# Protein Synthesis during Learning

Hydén and Lange (*1*) have reported that rats trained on the reversal of handedness exhibit increased protein synthesis in pyramidal neurons of the hippocampus. Their data, however, left the validity of this biochemical finding unresolved, for reasons discussed below. In particular, differences in the ³H-leucine concentrations suggest that the apparent increase in protein synthesis was an artifact, and that the only clear biochemical effect was the difference in the ³H-leucine concentration. Furthermore, even if these biochemical findings can be rigorously established, the behavioral conditions and meaning of the study were not adequately considered.

1) The ³H-leucine concentration (for which the units of measurement should be given) is of central importance to the results, since the apparent increase in protein synthesis only becomes manifest when the nondiffering uncorrected specific activities are converted to corrected specific activities by dividing by the ³H-leucine concentration. Although not pointed out in the study, the experimental ³H-leucine concentration (Table 1) was only one-half that of the control, a difference which is clearly significant. This raises two issues. First, if the difference occurred because greater amounts of protein were present in the experimental tissues as compared to the control, then the concentrations might represent a spurious indication of the tissue content of ³H-leucine, thereby invalidating the corrected specific activities. Presentation of data on the tritium and protein content of portions (100 μg) of hippocampal tissue might resolve this point. Second, if the difference in ³H-leucine concentrations arose as a function of the 30-minute training period, then the 30-minute ³H-leucine concentration measured in the experimental rats would underestimate the total ³H-leucine available during the 30 minutes, and the corrected specific activity would therefore overestimate the amount of protein synthesis. This could account for the entire apparent difference in protein synthesis. Additional data on experimental and control ³H-leucine concentrations at 0, 15, and 30 minutes would permit the use of temporal integrations of ³H-leucine concentrations as the basis for corrections.

2) Apart from the above issue, the data of Fig. 2 (as corrected in the erratum on page 201) do not clearly support the appropriateness of obtaining corrected specific activities based on the ratio of uncorrected specific activity to ³H-leucine concentration. From the number of data points and their means, it seems likely that the data of Fig. 2 represent the same data as those in Table 1. However, the apparent error in decimal point between the units of the y-axis (Fig. 2) and the units of uncorrected specific activity (Table 1) should be clarified. The origins of the two regression lines and the unidentified solid line (Fig. 2) are ambiguous, although our calculations indicate that the two regression lines most likely represent the y-on-x and x-on-y regressions of all 13 data points. However, the statement of Hydén and Lange that ". . . the specific activity of the protein . . . varies as a result of variations in the local concentration of ³H-leucine . . .," which appears to be a reasonable assumption, indicates that only the y-on-x regression, and not the x-on-y regression or the solid line, would have meaning. The apparent y-on-x regression (Fig. 2) approximates the conditions necessary for calculating the corrected specific activity, except that the regression appears to combine experimental and control groups which should not be combined because they differ markedly in ³H-leucine concentrations. The combined regression certainly offers no proof that the separate experimental and control regressions would each have significant slopes as well as intercepts of zero. Yet each of these regressions must satisfy both of these requirements in order to justify the ratio formula for obtaining the corrected specific activity (*2*). If there is not enough statistical power in these data to establish these requirements for the two groups separately, then additional data should be offered, particularly in view of the critical importance of the correction of the uncorrected specific activity in producing evidence for increased protein synthesis. In any event, the identity of the four control points (Fig. 2) should be indicated.

3) Data on specific activity were used without verification that ³H-leucine activity was directly proportional to the amount of protein. If these measures did not correlate significantly, then the specific activity ratio would provide no more information than ³H-protein activity alone and would be more variable, thereby increasing the likelihood of statistical β errors (failure to recognize a real effect). If there were linear regressions which did not pass through zero, then the specific activity ratio would be inaccurate and should be abandoned for a correction procedure such as covariance (*3*).

4) There are a number of statistical ambiguities which should be resolved. The meaning of n, defined in Table 1 as the number of experiments, is not clear, although we interpreted it to mean the number of replicate chemical determinations on each hippocampus. Assuming such replicate determinations, it is important for one to know whether the deviation value for each group mean in Table 1 was calculated from (i) the sums of the squares of the replicates about each hippocampus mean or about the group mean, or (ii) the sums of the squares of the nine experimental hippocampus means and the four control hippocampus means about their respective group means. Only (ii) would provide the proper deviation term for the statistical comparisons reported. The paper also failed to indicate whether the reported deviations were standard deviations or standard errors of the mean.

In addition, statistical tests reported (column 3, p. 1372) were not identified, although we assume the use of uncorrelated t-tests. However, the use of a correlated t to test the differences between sides would in general have been more powerful than the testing of contralateral to ipsalateral quotients with the uncorrelated t. In fact, uncorrelated t-tests applied to the reported quotients yield significance levels at P < .05 only for one-tailed tests. For example, a t of 1.93 with 8 degrees of freedom is obtained for the quotient 1.56 ± 0.29 (S.E.), which would be significant at P < .05 for a one-tailed t-test (for which the critical t = 1.86), but not at P < .05 for a two-tailed t-test (for which the critical t = 2.31). However, since the last sentence of the paper states that the laterality findings were unexpected, two-tailed tests should be used.

5) Finally, this study derives its main importance from the possibility that neurochemical changes may have occurred in response to behavioral training. The conditions of this training therefore deserve clear statement. Despite this, the behavioral test was de-

scribed as "modified" without identification of the modification, and the behavioral treatment of the control group was not specified. The paper also devotes considerable discussion to the speculation that the observed neurochemical changes were correlated with learning processes. This is only one possibility of several which bear mention. For instance, it is plausible that sensorimotor performance could induce the observed neurochemical effect; biochemical data on contralateral and ipsalateral hippocampi of control rats who reach for food without a forced change in handedness might help to settle this matter. It is also plausible that differences in the amount of obtained food, motor activity, and stress or frustration associated with forcing a change in handedness might affect hippocampal neurochemistry. Although controls for all these possibilities, and others, need not be expected in a preliminary report, nevertheless the consequences of the absence of these controls should be explicitly recognized.

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Bowman and Harding have suggested that the increased protein synthesis observed in hippocampal nerve cells is an artifact. The well-known behavioral test we used—the transfer of handedness in rats—was first described in the early 1930's. The experimental details (*1*) and an outline of modifications of the original experiment introduced by Peterson *et al.* have been described (*2, 3*). The major modification consisted of a sharpening of the task. We should emphasize that the animals were not forced to switch to the nonpreferred paw in retrieving food from the glass tube, as Bowman and Harding state. During the establishment of the new behavior an increased incorporation of $^3$H-leucine occurred in protein of the pyramidal nerve cells of the hippocampus (CA3 region).

Recently we have tried to answer the question whether the observed changes in the $^3$H-leucine concentrations were specific for the process. Rats were again trained with the nonpreferred paw and the protein analysis of the hippocampal nerve cells was performed on the 5th day. Some of these rats were returned to their cages and subjected to resumed training a fortnight after the initial training, after which the protein concentrations of some rats were again analyzed. Still others were returned to their cages and subjected to a third period of training 1 month after the initial training. At all periods of training the rats performed well. Increased synthesis of two acidic protein fractions in the pyramidal nerve cells of the hippocampus occurred on the 5th day of the original training and at resumed training after 14 days, but not at resumed training after 30 days. This result strongly suggests that the observed increase of protein synthesis in nerve cells was specific for the process. If protein synthesis were an expression only of a sustained neural function, it should also have been observed at resumed training after 30 days (*4*).

In their point 1, Bowman and Harding discuss whether the $^3$H-leucine concentration (in counts per minute in the supernatant divided by micrograms of protein in the sample) of the experimental tissue may be due to greater amounts of protein present in the experimental tissue as compared to the control, thereby invalidating the corrected specific activities observed. This cannot be the case because the same volume of cells was taken from both left and right CA3 regions of the hippocampus by a standardized method. The weights of protein for the three samples with their statistical variation (expressed as an average weight of protein with the standard error of the mean for the two halves of the hippocampus of the experimental and control animals) are: weight of protein from the right side of the hippocampus (contralateral to the used paw) was $32.6 \pm 2.1$ μg (15 animals); weight of protein from the left side (contralateral to the preferred paw) was $31.7 \pm 2.2$ μg (15 animals); weight of protein from the right and left sides of the hippocampus of the four control animals was $28.3 \pm 2.9$ μg. There were no differences in the weight of protein between the experimental and control tissues which could give rise to differences in $^3$H-leucine concentration of the magnitude shown in Table 1 of our paper. The postulate of Bowman and Harding would require a doubling of the protein content, and this did not occur.

Furthermore, these authors are of the opinion that the concentration of $^3$H-leucine measured in the experimental rats after the training period would underestimate the total $^3$H-leucine available during this period and that this could account for the difference in protein synthesis. The data presented above indicate that the weight of protein does not differ significantly in experimental and control tissue.

Such an underestimation of the $^3$H-leucine concentration would imply a higher incorporation of $^3$H-leucine in protein of the experimental tissue than in that of the control, resulting in higher (uncorrected) specific activities of the experimental tissues. The data observed (Table 1) contradict this part of the objection of Bowman and Harding. The values for the experimental animals were $5.70 \pm 0.84$ and $7.30 \pm 0.93$ counts per minute per $10^{-8}$ g of protein as compared to $8.12 \pm 0.96$ for the controls.

In points 2, 3, and 4, Bowman and Harding have misinterpreted the data in Fig. 2 and the details of our recently published method (*5*) as indicated by their statement that the data of Fig. 2 and Table 1 are the same. Furthermore, their suggestion that we have combined control and experimental data is surprising. The data of Fig. 2 were obtained on rats given varying amounts of $^3$H-leucine (5 to 60 μl, 1 μc/μl) by administration bilaterally into the ventricles. The results were plotted (Fig. 2) as uncorrected specific activities of unseparated protein against the $^3$H-leucine concentration. The amounts of radioactivity incorporated depend on the $^3$H-leucine concentration. However, both variables are subject to experimental errors. This gives rise to two regression lines, with a third line between them representing the (most probable) functional relation (*6*, pp. 153 and 164). Conventional statistical methods indicate that the intercept (Fig. 2) is not significantly different from zero (which is evident both from inspection and from the fact that, when the $^3$H-leucine concentration equals zero, the specific activity is zero).

It is of no practical importance for the correction procedure whether data are treated as a *y*-on-*x* regression or an *x*-on-*y* regression. The comment by Bowman and Harding that only *y*-on-*x* regressions have meaning is incorrect. Perhaps the best way to treat the data is to use the method of Bartlett (*7*),

that is, fitting the linear functional relation when the error variances of $x$ and $y$ are unknown. According to his method, the slope of the line is 1.74 with 95 percent confidence limits of 2.84 and 1.00 passing through $\bar{x}$ and $\bar{y}$; such a line is practically identical with the solid line (Fig. 2). In answering point 3 we would verify that the ^{3}H-leucine activity incorporated was proportional to the amount of protein.

Thus, the only way to compare specific activities of the protein from different samples is to use the correction for variation in ^{3}H-leucine concentration based on the linear relationship between (uncorrected) specific activity and ^{3}H-leucine concentration.

In Table 1, the sums of the squares are calculated about their respective group means. The errors given are standard errors of the mean.

Bowman and Harding object to the use of the single-sided $t$-test. We used this $t$-test because the specific activities of defined proteins of the hippocampal nerve cells are significantly higher in the experimental material than in the control. These data prompted the question whether one side of the hippocampus responded with a higher activity than the opposite side, in view of the fact that nerve cells in a control area of the sensorimotor cortex contralateral to the training paw respond on training with a synthesis of small amounts of DNA-like RNA (1, 8). Furthermore, Booth (9) conducted experiments on the transfer of handedness in rats in which he gave intracerebral pulses of radioactive orotate at various times before and after an hour of training. Autoradiographs showed a marked concentration of the labeled component only in pyramidal neurons of the anterior motor cortex and in the hippocampus, with greater intensity on the side contralateral to the practicing paw. Booth concluded that (9) "these results support and extend the RNA base composition analysis." Therefore we questioned whether the cells of the hippocampus contralateral to the training paw would show higher values of the specific activity than those of the ipsalateral side. By using the single-sided $t$-test, we found a trend to lateralization of the highest degree of protein synthesis on the side contralateral to the training paw, a result which requires further elucidation.

Bowman and Harding consider stress as a factor which induces biochemical changes in nerve cells. We found an increase of nuclear RNA in the nerve cells of rats during a stress experiment (10). This response was considered to be unspecific because of the RNA composition. The newly synthesized RNA was not characterized by high proportions of adenine and uracil, as in the establishment of a new behavior. Neither did training with the preferred paw give a specific response. Similar controls on the RNA response of nerve cells have been reported for five additional cases (11). We have thus performed the necessary control experiments.

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*Erratum:* In the report "Protein synthesis in the hippocampal pyramidal cells of rats during a behavioral test" by H. Hydén and P. W. Lange [**159**, 1370 (1968)], Fig. 2 [Linear relation between specific activity of protein and ^{3}H-leucine concentration in the CA3 region of hippocampus. Regression lines dotted.] should have been
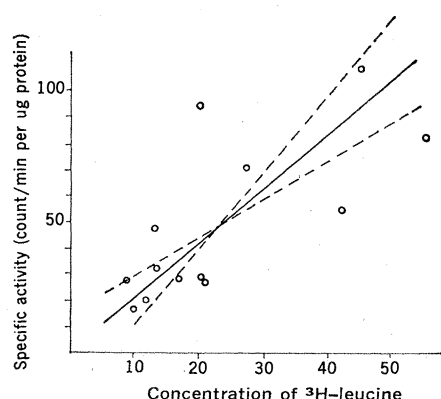


rather than
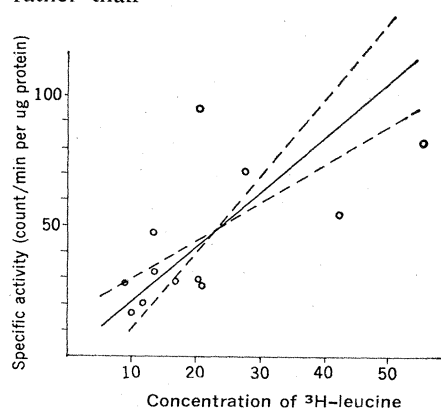


## Stratigraphic Data and Length of the Synodic Month

Pannella, MacClintock, and Thompson (1) presented evidence of variations in length of synodical month based on studies of growth bandings in living and fossil organisms, and suggested that the slowing down of the earth's rotation has not taken place at a uniform rate. A curve drawn on nine points derived from organisms ranging in age from Late Cambrian to the present appears to show that the length of the synodical month decreased rapidly through the Paleozoic, held steady through the Mesozoic, and decreased rapidly through the Cenozoic to the present. We would like to call attention to (i) errors in the age assignments of specimens due to incorrect stratigraphic data; (ii) discrepancies in radiometric dates due to differences between published time scales; and (iii) weaknesses in the primary growth banding data due to biological and statistical considerations.

The stratigraphic errors detected by us involve the Eocene point, which is based on growth increment counts from two specimens, probably neither of which is from the Eocene. The upper Eocene is represented in the data by *Crassatella mississippiensis* Conrad, a poorly known bivalve species described 120 years ago from "Newer Eocene" deposits at Vicksburg, Mississippi (2). There is no Eocene exposed at Vicksburg. It is the type locality of the Vicksburg Group (Oligocene), however, and prior to the proposal and general acceptance of Beyrich's Oligocene, rocks of that age were often included in the