325
9-27-66

MASTER

ENDOGENOUS BIOCHEMISTRY OF PLANARIANS CORRELATED WITH
LEARNING EXPERIMENTS AT BROOKHAVEN NATIONAL LABORATORY

SIMON FREED

RELEASED FOR ANNOUNCEMENT
IN NUCLEAR SCIENCE ABSTRACTS

December 1965

BROOKHAVEN NATIONAL LABORATORY
ASSOCIATED UNIVERSITIES, INC.

under contract with the

UNITED STATES ATOMIC ENERGY COMMISSION

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

H.C. \$2.00; MNr 50

ENDOGENOUS BIOCHEMISTRY OF PLANARIANS CORRELATED WITH LEARNING EXPERIMENTS AT BROOKHAVEN NATIONAL LABORATORY

SIMON FREED

RELEASED FOR ANNOUNCEMENT
IN NUCLEAR SCIENCE ABSTRACTS

December 1965

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

BROOKHAVEN NATIONAL LABORATORY
UPTON, NEW YORK 11973

LEGAL NOTICE

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or

B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

PRINTED IN USA

PRICE \$2.00

Available from the
Clearinghouse for Federal Scientific and Technical Information
National Bureau of Standards
U.S. Department of Commerce
Springfield, Virginia

August 1966

600 copies

ENDOGENOUS BIOCHEMISTRY OF PLANARIANS CORRELATED WITH LEARNING EXPERIMENTS AT BROOKHAVEN NATIONAL LABORATORY

Introduction

This report summarizes researches begun 4½ years ago by an interdisciplinary group which included a young physiological psychologist of considerable experience with invertebrates and animal psychology, a biologist with broad experience, a physical biochemist with an assistant to carry out biochemical analysis, and, as consultants, a psychologist whose specialty was planarians and a physical chemist with experience in methods utilizing radioactivity.

The first stage in these researches was reached about two years later and is summarized, for the most part, in the Ph.D. thesis of William Corning in the Department of Psychology, University of Rochester, the work for which was performed at Brookhaven National Laboratory.

When the program was initiated, evidence was beginning to accumulate, and has since become much stronger, that ribonucleic acid (RNA) may be of central importance in the storage of information gained in the behavioral processes of learning. The thesis work indicated that the changes in the nucleic acids within the animal during conditioning were different from the changes within control animals subjected to random light and shock stimuli rather than to paired stimuli as in conditioning. Radioactive phosphorus, P^{32} , has been present in the bath of planarians at the time of training and its incorporation within the nucleic acid molecules served to distinguish these nucleic acids from others laid down throughout the lives of the animals. In terms of the ratio of the specific activity of RNA- P^{32} to that of DNA- P^{32} , at stages of the conditioning process, the animals being conditioned showed ratios different from those of the control animals.

In Figure 1, the upper drawing gives the values of the ratios of the specific activities of the nucleic acids with increase in number of presentations of stimuli. The "curve" $L-S$ represents the ratios in animals subjected to paired light and shock stimuli, and the "curve" $L,S(R)$ gives the ratios for

worms subjected to the same number of randomly applied light and shock stimuli.

The lower drawing gives the number of unconditioned responses of the planarians to light alone, i.e., responses given as if they had been or were about to be shocked. They increasingly associated light and shock with continued training by paired ($L-S$) stimuli. If a similar growth in responses had occurred when light and shock were administered randomly, it would have indicated sensitization of the animal by shock. Figure 1 shows, on the contrary, that no trend in number of responses occurred with light alone under random stimuli from the beginning to the end of the experiment.

Figure 1. Changes in the responses and in the RNA/DNA specific activity ratio during training of planarians. Subjects receiving paired light and shock stimuli ($L-S$) are compared with controls receiving random stimuli (L,S). H on the abscissa marks the end of the period of habituation to light. Taken from W.C. Corning, Ph.D. thesis, University of Rochester, 1963.

By erecting vertical lines through both upper and lower drawings at fixed numbers of trials, it can be observed that at trial 160 the endogenous chemical differences precede behavioral differences, that is, statistically significant differences in the ratios of the nucleic acids appear before any statistical differences in behavior. This observation is reinforced at 320 trials, where again statistically significant chemical changes are well in advance of acquisition of the new behavioral pattern. At this stage the numbers of the behavioral responses between the randomly stimulated planarians and those receiving paired stimuli are statistically not yet distinguishable.

After the planarians had acquired the highest degree of conditioning, at about 450 trials, the ratio of the specific activities of the nucleic acids which incorporated the label during the conditioning period dropped and equaled the ratio prevailing in the control animals. It seems then as if the pattern of information now consolidated in the behavior of the animals has its counterpart in the pattern of information already laid down chemically without further distinction between the conditioned and the control animals.

The following pages describe efforts to repeat the above experiments under more favorable conditions of behavioral control and biochemical analysis. Improved electronic circuits guaranteed identical electric intensities of shock for conditioning and for control. In addition, they made the electric intensity of the shock less variable with the position and orientation assumed by the planarian in its bath. The problem of electronics may thus be regarded as having been solved.

New chemical procedures were devised for characterizing changes within the nucleic acids, for quantitative isolation of their four component mononucleotides, each of which contained P^{32} for radioactive counting. This ratio has a marked bearing on the informational content of the nucleic acids synthesized during stimulation. When William Corning's thesis was written, tissue from eight planarians had been required for isolating enough mononucleotides for analysis. Now only about $\frac{1}{8}$ of a planarian is required, and the chemical analysis may be related in principle to the observed learning behavior of a single particular planarian rather than being averaged over eight. Indeed, changes in nucleic acids can now be followed as a gradient in chemical composition laid down from head to tail, and the variation in this profile of compositions, as training proceeds, can

be observed. A parallel study of control animals would serve for comparison.

The possibilities of these advantages have not been realized. Up to the present, the improvements were not all available at the same time. When training was excellent, the electronic circuits for random stimuli were not reliable and the chemical methods had not yet reached good reproducibility. When the latter were functioning properly, and in fact were routine in performance, difficulties appeared in training.

Since the inception of these researches (spring and summer of 1961), papers based on other approaches¹⁻⁵ have been published whose results give strong support to the basic assumptions of the adopted program: (1) conditioning of animals importantly involves nucleic acids that animals lay down during the learning experience, and (2) chemical methods are sensitive and discriminating enough (or can be made so without inordinate expenditure of time) to analyze some basic features of the endogenous chemistry of an animal correlated with its conditioning or learning.

Previous Work

Planarians, flatworms inhabiting ponds and streams, are members of the phylum Platyhelminthes, class Turbellaria. They are usually 8 to 20 mm long and 2 to 4 mm wide at the widest extension near the head. They represent the emergence in evolutionary development of true nerve synapses, bilaterality, and cephalic enlargement of the nervous system, in short, the emergence of a "brain and a central nervous system." The nervous system is simple, typically containing two cephalic ganglia, from which two longitudinal strands emerge to traverse the length of the organism. In some species, transverse fibers connect the two longitudinal strands in a ladder-type configuration.

The flatworm possesses several types of sensory receptors. In *Dugesia dorotocephala* and *D. tigrina*, two eyes are prominent near the nerve ganglia. Tactile cells are concentrated in the anterior end. Chemoreceptors and rheoreceptors (current detectors) are also present.

When worms are sectioned transversely into two or more pieces, the sections regenerate in several weeks into complete organisms. Multiple-headed or multiple-tailed planarians can be produced by grafting and they survive actively. Their cellular tissues may be regarded as semiembryonic since

Figure 2. Top: an enlarged photograph of the head portion of a typical planarian. Insert: a whole worm, about $1.2\times$ natural size. Bottom: abnormal worms produced by sectioning and grafting.

cells may differentiate to acquire varied functions. (See Figure 2.)

A published review⁶ of the voluminous literature on learning by planarians makes it unnecessary to discuss the earlier work. Planarians can learn to perform a number of tasks. Recently they were trained not only to choose a particular arm of a T maze but also to discriminate between rough and smooth surfaces of the arms.⁷

A publication of importance for this discussion is that of Thompson and McConnell,⁸ who first

used controls in conditioning planarians. Somewhat as in classical conditioning, flashes of light were used as "neutral" stimuli (conditioned stimulus, *CS*) and a shock by direct current as the unconditioned stimulus (*US*), the stimulus that elicits the instinctive response, a longitudinal contraction or violent turning. After the two kinds of stimulus had been paired and repeatedly presented, the light flash alone that previously elicited few and mild responses now was increasingly followed by responses somewhat similar to those invariably induced by electric shock. Because this work may be regarded as the starting point of most subsequent researches, the following quotation is given:

"The subjects were divided roughly in terms of length into four groups of 10 subjects each: the experimental group (group *E*), the light control group (group *LC*), the shock control group (group *SC*), and the response control group (group *RC*).

"Group *E* was given a total of 150 trials, each trial consisting of a light-shock pairing. The duration of the light was 3 sec.; that of the shock was 1 sec. The *US* overlapped the light on the last second of its presentation. All responses occurring during the initial 2-sec. period of the light were recorded. Training was carried out in three blocks of 50 trials each. A 5-min. interval separated each block. The intertrial interval was approximately 20 sec.

"For group *LC*, 150 trials were administered in the same manner as for group *E*, except that the *US* (shock) was never given. The light lasted for 3 sec., and responses were recorded only if they appeared during the first 2 sec.

"To determine if the shock sensitized the animal to the light stimulus, group *SC* was given a total of 150 shock trials, each trial lasting for 1 sec. After every five shock trials, 1 test trial was given which consisted of the presentation of the light alone for 3 sec. In this case, three blocks of 60 trials were presented, 50 shock trials and 10 test trials in each block. Thus a total of 30 test trials was obtained. All responses occurring during the first 2 sec. of the light were recorded.

"Group *RC* was employed to determine the number of (spontaneous) responses which one might expect to occur without stimulation from either shock or light. For this group, 150 trials of 3-sec. duration were given during which no experimental stimulation of any kind was administered to the animals. All responses that occurred during the initial 2 sec. of each trial were recorded."

The positive responses to light in the first 25 trials were about 30%, while in the last 25 of a total of 150 trials, they amounted to 42%.

Procedures in training have since undergone many modifications to decrease the initial and increase the final percentages of response. Signs of fatigue were recognized and the number of trials was reduced from 150 trials a day with two rest periods of 5 min each to as few as 20 trials a day with a rest period of 10 min after 10 trials. The source of light continued to be two 100-W bulbs 1 ft away, with continuous background illumination from a 7½-W bulb for observing whether motions were begun before or after the incidence of the strong light flash. As criterion of achieved training the McConnell school accepted 18 positive responses out of 20 successive presentations of flash stimuli. The more recent apparatus consists of a narrow trough of Teflon, at the ends of which are platinum electrodes activated by an inductorium.

The care of the planarians is complex. These worms are subject to various diseases and unknown influences. While several authors have confirmed the results of Thompson and McConnell, others have failed to do so, sometimes ignoring factors known to be important. Clearly success depended on a number of factors, some unknown.

Before training is begun, reduction of spontaneous responses to light alone may be achieved by habituation, i.e., by presenting light flashes unaccompanied by shock, in the same sequence as in training. Habituation is a form of learning and was indeed the first form reported (1908). Recently a systematic study of habituation⁹ has been made. Figure 3 depicts responses planarians make to shock, to light, and to paired stimuli presented repeatedly for conditioning.

A next stage in the development of this field is described in the following observations, quoted from the review by A.L. Jacobson⁶:

"McConnell, Jacobson, and Kimble (1959) employed the light-shock paradigm of Thompson and McConnell, using training (50 trials a day) and establishing a definite criterion of conditioning (23 responses to the CS out of any 25 consecutive trials). Once a worm (*D. dorotocephala*) had reached criterion, it was immediately cut in half transversely, and the halves were isolated and allowed to regenerate. When regeneration was complete (about 4 weeks), all animals were retrained to the original criterion. Whereas an average of 134 trials had been required for initial training, only 40 and 43.2 trials were required for retrain-

ing in the original head- and tail-halves, respectively. These test-retest differences were highly significant. A trained but uncut group showed the same amount of savings as did the experimental animals. A group that was cut and *then* trained (after regeneration) actually took *more* trials than the initial training of the experimental group, thus eliminating the possibility of sensitization by the cutting and regeneration process. An independent replication of this experiment by Agostoni (1960) yielded identical results.

"Following up these findings and using the same conditioning arrangement, McConnell, R. Jacobson, and Maynard (1959) tested for regeneration in 'totally re-formed' worms. They reasoned that if worms totally re-formed from a conditioned 'ancestor' showed savings, this would provide evidence for a chemical process involved in the regenerative transmission demonstrated by McConnell et al. (1959a). McConnell et al. (1959b) *cut* large planaria (*D. dorotocephala* or *D. tigrina*) in half posterior

Figure 3. Top: sequence relationship between light and shock stimuli given to planarians during training. Bottom: typical responses observed during training. Head waving was frequently seen in naïve worms and it was not counted as a valid response. Both naïve and trained worms responded identically to electrical shock stimuli.

to the pharynx, conditioned the head end using relatively spaced trials, then cut off the regenerated tissue up to the pharynx, and isolated the head end. After the head had regenerated a new tail they again cut the worm posterior to the pharynx, and allowed both halves to regenerate. Finally, they tested both of these latter groups. It will be noted that one of these (referred to hereafter as group *A*) contained the head half of the original worm, whereas the other group (*B*) contained animals consisting completely of tissue re-formed or regenerated subsequent to conditioning. Nonetheless, both group *A* ($\bar{X}$ = 56 trials to criterion) and group *B* ($\bar{X}$ = 122 trials) showed significant savings as compared with the original training ($\bar{X}$ = 268 trials). The difference in savings between groups *A* and *B* was also significant."

Since there is concentration of nerve tissue in the head, which contains the only ganglia, the above results suggest a transmission of information from the "brain" to the tail portion and a retention of information by the tail during regeneration even in successive generations of "new" tissue.

Corning and John¹¹ supposed that the information of learning, in analogy with the transmission of genetic information, might be coded in nucleic acid, most probably in ribonucleic acid (RNA) rather than in deoxyribonucleic acid (DNA), the custodian of genetic information. As a test they exposed two transected halves of a trained worm to a dilute solution of the enzyme ribonuclease while these halves were regenerating into two complete worms. Ribonuclease accelerates the hydrolysis of the macromolecule RNA into small units and so destroys the information that is stored in the order in which the units are coupled.

They observed that the planarian regenerated from the head portion of the transected trained planarian retained about the same saving in trials to reach criterion as if it had regenerated in ordinary pond water, but that the planarian regenerated from the tail portion had its "memory" erased; this planarian required about the same number of trials to reach criterion as if it had been regenerated from a transected naïve worm. Thus it would seem that nucleic acids are importantly involved in the storage of behavioral information.

Current Study

Soon after completion of this experiment William Corning was invited to join Brookhaven's

Chemistry Department for the summer and participate in the researches described here. At the summer's end he abandoned a thesis in physiological psychology and performed the work for his doctoral thesis at this Laboratory.

Participating in this program, in addition to Corning and the author, who was assisted in chemical analysis by Raymond Goldberg, were Henry Quastler of the Biology Department and, as consultants, Norman Sutin of the Chemistry Department and James McConnell of the University of Michigan. Frank Grady, a medical student at Yale University, also participated for two summers. Plans were discussed in the spring of 1961 and experimental work was started that summer.

It should be kept in mind that the nucleic acids are copolymerized mononucleic acids or nucleotides. Four of these constitute RNA and four DNA. The nucleotides consist of two purine bases and two pyrimidine bases joined to phosphated pentose sugars. Three of the bases are identical in RNA and in DNA; the fourth in DNA is the methylated fourth base in RNA. The sugar in RNA is ribose, while in DNA one of the hydroxyl groups of ribose is replaced by a hydrogen atom, and the sugar formed is deoxyribose.

The four bases of DNA are shown in Figure 4. Typical nucleotides of RNA and DNA are adenylic acid and deoxyadenylic acid (see Figure 5).

The phosphate groups join the sugars of the nucleotides and form with them the backbone of the macromolecule, while bases in spatial proximity couple with each other by hydrogen bonding, as indicated in Figure 4.

The macromolecule does not exchange its phosphorus with phosphate ion in solution. This fact is primary in our experimental design. The presence of P^{32} within the structure of nucleic acids implies its incorporation during synthesis. If P^{32} as inorganic phosphate is restricted to the bath of planarians only while they are trained or stimulated, its subsequent finding in nucleic acid marks the synthesis of the nucleic acid as of the time of training or stimulation. This nucleic acid may then be differentiated from any other laid down by the animal at other times of its life.

The animal was killed and frozen at the end of training to avoid subsequent secondary enzymatic hydrolyses of the nucleic acid containing P^{32} and resynthesis while not in training.

Figure 4. Pairing of bases shown as it occurs in the polymer chain of DNA.

For the purpose of feeling our way in the first experiment, it was decided to avoid contaminating the electrodes, training dishes, etc., with radioactivity from P^{32} by preloading the planarians with P^{32} just before training and replacing the radioactive water with ordinary pond water. Training would then begin. The turnover of nucleic acids or incorporation of fresh nucleic acid during training would then show up as a reduction in the specific activity of the RNA- P^{32} . The results summarized in Figure 1 were obtained in this way. Only considerably after the thesis work was the original intention carried out, with the use of P^{32} as marker to permit in principle the isolation of the nucleic acids containing the information of interest.

In the beginning, it was assumed that DNA, supposedly representing only "inalterable" genetic material, would be synthesized to a trifling degree during the stimulation periods. The measured DNA could then be equated with the total number of cells within the worm. Planarians of the same size, appearance, etc., presumably would not have widely differing numbers of cells. In this way

Figure 5. Typical purine nucleotides of RNA (top) and of DNA.

the synthesized RNA might be normalized with respect to DNA content.

The facts contradicted these suppositions. Henry Quastler found indications that DNA was also present in the cytoplasm of planarian cells, that is, it was not entirely restricted to the nuclei where genetic DNA resides. He also discovered that the tritiated precursors of nucleic acids customarily utilized in autoradiography were not incorporated from the bath by planarians. He was able to inject these precursors into the animal and in this way induce the appearance of labeled nucleic acids. Injection in itself, by disturbing the animal, might affect the synthesis of nucleic acid and so vitiate the role of the precursor as a mere tag of nucleic acids associated with learning. However, P^{32} was rapidly incorporated from pond water, and autoradiography to localize nucleic acid synthesis could be carried out with this tracer which, although not as desirable as tritium, is still capable of yielding valuable information. As shown later, the decision to use P^{32} had important bearing on the selection of analytical procedures for determining ratios of types of nucleotides, i.e., "base ratios," within the nucleic acids of interest.

Figure 6 depicts the design of the apparatus for presenting stimuli to the planarians, a distinct departure from all others. This design resulted from many attempts to give the planarians shocks of greater uniformity, more or less independent of the position and orientation the animal could take in the vessel. Mass training was carried out in glass cylindrical crystallizing dishes. The worms were placed inside a porous earthenware ring that per-

Figure 6. Arrangement of the training vessel which was finally adopted.

mitted electrical conduction and yet kept the planarians from avoiding shock by sitting on the electrodes, a trick they quickly learned. Two pairs of electrodes at right angles to each other minimized the reduction in intensity of shock that planarians could produce by orienting themselves at right angles to the field resulting from the usual pair of electrodes.

In his thesis, Corning had observed that light of the intensity used in the Thompson and McConnell procedure, and presumably in all others since, was an aversive stimulus and would excite responses on its own. He therefore substituted one 15-W bulb for the two 100-W bulbs.

Prior to initiating the accepted training schedule some uniform basis for expressing analysis was sought. Intersubject variation of RNA per mg of tissue reached a rather stable average with eight planarians. Including an additional planarian to form an average was not likely to affect the average deviation already obtained, about 10%. Since the analyses for RNA entailed deviations of from 3 to 5% and for DNA of from 6 to 8%, the intersubject variation indicated that eight planarians should be regarded as a minimal number for any analysis. This number subsequently proved sufficient for the determination of the base ratios in RNA and DNA, but measurements of the ratios were not included in the thesis.

The experimental design followed by Corning was rather elaborate; more kinds of information were sought than have been indicated. For the present purpose, it may be described as follows:

The complete training group, as well as the controls, was divided into four stages: habituation, 120 trials; and training, 160 trials, 340 trials, and 460 trials. Forty trials a day were given, divided into 2 sections of 20 trials, with an intermediate 4-hr rest period. Mass training of all worms was employed, and at each stage about ten subjects were withdrawn for testing. Six of these were randomly selected for individual observation to determine the percentage response, i.e., the degree of conditioning. On each of three nights, before a given stage was reached, the ten planarians were placed in pond water containing P^{32} as phosphate ($20 \mu\text{Ci/ml}$). The following morning this water was replaced by fresh pond water and training continued. Again at night the ten planarians were surrounded by water containing P^{32} . When the desired state in training was reached, e.g., 160 trials, the animals were quickly plunged into liquid nitrogen. They were then freeze-dried at -20°C under vacuum, and the dried tissue was analyzed for nucleic acids.

There was no conclusive gross or total change in RNA or DNA within experimental error ($\pm 5\%$ RNA and $\pm 8\%$ DNA). At times the reproducibility was better than this. However, by radioactive counting changes were detected in the nucleic acids synthesized during the different stages in the two patterns of stimulation.

Figure 1 shows that when the results are expressed in terms of the ratios of the specific activity of RNA- P^{32} to that of DNA- P^{32} , differences emerge between the conditioned planarians and the controls (subject to randomly ordered stimuli). At 160 trials the difference is statistically significant, even more so at 340 trials. The controls were subjected to random stimuli because it had repeatedly been observed that stimulations, exercise, etc., affect the RNA content of cells. Since the same number and kind of stimuli were given to both groups of planarians, any differences would be attributable to differences in the temporal relations of the stimuli. The conditioned planarians responded to the light alone, which the randomly stimulated planarians failed on the average to do, and therefore could be expected to change their RNA content on the basis of muscular contraction. Some compensation for this was attempted

by tapping randomly on the vessel containing the control animals. The number of tappings was regulated according to the progress of the conditioning in order to make up the deficit in the number of muscular responses. The number of stimuli, both of light and of shock, in each training session was identical for the group undergoing conditioning and for the control group.

From the upper graph in Figure 1 it is evident that for a given value of DNA there is more turnover of RNA incorporating nonradioactive phosphorus from the bath during training than during the corresponding random stimulation. In addition, the graphs show that the chemical changes occurred before appreciable behavioral changes had set in. At 160 trials the difference in the ratios of specific activities is statistically significant ($p = 0.01$), whereas the lower graph shows that at 160 trials with stimuli of light and shock, randomly related, there is no significant differentiation in the percentages of response. At 340 trials these differences between chemistry and behavior are still more prominent, i.e., the chemical changes are still in advance of the behavioral. More than 340 trials are required before behavioral differences become unmistakably evident. After about 450 trials the ratios of the specific activities become about equal to those at the beginning, yet the trained animal continues to "remember" his previously learned pattern of behavior. There is no further distinction in new synthesis of nucleic acids as expressed by the ratios between the planarians receiving the stimuli associated in time and those receiving nonassociated stimuli. It would appear that the chemical information in molecules either remains unchanged or is self-replicating.

To put the above results on a more solid footing it was decided in the fall of 1963 to repeat these experiments under improved conditions with respect to analytical chemistry and controlled behavior. First, P^{32} would be introduced directly into the baths where stimuli occur. Second, miniaturization of the analytical chemical methods would be attempted to determine the content of nucleic acid and that of the separate nucleotides within a single worm in order to relate the chemical change within it to its particular behavior. Third, the electrical stimuli would come from the same power supply and would be of identical intensity for the worms receiving paired stimuli and those randomly stimulated. To simplify the fields supplying the electric shock, one pair of electrodes would be

activated first, and the other, at right angles to it, milliseconds later.

Just before this stage of the work Henry Quastler died and William Corning left to take a position at Michigan State University.

To reduce the number of planarians required for analysis, large planarians, *Dugesia dorotocephala*, 20 to 35 mm long and about 5 mm wide near the head, were obtained from California. When dried, these planarians weighed 7 to 9 mg instead of the usual 1 to 3 mg. They had the additional advantage of reproducing by laying eggs rather than dropping tails (these are sexual rather than asexual worms). They would usually remain intact throughout the entire training period. Tail dropping introduces regeneration of tissue, the production of new cells with attendant synthesis of DNA and RNA. Such synthesis acts as noise with respect to the RNA laid down as a result of the training process.

The California planarians were trained successfully by Claudia Ferguson, who had succeeded in training planarians as a hobby while a senior in a neighboring high school. She had fed her planarians egg yolk and continued to use this as food in her work here. Her equipment was similar to William Corning's. The flashes for 3 worms were produced by 7½-W bulbs and those for the 3 others by 15-W bulbs. A very small neon light furnished background illumination, although sometimes none at all was used. The electronics of the random shock and light stimuli was not yet functioning properly; hence there were no controls.

Habituation to light consisted in presenting 20 light flashes of 3 sec each every minute in the pattern that was to be used during the regular training sessions. The rest period of 10 min was inserted after 10 successive light flashes. These sessions were carried out at roughly the same hour of the day to avoid possible diurnal factors¹² in the habits of the planarians.

The graphs in Figure 7 indicate that in general the worms reduced the number of their responses to the light alone. A degree of habituation had occurred. This behavior already represents effective learning.

The broken lines are drawn through the total responses the planarian made to light, while the solid line gives what were named the *valid responses*. These omitted the simple turn, which was not infrequent before and during habituation. The other responses are given in Figure 3 under "trained."

Counted among the valid responses, but not shown, was a sudden start of movement after the onset of the light flash. The end of habituation is marked by the first vertical line at the left in each figure. The following day training started. During the third second of the light flash the electronic equip-

ment set off a momentary electric shock. The shock had a profile of voltage and time somewhat simpler than but resembling that of shock produced by an inductorium; however, both voltage and frequency were controlled. These adjustments were made to obtain distinct but not too violent

Figure 7. Total (broken lines) and valid (solid lines) responses of 6 worms during training. The vertical line at 100 trials marks the end of the period of habituation to light. The interval between the vertical lines at 380 and 400 trials was 39 days.

contractions in the planarians. A good response with subsequent undiminished activity of the planarian was the criterion of a good adjustment.

The drawings indicate a quick growth in the responses to light after successive pairings of light with shock. After about 100 of these combined stimuli, the number of valid responses to the light began to level off to 13 or 14 in worms *A*, *B*, *C*, and *D* and to 15 or 16 in worms *E* and *F*.

The time interval between the 2 vertical lines at 380 and 400 is 39 days.

Worm *D* was especially irregular after about 180 trials. At 240 trials the planarian made a sudden surge upward in its responses to 16; the responses then dropped to zero at 320 trials and remained there for 4 days, at 320, 340, 360, and 380 trials. In this period it dropped a tail.

After the 39-day interval a general drop in responses occurred. They still remained appreciably higher than the value after habituation or even before habituation to light. The small number of additional trials required to reach the former high plateau indicates to what extent the planarians had retained the results of their previous training. About 60 combined stimuli were sufficient to reach their previous plateau.

Worm *D* is outstanding. After 39 days, considerably after regeneration, the first training session increased its responses to values comparable with the highest at this stage of training, and in a total of 40 trials it reached levels of performance equal to the best. There seems to be a suggestion here that at the time of tail dropping, although its performance was poor, its conditioning or learning continued unabated.

Worm *F* died after 5 days of retraining.

In the summer of 1965, Miss Ferguson tried to train a set of planarians that were repeatedly dropping tails for reasons that could not be determined. Some worms dropped tails as many as four times during the course of training. The responses were very chaotic.

What is especially striking in the planarians trained the first summer is their uniformly good training. Unfortunately the controls of random light and shock were not functioning properly, nor were the analytical procedures yet developed. Both processes became routinely satisfactory more than a year later.

It should be added that this training was entirely manual and the operator did not adhere strictly to 3-sec periods of light. If toward the end

of 3 sec a planarian started to respond, the light was kept on until the response could be characterized. The same procedure was used on the worms that were repeatedly dropping tails.

CARE OF PLANARIANS

The planarians were fed liver in the early period of these researches, and the water had to be changed often to remove the remnants of food since bacterial growth occurred rapidly. If this was not done, scum would form on the surface of the water and the planarians would die. The likelihood of disease among planarians was high. Dishwashing entailed considerable labor and time. Similar results followed the use of egg yolk as food. The planarians exuded thick gelatinous waste which promoted bacterial growth. Dishwashing was, if anything, more demanding than when liver served as food.

Following the experience of others we turned to live food that contained inhibitors against bacterial infection and growth. Tubefex worms were eagerly digested by planarians in dramatic struggles. No work seems to have been reported on the training of planarians fed on tubefex. Bacterial infection of planarians was reduced greatly and along with this the inordinate amount of dishwashing. The bath water remained clear 2 or 3 times as long as previously. The worms thrived on the tubefex. However, it was later suspected that tubefex might have affected the learning capacity of the planarians because of the lack of some chemical ingredient required for synthesis of the macromolecules necessary for associating stimuli. With tubefex as food the pH of the water rose from 5.8 to 6.0; when liver was used, it rose to 7.0 to 7.3. On some occasions, the planarians fed with tubefex lived in an aquarium together with snails and algae, which created an almost ecological equilibrium. The presence of snails brought the pH to the highest levels. The planarians laid eggs, which probably hatched. Air from the compressor, after being washed, constantly bubbled through the aquarium.

Upon the advice of Dr. Eugene Sachs of the New York Medical College, water for the planarians was first left for a time in another ecological situation that included gold fish as well as snails and even some planarians that were not used for training.

The source of the water was changed from time to time as suggested by the degree of well-being of

the planarians. Once, when they were exceptionally sluggish, it was learned that Yaphank Lake, the source of the water, was badly contaminated with insecticide. Then water from the Village of Patchogue was tried until it was learned that the chemical content of the water was excessive. Finally, water that had not been chemically processed was obtained from wells at the Laboratory that were especially low in iron content. Analysis showed that the ratio of calcium to potassium was considerably lower than had been found favorable for the growth and regeneration of planarians. The addition of calcium chloride to this water to bring the ratio to 5:1 appeared to improve the daily activity of the planarians.

Janusz Gebicki joined our project in August 1963; Raymond Goldberg left. Dr. Gebicki had previously participated in biochemical researches and had prepared himself for this particular project by working for a time with Dr. O.H. Lowry of Washington University, St. Louis, in ultramicro-biochemical analysis. He was to spend most of his time developing micro- and submicroanalytical methods for the determination of nucleic acids in planarian tissue.

Dr. Gebicki also undertook, with the assistance of Patricia Donnelly, the training of planarians in new quarters and with the adequate new electronics for the training. The vertical cupboards containing 12 compartments each ("planarian classrooms") were placed in a horizontal position with the openings at the top rather than on the side. Visibility of the planarians was now much better than previously. Toward the top and center of the outer wall of each "classroom" was placed

Figure 8. Average values of the responses counted during the training of a group of 11 worms. Vertical bars indicate standard deviations. The vertical line on the left shows the end of the habituation period.

a 25-W bulb. Above it an aluminum reflector extending the whole length of the wall was bent to reflect much of the light toward the center of the compartment to be occupied by the training vessel with electrodes, etc.

The final electronics are described in a separate section. The electronics for random stimuli were not developed in time for the training experiment described below.

The planarians *Dugesia dorotocephala* had arrived from California July 7, 1965, and had been fed tubefex worms until the beginning of training, September 29. Their water had been obtained from a well at the Laboratory that was relatively free of iron, and some calcium chloride was added to make the ratio of calcium to potassium by weight between 4 and 5. After an habituation period of 20 trials, when the average response to light was <1 out of 20 trials, training began.

Figure 8 indicates averages of the "valid" responses, as defined in the description of the earlier training. The growth in the number of responses was slow and leveled off at about 13 to 14 out of 20 trials. The percentage of responses dropped as a rule over the weekend and rose to the higher level in a day or two. The percentage of positive responses was somewhat lower than that obtained by Claudia Ferguson. However, their value was decisively higher than the value at habituation.

The deviations in Figure 8 are somewhat higher than those obtained in Miss Ferguson's work but cannot be compared with the wild fluctuations re-

Figure 9. Average values of the responses counted during the training of a group of several worms. The light sources consisted of two 100-W bulbs and a 7½-W background light. Calculated from the data of Bennett and Calvin.¹³

ported by Bennett and Calvin¹³ which appeared so chaotic that the authors doubted that planarians could be conditioned. However, it seems that the fluctuations were random in character, since a plot of the average number of responses of all the worms^{13, p. 6} gives the graph in Figure 9, which has the characteristics of a typical conditioning curve, like those obtained by others and in the present work. The fluctuations in response, in fact, resemble the results obtained by Miss Ferguson when some disease seemed to affect the planarians.

Bennett and Calvin used the experimental devices of McConnell, two 100-W bulbs, with background illumination from a 7½-W bulb. A Harvard inductorium furnished the electric shock.

METHODS OF BIOCHEMICAL ANALYSIS

A published method¹⁴ for the microanalysis of nucleic acids in tissue was satisfactorily modified for application to planarian tissue.

To characterize changes in the composition within the nucleic acids, a first step is to determine the ratios of their component nucleotides. Differences in ratios of nucleic acids from trained and control worms would be vivid expressions of differences in information content and would be an important step in making correlations between chemistry and conditioning.

It was necessary to develop new analytical methods since no published methods for the determination of the nucleotides applied to the small quantities of planarian tissue of interest. A method for handling much smaller amounts of the bases had been developed¹⁵ at Gothenberg, Sweden. However, it could not be used in this work because the finally isolated and measured component of the nucleic acids no longer contained P³², the all-important tag of the nucleotides that designated them as having been formed during conditioning.

Our analytical procedure¹⁶ was an improved method of electrophoresis on cellulose acetate membranes which resolved the nucleotides more definitely and provided about 100 times the sensitivity of previous procedures. The present sensitivity appears adequate and is limited by conventional microchemical absorption spectroscopy. Should the need arise the method can be further refined.

In the work of Corning, about 8 planarians were required for determining the ratios of the nucleotides; at present the ratio can be measured within

a fraction of a planarian, < 1/10 of the larger animals. The base ratios can then be followed in the course of training in planarians sectioned from head to tail so that one can observe to what extent the changes in quantity and quality of the nucleic acids along the gradient correspond to transmission of information. At present the method can determine about 0.15 µg of each nucleotide in nucleic acid to ±3%.

This method is more convenient for our purpose than another method we devised after much labor.¹⁷ The approach was deemed for a time to be virtually impossible. It depends on thin-layer chromatography on cellulose mixed with silica. Other workers had used thin-layer chromatography successfully to separate synthetic mixtures of purified nucleotides taken from the shelf. Their methods failed to achieve separation between the nucleotides obtained by hydrolysis of RNA because of the presence of salts to which the systems were excessively vulnerable. To reduce the salt content it had been customary to depend on the rather low solubility of potassium perchlorate. Hydrolysis of RNA was carried out with potassium hydroxide which was subsequently neutralized with perchloric acid. The mixture was cooled to 0°C and potassium perchlorate was removed by filtration. We found that even at -20°C in an equivolume mixture of water and ethanol, the solubility of the salt was too great for effective use of thin-layer chromatography on cellulose. However, if the final salt arrived at was lithium perchlorate, its adequate removal became easy with organic solvents such as 1:1 ethyl ether and isopropanol. This was done after neutralization of excess lithium hydroxide with perchloric acid, evaporation of water by pumping, and extraction of the resulting dry residue with the solvents.

Another program of training and control was started November 10, 1965. By this time analytical procedures and the electronics in all circuits were on a reliable, routine basis.

However, the planarians did not respond to training to any significant degree for reasons altogether unknown. They showed somewhat larger variations than previously but not the widely fluctuating behavior found when planarians were dropping tails or such fluctuations as Bennett and Calvin described. In view of the paucity of responses the degree of conditioning was inadequate and the percentage deviations were so great as to make the low degree of training unusable. The

planarians subjected to paired light and shock gave an average after 480 trials of 6 valid responses (average deviation ± 2) out of 20 successive pairs of stimuli, a value they had attained also at 200 trials. At 280 the highest average was reached at 10 valid responses with an average deviation of 3.

The corresponding valid responses to random stimuli were 2 ± 1.3 at 480 trials, $0.6_{-0.6}^{+2.0}$ at 200 trials, and 2.0 ± 2 at 280 trials. From 300 to 420 trials there were virtually no responses and little variation (about 1), and at 440 and 460 trials responses were zero. Had the planarians become ill? It was indeed unfortunate that these were the only planarians on which there was time for the analysis of nucleic acids synthesized during training. Despite the poor conditioning, analyses were carried out. Also there was no time for taking advantage of our methods of analysis of base ratios within the nucleic acids.

The possibility existed that planarians could not be trained if they had been fed tubefex worms for a long time. The original appearance of the California planarians had altered considerably. They had become substantially smaller on the average and their pigmentation somewhat lighter. They were not, however, altogether inactive.

It has been recorded in McConnell's planarian handbook¹⁸ that planarians have been fed daphnia satisfactorily. On the assumption that some ingredient for ready nucleic acid synthesis was lacking in tubefex, we introduced live daphnia (water fleas) in an ecological environment in which both planarians and live daphnia coexisted. The planarians became very small. Under the conditions it became questionable whether the planarians were actually eating. After 3 or 4 weeks on this diet, planarians were subjected to training. Figure 10 shows the average number of valid responses.

A real improvement in training is visible even though the level obtained is not as high as in the early periods of the project. The reliability of the differences between the numbers of responses given by trained planarians and by controls is probably the highest ever measured at Brookhaven, because of the quality of the final electronics, which were devised by Ronald Withnell and Edward Nielson.

Because of the paucity of tests, it cannot be stated that some chemical deficiency in tubefex accounts for the planarians' "retardation" in learning, but the possibility exists.

There were no detectable differences within experimental error between the ratios of the specific

activities (RNA- P^{32} /DNA- P^{32}) of the trained planarians and their controls at any stage of the training program. The fact is that the degree of training achieved by the planarians was low and the average percent deviation in positive responses among the planarians was very high in this training program.

The following data on intersubject variation apply to untrained planarians (Table 1).

The head portion in worms 1, 2, 3, 4, 5, and 8 showed higher ratios than the middle and tail portions.

The subject variation among planarians in terms of average deviation from the mean in ratio of specific activity of RNA to that of DNA was relatively small. William Corning also found much less variation in this ratio than in the quantities.

In view of the extremely wide variation in the specific activities of RNA- P^{32} and of DNA- P^{32} and the moderate variation in their ratio, the conclusion follows that these variations are related and that cellular growth is basically responsible. If this is true, it would seem, in view of Corning's results, that paired light and shock stimuli have a different effect on the proliferation of cells in animals dur-

Figure 10. Comparison of the average responses of 6 worms receiving regular (paired) light and shock stimuli. The heavy line shows the changes in the number of responses of the group given regular stimuli after subtraction of the responses of the control group given random stimuli.

Table 1
Specific Activities of RNA-P³² and Ratios (RNA-P³²)/(DNA-P³²) in Untrained Planarians*

Planarian No.	Head		Middle		Tail	
	RNA-P ³²	(RNA-P ³²)/(DNA-P ³²)	RNA-P ³²	(RNA-P ³²)/(DNA-P ³²)	RNA-P ³²	(RNA-P ³²)/(DNA-P ³²)
1	28.6	4.03	12.1	2.05	15.6	3.00
2	257.5	8.08	17.2	3.13	95.6	2.39
3**	408	46.4	268.0	27.9	288.0	11.4
4	87.6	7.4	30.5	4.5	24.3	6.1
5	263	7.1	64.2	5.0	87.4	5.4
6	56.9	2.56	25.6	3.08	41.0	4.36
7	90.8	5.28	719	5.94	95.0	4.60
8	64.5	5.7	69.0	2.14	198.5	3.88
Av		5.74 ± 1.55		3.55 ± 1.23		4.28 ± 0.97

*This experiment was performed to obtain information on the degree of subject variability in measurements. The quantities of dried planarian tissue used for analysis varied from 0.115 to 2.10 mg. RNA per mg tissue was rather constant amidst all this variation: 26 µg/mg ± 20%.

**Not included in average.

ing a program of training than random light and shock stimuli.

This would lead to what seems at present the extreme conclusion that learning has been associated with cells created during the conditioning process. Such a possibility in these primitive animals requires much validation.

P³² LABELING PROCEDURE

Because only 12 separate training vessels were available for cells in the two training schemes (the random and the regular), 11 vessels were set up with one worm in each and the 12th contained a batch of about 20 animals to serve as a reserve. Training was begun and at desired stages 4 worms in each training scheme were exposed for 1 hr to labeled phosphorus (40 µCi/ml) in water containing about 0.1 µg phosphate per ml. During the exposure the worms were put through a normal training session which consisted of the usual 20 exposures to light and shock. After 1 hr the 4 worms from the regular training scheme together with their 4 controls from the random scheme were frozen individually in liquid N₂, freeze-dried, and stored at -80°C before analysis.

The killed worms were replaced by 4 worms taken from the reserve vessel. Each new worm had been exposed to the same training scheme as the

freshly killed animals but their individual responses had not been scored while they were being mass-trained.

Training was continued until a new batch of 4 worms given paired light and shock and 4 control animals given random light and shock were judged ready for exposure to P³². These were selected only from animals that had been under continuous observation for at least 2 weeks.

CIRCUIT FOR PLANARIA TRAINING EXPERIMENTS

Training Apparatus

The planarians were trained in circular glass vessels partly filled with water. Each vessel was equipped with 2 pairs of platinum electrodes (N-S and E-W) arranged along the edge of the training vessel (see Figure 6). The vessels were placed in wooden pigeonholes open only at the top and a 25-W light bulb was fixed above the vessel. There were two independent sets of 12 pigeonholes, one for regular, or paired, and the other for random training experiments.

Function of the Circuit

The circuit was designed to deliver ac or dc shocks to the 24 pairs of electrodes in 12 training

simultaneously and at regular intervals (1 min). The shock was delivered in the last ½ sec of the light exposure. (This method of operation is not recommended and was soon abandoned, because the simultaneous delivery of shock to 12 pairs of electrodes lowered the output voltage.)

In the manual operation, the delivery of shock and light were independent. Each training vessel was chosen by a selector switch (manual control box). Shock and light could then be delivered only to the selected vessel. Normally a 3-sec light exposure was accompanied in the last ½ sec by the shock.

Random Circuit. In normal use the shock circuit was operated automatically, so that shock was delivered in succession and at random intervals to each training vessel. Light was delivered manually, each vessel normally receiving a 3-sec exposure. In this procedure any overlap of light and shock was very rare and random. Provision was also made for automatic delivery of random shocks and light (timer for random light and shock). In this case the shocks were again delivered to each vessel in turn so that the power supply activated only two electrodes at any one time. This kept the quality of the shocks constant.

STATISTICAL ANALYSIS

Total amounts of RNA and DNA found in the planarians were analyzed statistically to determine the smallest number of animals needed to provide reasonably small and predictable deviations in the total amounts of nucleic acids which they contained, i.e., to arrive at a stable intersubject average.

Results are available for 23 worms. These were analyzed in 2 groups of 8 (Experiments *A* and *B*) and one group of 7 (Experiment *C*). Each group was subdivided into 4 worms which received random light and shock (ran.) and 4 worms (except in Experiment *C* where only 3 worms were in this group) which received paired light and shock (reg.).

The first section of Table 2 lists the analysis of the smallest groups of worms, namely, batches of 4 animals. The values for RNA and DNA are given for the regular and random group in each experiment. Figures are given in micrograms per milligram of dry weight of worm ($\mu\text{g}/\text{mg}$), together with the standard deviations for each group. The standard deviations as percentages of total analytical values are also shown. The second section

Table 2

Summary of Statistical Analysis

Experiment	No. of worms	Type of stimulus	RNA, $\mu\text{g}/\text{mg}$	DNA, $\mu\text{g}/\text{mg}$	Standard deviation, %	
					RNA	DNA
Worms Analyzed According to Experiment and Type of Stimulus						
A	4	Reg.	35.85 ± 7.32	10.16 ± 1.51	20	15
	4	Ran.	31.46 ± 3.90	11.72 ± 3.7	12	32
B	4	Reg.	31.57 ± 2.23	11.30 ± 1.12	7	10
	4	Ran.	30.07 ± 1.01	11.85 ± 2.03	3	17
C	3	Reg.	29.03 ± 3.92	9.63 ± 3.07	13	32
	4	Ran.	32.86 ± 3.46	13.68 ± 1.86	11	14
Worms Analyzed According to Experiment						
A	8	Reg. + ran.	33.66 ± 6.7	10.90 ± 2.1	12	19
B	8	Reg. + ran.	30.83 ± 5.4	11.58 ± 1.5	18	13
C	7	Reg. + ran.	32.22 ± 3.5	11.94 ± 2.9	11	24
Analysis of Total Group						
	23	Reg. + ran.	31.90 ± 3.06	11.74 ± 2.2	10	19

shows a similar analysis for groups of worms divided according to experiment (2 groups of 8 and 1 of 7 worms). The last section gives the analytical results and standard deviations for all 23 worms.

It appears that very high deviations occurred when worms were analyzed in groups of 4 animals and that they decreased as the size of the group examined increased.

Tables 3 to 5 summarize the total amounts of the two nucleic acids and their specific activities in groups of worms tested at different stages of learning.

For more than a year serious consideration has been given to starting a somewhat similar program with earthworms, which have been frequently conditioned and have greater reproducibility than planarians. However, these biochemical studies should not be limited to the primitive invertebrates.

The experiences with planarians have been recently applied to vertebrates, and startling results have been reported. In analogy with the work of Corning and John using ribonuclease on regenerating, sectioned trained planarians, Russian workers have reported that ribonuclease, when injected into rats that had elaborated a conditioned reflex, erases the memory of the conditioned or learned response but not the unconditioned response.

Even more striking is the work of A.L. Jacobson et al.,¹⁹ who report that RNA extracted from the brain of a trained rat, when injected into the brain of an untrained one, reduces the number of trials needed to reach criterion.

It seems then more timely than ever to continue researches on the endogenous chemistry of animals correlated with learning.

Table 3

Total Amounts of RNA and DNA and Their Specific Activities in Worms Tested at the 60-Trial Stage

Training	Worm No.	Portion	RNA, $\mu\text{g}/\text{mg}$	DNA, $\mu\text{g}/\text{mg}$	RNA, specific activity	DNA, specific activity	RNA/DNA, specific activity ratio
Reg.	1	Head	59.07	16.70	2.04	2.05	0.99
		Middle	39.60	10.19	0.78	0.74	1.06
		Tail	43.18	9.50	1.28	1.80	0.71
Reg.	2	Head	31.20	11.90	1.27	0.89	1.43
		Middle	29.27	8.41	0.81	0.93	0.87
		Tail	31.70	9.75	0.70	0.82	0.85
Reg.	3	Head	15.63	6.38	2.56	1.55	1.66
		Middle	33.69	9.66	1.13	1.05	1.08
		Tail	34.46	9.34	2.64	2.50	1.06
Reg.	4	Head	38.15	7.66	1.08	1.41	0.77
		Middle	31.40	10.32	0.97	0.95	1.02
		Tail	42.10	11.95	1.35	1.16	1.16
Ran.	5	Head	20.47	6.96	1.98	5.27	0.38
		Middle	28.63	9.96	0.94	0.64	1.46
		Tail	28.57	8.99	1.04	1.00	1.04
Ran.	6	Head	31.90	16.00	3.14	1.80	1.19
		Middle	31.84	9.26	2.31	2.13	1.08
		Tail	33.68	10.35	0.76	1.00	0.76
Ran.	7	Head	37.60	19.97	0.60	1.27	0.47
		Middle	29.86	10.12	4.96	1.04	0.82
		Tail	27.84	10.34	1.31	1.13	1.16
Ran.	8	Head	37.50	15.20	1.03	0.82	1.26
		Middle	35.19	11.19	0.70	0.91	0.77
		Tail	34.43	12.30	1.36	1.38	1.02

Table 4

Total Amounts of RNA and DNA and Their Specific Activities in Worms Tested at the 120-Trial Stage

Training	Worm No.	Portion	RNA, $\mu\text{g}/\text{mg}$	DNA, $\mu\text{g}/\text{mg}$	RNA, specific activity	DNA, specific activity	RNA/DNA, specific activity ratio
Reg.	1	Head	24.21	13.0	2.47	0.77	3.21
		Middle	32.13	9.8	66.24	9.92	6.68
		Tail	31.87	11.7	11.60	2.67	4.34
Reg.	2	Head	36.53	8.8	44.92	86.85	0.52
		Middle	33.47	10.3	1.08	33.25	0.03
		Tail	27.91	10.5	2.42	16.23	0.15
Reg.	3	Head	29.42	13.6	15.56	15.58	1.00
		Middle	28.43	9.0	1.69	11.19	0.15
		Tail	34.41	15.2	20.61	9.84	0.10
Reg.	4	Head	40.06	14.2	14.84	19.73	0.75
		Middle	29.45	8.3	12.70	1111.52	0.01
		Tail	31.23	11.2	58.09	102.52	0.57
Ran.	5	Head	42.93	18.1	0.66	15.38	0.04
		Middle	33.62	10.9	6.75	6.23	1.08
		Tail	30.33	9.9	9.71	47.85	0.20
Ran.	6	Head	10.78	13.9	15.66	43.63	0.36
		Middle	25.46	8.0	31.42	0.15	29.95
		Tail	27.02	10.3	14.69	30.23	0.49
Ran.	7	Head	29.68	13.1	6.48	17.74	0.37
		Middle	33.76	14.0	2.17	5.19	0.42
		Tail	37.41	15.1	2.14	9.04	0.24
Ran.	8	Head	33.02	12.2	5.91	8.33	0.71
		Middle	27.26	7.5	4.43	7.47	0.59
		Tail	29.55	9.2	17.29	37.13	0.47

Table 5

Total Amounts of RNA and DNA and Their Specific Activities in Worms Tested at the 260-Trial Stage

Training	Worm No.	Portion	RNA, $\mu\text{g}/\text{mg}$	DNA, $\mu\text{g}/\text{mg}$	RNA, specific activity	DNA, specific activity	RNA/DNA, specific activity ratio
Reg.	1	Head	27.42	12.36	5.88	2.39	2.46
		Middle	28.91	9.07	0.32	2.16	0.15
		Tail	24.36	9.22	2.15	1.40	1.54
Reg.	2	Head	42.91	18.99	0.22	0.62	0.35
		Middle	29.90	8.22	0.53	0.64	0.83
		Tail	27.96	9.88	1.33	1.77	0.75
Reg.	3	Head	26.24	3.29	32.16	9.74	3.30
		Middle	23.75	7.05	1.14	0.80	1.43
		Tail	29.93	8.59	1.34	0.50	2.68
Ran.	5	Head	30.97	12.74	0.31	0.82	0.38
		Middle	38.21	12.24	2.64	2.60	1.02
		Tail	31.25	11.09	1.44	1.43	1.01
Ran.	6	Head	36.82	13.92	0.33	2.52	0.13
		Middle	29.25	10.00	1.04	0.46	2.26
		Tail	35.31	12.69	1.03	1.36	0.76
Ran.	7	Head	30.91	14.81	0.35	0.76	0.46
		Middle	32.43	9.40	1.52	2.82	0.54
		Tail	20.59	20.02	3.87	1.78	1.61
Ran.	8	Head	27.65	15.76	2.06	0.56	3.68
		Middle	44.68		0.45		

REFERENCES

1. DINGMAN, W. AND SPORN, H.B., *J. Psychological Res.* **1**, 1 (1961).
2. FLEXNER, J.B., FLEXNER, L.B., AND STELLAR, E., *Science* **141**, 57 (1963).
3. HYDÉN, H. AND EGYHÁZI, E., *Proc. Natl. Acad. Sci. U.S.* **48**, 1366 (1962).
4. VLADIMIROVA, T.A., Change in ammonia content of the brain related to conditioning processes, in *Problems of Biochemistry of the Nervous System*, Institute of Biochemistry, Academy of Sciences, Ukrainian SSR, Kiev, 1957.
5. KHATCHATRIAN, G.S., Changes in some aspects of carbohydrate metabolism in brain during various functional states, Abstr. in *Proc. 5th Intern. Congr. Biochemistry, Moscow, 1961*, Vol. 9, p. 280, N.M. Sissakian, Editor, Pergamon, Oxford, 1963.
6. JACOBSON, A.L., Learning in flatworms and annelids, *Psychological Bull.* **60**, 74 (1963).
7. BEST, J.B. AND RUBINSTEIN, I., *Science* **135**, 916 (1962); BEST, J.B., *Sci. Am.* **208**, 54 (1963).
8. THOMPSON, R. AND McCONNELL, J.V., *J. Comp. Physiol. Psychology* **48**, 65 (1955).
9. WESTERMAN, R.A., *Science* **140**, 676 (1963).
10. McCONNELL, J.V., JACOBSON, A.L., AND KIMBLE, D.P., *J. Comp. Physiol. Psychology* **52**, 1 (1959).
11. CORNING, W.C. AND JOHN, E.R., *Science* **134**, 1363 (1961).
12. BEST, J.B., Diurnal cycles and cannibalism in *Planaria*, *Science* **131**, 1884 (1960); of interest also, ARBIT, J., Diurnal cycles and learning in earthworms, *Science* **126**, 654 (1957).
13. BENNETT, E.L. AND CALVIN, M., *Neurosciences Research Program Bulletin*, Vol. 2, p. 23, Massachusetts Institute of Technology, Cambridge, Mass., 1964.
14. SCOTT, J.F., FRACCASTORO, A.P., AND TAFT, E.B., *J. Histochem. Cytochem.* **4**, 1 (1956).
15. EDSTROM, J.-E., *J. Biophys. Biochem. Cytol.* **8**, 39 (1960).
16. GEBICKI, J.M. AND FREED, S., *Anal. Biochem.* **14**, 253 (1966).
17. GEBICKI, J.M. AND FREED, S., *Anal. Biochem.* **13**, 505 (1965).
18. McCONNELL, J.V., *A Manual of Psychological Experimentation on Planarians*, Univ. of Michigan, Ann Arbor, Mich., 1965.
19. JACOBSON, A.L., BABICH, F.R., BUBASH, S., AND JACOBSON, A., *Science* **150**, 636 (1965).