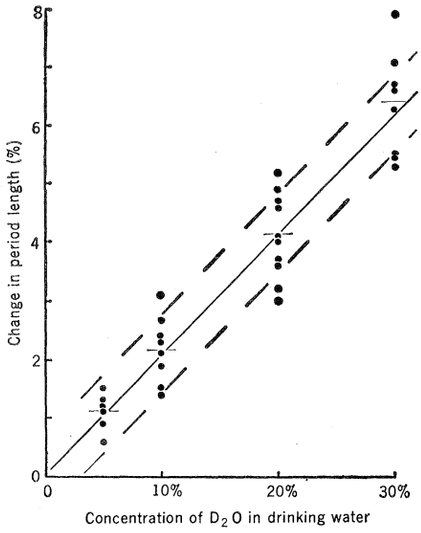

Fig. 3. Relation of D_2O concentration to period length of *Peromyscus* circadian rhythms. Solid line has been fitted by the method of least squares. Dashed lines represent ± one standard deviation. Short horizontal lines show average values.

effect of period lengthening in *Peromyscus*.

Much of the search for a "site" of the physiological clock of various organisms has centered around the nucleus and the nucleic acids [Bünning (*1*)]. The possibility that DNA, RNA, or protein synthesis is responsible for circadian rhythmicity was suggested by the work of Karakashian and Hastings with puromycin and actinomycin D (*1*). Dicken and Bray (*8*) found that DNA synthesis decreased in mammals drinking D_2O. Bray found a linear dosage effect in which the amount of P^{32} incorporated into rat liver DNA varied inversely with concentrations of D_2O up to 30 percent in the body water. Decreases in DNA synthesis were an order of magnitude greater than the changes found in this study with *Peromyscus*, which suggests that the effect of period lengthening on the circadian rhythm is not directly related to DNA synthesis.

The linear relation, without threshold, shown in Fig. 3 is consistent with variations in some of the physical properties of mixtures of D_2O and H_2O (*7*). Viscosity and positive ion mobilities match the data closely while changes in specific gravity and vapor pressure do not. That some physical property of H_2O or D_2O might control the length of the period of rhythm is in accord with the small, though measurable, effects of tissue temperature on these rhythms (*1*, *9*).

Although the effects of D_2O do not as yet identify any single mechanism as determining the period of circadian rhythms, D_2O should prove to be a useful tool for the further analysis of the physiological basis of these rhythms.

ROBERT B. SUTER
KENNETH S. RAWSON
Department of Biology,
Swarthmore College,
Swarthmore, Pennsylvania 19081

References and Notes

1. E. Bünning, *The Physiological Clock* (Academic Press, New York, 1964); J. W. Hastings, *Cold Spring Harbor Symp. Quant. Biol.* **25**, 131 (1960); M. W. Karakashian and J. W. Hastings, *Proc. Nat. Acad. Sci. U.S.* **48**, 2130 (1962); C. S. Pittendrigh, *ibid.* **40**, 1018 (1954); C. L. Ralph, *Physiol. Zool.* **32**, 57 (1959); K. S. Rawson, *Cold Spring Harbor Symp. Quant. Biol.* **25**, 105 (1960).
2. V. G. Bruce and C. S. Pittendrigh, *J. Cell. Comp. Physiol.* **56**, 25 (1960); E. Bünning and J. Baltes, *Naturwissenschaften* **50**, 622 (1963).
3. Mice were disturbed in order to clean the cages, replenish food and water, and to repair equipment failures. Such disturbances were usually made during the inactive phase of the activity cycle, when dim light has a negligible effect on the phase or period of the circadian rhythm [Rawson (*1*)]. When disturbances occurred during the active phase, continuous darkness was maintained. The adequacy of the conditions of isolation was shown by "free running" circadian rhythms that had different period lengths for each animal and gave no evidence for synchronization of one mouse with another or with an exact 24-hour day.
4. Days at the beginning of each run, in which the period of oscillation was clearly changing, were not included in the analysis since it was the "equilibrium" period length that was of interest.
5. P. G. Guest, *Numerical Methods of Curve Fitting* (Cambridge Univ. Press, London, 1961). A straight line was fitted to the onsets of activity by the method of least squares in accord with the suggestion of C. H. Lowe, *et al.* [*Science* **156**, 531 (1967)]. Their criticism of the previous method used [Rawson (*1*)] is of theoretical importance only as the differences in calculated period lengths by the two methods were less than 2.5 minutes per period, or 0.16 percent, for *all* of the 43 period lengths analyzed in this study (Table 1).
6. J. T. Enright, *J. Theoret. Biol.* **8**, 426 (1965). Our computer program used a 10-minute interval in scanning a range of period lengths. This limited the accuracy of the method to ± 5 minutes, which was less accurate than the derivations from onsets of activity. Consequently this periodogram analysis is not reported in detail, although it is the analytic method of choice when a precise measure such as the sharp onset of activity cannot be obtained.
7. J. F. Thomson, *Biological Effects of Deuterium* (Macmillan, New York, 1963); A. Farkas, *Orthohydrogen, Parahydrogen and Heavy Hydrogen* (Cambridge Univ. Press, London, 1935).
8. C. Dicken, T. R. Henderson, J. S. Dinning, *Proc. Soc. Exp. Biol. Med.* **110**, 208 (1962); D. A. Bray and J. F. Thomson, *ibid.* **111**, 380 (1963).
9. Deuterium oxide may also directly inhibit the activity of enzymes. Thomson (*7*) reports that catalase activity, as measured in vitro by the rate of decomposition of perborate, decreases linearly with increasing concentrations of D_2O. In 30 percent D_2O there is less than 10 percent inhibition, which is similar in magnitude to the change demonstrated for the period length of the *Peromyscus* circadian activity rhythm.
10. Supported by NSF grant GY 2628.

7 March 1968

Mixed Lymphocyte Reaction: An in vitro Test for Antilymphocytic Serum Activity

Abstract. *When Lewis rats were exposed to antilymphocytic serum, produced in rabbits, to Lewis lymphoid cells, the transformation of their thoracic duct lymphocytes in response to foreign lymphoid cells was markedly reduced in comparison with that in response to similar cells from donors treated with saline or normal rabbit serum. It is suggested that the mixed lymphocyte reaction may be used as an in vitro test for the in vivo activity of antilymphocytic serum.*

Allogeneic skin graft survival may be prolonged by pretreatment of a recipient with heterologous antiserum produced against the recipient's lymphoid cells or against cells from another donor of the same species or strain (*1*). This prolonged survival is accompanied by an inability of the animal's lymphoid cells to initiate graft-versus-host reactions and normal lymphocyte transfer reactions (*2*). The normal lymphocyte transfer reactions are produced by injecting lymphoid cells intradermally into genetically dissimilar recipients (*3*). In addition, antilymphocytic serum usually induces a circulating lymphopenia (*4*), although this is unnecessary for prolonged graft survival (*5*). It has also been reported that when lymphoid cells from two genetically dissimilar donors are cultured together, some of the small lymphocytes in the mixture will transform into large, immature-appearing blast cells. This mixed lymphocyte reaction represents an in vitro homograft reaction which correlates with the immunological status of the donors, as measured by the ability to induce runting or normal lymphocyte transfer reactions (*6*). Therefore, it seemed desirable to determine whether the mixed lymphocyte reaction (MLR) was effected by pretreating the cell donors with antilymphocytic serum. Results of the present study indicate that the mixed lymphocyte reaction was depressed and, hence, may serve as an in vitro test for antilymphocytic serum activity.

The antilymphocytic serum was produced in six adult, male New Zealand white rabbits by the intravenous injection of 30 to 80×10^6 thoracic duct lymphocytes obtained from male Lewis rats. Six injections were made over a period of 7 weeks, and 34 days after the

last injection the animals were exsanguinated by puncture of the inferior vena cava. The rabbit serum from all donors was then pooled and samples of the serum were absorbed with washed, rat red blood cells. The absorption was accomplished by thoroughly mixing the serum with packed red blood cells in a volume ratio of 5 : 1. After standing for 1 minute, the serum was separated from the agglutinated cells by gentle centrifugation. The procedure was then repeated twice and the serum was injected into two rats in the fashion described below. In this case, no detectable difference in the mixed lymphocyte reaction or the normal lymphocyte transfer reaction was noted when absorbed instead of nonabsorbed serum was employed.

Eighteen highly inbred Lewis rats, divided equally into six groups, were employed as the cell donors in this study. While the average weights per animal of the six groups varied between 140 and 265 g, the individual members of each group possessed approximately equivalent weights. Individual rats in four of these six groups then received five daily intraperitoneal injections of 1 ml of either saline, normal rabbit serum, or antilymphocytic serum. Twenty-four hours after the last injection, lymph was collected from the thoracic duct of each donor according to the method of Reinhardt and Li (*7*). From cell counts performed on the nondiluted lymph and from differential counts made on smears of the collected cells, the absolute number of large and medium, as well as small, lymphocytes was determined (*6*). After the cell counts, the animals were injected with 5 to 10 ml of 5 percent glucose in saline and lymphocytes were collected for culture. Thoracic duct lymphocytes for culture purposes were also collected from four F_1 (Lewis × Brown Norway) hybrids [F_1 (Lew × BN)] ranging in weight from 150 to 300 g. According to the method described previously (*6*), the cells from the injected donors were then either cultured alone as nonmixed controls, or exposed to pokeweed mitogen as a blastogenic control, or mixed in equal numbers with cells from the F_1 hybrid donors. In the latter case, as in normal lymphocyte transfer reactions, the response observed was "one way" with Lewis cells reacting against the BN elements but not vice versa. Three days after initiation, the cultures were exposed to H^3-thymidine (4 μc/ml; 6.7 c/mmole) for 12 hours. The cultures were then analyzed for the presence of transformed cells by determining the percentage of enlarged cells on smears and by assaying the amount of H^3-thymidine incorporation by scintillation counting (*6*).

Table 1. The average number of lymphocytes (range in parentheses) in the thoracic duct lymph of rats 24 hours after five daily injections of either saline (SAL), normal rabbit serum (NRS), or antilymphocytic serum (ALS).

Treatment of donor of cells	Millions of cells per milliliter	Percent of cells that are small lymphocytes	Millions of small lymphocytes per milliliter	Millions of LMC* per milliliter
SAL	93.7 (80.0 to 113.0)	84.8 (81.8 to 91.1)	79.2 (68.0 to 92.4)	14.5 (10.8 to 20.6)
NRS	100.0 (67.0 to 140.0)	83.1 (81.3 to 89.5)	83.2 (60.0 to 113.8)	16.8 (7.0 to 26.2)
ALS	35.7 (20.0 to 53.0)	76.2 (71.6 to 81.3)	27.6 (15.0 to 43.1)	8.1 (5.0 to 9.9)

*LMC, large and medium lymphocytes.

The remaining two groups of animals were injected with saline, normal rabbit serum, or antilymphocytic serum in a manner identical to that described above. In this case, however, immediately after a small sample of thoracic duct lymph for cell counts and smears was obtained, suspensions of mesenteric lymph node cells were prepared (*6*). Normal lymphocyte transfer reactions were then initiated by injecting 25×10^6 cells from each of the three donors (*3*, *6*) intradermally into the ventral abdominal wall of an F_1 Lew × BN) hybrid. Five days later, a 1 percent saline solution of Evans blue was administered intravenously in a dose of 0.4 ml/100 g. After 8 hours, the skin involved in the reactions was excised, and the reactions, as viewed on the everted skin, were measured and photographed.

The results of the cell counts on thoracic duct lymph of the injected animals are shown in Table 1. The thoracic duct lymphs of the saline-injected rats contained an average of 93.7 million cells. In contrast the lymphs of the donors treated with normal rabbit or antilymphocytic serum possessed 100.0 and 35.7 million cells, respectively. The diminution in absolute number of thoracic duct lymphocytes in the rats treated with antilymphocytic serum represented primarily a decrease in small lymphocytes, although the number of large and medium cells was also reduced. The slight but consistently observed increase in the two categories of cells in animals treated with normal rabbit serum most likely reflected the immune response to this serum.

Results of the normal lymphocyte transfer experiments revealed that lymph node cells from the animals treated with antilymphocytic serum did not produce detectable normal lymphocyte transfer reactions. In contrast, cells from the rats injected with saline or normal rabbit serum produced lesions measuring 7 to 9 mm and 8 to 10 mm, respectively. It is unlikely that the failure of cells from the donors treated with antilymphocytic serum to produce a reaction was due to the injection of an insufficient number of cells. Evidence supporting this view may be found in the following two observations: (i) Ten times the number of cells necessary to produce a demonstrable lesion were routinely employed; and (ii) sizable lesions were seen with an equivalent number of cells from donors treated with saline or normal rabbit serum.

Figure 1 demonstrates the results observed in the cultures. As may be seen: (i) The mixed cultures containing the cells from donors treated with antilymphocytic serum transformed five to eight times less well than comparable saline or normal rabbit serum × F_1 control mixtures, but 40 to 50 percent more than nonmixed cultures; (ii) the transformation of antilymphocytic serum cells induced by pokeweed mitogen was significantly reduced as compared to saline or normal rabbit serum controls; and (iii) the cultures containing normal rabbit serum evidenced greater degrees of transformation than did the cultures of cells from saline-treated donors. In addition, only rarely was a typical blast cell observed on smears from antilymphocytic serum × F_1 cultures. In contrast, large numbers of these cells were observed in either saline or normal rabbit serum × F_1 incubations. These results suggest that small lymphocytes

from donors treated with antilymphocytic serum have a diminished capacity to transform in response to blastogenic stimuli in general, and especially to foreign lymphoid cells. That this failure was not due to a diminished number of small lymphocytes was supported by the observation that when the number of small lymphocytes in the mixed cultures from donors treated with antilymphocytic serum was doubled, the H^3-thymidine incorporation increased an equal amount. This increase still represented only one-half to one-third the incorporation observed in saline × F_1 (Lew × BN) hybrid mixtures containing the usual number of small lymphocytes. Since the number of small lymphocytes from donors treated with antilymphocytic serum in these special cases exceeded by 80 percent the number in cultures with cells from saline-treated donors, the lack of an equivalent amount or more of H^3-thymidine uptake cannot be ascribed to a simple absence of small lymphocytes. More reasonably, it may have reflected a population of small lymphocytes, the majority of which were incapable of responding to foreign cells. Alternatively, it may have represented a reduced proliferation of the transformed cells after enlargement. From the findings of these studies, it may be suggested that the in vitro mixed lymphocyte reaction can monitor the in vivo effectiveness of antilymphocytic serum in prolonging grafts. Further evidence supporting this concept may be found in the observation that the mixed lymphocyte reaction correlates with the normal lymphocyte transfer reaction which, in turn, reflects the ability of the donor animal to reject foreign grafts (*6*). The test is also technically simple to initiate and, in addition to being sensitive, it is, within limits, reproducible and quantifiable. Moreover, because blood lymphocytes would undoubtedly demonstrate the same phenomenon, the few cells required could be obtained without extensively manipulating the donor animals. From other studies (*8*) it is apparent that 8 days after seven daily injections of antilymphocytic serum, the treated animal demonstrated some recovery from the effect of this serum as monitored by the mixed lymphocyte reaction. By 6 weeks after treatment, this reaction has returned to approximately 50 to 80 percent of normal. In addition, the transformation of thymus cells did not appear to be affected by treatment with antilymphocytic serum. This latter observation may explain the delayed immunological recovery of thymectomized animals after treatment with this serum (*9*).

The mode of action of antilymphocytic serum remains speculative, although a number of overlapping theories have been proposed (*10*). One theory suggests that this serum destroys a selective population of cells which normally are responsible for graft rejection. While the present study does not relate directly to this theory, it does suggest further studies that may be fruitful. This follows since it is known that the majority of small lymphocytes in the thoracic duct of the rat are long-lived with respect to circulating life span (*11*). Moreover, it has been reported that both short- and long-lived small lymphocytes transform in the mixed lymphocyte reaction (*12*). Since both this reaction and the thoracic duct content of small lymphocytes were depressed by antilymphocytic serum, it may be suggested that at least the long-lived cells are affected by this serum. Further studies are needed to determine whether this is the case.

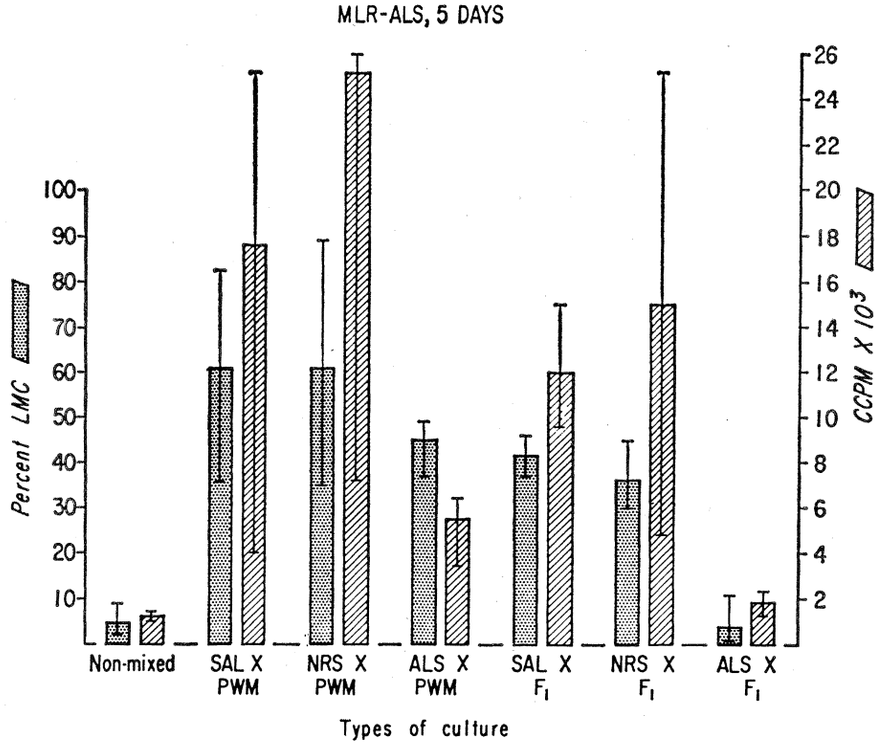

Fig. 1. Results of the mixed lymphocyte reaction involving thoracic duct lymphocyte, using as assays for transformation the uptake of H^3-thymidine and the percentage of cells which, after 3 days, had nuclear diameters greater than 7 μ (large and medium cells, LMC). The types of cultures are indicated on the horizontal axis and the narrow bars represent the range of values observed. Note the suppression of the ALS × F_1 mixed reaction to the levels slightly above nonmixed controls. Also note that when cells from ALS-treated donors were exposed to pokeweed mitogen (PWM), they evidenced a smaller amount of transformation than did cells from donors treated with saline or normal rabbit serum. *SAL*, saline-injected donor; *NRS*, normal-rabbit-serum-injected donor; *ALS*, antilymphocytic-serum-injected donor; *PWM*, pokeweed mitogen; F_1, F_1 (Lewis × Brown Norway) hybrid donors; *CCPM* × 10^3, thousands of corrected counts (for background) per minute.

M. Roy Schwarz
R. W. Tyler
N. B. Everett
Department of Biological Structure, University of Washington, Seattle

References and Notes

1. M. F. A. Woodruff and N. A. Anderson, *Nature* **200**, 702 (1963); *Ann. N.Y. Acad. Sci.* **120**, 119 (1964); J. G. Gray, A. P. Monaco, P. S. Russell, *Surg. Forum* **15**, 142 (1964).
2. R. H. Levey and P. B. Medawar, *Ann. N.Y. Acad. Sci.* **129**, 164 (1966); J. L. Boak, M. Fox, R. E. Wilson, *Lancet* **1967-I**, 750 (1967).
3. W. L. Ford, *Brit. J. Exp. Pathol.* **48**, 335 (1967).
4. W. B. Chew and J. S. Lawrence, *Immunology* **33**, 271 (1937); J. H. Sacks, D. R. Filippone, D. M. Hume, *Transplantation* **2**, 60 (1964); H. Nagaya and H. O. Sieker, *Proc. Soc. Exp. Biol. Med.* **121**, 722 (1966).
5. R. H. Levey and P. B. Medawar, *Proc. Nat. Acad. Sci. U.S.* **56**, 1130 (1966); H. M. Azaba *et al.*, *Transplantation* **4**, 618 (1966); T. E. Starzl, T. L. Marchioro, K. A. Porter, Y. Iwasaki, G. L. Cerilli, *Surg. Gynecol. Obstet.* **124**, 301 (1967).
6. M. R. Schwarz, *Am. J. Anat.* **121**, 559 (1967); *J. Exp. Med.*, in press.
7. W. O. Reinhardt and C. H. Li, *Proc. Soc. Exp. Biol. Med.* **58**, 321 (1945).

8. Details in preparation.
9. H. F. Jeejeebhoy, *Immunology* **9**, 417 (1965); A. P. Monaco, M. L. Wood, P. S. Russell, *Science* **149**, 432 (1965).
10. K. James, *Clin. Exp. Immunol.* **2**, 615 (1967).
→ N. B. Everett, R. W. Caffrey, W. O. Rieke, *Ann. N.Y. Acad. Sci.* **113**, 887 (1964).
12. J. Clancy and W. O. Rieke, paper presented at Third Leucocyte Culture Conference, University of Iowa, November 1967.
13. Supported in part by PHS grant No. AI-07509 and in part by PHS grant No. GM-06309. We thank Miss Diana Carolin and Miss Ruth Knoph for their superior technical assistance.

1 April 1969

Neurohumoral Interaction in the Rat Amygdala after Central Chemical Stimulation

Abstract. *The interactions of chemically induced thirst and hunger with deprivation-induced hunger and thirst, respectively, were studied in the amygdala. The results suggest direct neurohumoral blocking at this locus, rather than mediation through activated circuits.*

The rat's amygdala shows a functional characteristic that makes it a particularly appropriate locus for investigation of the interaction of hunger and thirst by central chemical stimulation. Neither adrenergic nor cholinergic stimulation of the amygdala has significant effects on intake of food or water, respectively, in satiated animals (*1*). In suitably deprived animals, however, adrenergic stimulation greatly augments food intake and reduces water intake; and cholinergic stimulation greatly augments water intake and reduces food intake. Thus the amygdaloid "thirst" and "hunger" circuits in the rat display a modulating, but not initiating, function. They are apparently not functional unless the animal has been appropriately deprived for a sufficiently long time.

For this reason we chose the amygdala as the locus of stimulation in this investigative study of the interactions of adrenergically induced "hunger" with deprivation-induced thirst, and of cholinergically induced "thirst" with deprivation-induced hunger. Grossman (*2*, *3*) originally hypothesised a direct neurohumoral blocking effect in at least partial explanation of the depression of consummatory behavior (drinking, for example) by some other, chemically enhanced drive (adrenergic "hunger," for example). However, on the basis of his findings in the septal area (*4*), he later proposed the alternative hypothesis that the observed depression of consummatory behavior was mediated by inhibitory circuits activated, if indirectly, by the chemical stimulation. As a test of this hypothesis we predicted a greater depression of consummatory behavior after amygdaloid chemical stimulation in deprived rats (that is, where the amygdaloid circuits have been sensitized by deprivation) than in satiated rats (that is, where the amygdaloid circuits are apparently not responsive to chemical stimulation).

Of the two experiments that tested the hypothesis, one failed to establish the predicted increase in adrenergic depression of drinking in food-deprived rats over that in food-satiated rats. Thus we felt that our results gave more, if limited, support to Grossman's original hypothesis of a direct neurohumoral blocking effect at the locus of stimulation. The second experiment failed to replicate the cholinergic depression of eating behavior and thus did not contribute to decision between the two hypotheses.

Eleven male albino rats (Sydney University Wistar strain; 70 to 120 days old at the time of operation) were cannulated during the experiments. Eight were used at any one time; some replacements were necessitated by sickness or death.

Throughout each experiment the subjects were kept singly in test cages designed to permit accurate measurement of intake of food and water. Availability of water was automatically controlled by an electric motor; food supply was manually controlled. Cannulas were made from modified gauge-19 needle tubing, cut to the appropriate length, and sealed at the end with silicone rubber that permitted repeated injections but acted as a barrier to external infection. Injection needles were modified gauge-26 hypodermic needles attached by polyethylene tubing to an Agla micrometer syringe. The cannulas were implanted bilaterally by means of a stereotaxic device; the intended locus was the amygdaloid cortical nucleus. A subject was allowed to recuperate for at least 7 days after the operation before it was placed in the experimental squad.

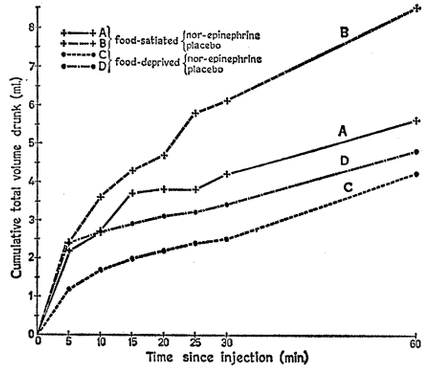

Fig. 1. Amounts of water drunk after injection, versus time; four different conditions of treatment (*A*–*D*).

For study of the interaction of chemically induced hunger and deprivation-induced thirst, all subjects were deprived of water for 3 hours, and half were food-satiated and half were food-deprived for 3 hours. They were injected with either norepinephrine ($648 \times 10^{-4}M$, made isotonic to 0.9 percent saline by addition of NaCl) or placebo (0.9 percent saline) (*5*). Orders of treatment were counterbalanced over subjects, allowing each subject to be used as its own control. Subjects had 1-hour access to water but no access to food. This procedure avoided possible confounding of a behavioral interaction with the predicted effect, a procedural error that may have occurred in Grossman's two later studies (*1*, *4*) in both of which the subject had only a limited time in which to satisfy both hunger and thirst, while one of these conditions was enhanced by chemical stimulation.

The results appear in Table 1. In addition to recording the total volume drunk during the 60-minute test period, we made cumulative measurements of water intake at 5-minute intervals for 30 minutes after injection; these time curves for water intake are graphed in Fig. 1. Analysis of these results, with *t*-tests (*6*), showed significant differences in mean water intake (i) between food-deprived and food-satiated subjects —to be expected; and (ii) between adrenergically stimulated and placebo-injected subjects [t (7) = 3.37, $P < .02$], so that Grossman's finding is confirmed (*1*). However, no significant difference was found in adrenergic depression of water intake between food-satiated and food-deprived subjects.

The second experiment employed the converse procedure, involving cholinergic stimulation with all subjects de-