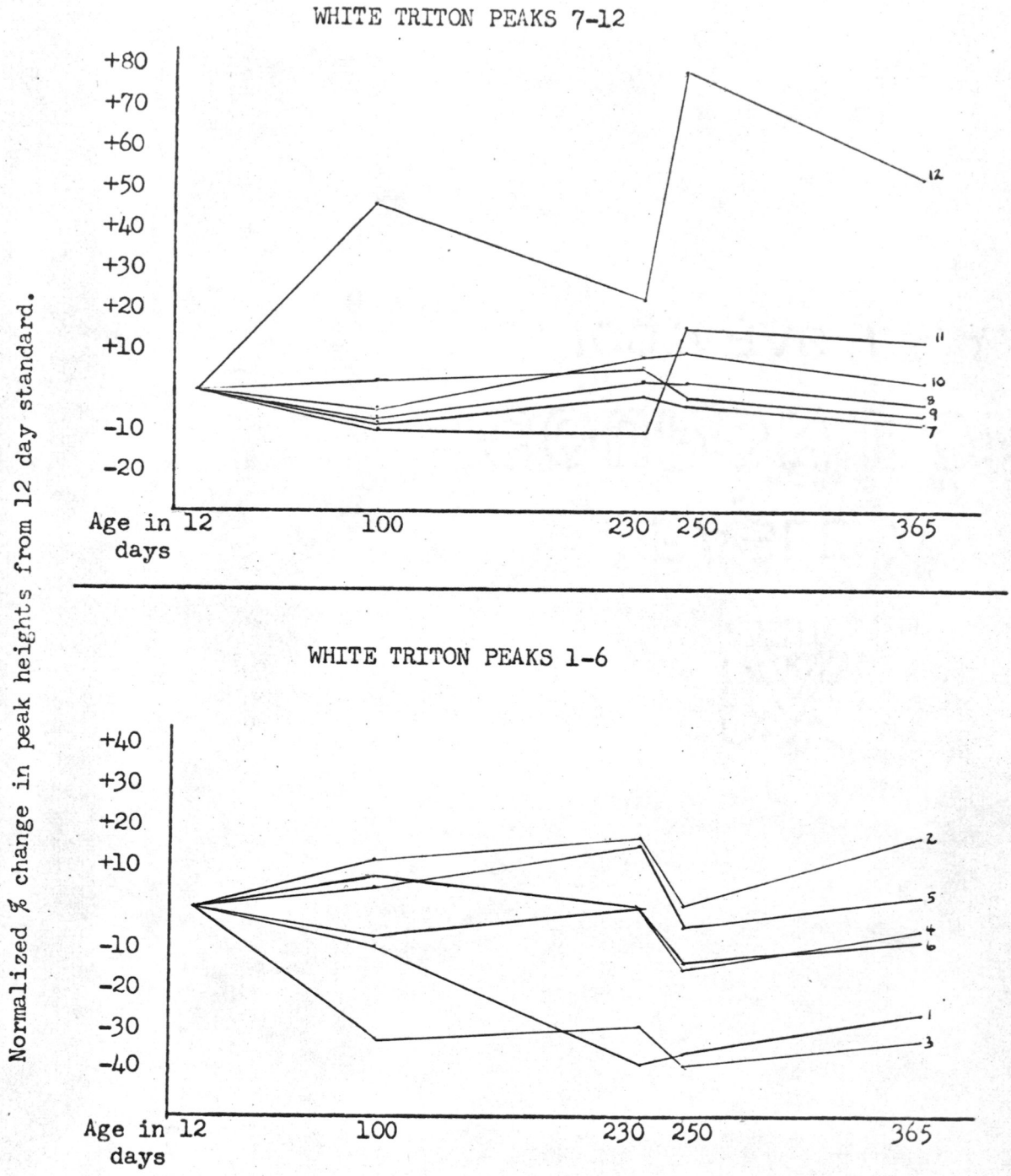

Figure 5: Changes in Peak Heights in Triton Extracts from Cerebral White Matter.

day group.

Comparison of White and Grey Matter

Phosphate Extracts. - Tracing of white and grey extracts showed many similarities in both number and position of peaks. However, peak four point five was present in white matter but not in grey. Peak one showed large increases in all age groups beyond the twelve day standard in white matter and large decreases in all age groups beyond the twelve day in grey matter. Peak twelve showed a large increase in both white and grey extracts in the older age groups when compared to the twelve day standard. Figure 7 shows the large increase in peak twelve in phosphate extracts of grey matter from a young and old rat.

Triton Extracts. - White and grey triton extracts also showed many similarities in number and position of peaks. Both white and grey triton extracts exhibited large increases in peaks twelve. Peak one showed a large decrease in the white matter and a smaller decrease in grey matter. Peak two showed a large increase in grey matter and a smaller increase in white matter. Other peaks appeared to be very similar.

Variation in Experience in Experimental Animals

The 250 day rat had been trained to perform simple tasks using electrical shock and food reward techniques. Only one rat was used in this preliminary study of the effect of training on protein pattern. This training may account for some of the differences observed in the 230 and 250 day rats in peak twelve in both white and grey triton extracts (figures 5 and 6). Peak eleven also

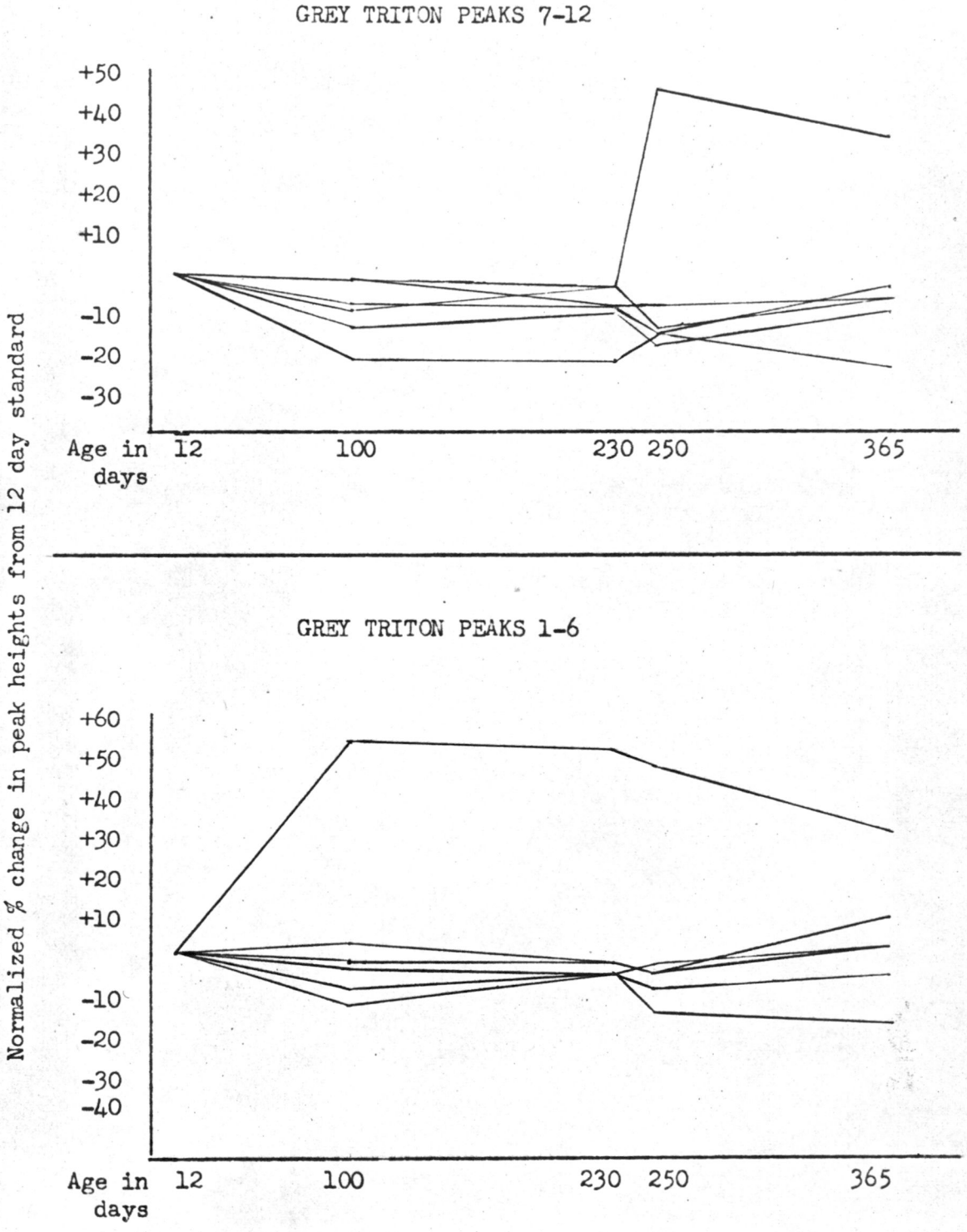

Figure 6: Changes in Peak Heights of Triton Extracts from Cerebral Grey Matter.

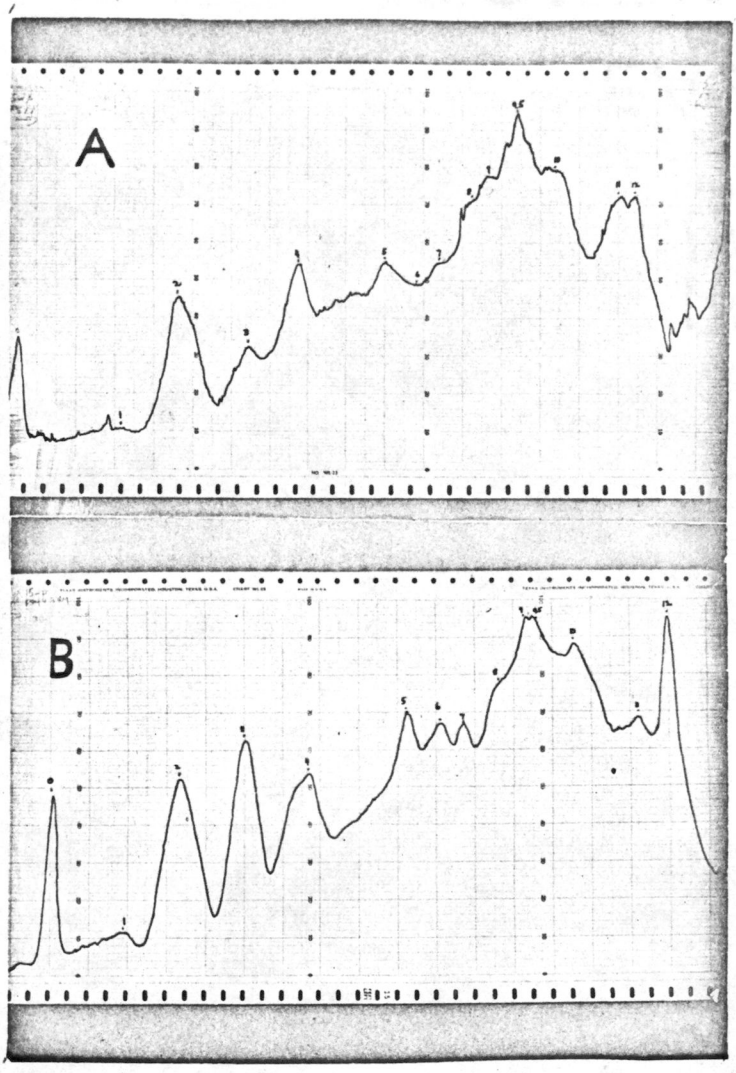

Figure 7: Comparison of tracings of phosphate extracts of cerebral grey matter from rats of age 12 days (A) and age 365 days (B).

exhibited this increase but to a lesser extent. These large variations may be explained in part by the difference in experience between the two groups of animals.

Animals in the 365 day group were retired breeders and, therefore, had a wider range of experience than the non-breeders. The closest controls for the 365 day group would be the 230 day group, since no non-breeders beyond age 230 days were tested.

All other animals had been segregated on the basis of sex since birth and were naive.

DISCUSSION

Incubation

Although some of the technical problems of this portion of the experiments remain unsolved, our results have shown that we are able to obtain healthy brain slices of uniform thickness which consume oxygen for several hours and which yield consistent electrophoretic patterns. Incubation studies yield information about the synthesis of each protein peak on a short term basis (several hours), while age group studies yield information about the changes in protein patterns occurring over long periods of time. Autoradiograph patterns of gels prepared using incubated tissue yield faint bands which, though unsuitable for densitometer tracings, indicate the feasibility of pursuing this line of experimentation. Other means of measuring the radioactivity levels of the protein bands, such as scintilation counting, might be employed. If this method were used the individual protein bands in each gel could be isolated by slicing gels crosswise and the radioactivity of each band then measured. This method would allow a better comparison between radioactivity levels present in the various bands. It would also help provide correlation between densitometer tracings and actual amounts of protein present, since the density of a stained protein band is not completely proportional to the amount of protein present. This is due to the fact that when large amounts of protein are present the dye molecules used to stain and fix the protein may not be bound equally to all of the protein molecules. The results

of our studies on incubation indicate that further investigation of this area is needed. In any further studies variations in incubation techniques such as using high levels of radioactivity in the media or increasing concentrations of neurotransmitters should be considered.

Age and Tissue Studies

These experiments establish that a difference in protein pattern occurs with age in both white and grey cerebral tissue in rats. The fact that a few peaks show significant increases while the majority remain relatively constant in all age groups tested would be expected when physiological considerations are examined. Many enzymes are known to be necessary for normal cell function, and these must be maintained at relatively constant levels throughout life.

Some proteins however might be expected to vary as the animal increases in age due to any one of several factors. Ageing, experience, training, and hormonal effects on growth, as well as any pathological effects arising during the life span, might affect the protein present in cerebral tissue. Similar changes have been noted in electrophoresis studies of the developing chick embryo (22). The precise effect of these factors on cerebral protein has not been determined. However, it would seem that the consistent increase in protein content of some peaks, such as is shown in peak twelve, is a result of one or a combination of these kinds of factors. The large increase in peak heights between the untrained 230 day

rat and the trained 250 day rat might be indicative of the effect of learning activity on protein content.

The single study on trained versus untrained rats presented here was not an attempt to provide conclusive results. However, as a preliminary study it has shown that further studies on the effect of specfic training on protein patterns are warranted. It should be noted here that in a parallel study of cerebral protein patterns using unseparated white and grey tissue a large increase in the same peaks also was observed between an untrained and trained rat (23).

The use of breeders as experimental animals in the 365 day rats has been noted earlier. This may have affected the results of these studies by introducing another variable, since all other rats were naive and were never allowed to breed. However, since we were not trying to distinguish the cause of the variations in protein pattern except as a function of age and experience, it was felt that retired breeders, with increased experience, would be suitable.

These experiments also show that protein patterns in white and grey tissue are basically similar. Some differences between the two tissues do occur both in number of peaks present and in quantity of protein in the peak. One peak, four point five, present in phosphate extracts of white cerebral tissue was not measurable in grey cerebral tissue. This was the only peak that was not present in both types of tissue.

Large differences also were noted in some peaks present in both white and grey matter. Protein composing peak one, for example, was present in much greater quantities in white matter phosphate extracts than in grey matter extracts. Triton extracts showed no significant difference in peak one between white and grey matter, and protein was present in much smaller amounts. This may indicate that peak one is present in largest quantities as a soluble protein in white matter.

Since separation of the cerebral tissue into white and grey matter was not complete, the protein patterns represent only major differences. Procedures to separate the cerebral tissue more completely might yield somewhat different patterns. However since the electrophoretic method measures only major proteins or groups of proteins, small amounts are not detected and considerable differences may exist in white and grey tissue that have not been detected here.

Differences were noted also in the phosphate and triton extracts. Two peaks present in the phosphate extracts, four point five and nine point five, were not present in the triton extracts. This indicates that the extracts are yielding some different proteins. It is possible however that some of the soluble proteins which are thought to be removed in the phosphate extract are incompletely removed and may be present in the triton extracts. This problem of extraction requires further investigation to substantiate the effectiveness of the two extract procedures.

Methodology

It should be pointed out that all peaks were measured in the same manner by measuring the distance from the base line to the top of eack peak. This procedure introduces some error into the results since the exact height of individual peaks are influenced by the height and background of other neighboring peaks. Thus, the difference between two peaks, such as peak seven and eight, present on a high background may appear quite minimal. However, due to the lack of resolution between peaks no other method is presently available to measure the individual peaks. Smaller peaks could also be made to vary without actually varying the amount of protein present due to slight differences in the distance between neighboring peak height. Greater resolution between peaks would also minimize this effect.

SUMMARY

Brain slices incubated for four hours in a media containing uniformly labelled C^{14}L-leucine and noradrenalin yielded faint autoradiograph patterns unsuitable for conclusive interpretation of the synthesis of individual proteins. Protein synthesis was shown to continue *in vitro* as indicated by the autoradiograph patterns.

Differences in protein patterns of white and grey cerebral tissue were shown to occur with age and a considerable increase in some peaks was noted with advancing age. This increase was most pronounced in oldest rats and in rats that had undergone a learning experience. A preliminary study of trained versus untrained rats indicated that a significant difference in brain protein patterns may exist and that further study is needed.

Phosphate and triton extracts from cerebral white and grey tissue were similar in number and position of peaks. Phosphate extracts had two additional peaks not present in triton extracts.

White and grey matter extracts were also basically similar in size and position of peaks. Phosphate extracts of white matter had one peak present not found in grey matter, and one peak was found in much greater amounts in white matter than in grey matter. Triton extracts showed less difference between white and grey matter than did the phosphate extracts.

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