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# Time-Dependent Results of Amino Acid Uptake Studies of Learning in Frogs[1]

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PETERS, R. I., JR. *Time-dependent results of amino acid uptake studies of learning in frogs.* PHARMAC. BIOCHEM. BEHAV. 4(1) 7–11, 1976. – Myelencephalic grass frogs were trained to elevate the right forelimb to avoid a shock plate at several times in relation to administration of $^3$H-leucine and sacrifice. Total radioactivity (excluding $^3H_2O$) and radioactivity in the soluble pool were significantly greater in the trained animals. The differences between trained and yoked animals decreased with increasing time, up to 30 min after training. The opposite trend was apparent in TCA insoluble material; differences between trained and yoked animals increased with increasing time after training. Similar experiments with $^3$H-inulin provide evidence that the effects were not due to generalized permeability increases or circulatory alterations.

Learning Leucine uptake Neurochemistry Biochemistry of behavior

INVESTIGATION of macromolecular correlates of learning behavior is certainly one of the more interesting and challenging areas of current neurochemical research. Numerous reports have appeared on changes in RNA metabolism concomitant with learning, while information concerning subsequent changes in protein metabolism is limited. Glassman [4], and more recently Uphouse [15], have presented excellent reviews on the subject.

Most reports of macromolecular correlates of learning have utilized rodents as experimental animals, and the behaviors investigated have necessarily been rather complex. The rodent brain is certainly a very heterogeneous mass; thus, most reports have focused interest on the hippocampus for theoretical and practical reasons: changes in amino acid incorporation with learning have been observed in this region [2, 6, 7, 8, 9, 18]. The mere presence of changes in metabolism in a region of the mammalian brain, while interesting, is not sufficient evidence that these changes are directly associated with learning and, or, memory. The search for a more simple vertebrate preparation capable of associative learning led me to the myelencephalic frog trained to avoid shock to a forelimb, in the paradigm of Horridge [5]. This system offers the advantages of quantifiable behavior and relatively limited neuronal circuitry, and the yoked animal serves as a control for any nonspecific stimuli associated with the training routine.

Previous investigations of radioactively labelled precursor uptake with training have involved administration of the precursor by various routes at different times in relation to the actual training, different durations of training, and sacrifice of the animals at various times thereafter. Since one would expect any changes in protein metabolism to be time-dependent processes, accurate interpretation of results from such experiments is sometimes difficult. Time-dependent changes in incorporation of labelled amino acids have been reported in quiet [10] and exercised [14] animals, but to date there has only been one reported systematic investigation of possible changes in incorporation of labelled precursor with different injection-training-sacrifice schedules [13]. The present study was undertaken in order to determine the time course of incorporation of tritiated leucine into a simple vertebrate system in conjunction with training.

## METHOD

### *Animals and Procedure*

Neuraxes of adult *Rana pipiens* (northern variety) of around 20 g body weight were sectioned immediately caudal to the optic bulbs, and all nervous tissue rostral to the section was mechanically destroyed. Thus, the spinal cord and the majority of the medulla were the only parts of the central nervous system remaining with intact connections with the limbs. Two hr later the animals were clamped into the training apparatus so that withdrawal of the right forelimb of the trained animal by at least three millimeters was required to avoid a 15 V, 60 HZ shock. The criterion for conditioning was set as 3 consecutive min of receiving no shocks. Criterion was usually reached in less than 7 min, and in the majority of animals previously tested, effects of the training experience were present 45 min later. A yoked control animal was wired in series into the circuit controlled by the trained animal so that it received the same number, pattern and intensity of shocks but the shocks were not contingent upon its limb position.

Twenty microcuries of L-(4,5-$^3$H) Leucine or (Methoxy-$^3$H) Inulin (New England Nuclear) were injected into the

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dorsal lymph sac in the vicinity of the iliac lymph hearts at several times in relation to the training experience, and the animals were sacrificed by placing them in a freezer maintained at −65°C. Pairs of frogs were randomly assigned to one of the following schedules: (1) Injected 30 min prior to training and placed in the freezer immediately after training (30-PRE). (2) Injected 15 min prior to training and placed in the freezer 15 min after training (15-15). (3) Injected immediately after training and placed in the freezer 5 min later (5-POST). (4) Injected immediately after training and placed in the freezer 30 min later (30-POST). These schedules were designed so that, with the exception of the 5-POST schedule, the tracer would be in the animals the same length of time but the time between training and sacrifice would be varied.

*Assay Procedure*

The central nervous systems between the destroyed cerebelli and the 7th spinal nerves were removed while still frozen, and adhering blood and cerebrospinal fluid were scraped from the dorsal and lateral surfaces. The excised tissue was then homogenized in ice-cold 0.05 M Tris Borate buffer, pH 8.9, containing 0.3 percent Triton X-100. A segment of the right lobe of the liver weighing approximately 200 mg was removed and processed simultaneously. The samples labelled Dried Homogenate were prepared by placing 0.2 ml of the homogenate in a scintillation vial and drying for 2 hr at 100°C. The trichloroacetic acid (TCA) precipitate samples were prepared by spotting 0.2 ml of the homogenate on circles of Whatman GF/C paper and incubating in ice-cold 5 percent TCA for 2 hr. The samples were then treated as described by Rees *et al.* [13], with the exception that they were incubated in Protosol at 50°C for 12 hr, and at room temperature (21°C) for 24 hr. The difference between dried homogenate and TCA precipitate was counted as TCA soluble pool. The ratio of TCA insoluble to TCA soluble will be referred to as relative radioactivity (RR). Samples were prepared for liquid scintillation counting by adding 10 ml of toluene-based cocktail (PPO: 4 g/l; POPOP: 0.05 g/l) and dark adapting for 2 hr at 4°C prior to counting. Each sample was counted at 4°C for three consecutive 20 min periods, and the median value was used in calculations. The maximum counting error was less than 3 percent as calculated from the formula of Van Slyke and Sinex [16]. Counting efficiency varied between 12 and 25 percent as determined by the channels ratio method.

Since the animals were sacrificed by cooling, it was desirable to know how much time elapsed between when the animals were placed in the freezer and when their intravertebral temperatures dropped to the point of cessation of coordinated neuronal function. To this end, copper-constantan thermocouples were inserted into the vertebral canals of several double-pithed frogs and intra-vertebral temperatures were monitored remotely (Bailey Instruments Thermometer model BAT-4) as the animals cooled.

## RESULTS

The cooling rate experiments revealed that frogs of 18–25 g body weight cooled linearly at approximately 2°C/min. Thus, 6 min, ± 30 sec, were required for frogs of this size to cool to 10°C. It has been reported that conditioned reflexes in goldfish acclimated to 25°C disappear at 10–15°C [12], but no comparable data are available on frogs.

Preliminary experiments with quiet frogs subjected to neuraxis section, given the tracer, and placed in the freezer at various times thereafter, revealed that the counts in the TCA soluble pool rose approximately linearly with time at a rate of about 227 normalized CPM/minute for the first 30 min. The counts in the TCA precipitate of these quiet animals also rose approximately linearly over this time, at a rate of about 118 normalized CPM/minute.

Although trained (T) and yoked control (Y) animals were matched according to body weight to within 5 percent, Wilcoxon's Matched Pairs test revealed that the lighter of the 2 frogs consistently showed greater incorporation of label into the TCA precipitate; the difference was significant at the 0.05 level in a two-tailed test. In an attempt to correct for dilution differences, counts were normalized to the body weight of the animal by multiplying the disintegrations per minute (DPM) or counts per minute (CPM) by the animal's body weight in grams. To facilitate comparisons, results are expressed as the ratio of normalized radioactivity in the T animal to the normalized radioactivity in the corresponding Y animal. This will be referred to as the T/Y ratio.

The liver samples from T and Y animals were compared in order to determine the direction and magnitude of any differential hormonal effects, since protein metabolism has

TABLE 1

RADIOACTIVITY IN LIVERS OF TRAINED AND YOKED ANIMALS INJECTED WITH $^3$H-LEUCINE

| | TCA Soluble | | TCA Insoluble | | RR | |
|---|---|---|---|---|---|---|
| | T | Y | T | Y | T | Y |
| Mean | 21,202 | 21,151 | 4,989 | 7,948* | 0.31 | 0.26 |
| SEM | 1,304 | 1,816 | 700 | 1,450 | 0.06 | 0.05 |

Data presented are means of animals on all injection schedules (17 pairs), expressed as disintegrations per minute normalized to the body weight of each animal.

*Y>T, $p<0.01$, Wilcoxon's Matched Pairs, 2-tailed

TABLE 2

RADIOACTIVITY IN SPINAL CORDS OF FROGS INJECTED WITH $^3$H-INULIN OR $^3$H-LEUCINE

| | Inulin | | | Leucine | | |
|---|---|---|---|---|---|---|
| | T | Y | T/Y | T | Y | T/Y |
| Mean | 38,788 | 39,082 | 1.00 | 227,000 | 160,888 | 1.19* |
| SEM | 3,969 | 4,802 | 0.28 | 27,637 | 13,024 | 0.08 |

Frogs were injected with 20 $\mu$Ci of $^3$H-Inulin (6 pairs) or $^3$H-Leucine (8 pairs) into the dorsal lymph sac immediately after training, and placed in a −65°C freezer 30 min later. Counts are those in the dried homogenate samples, expressed as disintegrations per minute normalized to body weight.

*T>Y, $p<0.01$, Wilcoxon's Matched Pairs, direction predicted

been shown to be affected by the hormonal state of the animal [11,17]. Since there were no significant differences between injection-training-sacrifice schedules, data from all schedules were pooled in Table 1. Due to the nature of the data, Wilcoxon's Matched-Pairs, Signed-Ranks test was used to analyze differences between T and Y animals. Since this test utilizes both the direction and the magnitude of differences between paired samples, one extreme value may markedly affect the significance level of differences. Thus, differences must be very reproducible in order to reject the null hypothesis at the 1 percent level. The only significant difference between the livers of T and Y animals was in the TCA precipitate of animals injected with $^3$H-Leucine, where the normalized DPM in the Y animal was consistently greater than that in the T animal ($p<0.01$, Wilcoxon's Matched-Pairs, 2-tailed).

Only dried homogenate samples were prepared from those animals injected with $^3$H-inulin. It was found that the counts were only around 20 percent of those of the $^3$H-leucine injected animals, and there were no significant differences in radioactivity between trained and yoked animals. Table 2 illustrates the differences between T and Y animals injected with $^3$H-inulin or $^3$H-leucine immediately after training and placed in the freezer 30 min later.

Figure 1 summarizes the findings in the neuraxes of T and Y animals, as the mean T/Y ratio is plotted as a function of time between training and placing the animals in the freezer. The T/Y ratio in the dried homogenate was greatest in those animals sacrificed immediately after training, and fell significantly ($p<0.025$, Difference of Means, 1-tailed) with time after training. The ratio in the TCA soluble pool followed the same pattern, with the T/Y ratio on the 30-POST schedule being significantly lower than that on the 30-PRE schedule ($p<0.01$, Difference of Means, 1-tailed). The ratios with respect to TCA precipitate and relative radioactivity followed the opposite pattern; the ratios increased significantly ($p<0.05$, Difference of Means, 1-tailed) with increasing time after training. These differences between schedules were apparent only in the T/Y ratios; there were no significant differences between schedules with respect to either T or Y animals alone.

On each schedule the T animal had a significantly greater amount of radioactivity in the dried homogenate and TCA soluble pool samples. The level of significance by Wilcoxon's Matched Pairs test was always less than 0.05, becoming smaller on the 5-POST and 30-PRE schedules. On no schedule were there significant differences between T and Y animals with respect to TCA insoluble samples and relative radioactivity, although these differences approached significant levels on the 30-POST schedule.

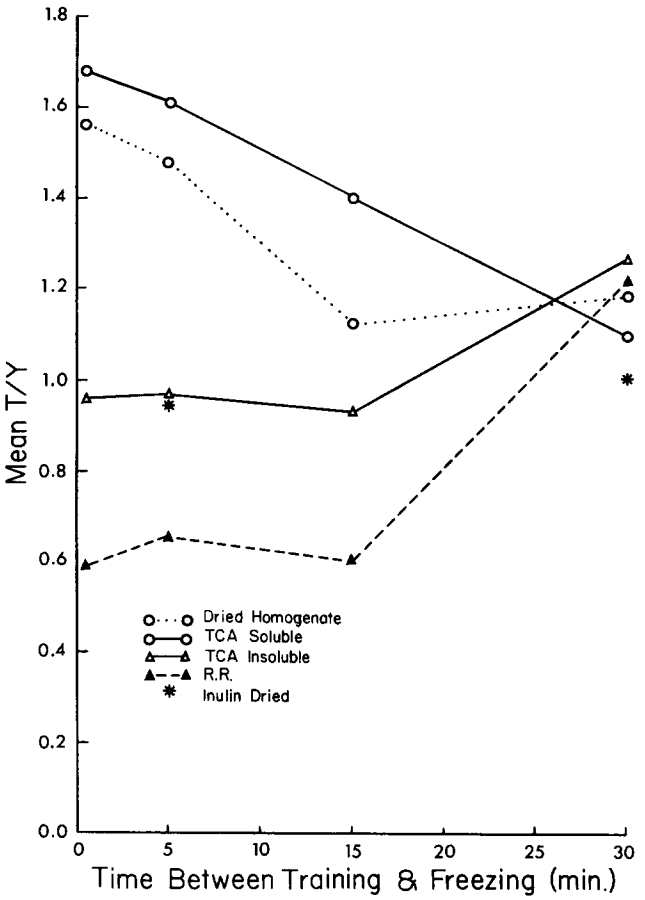


FIG. 1. Changes in the mean T/Y ratio as a function of time between training and placing the animals in a freezer maintained at −65°C. Ratios are of counts per minute normalized to the body weight of each animal.

## DISCUSSION

The experiments with quiet control animals showed that the radioactivity in the TCA soluble pool rose more rapidly than that in the TCA precipitate, at least over the first 30 min. These data are qualitatively similar to those reported by Tiplady for $^3$H-Lysine incorporation into rat cerebral cortex after mild exercise [14].

One recurrent problem complicating the interpretation of precursor uptake studies has been lack of knowledge of the size and specific activity of the TCA soluble precursor pool over the time allowed for incorporation of labelled precursor into TCA insoluble material. Increases in soluble pool counts as a result of training have been reported by several other workers [9, 13, 18], but little attention has been attracted to this point. Since it is technically impossible to measure the endogenous precursor pool size or radioactivity in a given animal at more than one point in time, the approach taken in this study was to train animals to the same criterion and note the amount of label present in the TCA soluble pool and TCA precipitate at several times in relation to the training experience. Since the first marked increase with training in this study was seen in the TCA soluble pool, and since the incorporation of radioactivity into protein is presumably dependent upon the presence of label in the endogenous pool, the observed differences in labelling of TCA insoluble material could have been at least partly due to different amounts of label in the endogenous pool.

It should be noted that in this study TCA insoluble material is not necessarily synonymous with protein, since the routine analysis did not involve steps to remove aminoacyl charged t-RNA. This ommission was not considered to be a major source of error, since control experiments showed that only about 3 percent of the radioactivity on these filter paper discs was removed by a 20 minute wash in 5 percent TCA heated to 60°C.

To this author's knowledge, the only other report of a systematic study of time-dependent changes in precursor uptake with learning is that of Rees and his coworkers [13]. They administered a 10 min pulse of $^3$H-lysine to mice 5, 20, or 35 min after shock avoidance training. They reported a significant increase in radioactivity in the soluble pool in trained animals given the tracer pulse 20 min after training, but did not report the condition of the pool at the other times. However, they did report no significant differences between relative radioactivities at these times. It should be noted that their analyses were of whole mouse brain; thus, regional differences may well have been obscured.

Recently, Bondy and Morelos [3] have reported circulatory alterations in chick brain as a result of functional activity. Changes in blood flow or permeability of the vessels to labelled molecules could explain the increases in tissue pool radioactivity seen in trained animals. The experiments with $^3$H-inulin provide evidence that this was not the case. Assuming that the permeability of the vasculature in the spinal cords remained constant, one would have expected that more inulin would have appeared in the nervous tissue if more had been presented to it by increased blood flow. Alternatively, if the permeability of the vasculature were altered as a result of training, one would have expected that the amount of inulin found in the nervous tissue would have directly reflected such permeability changes.

The data on incorporation into liver tissue of T and Y animals do not support the hypothesis that the effect was a generalized hormonal one, although there are no assurances that hormonal effects would have been the same on nervous tissue as on liver.

Spurred by the report of Banker and Cotman [1], there has been an increasing awareness of the possible lability of tritium. Considering this, one might propose that the reason that the T/Y ratio was small in the TCA precipitate counts of the 15-15 and 30-PRE schedules was that the label was not on amino acids by the time of training. Thus, protein synthesis would have been undetectable by radioactivity measurements. Such an argument may be valid, but a counter argument is presented by the 5-POST schedule. The tracer was in these animals only 5 min at room temperature, yet the T/Y ratios fit neatly into the pattern established by the various 30 min incorporation schedules. Additionally, Banker and Cotman reported that the major acceptor of $^3$H from labelled leucine was the $H_2O$ molecule [1]. Since the same pattern was present in the dried homogenate samples from which all $H_2O$ had been evaporated, it seems probable that much of the measured pool radioactivity was on amino acids, presumably available for protein synthesis.

The primary goal of this work has been to determine the importance of the temporal sequence of injection-training-sacrifice in determining the outcomes of precursor uptake studies of macromolecular correlates of learning. It appears that this sequence can have significant effects, and that these effects should be more fully investigated before theories of macromolecular synthesis related to learning are solidified.

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