RESEARCH REPORT

MPL 12

THE DETECTION OF RNA SPECIES UNIQUE TO A BEHAVIOURAL TASK

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The Detection Of RNA Species Unique To A Behavioural Task

by

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I. INTRODUCTION

A variety of experimental studies has been conducted in this Laboratory in an attempt to understand the relationship between neurochemistry and behaviour (see MPL 11). The present study is the first of a series that is concerned with specific molecular events at the DNA-RNA complex level.

A number of individuals have suggested that RNA plays a unique role in learning phenomena (e.g., Cameron, 1963; Corning and John, 1961; Hyden and Egyhazi, 1962; Landauer, 1964; numerous others). In spite of many research efforts, however, there is still no conclusive evidence to implicate RNA in learning (Dingman and Sporn, 1964; Gaito, 1966). Unfortunately, it is difficult to evaluate the role of RNA in learning because of the many contradictory experimental results (Gaito, in press).

One means of attempting to determine the role of RNA in learning events is by DNA - RNA hybridization procedures (Gillespie and Spiegelman, 1964; Bonner, 1966; Gaito, 1966). If there exist unique species of brain RNA which are involved during learning, and RNA from the brain of a nonlearning animal is hybridized with DNA, then when RNA from the brain of a learning animal is added to this hybrid, the unique RNA species should adhere to the DNA. An important aspect of this successive competition hybridization procedure is that only the RNA from learning animals is labelled. Thus the presence of label in the twice hybridized DNA would suggest that RNA species not present in the brain of nonlearning animals has been synthesized in

learning animals during the learning task.

In this study a learning task was chosen which included components that would maximize sensory stimulation (a shock avoidance task) and involve some motor activity as well so as to maximize the behavioural differences between learning and non-learning animals. Thus, although the basic question "Are there unique RNA species for learning?" was of interest, this study was attempting to answer first a more gross question "Are there unique RNA species produced during a gross behavioural event?"

II. EXPERIMENT I.

As a first step it was necessary to determine if denatured DNA (single stranded) adhered to nitrocellulose membranes (Schleicher and Schuell, B-6, 25mm). Thus a preliminary experiment with gastrointestinal DNA labelled with thymidine -H^3 was conducted. It was found that all, or most all, of the labelled DNA adhered to the membrane following the pouring of heat denatured DNA on these membranes. This result occurred with amounts of DNA varying from 50 to 1000 micrograms (µg). In all later experiments 50 µg of DNA was used for each hybrid.

In this preliminary experiment and in later ones the chemical procedures were the following. DNA was extracted using a phenol procedure. The tissue was homogenized with a solution containing cold 90% phenol and 6% sodium para-aminosalicylate. Three further deproteinizing steps with phenol were instituted. DNA was precipitated with 95% ethanol, wound around a glass rod, and dissolved

in 1xSSC (0.15 M Na Cl and 0.015 M Na citrate). It was reprecipitated with 95% ethanol, washed with 50%, 75%, and 95% ethanol, and dissolved again in 1xSSC.

The tissue from which RNA was obtained was homogenized with cold 90% phenol containing physiological saline and 0.5% sodium lauryl sulfate. Further phenol deproteinizing steps were used (one with hot phenol and two with cold). The RNA was precipitated two times with 95% ethanol, treated with ether to remove phenol, and dissolved in 1xSSC.

The hybridization procedure involved denaturing DNA by heating at 95° C for five minutes, chilling the DNA, and pouring the denatured DNA onto nitrocellulose membranes during vacuum filtration. The membranes were washed on both sides with 6xSSC, dried for four hours at room temperature, and immersed in a 6xSSC solution containing RNA in scintillation vials within a water bath maintained at 66° C for 12 or 24 hours. The vials were placed in ice. The membranes were removed, both sides washed with 6xSSC, and immersed for one hour in 5 ml of 2xSSC containing 25 µg/ml of RNase at 37° C, shaking gently. The vials were placed in ice, membranes removed, and both sides washed with 6xSSC. After drying four hours the membranes were placed in scintillation vials with scintillation fluid and dpm determined in a Beckman Liquid Scintillation Spectrometer (LS-100) with a DPM 100 attachment.

A further step was required before behavioural experiments could begin. It was necessary to determine a DNA-RNA saturation curve, i.e., the amount of RNA hybridized with 50 µg of DNA beyond

which further amounts of added RNA would not increase the degree of hybridization. In one portion of an experiment, two littermate rats (Wistar strain, aged 90 days, weight approximately 300 grams) were injected intracranially with 200 μc of orotic acid 5-H^3 in 100 microliters of physiological saline (specific activity, 13.8 c/mM) and sacrificed 90 minutes later. This period was chosen because previous work had indicated that this time period allowed the RNA precursor to spread rather evenly throughout brain areas. The brain was removed and separated into approximately equal parts by a vertical cut down the middle. Each of the two included a cerebral hemisphere and one half of the cerebellum and brain stem. DNA was extracted from one portion and RNA from the other. For each rat, 50 μg of DNA was hybridized with 10, 25, 50, and 75 μg of RNA for 12 hours. Two samples were obtained from each rat, thus providing four observations for each of the four points.

A second portion of this experiment was similar to the first except that two samples of 50 μg of DNA hybridized with 0 μg of RNA for each rat were obtained as well as the samples at the other points. All hybridization was for a 24 hour period.

In both parts of the experiment, each hybrid was counted for two 20 minute periods to check consistency; the data of the second 20 minute period was utilized for the analysis.

The dpm in 50 μg of DNA, 25 μg of RNA, and in the SSC washes was determined also. No or negligible counts above background appeared in DNA; this result is expected inasmuch as little DNA

turnover occurs in brain tissue and only small amounts of orotic acid is converted into uridylic acid which ultimately ends up as thymidylic acid in DNA. Furthermore, in all cases the 50 µg of DNA adhered to the membranes.

The dpm in the 25 µg of RNA was used to determine the µg of RNA complexed with the 50 µg of DNA (dpm in sample hybrid/dpm in 50 µg of RNA x 50 = µg of RNA in hybrid). The ratio, µg RNA/50 µg DNA, multiplied by 100 for each hybrid (the percent DNA hybridized) constituted the dependent variable. The dpm (above background) in the 25 µg of RNA for the four animals varied from 383 to 935.

There was little difference in the results in the two experiments (Table 1); thus the results of the two experiments were combined to provide eight observations at 10, 25, 50, and 75 µg points and four at 0 µg. The means and standard deviations are shown in Table 1. The degree of hybridization (percent DNA hybridized) increases at 10 and 25 µg and appears to reach a plateau at 50 µg of RNA. At this point and at 75 µg, approximately 1.1 µg of RNase resistant RNA is hybridized with the 50 µg of DNA. Thus, at the incorporation period of 90 minutes, about 2.2% of the brain DNA appears to be complexed with brain RNA at these points.

III. EXPERIMENT 2. BEHAVIOURAL TASK

The results of Experiment 1 suggested that in the behavioural experiment, 50 µg of RNA should be hybridized with 50 µg of DNA. In Experiment 2, eight pairs of littermate rats similar to those in Experiment 1 were used. All experimental rats were

TABLE 1

Percent DNA Hybridized In Experiment 1

	Amounts of RNA (in μg)				
	0	10	25	50	75
Part 1. Means		1.11	1.76	2.33	2.05
Part 2. Means	0.06	1.22	1.79	2.21	2.16
Combined Means	0.06	1.17	1.78	2.27	2.11
Combined St. Dev.	0.36	0.31	0.22	0.30	0.40

injected intracranially with 200 μc of orotic acid 5-H^3. Control animals were injected with unlabelled orotic acid. Sixty minutes later each animal was placed in a one way active shock avoidance apparatus. (See MPL 4 for a description of this apparatus.) After fifteen minutes of adaptation in the shock chamber of the apparatus, the experimental animal (learning-L) was given 15 trials in 15 minutes and sacrificed by immersion in liquid nitrogen for 10 seconds. The control rat (nonlearning-NL) did not receive this training and was sacrificed at the end of 30 minutes in the shock chamber. All eight learning animals showed eight or more avoidance responses in the 15 trials (Mean, 10.1; Standard Deviation, 1.2).

With the two rats in each of the eight pairs, four hybrids were obtained as shown in Table 2. The DNA and RNA of the learning rats were hybridized (1 - intraanimal hybridization); the same event occurred with the nonlearning animals (2 - intraanimal hybridization); DNA of the learning animal was hybridized first with RNA from the nonlearning animal and then with labelled RNA from the learning animal (3 - inter and intraanimal hybridization); and another double hybrid was used in which L and NL were interchanged for the DNA and RNA's (4 - inter and intraanimal hybridization). Hybrid 3 was the crucial one for an evaluation of the question "Are there RNA species produced during this behavioural event?" Each hybridization event was for 12 hours.

The dpm of the membranes for Hybrids 1, 3, and 4 were

TABLE 2

Hybridization Procedures In Experiment 2 and Micrograms Of RNase Resistant RNA And Percent DNA Hybridized In Each Hybrid

Hybrid	µg RNA Hybridized Mean	Percent DNA Hybridized Mean	S.D.	Expected Mean	t	p
1. DNA_L - RNA^*_L	1.55	3.10	0.24	2.20	6.13	<.001
2. DNA_{NL} - RNA_{NL}	0.99	1.98	0.16	2.20	4.30	<.001
3. DNA_L - RNA_{NL} - RNA^*_L	0.70	1.40	0.21	0.00	6.06	<.001
4. DNA_{NL} - RNA^*_L - RNA_{NL}	1.45	2.90	0.25	2.20	2.54	<.05

DNA_L and RNA_L, DNA and RNA from brain of learning animal; DNA_{NL} and RNA_{NL}, DNA and RNA from brain of nonlearning animal; asterisk indicates the presence of labelled precursor in RNA; S.D. is standard deviation. Percent DNA hybridized and µg RNA hybridized determined by presence of label in Hybrids 1, 3, and 4; for Hybrid 2, by elution of hybridized RNA.

obtained by two 100 minute runs in the liquid scintillation spectrometer; the second one was used for the analysis. The RNA in Hybrid 2 was eluted with 0.01 M Tris buffer (pH 7.3), shaking vigorously for 24 hours, and determined by absorbancy readings in a Zeiss PMQ II Spectrophotometer. The µg of RNase resistant RNA and percent DNA hybridized for each hybrid was obtained as in Experiment 1. This information is percented in Table 2.

The main statistical analysis involved the use of a one sample t test to determine whether the degree of hybridization (indicated by labelling) with the previously hybridized DNA (3) was significantly different from zero, the expected value under the null hypothesis. One sample t tests were used also for Hybrids 1, 2, and 4. In these cases the expected value was 2.20, i.e., the value obtained from the work obtaining the saturation curve. Two sample t tests were used to check for significant differences between Hybrids 1 and 4, 1 and 2, and 2 and 4.

All one sample t tests indicated that the null hypothesis of concern should be rejected. The result with Hybrid 2 was unexpected. There are two possible reasons for this finding. First, the value of 2.20 percent DNA sites occupied as predicted in Experiment 1 was an over-estimation. Second, the elution process did not remove all of the RNA from the membrane. A check was performed later to evaluate the second possibility. The check consisted of eluting a radioactive hybrid based on nonlearning rats injected with labelled orotic acid (DNA_{NL} - RNA^{*}_{NL}). After the elution procedure had continued for 24 hours,

the membrane was removed from the elution solution, dried, and tested in the liquid scintillator spectrometer. The reading was not above background, showing that complete elution had taken place. This result seems to suggest that the 2.20 value was an over-estimation. As can be seen from Table 2, the value received in this experiment for Hybrid 2 was 2.00. If 2.20 is an over-estimation and 2.00 is a better estimation, the one sample t tests for Hybrids 1 and 4 would not change except to be significant at a lower probability level.

Null hypotheses were rejected in the comparisons of Hybrids 1 and 2, and 2 and 4, but not with 1 and 4. In each of the eight replications, the values for Hybrids 1 and 4 were greater than those for Hybrid 2. The lack of significant differences between Hybrids 1 and 4 is very important in order to determine if unique species occurred during hybridization. This was due to the fact that in preparing Hybrid 3, DNA from the learning animal was first hybridized with RNA from the nonlearning rat. This hybrid was then hybridized with RNA from the learning animal. If interanimal hybridization (within littermates) did not give the same results as intraanimal hybridization, the question could have been raised as to whether incomplete hybridization in the first step had occurred because the RNA and DNA came from different animals. If this were the case, results indicating unique species in the learning rat would have been obtained, but would be an artifact. This possibility, however, is less likely because of this result.

Thus, these results (Table 2) suggest that during the last

15 minutes of the 90 minute incorporation period, additional species of RNA appeared in the brain of the learning animals (Hybrid 3). This conclusion is corroborated by the significant differences between Hybrid 1 and 2, and Hybrid 2 and 4; Hybrids 1 and 4 show a greater amount of RNase resistant RNA and thus a greater degree of hybridization than does Hybrid 2. The difference between the two in each case is approximately as great as the value in 3.

To check on the results of Experiment 2, a simple experiment was performed with two pairs of littermate rats. This check consisted of reversing the entire procedure by injecting the nonlearning animal with the labelled precursor and the learning animal with the non-labelled material. In this case the hybrid comparable to Hybrid 3 in Experiment 2 should read zero. The count should be zero because the RNA from the learning animal should fill up the sites on the DNA from the nonlearning animal, therefore making it impossible for the labelled RNA from the non-learning animal to find unoccupied DNA sites.

Two crucial Hybrids were 2 and 3. In 2, DNA from the nonlearning animal was hybridized first with RNA from the learning rat and then with RNA from the nonlearning animal. In 3, DNA was from the learning animal. If the results in Hybrid 3 in Experiment 2 were an artifact, one would expect that label would be detected in both hybrids. The dpm in each case was not above background.

Two other double hybrids were formed (4: DNA_L - RNA^*_{NL} -

RNA_L and 5: DNA_{NL} - RNA^*_{NL} - RNA^*_{NL}); both showed values close to 2.00. Another hybrid (1: DNA_{NL} - RNA^*_{NL}) checked on the degree of hybridization for nonlearning rats. The value obtained (2.50) was greater than values for this hybrid in Experiment 2 but the difference was probably of random error nature.

IV. DISCUSSION

The results of Experiment 2 suggest that during the behavioural task in this study, RNA species were produced which are different than those present in the brains of nonlearning rats. During the first 75 minutes of the incorporation period the two groups of animals were treated the same. Thus the differences probably reflect the synthesis of RNA during the last 15 minutes in the shock avoidance apparatus during which learning was occurring. Presumably the labelled RNA during this 15 minute period is of the messenger type although different species of ribosomal and transfer RNA's can not be excluded because the extraction procedures did not differentiate these three types.

One might suggest that these results indicate only that quantitative changes in RNA occurred during the behavioural task. However, increased amounts of RNA of the same species as were present in the brain of the nonlearning animal would not find unoccupied sites on the DNA. Thus no label would be found in Hybrid 3. Furthermore, studies reported previously with this shock avoidance task (MPL 4) indicated that there were lower amounts of RNA per unit DNA (i.e., RNA/DNA ratio)

TABLE 3

Hybridization Events For The Check Procedure
And Micrograms Of RNase Resistant RNA
And Percent DNA Hybrided In Each Hybrid
According To Amount Of Label Present

Procedure	µg RNA Hybridized Mean	Percent DNA Hybridized Mean	S.D.	Expected Mean
1. $DNA_{NL} - RNA^{*}_{NL}$	1.25	2.50	0.07	2.20
2. $DNA_{NL} - RNA_{L} - RNA^{*}_{NL}$	0.00	0.00	0.00	0.00
3. $DNA_{L} - RNA_{L} - RNA^{*}_{NL}$	0.00	0.00	0.00	0.00
4. $DNA_{L} - RNA^{*}_{NL} - RNA_{L}$	1.11	2.23	0.03	2.20
5. $DNA_{NL} - RNA^{*}_{NL} - RNA^{*}_{NL}$	0.93	1.85	0.14	2.20

See Table 2 for description.

for learning rats.

The results are consistent with the ideas of Hyden and others who stress the importance of RNA in learning events. For example, Hydén and Lange (1965) reported that different types of RNA's (i.e., varying in base ratios) were produced during the early period of learning than was the case a few days later. Although these results are consistent with the expectations of advocates of the "RNA hypothesis," the results do not indicate the significance of RNA in learning.

There are a number of possible events which could precipitate the increase in RNA, e.g., the learning process, the electric shock, sensory stimulation, and motor activity. A separation of contributions, and their effect on RNA synthesis, will be a subject for further experimentation along these lines. It is important to remember that the present experiments are the first in a number of experiments attempting to answer the precise question, "Are there unique species of RNA in learning?" The present results suggest that unique species have been found in a behavioural task in which learning processes are involved. But it is important to realize that these findings do not mean that these species are unique for only learning. It is possible that these species are common to many behavioural tasks.

Only through exhaustive experimentation will refined answers and results be obtained. Some of these endeavors have been started: the first one being a motor activity experiment, similar to the present experiment. Later experimentation will

deal with successive competition of RNA from animals in a motor task and those in a learning task. As the results of each experiment are evaluated, further experiments will be planned to answer other relevant questions. These endeavors may give the following information: the chemical mechanisms of learning, the characteristics of "learning" RNA, and the answer to the basic question, "Are there unique species of RNA in learning?"

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13. ABSTRACT

A shock avoidance experiment was conducted in which the RNA from learning rats was labelled with orotic acid -5-H^3; the RNA of nonlearning animals was not labelled. A successive competition DNA-RNA hybridization procedure indicated that unique RNA species were developed during this task. However, the experiment could not show that the unique species were specific for learning; the result may indicate only that these RNA species are involved generally in behaviour.