References and Notes

1. H. Brem and J. Folkman, *J. Exp. Med.* **141**, 427 (1975).
2. N. Sorgente, K. E. Keuttner, L. W. Soble, R. Eisenstein, *Lab. Invest.* **32**, 217 (1975); N. Sorgente *et al.* [in *Protides of the Biological Fluids*, H. Peeters, Ed. (Pergamon, New York, 1976), vol. 23, pp. 227–230)] report purification of a protease inhibitor from cartilage.
3. S. C. March, I. Parikh, P. Cuatrecasas, *Anal. Biochem.* **60**, 149 (1974).
4. The Elvax 40 was supplied through the courtesy of Alza Corporation, Palo Alto, California. Crude polymer was washed exhaustively in absolute ethanol to remove inflammatory impurities.
5. L. Carlsson and B. Karlsson, *Experientia* **28**, 990 (1972); W. B. Schill and G. F. B. Schumacher, *Anal. Biochem.* **46**, 502 (1972); G. F. B. Schumacher and W. B. Schill, *ibid.* **48**, 9 (1972).
6. M. A. Gimbrone, Jr., R. S. Cotran, S. B. Leapman, J. Folkman, *J. Natl. Cancer Inst.* **52**, 413 (1974).
7. J. Folkman, E. Merler, C. Abernathy, G. Williams, *J. Exp. Med.* **133**, 275 (1971).
8. J. Folkman, *Adv. Cancer Res.* **19**, 331 (1974); *Ann. Intern. Med.* **82**, 96 (1975).
9. Since this work was completed, Eisenstein *et al.* [*Am. J. Pathol.* **81**, 337 (1975)] have reported that 1*M* guanidine extracts of cartilage inhibit aortic endothelial cells (and to a lesser extent fibroblasts) in vitro.
10. We thank Ivan Preis, Germaine Grant, Susan Clark, Paul Wesley, Steven Fleit, and Dr. David Tapper for valuable technical assistance.

17 February 1976; revised 30 March 1976

Conditioning of *Pleurobranchaea*

The development of the mollusk *Pleurobranchaea californica* as a preparation for the study of brain-behavior relationships was a notable achievement of Davis and Mpitsos (*1*). *Pleurobranchaea* has large identifiable nerve cells, has complex behaviors (*2*), and can be studied as a "whole-body preparation" in which electrophysiological manipulations can be performed on the nervous system of an animal with almost complete freedom of action (*3*). Reports of conditioning with this species are, therefore, of considerable interest because they suggest the possibility of a neurophysiological analysis of learning at the cellular level.

The recent report of Mpitsos and Collins (*4*) and an earlier report of Mpitsos and Davis (*5*) are concerned with this problem, but should not yet be accepted as incontrovertible proof that *Pleurobranchaea* is capable of higher forms of learning. In the earlier work, on classical conditioning (*5*), the unconditioned stimulus (US) and conditioned stimulus (CS) were combined. A glass rod (providing the CS) was dipped into a liquid food substance (squid homogenate, the US) and then stroked on the chemosensitive oral veil. Although the response of the experimental group was appropriate for classical conditioning, a likely alternative explanation for the results is sensitization. Findings suggesting sensitization are that the CS occasionally (4.5 percent of the time) produced the same response as the US and that control subjects, with separate presentation of CS and US, showed a marked increase in response. The control groups used by the authors—CS alone, and CS alone followed in 3 to 4 hours by US alone—were inadequate to eliminate sensitization as an explanation. The required control group to demonstrate true classical conditioning is one in which the US and CS are presented alternately; and, in such controversial experiments as these, additional control procedures would be valuable.

Two other weaknesses of the experimental design should be noted. A particularly serious problem was the short intertrial interval, which was 30 to 60 seconds. We have observed, in attempting to replicate this experiment and in other work (*6*), that squid-elicited feeding responses last on the order of 30 to 60 seconds, or even longer. Therefore, the unconditioned response of the previous trial might often have occurred during the CS of the following trial. Another procedural difficulty was that the US used for the controls was not the same as that used for the experimentals because it was delivered by "decanting in the vicinity of the oral veil" rather than being carried directly to the surface of the oral veil by the rod. The CS also was not the same, since it would have a different texture due to the squid homogenate.

In the Mpitsos and Davis report (*5*) an avoidance conditioning procedure was described. Animals previously "conditioned" were subsequently divided into two groups: an avoidance group, in which animals failing to withdraw within 5 seconds after rod stimulation (previous CS) were shocked, and a control group which received a series of shocks followed in 2 to 3 hours by the CS test stimuli. Here again, the control group used was not appropriate. For a control, the shocks should have been alternated with the CS so that one could see if the avoidance contingency and not just the general effect of the shocks was the factor in decreasing the response.

One possible alternative explanation for the results that the authors cited was that the decreased feeding rate was caused by short-term shock-induced inhibition. This is a likely alternative explanation and the only argument that the authors can make on this point is that the depressed feeding rate—for two subjects—persisted for 4 days during a control procedure involving touch alone. Even if more subjects were used, this manipulation still would not provide the rigorous kind of evidence that appropriate control groups would, and it does not necessarily have a bearing upon other possible explanations. Furthermore, even if the experiment were improved with proper control groups, what would be demonstrated is only the facilitation of the extinction of a classically conditioned—or perhaps, sensitized—response.

In the more recent report (*4*), an "aversive conditioning" paradigm was used. The entire training consisted of ten trials spaced 1 hour apart. On each trial, the subject was presented with squid homogenate and was immediately shocked if it responded with the usual response of a bite or strike. This is very clearly a punishment procedure. However, there was an additional contingency: if there was no bite or strike, or "sustained" withdrawal response within 180 seconds, then a shock was delivered at the end of this 180-second period. This is clearly an avoidance contingency in which the response is a withdrawal and the warning signal is the squid stimulus.

Claims by the authors that these procedures particularly "resemble" classical conditioning are misleading. *All* signaled avoidance paradigms, not just this one, have some elements in common with classical conditioning. The procedure is instrumental conditioning (actually a combination of two paradigms), because the subject's response determines the occurrence of the reinforcer.

Let us now consider whether the authors have rigorously demonstrated a behavioral change classifiable as "conditioning." First of all, there seems to be some inconsistent information regarding the control procedure. Controls "received as much stimulation" as the experimentals, "but they were given food and shock alternately (unpaired) every half hour." Note that experimentals were not always shocked and there was some variability in the shocking procedure: "contact was often unavoidable." We have found that the exact location of electrodes can have a profound effect on the efficacy of the shock. Were these factors taken into consideration when the controls were run? Another point is that although the "observations were conducted blind," the person presenting the shocks had to be aware of the contingencies, and, because of the variable na-

ture of the shock, could have introduced some bias. Although this experiment is better designed than the earlier one, I think that its procedures still leave some doubt as to the learning capabilities of *Pleurobranchaea*. We must await more carefully controlled experiments with completely objective recordings of behavior and consistent, perhaps automated, stimulus presentations.

RICHARD M. LEE
Department of Neurological and Behavioral Sciences, Edsel B. Ford Institute for Medical Research, Detroit, Michigan 48202

References

1. W. J. Davis and G. J. Mpitsos, *Z. Vgl. Physiol.* **75**, 207 (1971).
2. R. M. Lee, M. R. Robbins, R. Palovcik, *Behav. Biol.* **12**, 297 (19 ➡ W. J. Davis, G. J. Mpitsos, M. V. Siegler, J. M. Pinneo, K. B. Davis, *Am. Zool.* **14**, 1037 (1974).
3. R. M. Lee and R. J. Liegeois, *J. Neurobiol.* **5**, 546 (1974).
4. G. J. Mpitsos and S. D. Collins, *Science* **188**, 954 (1975).
5. G. J. Mpitsos and W. J. Davis, *ibid.* **180**, 317 (1973).
6. R. M. Lee and R. A. Palovcik, *Behav. Biol.*, in press.

20 August 1975; revised 14 January 1976

In his review of our reports (*1, 2*) Lee states that the described results "should not yet be accepted as incontrovertible proof that *Pleurobranchaea* is capable of higher forms of learning." He states also that "the required control group to demonstrate true classical conditioning is one in which the US [unconditioned stimulus] and CS [conditioned stimulus] are presented alternately." This is the "traditional" interpretation of Pavlovian conditioning which emphasizes CS-US pairing as the essential feature for "true" conditioning (*3–5*). However, not all theorists would agree with this interpretation. An opposing theory places the emphasis on CS-US contingencies (*5*). By this theory the "proper" control is one in which the CS and US are presented in a random fashion. The traditional control procedures, alone or together, are said to be insufficient since they all contain CS-US contingencies. For example, the alternating CS/US control contains the contingency that the CS may "signal the absence of the US" (*5*). Traditional theory, on the other hand, does not accept the alternating control as a true conditioning procedure. In view of such fundamental disagreement on the criteria for conditioning and the proper control, it seems to us that attempts to provide the "incontrovertible proof" of "true conditioning" would be extremely difficult if not impractical.

Is *Pleurobranchaea* capable of learning? Since Lee has used traditional theory, the key issue is whether the reported experimental-control differences are attributable to CS-US pairing or to some nonassociative phenomenon. In our more recent experiments we employed the alternating CS/US control group, and, therefore, Lee's questioning as to whether we have "rigorously demonstrated a behavioral change classifiable as 'conditioning' " arises from the procedures rather than the design of the experiments. The most important of Lee's objections is that we did not use "blind" procedures to electrically shock the animals. The described method of electrical stimulation of the oral veil and head region was used in order to provide the animals a relatively specific stimulus from which to withdraw so that withdrawal responses as well as the suppression of feeding could be conditioned. The application of such a relatively localized stimulus required an "unblind" procedure since the person who directed the shocks had to know whether the animal belonged to an experimental or control group. With the unblind procedure there is the possibility that either the experimental or control group might have been shocked more than the other and that this bias might have caused the observed experimental-control behavior differences. We seriously doubt this possibility, however, since we minimized differential stimulation by requiring that the shocks always produced similar violent aversive behavior from all animals. The effectiveness of this measure in producing equivalent stimulation of both the experimental and control animals has been demonstrated in part by experiments in which we used completely blind and automated procedures to shock the whole animal rather than restricting the shock to the oral veil and head. Interestingly, the automated shock parameters we have used so far (biphasic, constant current pulses) effectively suppress feeding behavior and stimulate the body of the animal but have little effect on the oral veil and head regions; that is, not only is there an absence of an obvious stimulus to the oral veil and head from which the animal can withdraw, but, because of the biphasic pulses, the stimulus that is effective on the body is nondirectional. As suspected, training with this type of stimulus as the US provides evidence of conditioning in the suppression of feeding, but has little effect on the withdrawal behavior.

In the first two paragraphs of the discussion on the recent report, Lee correctly summarizes our procedures. He states, however, that "Claims by the authors that these procedures particularly 'resemble' classical conditioning are misleading. *All* signaled avoidance paradigms, not just this one, have some elements in common with classical conditioning." We made no statement that our procedures *particularly* resemble classical conditioning, but we did state accurately that "during the early stages of conditioning, our paradigm resembled classical conditioning," and we did so in order to place into perspective the stimuli and responses we used. Lee appears to be questioning whether our results can be classified as conditioning because our food-shock paired procedure was also "a combination of two procedures," punishment and avoidance conditioning. Both procedures were involved. Nonetheless, it seems to us that the results are classifiable as conditioning, even though we did not use a "pure" classical or operant training procedure to obtain them.

In the third and final paragraph of the discussion on the recent report, Lee questions whether the control animals received as many shock presentations as the experimentals and whether the shocks were equally effective for all animals since the exact location of the electrodes seems to be a critical factor. We stated that each experimental animal was matched with a control animal and that each matched-pair received as much stimulation. Thus if an experimental animal was shocked, its matched control was shocked; or if the experimental avoided shock, its matched control was not shocked. We do not believe that electrode position was a critical factor in our experiments since our definition of effective shocks consisted of producing the same violent aversive behavior from all animals rather than reproducing the exact location of the electrodes.

As discussed above, our reported procedures controlled for CS-US pairing. We have now completed studies using random CS/US procedures to better control for CS-US contingencies. The direction of CS-US pairing is also important, so we have also conducted an analysis of backward and forward conditioning, using only one trial and one training day. We have also obtained some evidence of CS specificity by comparing the responses of experimental and control animals to food and other stimuli before and after aversive conditioning. The results of these experiments are consistent with our reported findings and conclusions. Thus, while we may not have provided "incontrovertible proof" of conditioning, we feel that our reported findings can be "classified as condi-

tioning'' and that they provide evidence that ''*Pleurobranchaea* is capable of higher forms of learning.''

Turning to the earlier experiments (*1*), we generally agree with Lee's procedural comments, and we agree that the experimental design was less adequate than in the more recent study since the ''unpaired'' control group did not receive the CS and US alternately and within the span of time when the experimentals were conditioned. However, the ''unpaired'' control group did receive both the CS and US separately. Of these experiments, Lee raises three major issues dealing with nonassociative phenomena: sensitization, shock-induced inhibition, and facilitated extinction. We mentioned that sensitization may have been a factor underlying the behavior changes of the animals. In both the classical and avoidance conditioning procedures we attempted to minimize sensitizing the experimental or control animals differentially by seeking to apply equivalent amounts of the US; we felt that similar responses executed over the same period of time could be taken as a measure of equivalent stimulus strengths. For example, in the classical conditioning experiments we presented the food US to the control animals differently than to the experimentals in order to avoid exposing the control animals to tactile stimuli concomitantly with the food. Since the US in both cases elicited roughly the same number of feeding responses over the same period of time, we felt that the US's were equivalent. While this does not completely eliminate the possibility that the two methods of US presentation differentially sensitized the animals, it provides some evidence that the experimental-control differences were caused by CS-US pairing.

We also considered the possibility of shock-induced inhibition as the cause of the behavior changes of the experimental animals during avoidance conditioning. However, there are several lines of direct and indirect evidence that support the argument against it. Figure 2C in our earlier report (*1*) shows two groups of animals that were switched from conditions of experimental-avoidance to touch-control, one group on day 11, which Lee mentions, and another on day 15. Both groups of animals continued to withdraw from tactile stimuli for several days after the switch, indicating that some long-term effect had been conditioned. Figure 2, A and C, in (*1*) shows shock-control groups that received as much electrical stimulation as the experimental animals but continued to exhibit vigorous feeding responses to tactile stimulation for many days. The lack of long-term inhibition in these animals is inconsistent with the hypothesis of shock-induced inhibition. Our argument against shock-induced inhibition is indirectly supported by published observations that the normal feeding behavior of *Pleurobranchaea* is not suppressed even after strong electrical stimulation (*6*), and by the behavior of the control animals in the aversive conditioning experiments, which continued to feed despite the fact that they were exposed repeatedly to great amounts of electrical stimulation.

The statement that ''even if the experiment were improved with proper control groups, what would be demonstrated is only the facilitation of the extinction of a classically conditioned—or perhaps, sensitized—response'' is inconsistent with Lee's own use of the ''required control,'' and contains the unsupported implication that conditioned responses cannot be superimposed on previously altered behaviors: if Lee's proper control group were conducted, differences between the experimentals and controls would have to be accepted by definition as true associational conditioning, regardless of how the original response was established before the avoidance conditioning.

In conclusion, we feel that our reports, especially the more recent one, provide evidence that *Pleurobranchaea* is capable of associative learning. In his closing remarks on the recent report, Lee asks for objectivity and automated procedures. It was, in fact, for the purpose of objectivity that we specifically selected strong, identifiable, and reproducible behaviors, measured them quantitatively under blind conditions, and repeated the measurements thousands of times. Automation would certainly be helpful, and we have begun to develop some appropriate techniques. In the meantime, we believe that much of the fundamental biology has already been uncovered objectively and effectively by means of simple and inexpensive techniques.

GEORGE J. MPITSOS
Department of Anatomy,
School of Medicine,
Case Western Reserve University,
Cleveland, Ohio 44106

References

1. G. J. Mpitsos and W. J. Davis, *Science* **180**, 317 (1973).
2. G. J. Mpitsos and S. D. Collins, *ibid.* **188**, 954 (1975).
3. G. A. Kimble, *Hilgard and Marquis' Conditioning and Learning* (Appleton-Century-Crofts, New York, 1961), pp. 47–77.
4. I. P. Pavlov, *Conditioned Reflexes*, G. V. Anrep, Transl. (Oxford Univ. Press, London, 1927).
5. R. A. Rescorla, *Psychol. Rev.* **74**, 71 (1967).
6. W. J. Davis, G. J. Mpitsos, J. M. Pinneo, *J. Comp. Physiol.* **90**, 207 (1974).
7. I thank Dr. P. Sheafor for his critical discussions.

30 March 1976

Of Stress, Vitamin A, and Tumors

In two recent *Science* reports (*1, 2*) there are serious omissions of references to prior literature directly related to the work reported.

Riley (*1*) demonstrated an increased tumor incidence in mice infected with the mammary tumor virus as a result of chronic exposure to environmental stresses. He hypothesizes that the host response to stressful stimuli results in adrenal cortical hypersecretion of corticosterone which has marked thymolytic and lympholytic actions in mice and results in a depression of cell-mediated immune reactions. The immunodepression is then held responsible for increased tumor development in stressed mice exposed to an oncogenic virus.

My colleagues and I have tested this hypothesis directly and have demonstrated that in mice inoculated with a murine sarcoma virus (MuSV-M), physical stress increases the incidence and severity of tumor development (*3*). We discussed the importance of prior history and environmental conditions on tumor incidence in mice exposed to oncogenic viruses (*3, 4*). We demonstrated the influence of stress on thymic size and cellularity and hypothesized that stress reduced immunocompetence through increased adrenal corticosterone secretion.

Furthermore, we showed that metyrapone, a chemical that inhibits corticosterone production, prevents the typical stress reaction of adrenal hypertrophy and thymic involution and increases the resistance of stressed and nonstressed mice to MuSV-M (*5*). Also, steroids such as deoxycorticosterone, which can compete with corticosterone for some tissue receptor sites, prevent the adverse effect of stress in mice inoculated with MuSV-M (*6*). Finally, vitamin A, which blocks some host responses to stress or cortisone administration, is remarkably protective against both thymic involution due to these agents and tumor development following inoculation with MuSV-M (*4, 7*). Thus, some of the main