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AUTHOR Braud, William G.
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ABSTRACT

If biochemical substrates and mechanisms could be identified, progress might be made in the detection and remediation of certain learning and memory disabilities. "Memory transfer" or "behavioral bioassay" methodology is a new technique developed for this purpose. It uses the behavior recipient animals to detect whatever chemicals are synthesized in the brains of donor animals during learning. This document is a research report which attempts to delineate the limits to which the bioassay may profitably be extended by studying its behavioral specificity. In four experiments, it shows that the behavioral bioassay effect is stimulus specific, response specific, process specific, and task specific. Strong support is provided for the position that the information conveyed via brain extracts is quite specific and not generally facilitating or depressing, as certain critics have argued. Results indicate the appropriateness of the method for the study of important learning and memory phenomena. A bibliography is provided for reference. (Author/GA)

Final Report

Project No. 10515F
Contract No. OEC-6-71-0524-(509)

William G. Braud
University of Houston
3801 Cullen Blvd.
Houston, Texas 77004

STUDIES OF THE STIMULUS SPECIFICITY, RESPONSE SPECIFICITY, PROCESS SPECIFICITY, AND TASK SPECIFICITY OF THE BEHAVIORAL BIOASSAY PHENOMENON

April 1973

Task Force on Field Initiated Research
National Institute of Education
Code 600
Washington, D. C. 20202

Author's Abstract

If biochemical substrates and mechanisms could be identified, progress might be made in the detection and remediation of certain learning and memory disabilities. A new technique which has been developed for use in this biochemical quest is the "memory transfer" or "behavioral bioassay" methodology, which is an extension of the biological assays which have played important roles in the discovery and study of major biochemical systems (hormones, vitamins, neurotransmitters). The behavioral bioassay uses the behavior of recipient animals to detect whatever chemicals are synthesized in the brains of donor animals during learning. The present research attempts to delineate the limits to which the bioassay may profitably be extended by studying its behavioral specificity. In four experiments, it was shown that the behavioral bioassay effect is stimulus specific (conveys specific information about the particular cues used in donor training), response specific (behavioral output coding is specific; recipients make the same responses the donors would have made), process specific (processes within the same task, e.g., acquisition and extinction, transfer independently and specifically), and task specific (brain extracts from donors trained on one task facilitate learning of recipients in different tasks as a function of their similarity to the original donor task). Strong support is provided for the position that the information conveyed via brain extracts is quite specific and not generally facilitating or depressing, as certain critics have argued. Results indicate the appropriateness of the method for the study of important learning and memory phenomena.

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William G. Braud

University of Houston
3801 Cullen Blvd.
Houston, Texas 77004

April 15, 1973

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Introduction

A central problem in education concerns the biochemical basis of learning and memory. If biochemical substrates and mechanisms could be identified, progress might be made in the detection and remediation of certain learning and memory disabilities. Evidence is accumulating which increasingly suggests the importance of biochemical and metabolic errors in mental retardation (e.g., PKU). When the faulty mechanisms are understood, the chemical deficiencies may be corrected by dietary or other means, and the retardation may be prevented. It is quite likely that certain learning disabilities and memory or attention deficiencies have metabolic bases. The research described herein hopefully will aid in a more rapid understanding of not only normal but abnormal biochemical substrates involved in certain types of learning and memory. If normal mechanisms are understood, faulty biochemical processes may be discovered, opening the possibility of correcting metabolic defects and resulting in increases in learning and memory ability. Ways might also be found to increase even normal learning and memory functions in biochemical ways. More specifically, the research program described herein delineates the limits to which we may profitably extend biochemical theories of learning and memory by determining exactly the limits of coding possibilities in which environmental events are chemically specified.

An exciting new technique which has been developed for use in the quest for biochemical substrates of learning and memory is the so-called "memory transfer" or "behavioral bioassay" methodology. This methodology represents an extension of the biological assays which have played important roles in the discovery and study of major biochemical systems, being used whenever a chemical process was suspected but the nature of the substance involved was either unknown or inaccessible to the chemical or physical means of detection available at the time. Bioassays were useful in the discovery of the hormones, vitamins, and neurotransmitters. Similarly, bioassay techniques would be expected to be useful in the detection of chemicals and chemical systems involved in attention, learning, and memory.

Whereas the traditional bioassay technique employs a physiological indicant of the presence of a suspected chemical (e.g., contraction of a smooth muscle preparation), the behavioral bioassay procedure involves measurement of the overt behavior of an intact living organism. Here, information communicated to donor animals results in the appearance of new behavior. When the new pattern is consolidated, the brains of the trained animals are removed and an extract of these brains is administered to naive recipient animals. If the brain extract contains the information in a chemical code, the recipients should exhibit behavior similar to that of the donors without ever having received the original information in a direct, experiential way. It then becomes possible to use this behavior as an assay to guide the successive steps of identification, purification, and synthesis of the active substance or substances. The chemicals involved in different kinds of learning and memory then may be delineated. Radioactively labeled forms of those chemicals may be traced through the nervous system and their loci and mechanisms of action may be discovered. The ultimate outcome of such research would be an understanding of the manner in which information becomes coded, stored, and decoded and used in the brains of the donor animals.

The Reality of the Phenomenon

The first of three general questions about the behavioral bioassay phenomenon concerns its reality. The behavioral bioassay was first used in the study of "transfer of learning" in planarians by McConnell in 1962. In 1965, the phenomenon was successfully demonstrated at the mammalian level (Fjerdingstad, Nissen, and Rojgaard-Petersen, 1965; Jacobson, Babich, Bubash, and Jacobson, 1965; Reinis, 1965; Ungar and Oceguera-Navarro, 1965). Most recently, the methodology has been extended to goldfish (Braud, 1970; Bryant, Santos, and Byrne, 1972; Fjerdingstad, 1970; Ungar, Galvan, and Chapouthier, 1972; Zippel and Domagk, 1969). Although the reality status of the phenomenon was at first quite questionable (as a result of failures to replicate some of the earlier work), it now seems as though the reliability and replicability of the phenomenon is firmly established. At the time of the present writing (April, 1973), at least thirty-four independent laboratories are reporting positive results. A number of neeessary conditions for a successful behavioral bioassay have only recently been identified. It can now be shown that in the early failures to replicate, these essential conditions were not met. Thus, the various negative results can be understood in terms of the experimenters' failure to follow the "recipe" necessary for a successful experiment. The recipe is complex because it includes not only behavioral variables, but physiological and biochemical variables as well.

Optimal values must be selected for each of the following parameters: (a) appropriate selection of species and individuals within species, (b) assessment of pre-training behavioral preferences and response biases of both donors and recipients, (c) complete specification of the organisms' internal and external environments, (d) adequate donor training, (e) appropriate time must elapse following the donor's last training session before sacrificing, (f) proper biochemical extraction procedures, (g) proper dosage of brain extract must be injected into recipients, and (h) appropriate post-injection testing intervals must be used. It may be shown that in the early reports of negative results, the experiments failed to satisfy one or a number of the critical conditions just mentioned (e.g., donors were not trained long enough, brain extract doses were too low, testing was done only once and too soon following injection, the motivational state of the recipients did not match that of the donors, etc.). For details, the reader is referred to five excellent recent summaries (Adam, 1971; Byrne, 1970; Fjerdingstad, 1971; Hoffman, 1971; Ungar, 1970).

Biochemical Specificity

A second major question about the behavioral bioassay concerns its chemical specificity. Ungar (1970b) suggests that different molecules are associated with the learning and memory of different tasks, and that these molecules are probably polypeptides. Ungar reports that the active materials extracted from brains of animals learning different tasks have different properties in terms of their dialyzability, partitioning in organic solvents, nolecular size, sysceptibility to enzymic destruction, etc. Ungar and his colleagues are not in the process of identifying the amino acids and the sequences of these amino acids in the active material from brains of rats and fish trained on different behavioral tasks such as: morphine tolerance, sound habituation, black-box avoidance, blue-avoidance, green-avoidance, and learning of a new swimming skill by fish whose buoyance has been altered. Ungar, Desiderio, and Parr (1972)

have already already identified the sequence for the molecule involved in the black-box avoidance task, and have synthesized this molecule which has properties (both chemical and behavioral) identical to those of the natural substance. This substance has been named "scotophobin" ("fear of the dark"). Research is now in progress in Ungar's lab which involves testing altered forms of the molecule and tracing isotopically labeled forms of scotophobin through the nervous system. Biochemical techniques have been developed which now allow the detection of small quantities of scotophobin in the brains of individual animals. Very recently, a molecule associated with sound habituation has been isolated and now awaits synthesis. The biochemical specificity issue will be resolved only when a large number of behavior-linked molecules have been successfully synthesized, tested in a wide variety of situations other than that in which the donors had been trained, and found to selectively affect certain behaviors but not others. Ungar and others are rapidly approaching this goal.

Behavioral Specificity

The third general question concerning the behavioral bioassay phenomenon is its behavioral specificity. It is towards this question that the present work is directed. One interpretation of the behavioral bioassay is that the chemical changes observed are non-specific, i.e., the material synthesized in the course of learning is the same, irrespective of the information acquired. On this view, the same chemical is produced whenever any learning occurs, regardless of its nature, and the chemical does not encode any particular information. According to this interpretation, recipients injected with brain extracts from trained donors would be expected to learn any tasks more rapidly: nonspecific learning and memory processes would be facilitated. This possibility could not be ruled out in the early bioassay designs in which recipients were tested with reinforcement (i.e., they were trained) on the very tasks learned by the donors. At their very best, such experiments could demonstrate only that learning might be facilitated; the results would have no bearing on the problem of specific coding of information.

An alternative hypothesis is that specific biochemical changes occur with different types of learning; the biochemical changes encode specific acquired information in some way. On this view, recipients would evidence appropriate performance only on the very same task on which the donors were trained, and they should show appropriate behavior even without reinforcement (i.e., without training). To adequately test this specificity hypothesis, two experimental conditions are necessary: (a) the recipients must be tested without reinforcement, and (b) the recipients should be tested on a variety of tasks differing in their similarity to the task on which the donors had been trained. Both of these conditions must be satisfied if the nonspecificity interpretation is to be ruled out. Condition (a) has been met in several experiments involving nonreinforced testing procedures (Braud, 1970; Fjerdingstad, 1970; Ungar, Galvan, and Clark, 1968). Results indicate that recipient animals perform appropriately to the cues of the situation without benefit of direct training. However, one could still argue, in the absence of condition (b), that "trained brain" extracts might simply result in some sort of "sensitization" of the recipients to a variety of cues. Recipients might over- or under-respond to cues and, if such behavior happens to match that acquir-

ed by the donors, it might be erroneously concluded that specific information had been transferred. To deal with this possibility, "cross transfer" studies are needed in which recipients are tested in situations other than that in which the donors were trained.

Very few cross transfers have been reported to date. Ungar (1967) was able to show that recipients treated with brain extracts from donors habituated to sound showed habituation to the sound stimulus only; their startle reactions to an air-puff were still present. Conversely, mice injected with material extracted from brains of air-puff-habituated rats showed habituation only to the air puff. Ungar (1970b) has obtained interesting results in an experiment involving cross transfer between two passive avoidance situations. One group of donor rats was trained for dark avoidance, while another group was trained for the avoidance of step-down from a small platform to a wider grid-floored area. Electric shock was used in training both behaviors. Recipients of brain material from dark-avoiding donors exhibited "fear of the dark" but their step-down latency was unchanged. Conversely, mice given extracts of brain from donors trained to avoid stepping down had a markedly increased step-down latency, but no avoidance of the dark. Thus, while very few in number, the extant cross transfer studies do strongly suggest specificity.

In the present four experiments, the specificity issue is investigated on a variety of dimensions and in a more systematic manner. First, process specificity is investigated. Is the biochemical transfer effect specific to different processes within the same task, e.g., are acquisition and experimental extinction of some given response transferred independently and specifically? Second, response specificity is studied. Will recipients make only the responses the donors have learned, or will they exhibit other behaviors as well? Stated otherwise, what sorts of response differentiation and response generalization will be shown by the recipients. Here, results are relevant to the degree of specificity of output coding in molecular terms. Third, stimulus specificity will be considered. Here, questions such as the following are answered: how closely does recipient behavior exhibit control by the actual stimuli used in donor training? what degree of stimulus generalization and discrimination is shown in the recipients' behavior? can very fine discriminations be shown within the same (visual) sensory modality (i.e., discrimination of very close wavelengths)? Finally, the task specificity of the behavioral bioassay phenomenon is studied. Do recipients perform appropriately only on those tasks on which the donors had been trained? What degree of discrimination and generalization will be shown? Do recipients show improvement on tasks very similar to the donors' training task and show no improvement on very different tasks?

Methods

Experiment 1: Process Specificity of the Behavioral Bioassay

In a previous paper (Braud, 1970), it was shown that extracts from brains of donors given acquisition training in a two-way active avoidance task significantly increased the probability of avoidance responses in naive recipients when the latter were given nonreinforced test trials following injection. Similarly, extinction was facilitated in recipients of extracts from brains of extinguished donors. Two possible criticisms of that study are; (a) perhaps the extracts, rather than providing some

sort of information for the recipients, merely facilitated learning of the appropriate tasks, and (b) perhaps either the facilitation or information provided is general enough to affect behavior on tasks other than the ones on which the appropriate donors were trained. The first argument may be dismissed since recipients were tested in the absence of any reinforcement; thus learning, in any of the usual senses of this term, was prevented. The experiment reported here bears on the second criticism. Evidence is presented which suggests that brain extracts affect recipient performance selectively: facilitating the process corresponding to that active in the donors, but not facilitating an antagonistic process.

Subjects. The subjects were 120 common Comet goldfish, 7.5 - 10.0 cm. in body length, obtained from Ozark Fisheries, Stoutland, Missouri. The fish were maintained in the laboratory for seven days before the experiment began; maintenance conditions were identical to those described by Braud (1970). Goldfish were chosen as experimental animals because of their unique advantages in behavioral and biochemical research (Braud, 1970b).

Apparatus. The training/testing apparatus was a clear plastic aquatic shuttle-box, equipped with completely automated stimulus presentation and response recording devices; see Braud (1970) for a detailed description of the apparatus. The box was programmed so that a fish might terminate or avoid a pulsed 9 V dc electric shock by swimming from the light to the dark compartment.

Procedure. A group of 24 donor fish was given 11 days of avoidance acquisition training in the shuttle box. On each day, 20 training trials were administered. A trial consisted of a 12½-sec. presentation of light in the compartment occupied by the fish, then a 12½-sec. simultaneous presentation of light plus pulsed shock, then a 35-sec. period of darkness and no shock. Light- and shock-offsets were response-contingent. A second group of 24 donor fish was given 11 days of avoidance acquisition training followed by 7 days of extinction training. Twenty extinction trials were given daily, in a paradigm identical to that used in acquisition training, but with shock omitted. A third group of 24 donor fish was never exposed to the apparatus and served as a naive control. Donor animals were sacrificed 20 hr. after their last session, their brains were quickly removed, and an RNA-protein extract was prepared by the cold phenol method. A detailed description of these physiological and biochemical phases is given by Braud (1970). The lyophilized extract was concentrated so that a single 40 ul injection would contain 1.5 brain equivalents of material.

Recipients of the three different extracts were tested under one of two different conditions: acquisition testing or extinction testing. For acquisition testing, 24 recipient fish were randomly assigned to three groups of eight animals. The animals were injected intracranially (see Braud, 1970, 1970b) with 40 ul of either acquisition-trained, extinction-trained, or control donor brain material and were tested 24, 48, 72, and 96 hr. following injection. Testing consisted of 20 daily nonreinforced (nonshock) "acquisition" test trials. Both injection and testing were done blind. An "avoidance" was any response occurring within the first 12½ sec. of light presentation.

For extinction testing, 24 recipient fish were assigned randomly to

three groups of eight animals. These groups were given three days of avoidance acquisition training, which resulted in a terminal acquisition level of approximately 65 % correct avoidances. This intermediate training level was chosen to allow considerable room for both improvement and depression of performance. As may be seen later in Figure 2, the three groups did not differ in acquisition rate. Twenty-four hr. after the last training session, these recipients were injected intracranially with 40 ul of either acquisition-trained, extinction-trained, or control donor brain material and were tested 24, 48, and 72 hr. following injection. Testing consisted of 20 daily nonreinforced (nonshock) "extinction" test trials. An avoidance response was as defined above. Again, both injection and testing were done blind.

Experiment 2: Response Specificity of the Behavioral Bioassay

This second experiment is concerned with another aspect of the specificity issue: response specificity. The experiment was conducted at the same time as Experiment 1 and is presented in the same Figure; however, it will de discussed as a separate experiment in order to facilitate communication. At issue is the importance of the animal making a particular response during training. When confronted with the stimulus conditions of Experiment 1, most donor animals learn quickly and well. However, a small number of animals do not make the "correct" (hurdle-crossing) response, but rather engage in some "incorrect" behavior such as swimming away from the hurdle and into the lighted end of the compartment. Because of the brevity of the shock period, such incorrect behavior nonetheless will be reinforced by shock termination and will be acquired superstitiously. Such "nonlearners" are usually discarded from an experiment, but this time they were accumulated and constituted a special donor group. It is important to note that these animals are not unable to learn (i.e., they are not "brain-damaged" in some way); they simply learn something other than the correct response. It can be shown that even these animals are capable of learning the correct response: if the shock period is lengthened, the superstitiously acquired behavior will be punished consistently and will eventually be replaced by the correct hurdle-crossing pattern which is actually effective in terminating and avoiding shock. The interesting thing about the nonlearner group is that it serves as an effective control for a test of the importance of making a particular response in a given environmental setting.

Subjects. Subjects were the same 60 fish used in the first phase of Experiment 1, plus 20 additional fish. Supplier and maintenance conditions were identical to those reported above.

Apparatus. The apparatus was the same shuttle-box described above.

Procedure. Along with the acquisition and extinction donors trained in Experiment 1, a group of 12 fish were given 11 days of "acquisition" training in the shuttle-box, but these were fish that did not learn the correct response. These nonlearners were sacrificed 20 hr. after the last session and an RNA-protein extract was prepared from their whole brains by means of the cold phenol method. The resultant material was concentrated so that 40 ul contained 1.5 brain equivalents. Material was injected intracranially into each of eight recipient fish, which

were tested 24, 48, 72, and 96 hr. after injection. Testing conditions were identical to those of the first phase of Experiment 1. Both injection and testing were done blind.

Experiment 3: Stimulus Specificity of the Behavioral Bioassay

Fay and MacKinnon (1969) have described a technique in which a stimulus paired with electric shock comes to elicit a conditioned respiratory mouth movement in the goldfish; such movements are detected by a sensitive movement transducer and recorded via an amplifier and ink-writing polygraph. Goldfish have been shown to possess excellent color vision throughout the visual spectrum (Yager, 1968). Thus, in this third experiment, classical conditioning techniques were used to train large goldfish to make a particular respiratory response (diminution or cessation of ongoing movements) to a stimulus consisting of a given wavelength of light. After receiving injections of brain extract from appropriate donors, recipient fish were tested with the original training wavelength (CS) as well as other wavelengths (generalization test stimuli or GSs) varying in similarity to the CS. A stimulus specificity hypothesis would predict that the generalization functions generated by the recipients would mimic those of their respective donor groups.

Subjects. The subjects were 64 common goldfish, 6-7 in. long, obtained from Ozark Fisheries. They were housed in filtered and aerated aquaria and were given two daily feedings throughout the experiment.

Apparatus. The apparatus was suitable for differential classical respiratory conditioning of individual goldfish. The fish is restrained between two contoured sponges mounted in a harness inside of a larger water-filled container (a rectangular glass aquarium 30 x 20 x 18 cm.). Nylon monofilament thread connects the fish's lower lip to a Harvard Apparatus Model 352 movement transducer. Output from the transducer element goes to a Harvard Model 355 bioamplifier, and then to a Model 350 electronic recording module to be recorded on a Harvard Model 485 chart mover. Stimulus events (CS plus, CS minus, and US) were recorded by Harvard Model 280 and Model 283 event markers and event/time markers. Stimulus events were programmed by a Lafayette Model 5500 programmer-timer. The conditional and generalization stimuli were five wavelengths produced by incandescent lamps and narrow band filters; light intensity was equated for the various hues. The wavelengths chosen corresponded to the hues: red, orange, yellow, green, and blue. The unconditional stimulus (US) was a 50-msec. duration dc electric shock, pulsed at a rate of one per sec., with voltage adjusted for each fish so as to elicit a consistent cessation of respiration (unconditioned response or UR). Shock electrodes consisted of wire mesh attached to the long sides of the aquarium.

Procedure. A pilot study was conducted first, in order to insure that the conditioning procedure was effective in producing good stimulus generalization curves. Ten fish were given 15 differential classical conditioning trials per day for seven days in which one wavelength was consistently paired with shock and the other wavelength was presented in an unpaired fashion. For five subjects, the paired wavelength (CS plus) was red; for five subjects, the CS plus was blue. The unpaired stimulus (CS

minus) was blue for red-trained fish and red for blue-trained fish. A training trial consisted of the following events: 20 sec. of darkness and no shock; a 10-sec. presentation of CS minus; 15 sec. of darkness and no shock; a 10-sec. presentation of CS plus, the offset of which overlapped a 5-sec. shock presentation. The shock was pulsed at a rate of one pulse per sec. Twenty-four hr. after the last training session, a generalization testing session was given consisting of four non-reinforced presentations of each of five wavelengths, the two used in training plus three novel ones (orange, yellow, and green). Stimulus duration was ten seconds in each case, with an inter-stimulus interval of 60 sec. Stimuli were presented in five "blocks", with four stimuli of the same color presented within each block. Block order was randomized through use of a table of random numbers. As expected, these pilot fish exhibited typical stimulus generalization behavior, responding most to CS plus, least to CS-minus, and to the intermediate novel stimuli in proportion to their similarity to CS plus and CS minus.

In the experiment proper, one group of ten goldfish was given ten days of classical differential conditioning, as described above, with the red light as CS plus. A second group of ten fish received identical training, but with the blue light as CS plus. Ten naive control fish remained in their home tanks and had no experience with the conditioning stimuli. Generalization test trials were not given to these groups of donor fish. Twenty hr. following the last training session, all donor fish were sacrificed, their brains removed and extracted biochemically for RNA and protein. Three recipient groups of eight naive fish each received intracranial injections (50 ul of solution for each fish, each containing 1.25 brain equivalents of material) of either "red-trained", "blue-trained", or "control" brain extract. The procedures involved in sacrificing, extraction, and injection were identical to those described by Braud (1970). Beginning 24 hr. after injection, and continuing for four days, all recipients were given 20 daily generalization test trials consisting of four nonreinforced presentations of each of the five test stimuli (red, orange, yellow, green, and blue lights), as described above. Respiratory activity during each stimulus event was recorded. Injection, testing, and reading of the polygraph records were all done in a "blind" fashion, in order to avoid experimenter bias.

Experiment 4: Task Specificity of the Behavioral Bioassay

A popular argument against the specificity hypothesis of the behavioral bioassay is that the extracts do not convey specific information in some coded form but rather facilitate learning in a very general, nonspecific way. The assumption is that recipients of experimental extracts would learn any task better than recipients of control extracts. In this final experiment, an attempt was made to determine whether recipients of brain extracts from donors trained on Task X would indeed learn Tasks Y and Z significantly faster than recipients of control extracts. The finding that extract X facilitates learning of Task X but does not facilitate learning of other tasks would provide strong evidence for a specificity hypothesis. Also studied was the question of whether any task-specific effect might generalize to very similar tasks, but not to dissimilar ones.

Subjects. The subjects were 132 common goldfish, 3-4 in. long. The supplier and maintenance conditions were as described in Experiments

1 and 2.

Apparatus. In this final experiment, use was made of three different training apparatuses. The first apparatus was the same shuttle box described in Experiments 1 and 2. The only difference was that the dangerous compartment was signalled by colored light and the safe compartment by a different colored light instead of white light and darkness, respectively, as in Experiments 1 and 2. The second apparatus was a "colored photobeam escape apparatus". This consisted of a rectangular glass aquarium 30 cm. long, 20 cm. wide, and 18 cm. high, with wire mesh shock electrodes attached to its sides. A red photobeam projects horizontally through the water near one end of the tank; a green beam projects near the other end. Occlusion of the red photobeam by the fish activates a photorelay and associated equipment to terminate electric shock for a 30-sec. period. Otherwise, shock is continually pulsed through the water at the rate of one 50-msec. pulse per second. Thus, the fish may escape shock by swimming into the red beam, which is randomly switched from end to end of the tank to prevent learning of a position habit. The third apparatus was to be an appetitive lever-press apparatus in which the fish may obtain food by pressing a sensitive inertial switch (Gibbs Manufacturing Corporation) centered in front of a red background. Presses on a similar switch in front of a green background are recorded but have no other effect upon the environment. These manipulanda, along with a solenoidcontrolled food-delivery mechanism, are supported from the top of a 30 x 20 x 18 cm. glass aquarium. Responses were recorded by event markers as were described in Experiment 3.

Procedure. A group of 36 donor fish was given eight days of acquisition training in the shuttle box as described in Experiments 1 and 2. During donor training, swimming from the red to the green compartment would result in termination or avoidance of shock. A group of 36 control fish were never exposed to the shuttle apparatus. All fish were sacrificed 20 hr. following the last training session and the brains were extracted for RNA and protein as described above. Thirty naive recipient fish were injected with extract (50 ul; 1.2 brain equivalents) from "shuttle-trained" donors; thirty recipients received control extract. One third of each group was tested on learning of the shuttle task (with reinforcement); one third of each group learned the "colored photobeam escape task" (with reinforcement); one third of each group of recipients was to learn the lever-press appetitive task (with food reinforcement). An important feature of this experiment was that during recipient testing, approaching a red light was associated with the correct response. Although the donors had learned to avoid red during training, the recipient testing paradigms were set up so that approach to red was correct: approach the red compartment in the shuttle-box, approach the red photobeam in the escape apparatus, and approach the red lever in the appetitive situation. Such a negative transfer design was used in order to attack the task specificity hypothesis in still another way. The nonspecificity hypothesis would predict that trained extracts would facilitate any type of learning; it should not matter than the test learning would be opposite that learned by the donors. Thus, the recipients of trained brain extract should do better on all tasks than the recipients of control extract. On the other hand, the specificity hypothesis would predict that performance of "trained brain" recipients should be impeded, rather than facilitated,

relative to control recipients. This interference effect should be especially great on the shuttle test (the very task on which the donors had been oppositely trained), less dramatic on the escape task (not the same task on which donors had been trained, but sufficiently similar to yield some interference), and perhaps less dramatic still on the appetitive task (more dissimilar than the escape task due to its appetitive, rather than aversive, nature). In all cases, training sessions were limited to a single 10-min. session per day for three days, beginning 48 hr. after injection.

It turned out that the appetitive task was inappropriate for use in this sort of design. It had been expected that the fish would learn the appetitive response rather quickly, while the extract would still be active and before extinction to the colored lights would have time to develop. This expectancy was not confirmed. Learning the lever-press task was quite difficult and time consuming. In fact, it was almost impossible (under these sets of parameters) for the fish to learn the appetitive response without the introduction of a careful shaping procedure. Since the shaping procedure had to be taylored to the individual fish (thus a poor learner might learn just as well as a good learner with appropriately more help from the shaper), and since the entire procedure consummed so much time (days of shaping were necessary, resulting in the possibility of dissipation of the transfer-effect as well as the possibility of considerable extinction to the relevant cues), it was decided to abandon the appetitive component of this test. A way out of this problem would have been to pre-shape and/or pre-train the fish so that they had already learned the appetitive habit at the time of injection. But this would have eliminated the possibility of measuring the effect of the extract on the early learning process itself, which, after all, was what we were really interested in. Thus, it was decided that we substitute a simple color preference task for the inappropriate appetive one. The photobeam apparatus was used, but this time shock was never administered. The experimenter simply recorded the amount of time (out of a possible ten minutes) spent by each fish in the red half of the tank. This task was dissimilar from the other two tasks in that: (a) no aversive stimuli were introduced, and (b) making the "correct" response (staying in the red end of the tank) did not "make anything happen" (e.g., shock go off) as was the case in the other two tasks.

Results

Experiment 1: Process Specificity

Performance of recipients on the acquisition test may be seen Figure 1. The probability of an avoidance response during acquisition testing was increased by an injection of brain material from acquisition-trained donors, but not by brain material from extinction trained or control donors. Recipients of acquisition material were significantly superior to control recipients at the 24 hr. (Mann-Whitney $U = 14$, $p = .032$) and 48 hr. ($U = 7$, $p = .003$) tests. These same acquisition recipients were superior to extinction recipients at the 24 hr. ($U = 12$, $p = .019$) and 48 hr. ($U = 11$, $p = .014$) tests. The extinction recipients did not differ significantly from control recipients at any of the four test periods.

Performance of recipients on the extinction test may be seen in Figure

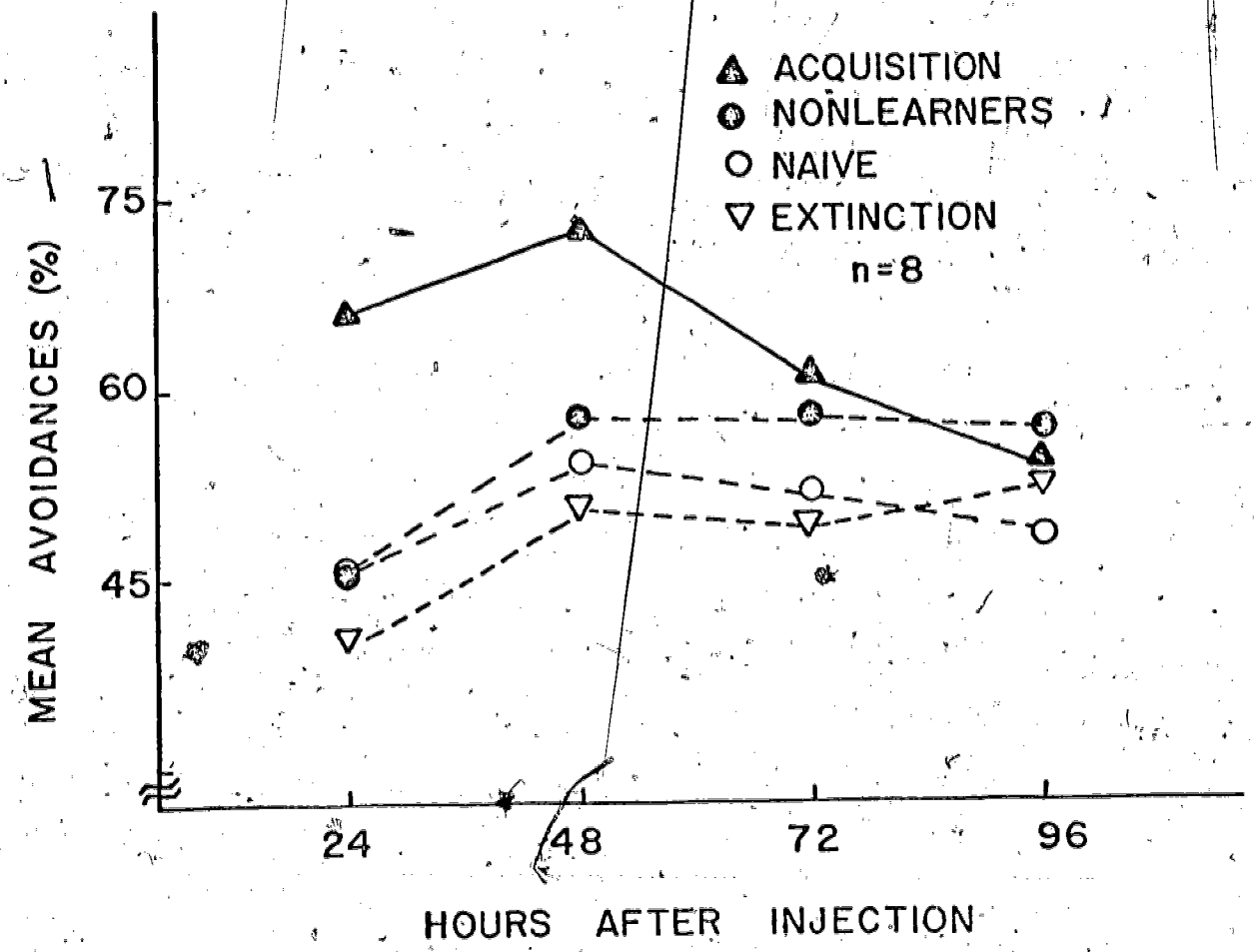

Figure 1. Mean percent avoidance responses during "acquisition" testing for naive recipients injected with either acquisition-trained, extinction-trained, "nonlearner", or naive control donor brain material.

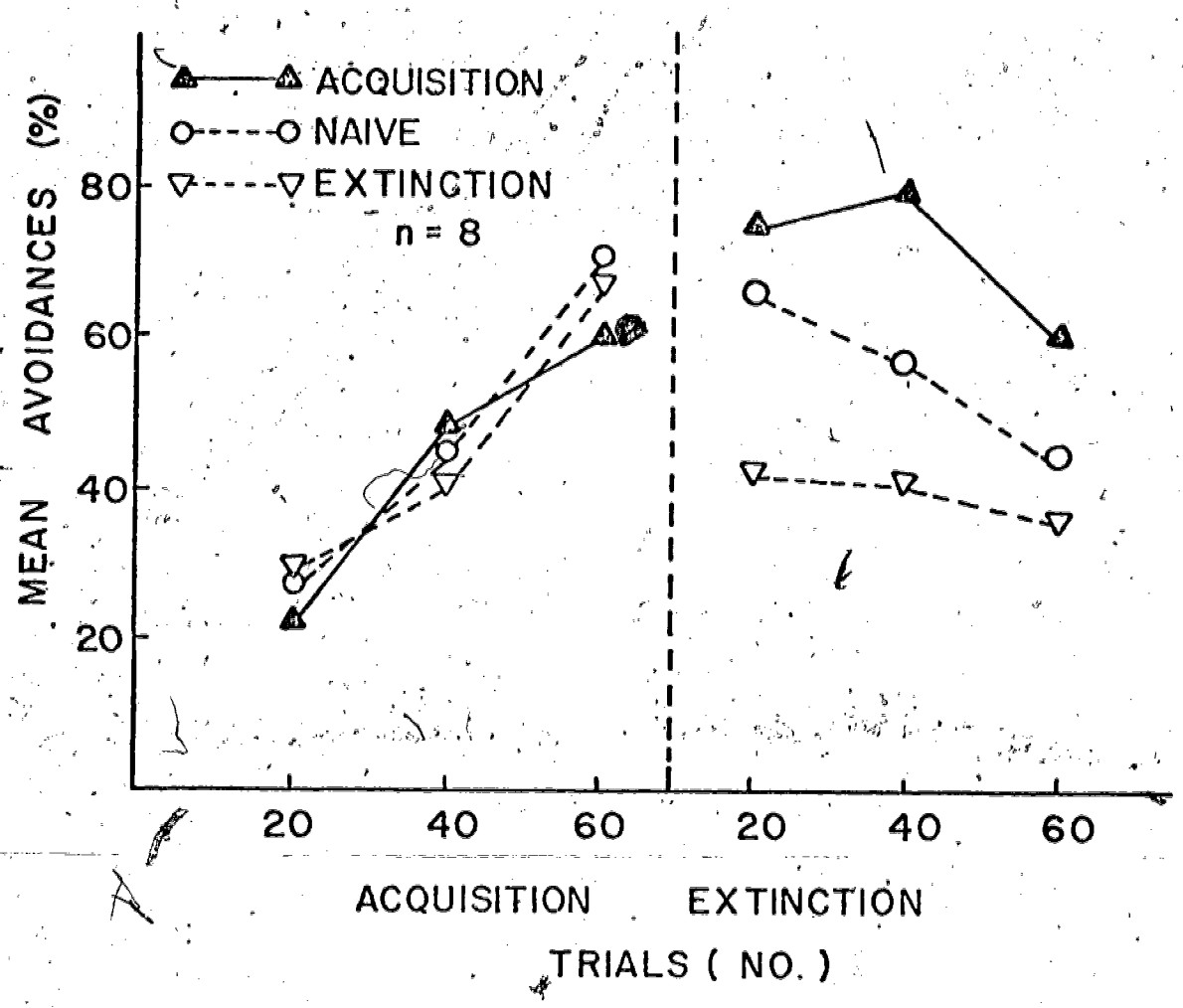

Figure 2. Mean percent avoidance responses during "extinction" testing; the vertical dashed line indicates time of injection of either ecquisition-trained, extinction-trained, or naive donor brain material.

2. Here, the extinction process was facilitated by brain material from extinguished donors, but impeded by material from acquisition-trained donors. Extinction proceeded normally in recipients of control extract. The avoidance behavior of acquisition recipients was significantly superior to that of control recipients at the 48 hr. ($U = 13$, $p = .025$) and 72 hr. ($U = 14$, $p = .032$) tests. The avoidance behavior of extinction recipients was significantly inferior to that of control recipients at the 24 hr. ($U = 7$, $p = .003$) and 48 hr. ($U = 13$, $p = .025$) tests. Acquisition recipients differed significantly from extinction recipients at all three tests.

The findings of these two phases of Experiment 1 replicate and extend those reported by Braud (1970). Not only do acquisition and extinction processes both "transfer" in some way to recipients, but these two processes fail to "cross transfer". Taken together, the results of these two phases strongly suggest process specificity: the acquisition extract facilitated acquisition but not extinction, while the extinction extract facilitated extinction but not acquisition. Thus, it is not the case that the extracts have very general facilitating properties.

Experiment 2: Response Specificity

The performance of the recipients injected with nonlearner material may be seen in Figure 1. The avoidance behavior of nonlearner recipients did not differ from that of control recipients, nor did it differ from that of the extinction recipients at any of the four test sessions. Nonlearner recipient performance was significantly inferior to that of acquisition recipients at the 24 hr. ($U = 13$, $p = .025$) and 48 hr. ($U = 14$, $p = .032$) tests.

The results of this second experiment suggest response specificity: only the fish that learned to make the hurdle-crossing response were effective donors. Extracts from brains of fish that experienced the same stimulus events but did not make the appropriate response were not effective. Besides demonstrating response specificity, the nonlearners constituted a good control, the likes of which is rarely seen in behavioral bioassay studies. A naive control is the crudest possible control and does not allow an investigator to determine which of the many variables to which the experimental donor group is exposed are really responsible for the biochemical changes assessed. This experiment has shown that learning of a particular response is important, apart from any exposure to light, shock, the apparatus, etc. (since all of these latter factors are equated for the nonlearners and the learners). Finally, the present results suggest the possible biochemical transfer of the substrate for superstitious behavior in the nonlearner recipients.

Experiment 3: Stimulus Specificity

Representative records illustrating a variety of unconditional and conditional respiratory reactions are presented in Figure 3. Panel (a) illustrates regular periodic mouth movements as they occur in the absence of exteroceptive stimulation. Panel (b) depicts a UR (marked diminution of mouth movement amplitude) elicited by a US (electric shock). Panel (c) illustrates a UR to shock, the beginnings of a CR to CS plus, and either an orienting reaction (OR) or generalized CR to CS minus; this record is typical of patterns which occur early in training. In Panel

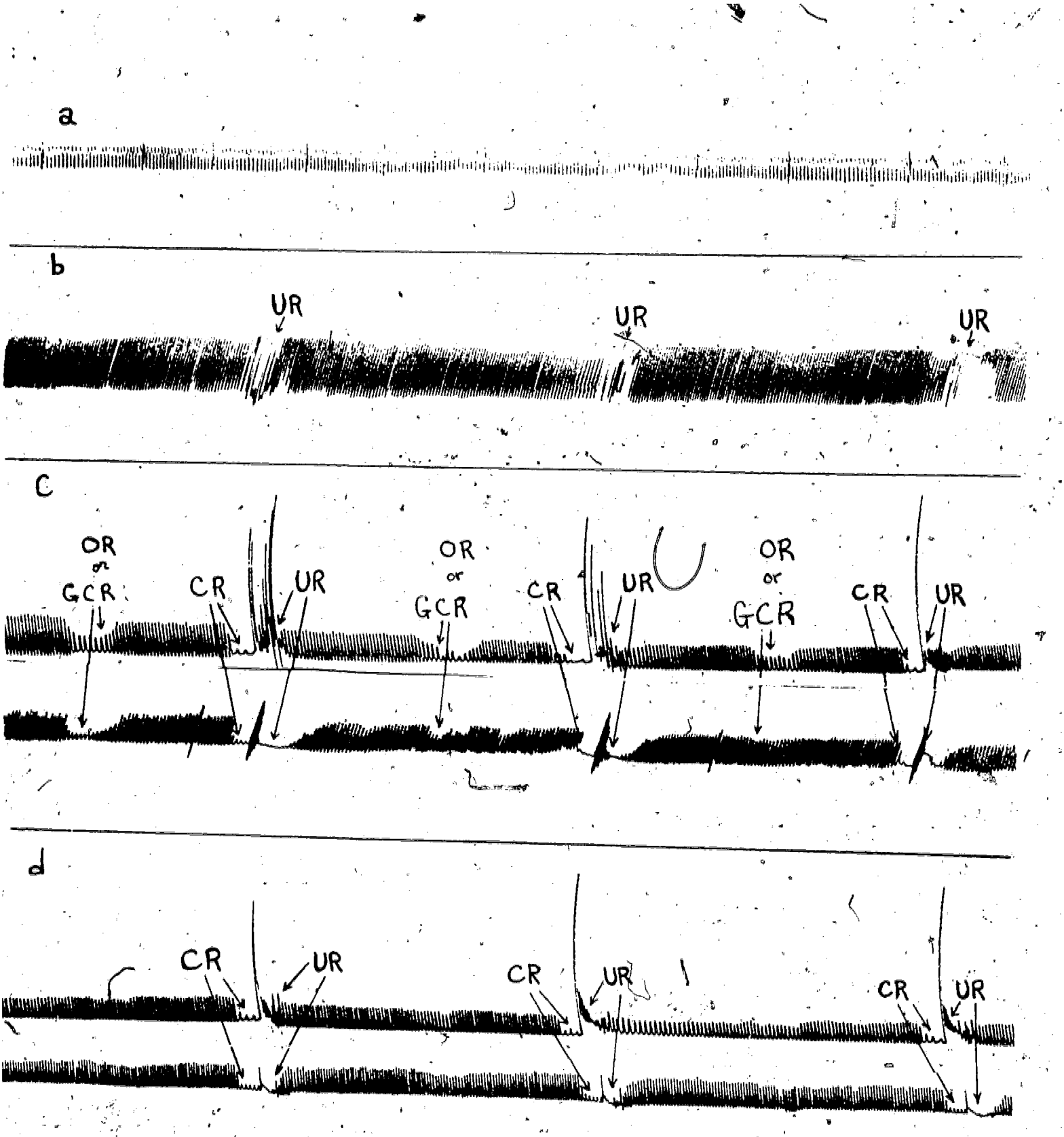

Figure 3. Representative polygraph tracings of respiratory activity in the goldfish. Panel (a) depicts baseline activity. A UR is shown in panel (b). URs, CRs, and either orienting reactions (ORs) or generalized CRs (GCRs) to CS minus, CRs to CS plus, and URs to USs are shown in Panel (c).. Late in training, Crs are well established, while the reactions to CS minus have disappeared (Panel d).

(d), which occurred late in training, the CR to CS plus is now firmly established, the reaction to CS minus has disappeared, and the UR is evident. The respiratory data were quantified by computing a percent suppression score for each fish, for each trial, for each day of training. Such a score was computed by first measuring the smallest amplitude mouth movement during the 10-sec. CS plus and CS minus intervals, and also during a 10-sec. baseline period immediately before the onset of CS plus. The percent suppression score was defined as the smallest baseline amplitude minus the smallest CS amplitude, with that difference divided by the baseline amplitude and multiplied by 100 percent. Separate suppression scores were computed for CS plus and CS minus. The mean suppression scores for the two CSs, as functions of successive training days, are presented in Figure 4. Panels (a) and (b), respectively, represent the development of a differential conditional response in the red-trained and blue-trained pilot subjects. Clearly, the conditioning technique is effective. Learning a CR to the red stimulus proceeds slightly faster than the learning of a similar response to the blue stimulus.

Figure 5 presents the results of the discrimination/generalization tests carried out one day following the last day of training. Fish trained with red as their CS plus respond most to red, least to blue, and at intermediate graded levels to the novel (orange, yellow, green) stimuli in proportion to their similarity to the two training stimuli. The pattern is reversed for those fish trained with blue as their CS plus. These fish respond most to blue, least to red, and in a graded fashion to the novel test stimuli, again as a function of their similarity to the blue CS plus.

In Figure 6 are plotted the performance curves for the three recipient groups for the four test days. Biochemical transfer of classical conditioning was not effective in these preparations. Mouth movement suppression was not evident in response to any of the five test stimuli. The red-trained, blue-trained, and control recipient groups never differed significantly from one another at any stimulus or at any test day. Since classical conditioning itself failed to transfer, no conclusions may be drawn from these present data about the stimulus specificity of reactions that do transfer.

The reason classical conditioning failed to transfer in this experiment is unclear. The donors certainly learned sufficiently well. It should be noted, however, that we know very little about the optimal parameters (the proper recipe) for classical, as opposed to instrumental, transfers. To my knowledge, no one has been able to chemically transfer direct classical conditioning to date. (The foregoing statement refers to vertebrate work; several planariah studies have been done in which classical conditioning was successfully transferred.) It may well be that classical conditioning transfers might require an optimal recipe which differs from the recipe found to be effective for instrumental transfers. Modification in variables such as degree of donor training, extract dosage, post-injection testing interval, etc., may well result in adequate classical transfers. Such a search for optimal parameters is, however, clearly beyond the scope of this grant proposal. Some additional information on both classical transfer and stimulus specificity will be presented later in this report.

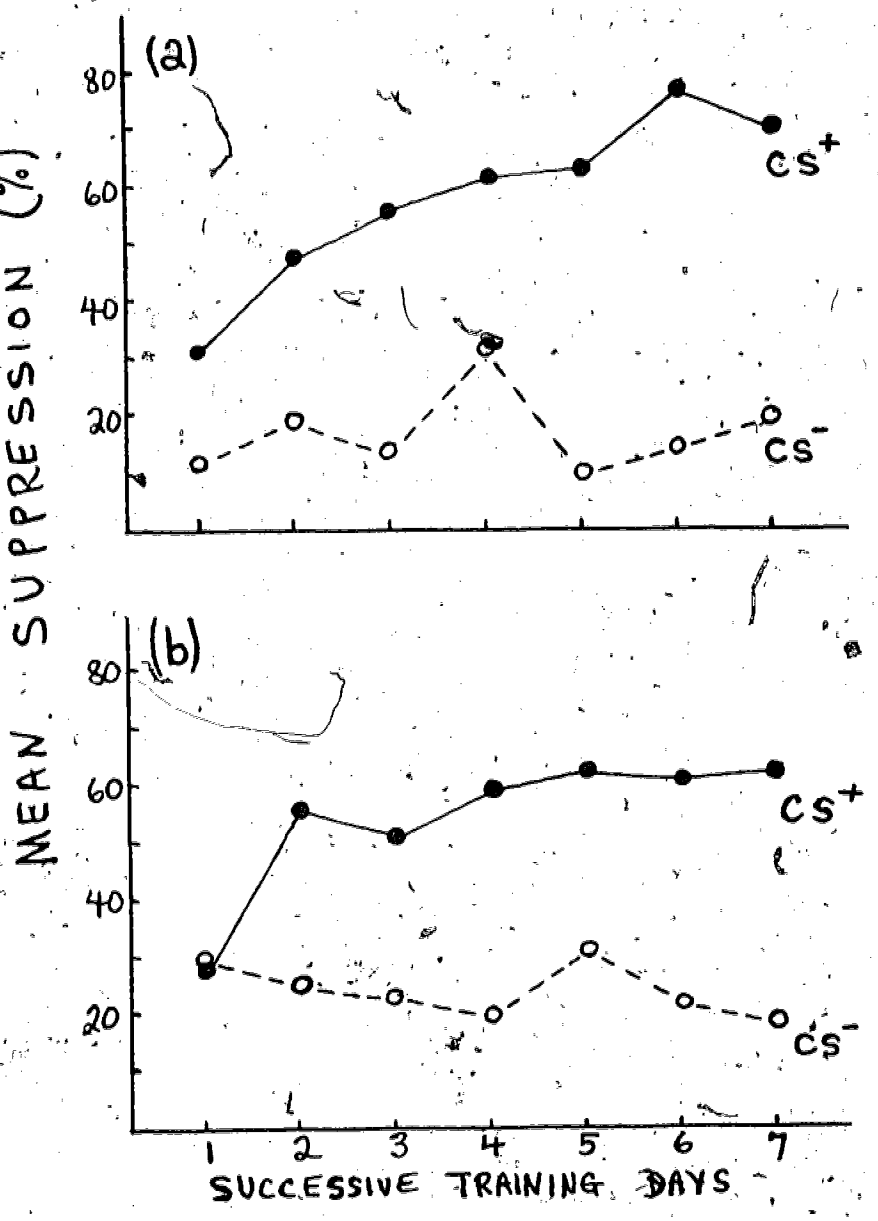

Figure 4. Mean percent suppression of respiration produced by CS plus and CS minus, relative to baseline. Panel (a) shows the performance of five fish trained with red paired with shock and blue unpaired. Panel (b) shows the performance of five fish trained with blue paired with shock and red unpaired. Fifteen reinforced differential classical conditioning trials were administered each day.

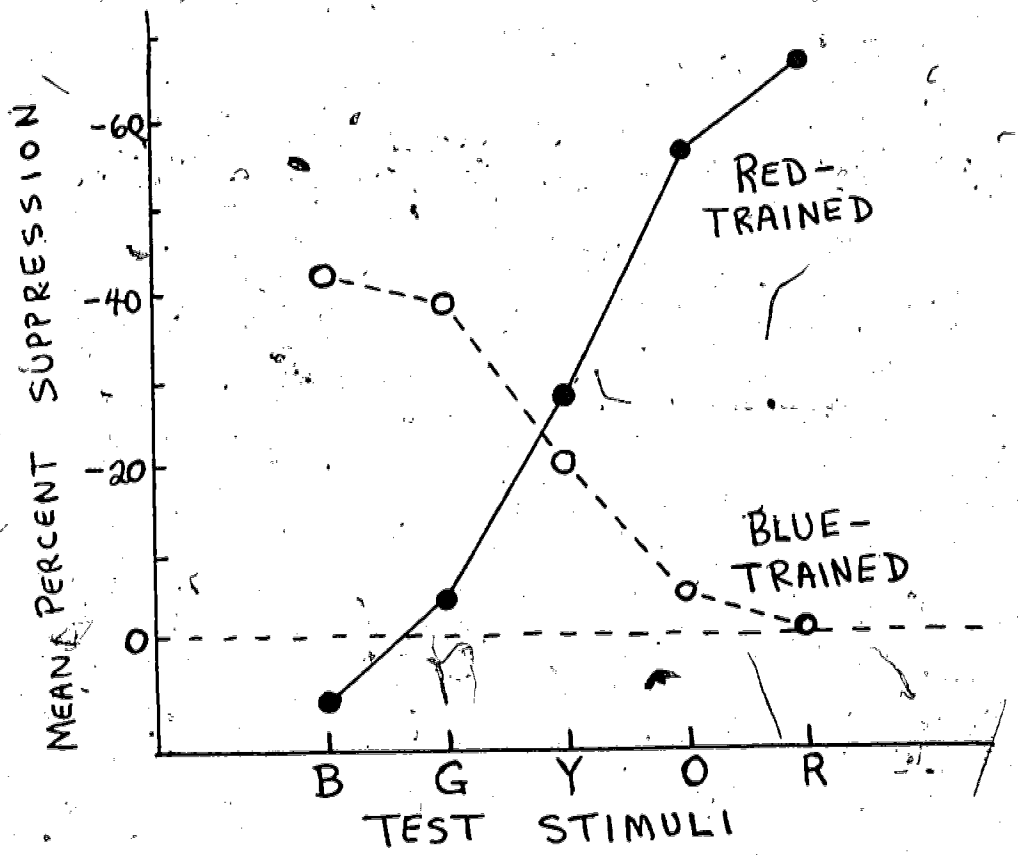

Figure 5. Mean percent suppression of respiration produced by the five test stimuli, relative to baseline. One group of five fish was trained with red; another group of five was trained with blue. Test stimuli include blue, green, yellow, orange, and red lights.

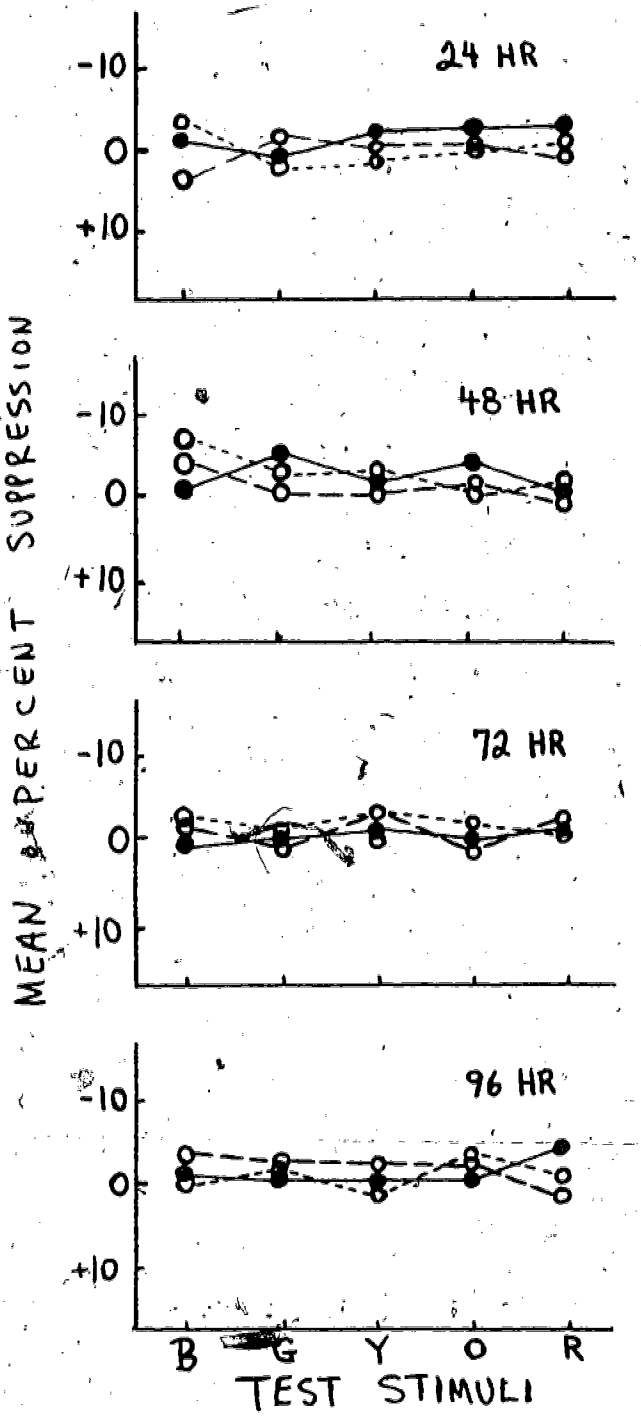

Figure 6. Mean percent suppression of respiration produced by the five test stimuli, relative to baseline. Nonreinforced tests were given 24, 48, 72, and 96 hr. after injection of recipients with red-trained (dotted line), blue-trained (dashed line), or control (solid line) donor brain extracts.

Experiment 4: Task Specificity

Braud (1970) has shown that extracts from brains of donors trained on shuttle-box light-avoidance facilitate recipient performance when the latter were tested on a similar light-avoidance task. Bryant, Santos, and Byrne (1972) and Bryant (1972) have shown that brain extracts from donors trained on light-avoidance facilitate light-avoidance learning but impede dark-avoidance learning in recipients, while dark-avoidance extracts facilitate dark-avoidance learning but impede light-avoidance. The results of the first phase of Experiment 4 suggest that training donors to swim from red to green to avoid and escape shock results in significant interference with green to red escape/avoidance learning in recipients that received those donor extracts. In Figure 7 are presented the green to red learning curves for recipients of "trained" and control extracts (expressed as mean percent avoidances on each of the three test days). Experimental recipients (of oppositely trained donor brain extract) are inferior to control recipients at every test day, and are significantly inferior at the 72 hr. test ($U = 18$, $p < .01$). These differences could not have been due to a general lowering of overall activity or general debilitation of the experimental recipients, since there was no significant difference between experimental and control recipients at any of the three test points in terms of a measure of general activity (number of hurdle-crossings in the absence of any stimulation, i.e., number of inter-trial responses). Thus, task specificity has been shown: brain extracts facilitate learning of identical or homologous tasks, but impair learning of "opposite" tasks.

Training donors to avoid red in the shuttle-box inhibits the ability of recipients to learn to approach red in the same shuttle-box. Red-avoidance donor training also appears to yield a brain extract which inhibits red-approach learning even when that learning occurs, not in the original shuttle-box, but in a similar "red photobeam escape apparatus". In Figure 8 are plotted the learning curves of experimental (red shuttle-box compartment avoidance trained extract) and control recipients. Here, approach and occlusion of the red photobeam terminated electric shock. At the 72 hr. test, the experimental recipients perform significantly less well than control recipients ($U = 24$, $p < .05$).

Performance of recipients in the third phase of Experiment 4 is shown in Figure 9. It can be seen that all fish display a preference (in terms of total amount of time spent in that area) for the green end of the compartment. This green-preference is greater in experimental recipients, but not significantly so. Apparently, the similarity of the two tasks (shuttle-box learning vs. color preference in the absence of shock) is not great enough to allow the green-approach trend to become significant.

It may be concluded from the combined results of the various phases of Experiment 4 that trained brain extracts do not facilitate learning or performance indiscriminately, but only in situations identical or similar to the situation encountered by the donors during original training.

Conclusions

In the four experiments reported here, four different sorts of specificity were evaluated. Care was taken to prepare all extracts at the same time, using the same extraction procedures. Since different aliquots of material from the same extract sample were shown to be effec-

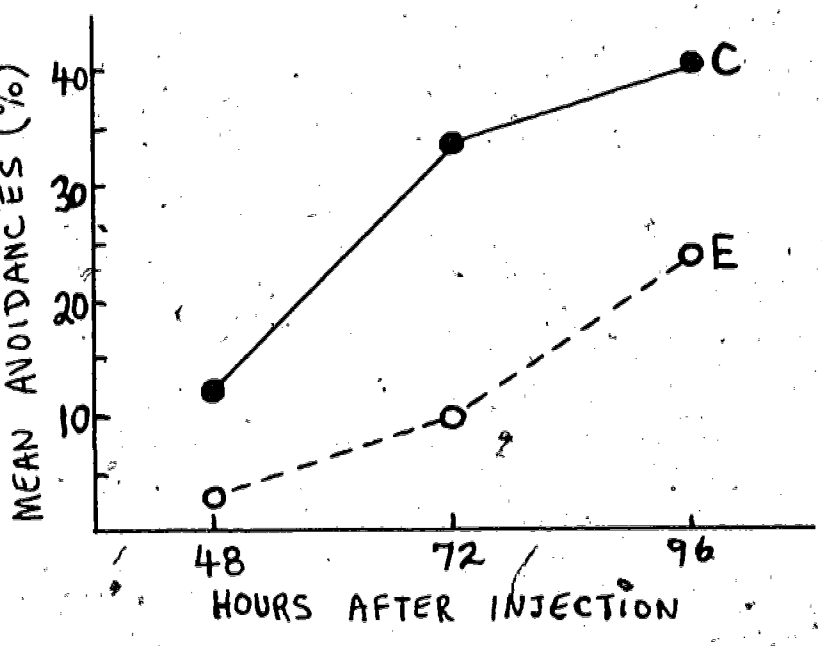

Figure 7. Inhibition of green to red escape/avoidance learning in the shuttle-box in recipients of brain extracts from red to green trained donors, relative to the learning curve of recipients of naive control extract. Ten reinforced trials are given on each of the three test days.

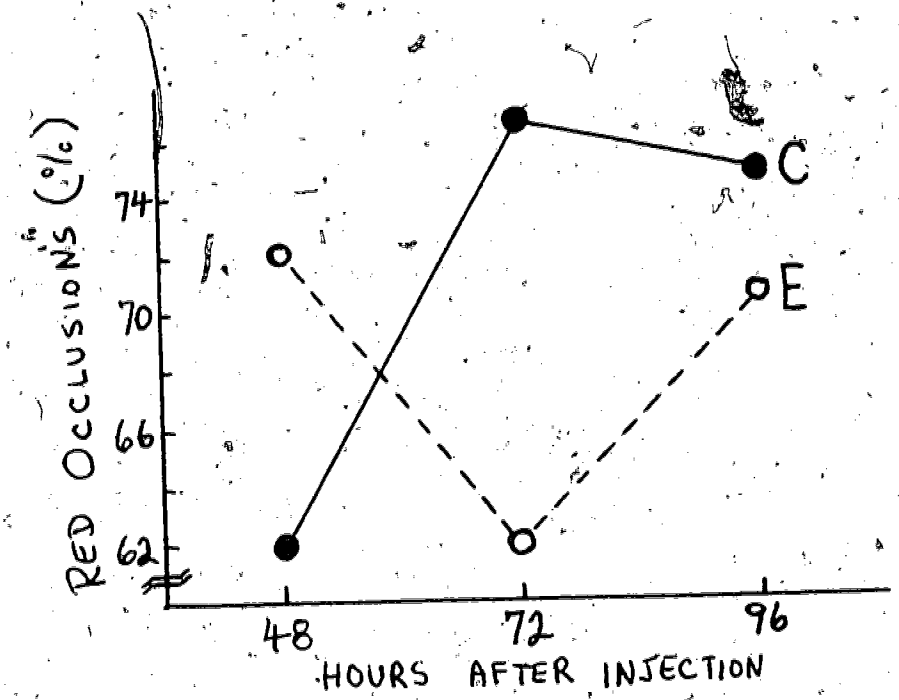

Figure 8. Inhibition of red photobeam occlusion behavior in the shock-escape apparatus at 72 and 96 hr. following injection of experimental (E) recipients with brain extract from donors trained to avoid red in the shuttle-box, relative to performance of recipients of naive control (C) brain extract. Data points represent mean percent occlusions of the red photobeam which terminated shock.

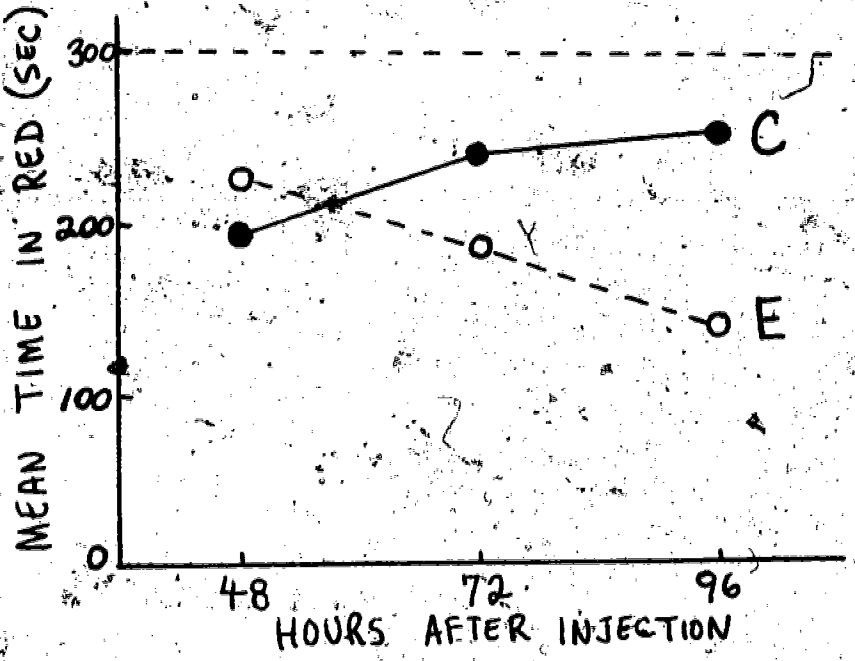

Figure 9. Mean time (in sec. out of a possible 600 sec.) spent in the red illuminated half of the color preference apparatus by recipients of extract from brains of donors trained to avoid red in the shuttle-box (E) and recipients of naive control extract (C). Scores lower than the horizontal dashed line indicate green preference.

tive or noneffective depending upon the testing conditions (which process, response, or task is considered), it cannot be argued that apparent specificity was an artifact of differentially effective extraction procedures for different extracts tested once and only once in different situations. Evidence was presented in support of three distinct types of specificity. It appears that extracts prepared from brains in which one process (acquisition vs. extinction) is active facilitate that same process in recipients, but do not facilitate antagonistic processes. Second, it was shown that it was necessary for the donors to make a specific response if extracts from their brains were to prove effective in increasing the probability of that response in recipient animals. Third, it was shown that the degree to which brain extracts from donors trained on Task A facilitated learning of Tasks B, C, etc. in recipients depended upon the similarity of the tasks involved: antagonistic behaviors were impeded, while identical or similar behaviors were facilitated. Due to the failure of the classical conditioning task of Experiment 3 to transfer, no evaluation of stimulus specificity could be made in that case.

Fortunately, other experiments conducted in our laboratory during the time period of this grant (although not directly sponsored by the grant and, hence, done independently) strongly suggest stimulus specificity. In three experiments, it was shown that recipients responded to an array of stimuli in a manner that mimiced the donors' response pattern. Goldfish donors were trained to approach either an upright or an inverted isosceles triangle for a food reinforcer; a third group of donor fish were never trained. Recipients of upright-triangle trained brain themselves approached the upright triangle during nonreinforced test trials. Recipients of inverted triangle trained brain extract chose the inverted triangle. Recipients of naive control extract showed no preference for either triangle. A report of these findings is now in press (Braud and Hoffman, 1973). In a second experiment (Braud and Braud, 1972), hooded rat recipients of brain extract from trained donors chose a circle size appropriate to that which would have been chosen by the donors; again, nonreinforced test trials were used. In the third experiment (Braud, Kuttner, Ginsburg, Woody, Hoffman, and Laird, 1973), neonatal quail donors were imprinted to either a red cylinder or a green cube; recipients preferred, followed, and emitted fewer distress calls in the presence of the object to which their respective donors had been imprinted, when the recipients were confronted with both stimuli during nonreinforced test trials. Thus, the case for stimulus specificity is quite strong.

Recently, still another type of process specificity (what might be called "motivational" process specificity) has been successfully demonstrated in our lab. In this study (Braud, Galvan, and Clark, 1973), brain extracts from donor rats given forward conditioning (to establish the motivational state of fear), backward conditioning (to establish the motivational state of relief or relaxation), or no conditioning (control) trials in a buzzer-shock paradigm were injected intraperitoneally into three groups of naive recipient mice. The mice were tested for amount of licking suppression (an index of emotionality) occurring in the presence of the buzzer. Licking behavior was facilitated in Backward extract recipients but suppressed in Forward extract recipients, relative to Controls. Thus, the antagonistic "fear" and "relief" processes transferred independently and specifically. This paradigm, by the way, involved an indirectly measured transfer of a classically conditioned effect. Pavlovian pairings were used in training the donors. No particular res-

ponse was measured in the donors, as had been done in the case of the direct classical conditioning transfer attempt of Experiment 3 reported above. Another indirect classical conditioning transfer was recently completed by Laird and Braud (1973). Here, goldfish were shocked in the presence of a blue light (or green light) but never shocked in the presence of a green light (or blue light). The paradigm was a differential classical conditioning one, similar to that of Experiment 3, but no responses were measured during conditioning itself. Recipients were given a brief reminder shock associated with the donor-appropriate color, then half were trained to avoid blue and half trained to avoid green in a shuttle-box. Controls learned each task equally well, while blue-trained recipients learned to avoid blue significantly faster than they learned to avoid green; green-trained extract recipients learned green-avoidance faster.

Recommendations

The present research, together with other recently completed experiments, makes a very strong case that the biochemical "memory transfer" experiments really involve a transfer of specific information, rather than some general facilitating or inhibiting factors as certain critics have supposed. Thus, it is seen as quite profitable that researchers continue to explore these "memory transfer" or "behavioral bioassay" experiments, especially in terms of the specific biochemical changes which occur in donor organisms as they attend to, learn, and remember specific tasks. It would appear that the general paradigm has no great behavioral limitations. Considerable efforts should be expended to elucidate the molecular mechanisms of learning and memory (using the behavioral bioassay strategy as well as others) so that normally acting processes be detected, abnormalities corrected, and progress made in the possible detection and remediation of attentional, perceptual, learning, and memory disorders in man.

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