different from that of denitrification. Much remains to be understood about microbial N_2O production and consumption in soils and in freshwater and marine environments before a cause-and-effect relationship can be established between man's activities and perturbation of the earth's stratospheric chemical reactions.

MARY K. FIRESTONE*
Department of Crop and Soil Sciences, Michigan State University, East Lansing 48824
RICHARD B. FIRESTONE†
Heavy Ion Laboratory, Michigan State University
JAMES M. TIEDJE
Departments of Crop and Soil Sciences and *Microbiology, Michigan State University*

References and Notes

1. P. J. Crutzen, *Q. J. R. Meteorol. Soc.* **96**, 320 (1971); H. Johnston, *Science* **173**, 517 (1971).
2. M. B. McElroy, S. C. Wofsy, Y. L. Yung, *Philos. Trans. R. Soc. London Ser. B* **277**, 159 (1977); P. J. Crutzen and D. H. Erhalt, *Ambio* **6**, 112 (1977).
3. W. C. Wang, Y. L. Yung, A. A. Lacis, T. Mo, J. E. Hansen, *Science* **194**, 685 (1976).
4. P. F. Pratt *et al.*, *Climatic Change* **1**, 109 (1977).
5. J. M. Bremner and A. M. Blackmer, *Science* **199**, 295 (1978); J. W. Elkins, S. C. Wofsy, M. B. McElroy, C. E. Kolb, W. A. Kaplan, *Nature (London)* **275**, 602 (1978).
6. T. Yoshida and M. Alexander, *Soil Sci. Soc. Am. Proc.* **34**, 880 (1970); J.-M. Bollag and G. Tung, *Soil Biol. Biochem.* **4**, 271 (1972); J. M. Tiedje, N. V. Caskey, M. S. Smith, B. H. Bleakley, R. B. Firestone, American Society of Agronomy Meetings, Fort Collins (1979), abstracts, p. 165.
7. J. M. Tiedje, R. B. Firestone, M. K. Firestone, M. R. Betlach, M. S. Smith, W. H. Caskey, *Soil Sci. Soc. Am. J.* **43**, 709 (1979).
8. T. Yoshinari, R. Hynes, R. Knowles, *Soil Biol. Biochem.* **9**, 177 (1977); W. L. Balderston, B. Sherr, W. J. Payne, *Appl. Environ. Microbiol.* **31**, 504 (1976).
9. M. S. Smith, M. K. Firestone, J. M. Tiedje, *Soil Sci. Soc. Am. J.* **42**, 611 (1978).
10. M. K. Firestone, R. B. Firestone, M. S. Smith, J. M. Tiedje, *ibid.* **43**, 1140 (1979); M. K. Firestone and J. M. Tiedje, *Appl. Environ. Microbiol.* **37**, 673 (1979).
11. Earlier work with a culture of *Pseudomonas aeruginosa* indicated that N_2O was a free intermediate in that organism [R. T. St. John and T. C. Hollocher, *J. Biol. Chem.* **252**, 212 (1977)]. We attempted to determine if this result could be extrapolated to the naturally occurring microflora of soil.
12. About 1 mCi of ^{13}N substrate (without added carrier) was added to soil slurries (50 g of soil + 40 ml of H_2O) contained in sealed 125-ml Erlenmeyer flasks, which had been anaerobic for 48 hours to deplete the indigenous soil NO_3^-. After the desired time of incubation on a rotary shaker, the headspace gas was sampled and analyzed with a gas chromatograph-proportional counter system (*7*).
13. The ^{13}N substrate plus carrier KNO_3 or KNO_2 was added to soil slurries (75 g of soil + 50 ml of H_2O), which were incubated in flasks on magnetic stirrers. Helium bubbling through the slurries at approximately 100 ml/min continuously stripped the product gases and transported the N_2O and N_2 to a differential trapping system for separation and quantification of $[^{13}N]N_2O$ and $[^{13}N]N_2$ (*7*, *10*). When no carrier NO_3^- or NO_2^- was added, the substrate consisted of about 68 fg of $[^{13}N]NO_3^-/NO_2^-$-N, plus any contaminating NO_3^-/NO_2^- in the preincubated soils or water (approximated as 0 ppm in Fig. 1, B and C).
14. A. M. Blackmer and J. M. Bremner, *Soil Biol. Biochem.* **10**, 187 (1978).
15. This has been reported numerous times in the literature. Two of the earliest reports are as follows: J. Wijler and C. C. Delwiche, *Plant Soil* **5**, 155 (1954); H. Nommik, *Acta Agric. Scand.* **6**, 195 (1956).
16. The *p*H of the Brookston soil was lowered by the addition of 1.0*N* HCl 3 to 4 hours before the experiment. Nitrate carrier was added with the ^{13}N substrate to two soil slurries of different *p*H, and only ^{13}N substrate (without added carrier) was added to a duplicate set of flasks. A 48-hour anaerobic preincubation had reduced the indigenous soil NO_3^- and NO_2^- to a concentration below that detectable by colorimetric procedures (below 0.5 ppm NO_3^--N). the gas sparging apparatus was used for incubation and gas analysis (*7*, *13*).
17. D. D. Focht, *Soil Sci.* **118**, 173 (1974).
18. The experimental procedure was similar to that reported for the label exchange experiments except that O_2 rather than N_2O was added to the headspace of the slurry (*12*).
19. It is well known that O_2 inhibits denitrification [W. J. Payne, *Bacteriol. Rev.* **37**, 409 (1973)].
20. Triplicate determinations of quantities and rates of N_2O production were made for approximately 50 hours after anaerobic conditions were imposed on soil slurries (50 g of soil + 50 ml of H_2O) contained in sealed 125-ml Erlenmeyer flasks in the presence or absence of 7 ml of acetylene. For the study shown in Fig. 1F, 100 ppm of NO_3^--N was added at 0 hours; other studies in which NO_3^- was sequentially added showed that the temporal pattern observed was independent of NO_3^- concentration (*10*).
21. M. S. Smith and J. M. Tiedje, *Soil Biol. Biochem.* **11**, 261 (1979).
22. J. Sørensen, J. M. Tiedje, R. B. Firestone, *Appl. Environ. Microbiol.* **39**, 105 (1980).
23. We thank M. S. Smith and M. R. Betlach for helpful discussions and technical assistance. This work was supported by NSF grants DEB-77-19273 and PHY-78-01684 and the Department of Agriculture under Regional Project NE-39. Journal article 8808 of the Michigan Agricultural Experimental Station.

* Present address: Department of Soils and Plant Nutrition, University of California, Berkeley 94720.
† Present address: Lawrence Radiation Laboratory, University of California, Berkeley 94720.

22 October 1979; revised 21 February 1980

Impaired Brain Growth in Neonatal Rats Exposed to Ethanol

Abstract. *Infant rat pups, fed through intragastric cannulas from postnatal day 4 through day 18, showed a 19 percent reduction in total brain weight when ethanol was included in their diet on days 4 through 7. This reduction in brain weight occurred even though body growth in the experimental rats was equal to that of their littermate controls. The ethanol-exposed animals were markedly hypoactive during the period of drug administration, then displayed gross body tremors for 3 to 5 days. Throughout the study, the animals treated with ethanol had poor motor coordination and were hyperresponsive. These brain and behavioral effects appear similar to those seen in fetal alcohol syndrome.*

Consumption of alcoholic beverages by women during pregnancy has been widely described as a significant threat to normal fetal development, and the constellation of anomalies in infants born to women who have done so has been labeled fetal alcohol syndrome (FAS). Three major signs are considered necessary for the diagnosis of FAS: (i) central nervous system (CNS) dysfunction, (ii) growth deficiencies, and (iii) a specific facial dysmorphology (*1*). In children with FAS, the CNS dysfunction may be a result of microcephaly and abnormal brain development (*2*). It is not yet clear how much and how often ethanol must be consumed by pregnant women to cause impaired brain development in their children.

In the course of development, the brain goes through several periods of rapid growth during which it is extremely vulnerable to exogenous insults (*3*). In humans, the period of rapid development known as the brain growth spurt begins at mid-gestation, peaks in the third trimester, and ends by the third postnatal year (*3*). Although this period occurs in all mammals, its timing relative to birth varies among species (*4*); in the rat, the brain growth spurt occurs during the first 15 days after birth, with a peak at postnatal days 6 to 8. This variable timing poses a considerable problem if the rat is used to model the effects of ethanol on the brain development of offspring of human mothers. Also, ethanol administered directly to rat pups may interfere with their ability to feed properly, resulting in a nutritional deficiency. Alternatively, if ethanol is administered to the mother and subsequently to the pups through her milk, two additional experimental complications can occur. First, the amount of ethanol each pup receives daily could be variable, and second, ethanol could interfere with lactation (*5*). In the present study, we used an artificial rearing procedure in which the neonate is provided the required total daily nutrition independent of the mother (*6–8*). This method of artificial rearing is an excellent means of examining accurately the effects of ethanol on brain development of animals at times comparable to those at which human fetuses might be exposed.

Adult female rats (Long-Evans) were individually housed with adult males and checked daily for the appearance of copulatory plugs. The day of plug appearance was designated as gestational day 0 (GD 0). (All further age references are based on this day.) The females were then individually housed in breeding cages and given free access to food and water for the remainder of their pregnancy. At parturition, the eight largest pups were culled from each litter. Litters of less than eight pups were not used. On

0036-8075/80/0516-0751$00.50/0

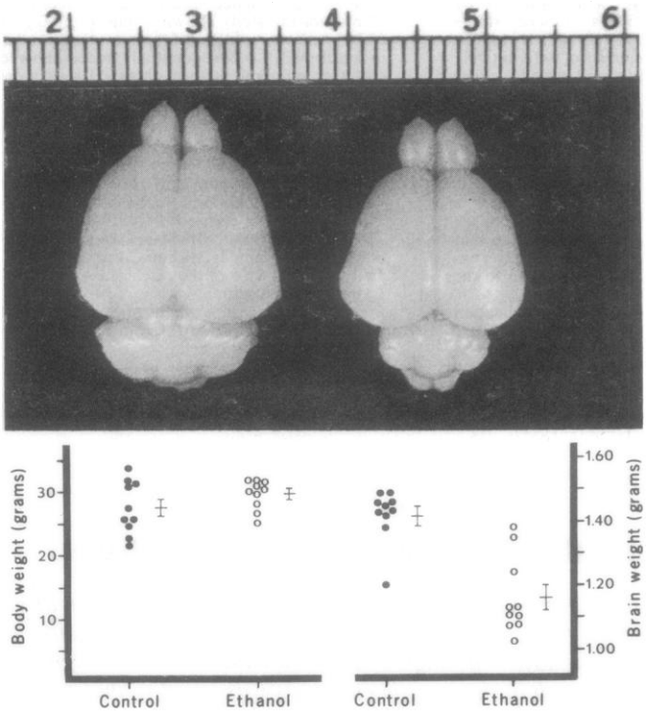

Fig. 1. (A) Representative brains from two 18-day-old rat pups of comparable body weight. The brain at left was removed from a control pup; the brain at right, from an ethanol-exposed pup. (B) Body and brain weights for 18-day-old control and ethanol-exposed pups. The mean and the standard error is indicated by bars for each data set.

GD 26 (approximately postnatal day 4), the pups were lightly anesthetized with halothane and implanted with intragastric cannulas (*6–8*). Each pup was then placed in a plastic cup (11 cm in diameter, 7.5 cm deep) floating in a water bath (40°C). The cannulas were connected to syringes (10 cm^3) that were filled with milk formula and mounted on infusion pumps (*9*). The infusion pumps were programmed to infuse the milk for 20 minutes every 2 hours, 24 hours a day. After the 12th infusion each day, the pumps were disconnected, the syringes washed and refilled, and the cannulas flushed with demineralized water. In addition, each animal's cannula assembly was checked and adjusted as necessary to accommodate the animal's growth. On GD 26 through 29, half the littermates were infused with an ethanol-milk formula [3 percent ethanol (by volume) on the first day, 5 percent on the second, 1 percent on third, and 5 percent again on the fourth (*10*)]. The other half received only the milk formula. In order to determine blood ethanol levels, a 10-μl blood sample was taken from the tip of the tail after the second day of ethanol administration (*11*).

After the 4-day exposure to ethanol, all the rats were infused with only the milk formula for the rest of the study. Beginning on GD 27, we gave the animals a battery of reflex tests: righting, negative geotaxis to a 30° slope, free-fall righting, and cliff avoidance (*12*). On GD 40 the animals were killed and, after cardiac perfusion with buffered Formalin, their brains were removed. The brains were stored in buffered Formalin for 5 days before being weighed on an analytical balance. Size measurements with a vernier caliper (accuracy, 0.05 mm) were made of total anterior-posterior length (from the posterior cerebellum to the frontal lobes), maximum cerebral width, anterior-posterior cerebral hemisphere length, cerebellar width, and vermis length. Brain volume was measured by liquid displacement in a 25-ml graduated cylinder (accuracy, ± 0.1 ml).

Three litters (a total of 24 animals) were used in three experiments, with one litter per experiment. During the experiments, four animals died (two controls and two experimentals), primarily as a result of unsuccessful attempts to replace pulled cannulas.

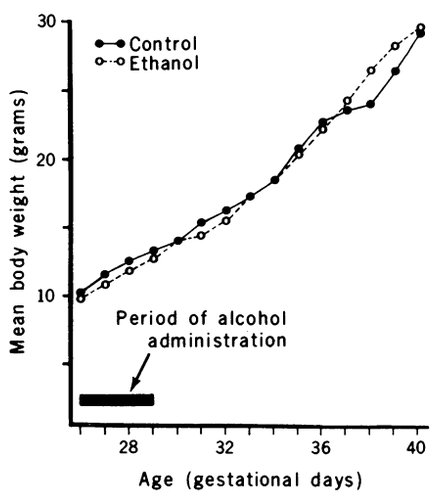

Fig. 2. Daily mean body weights for the ethanol-exposed and control groups.

Total weight of brains from the ethanol-exposed rats was 19 percent less than that of the control brains ($P < .01$) (Fig. 1). Also, the volume displaced by the brains of the experimental animals was 31 percent less than that displaced by the control brains ($P < .001$). These deficits were reflected in the size measurements, with the ethanol-exposed animals having smaller cerebellums (15 percent narrower, $P < .001$; vermes were 13 percent shorter, $P < .01$) and smaller cerebral hemispheres (6 percent shorter, $P < .05$) than the controls.

Daily body weights of the experimental rats did not differ significantly from those of the controls during the study (Fig. 2). Blood ethanol concentration after the first 2 days of ethanol administration ranged from 171.5 to 278.6 mg/dl (mean, 221.1 ± 36.64).

There were striking behavioral differences between the two groups. During ethanol administration [particularly on the days following 5 percent ethanol infusion (GD 27 and 29)], the experimental animals appeared heavily sedated, performing the reflex tests with great difficulty or not at all. For example, on the righting task, all control animals righted themselves, whereas only 25 percent of the ethanol-administered animals were able to do so, and even then they took significantly longer than the controls (mean time for experimentals, 13.9 seconds; for controls, 2.1 seconds; $P < .01$). Impaired performance on all the reflex tests persisted for several days after exposure to ethanol, but by GD 39 there were no differences between the experimental and control groups in the righting and cliff-avoidance tasks; however, the former were still significantly slower at performing negative geotaxis ($P < .05$), and fewer righted themselves during free fall (three of ten experimentals compared with seven of ten controls). Of the various reflex tests, these last two require the greatest motor coordination, indicating a possible motoric deficit in the ethanol-exposed animals.

Immediately after the end of ethanol administration, the pups experienced continuous body tremors that at times were quite severe and that occurred not only during the daily testing but also when the animals were in the water bath. This period of tremors lasted 3 to 5 days after discontinuation of ethanol administration. Similar tremors during the first few days of life have been reported for human infants born to alcoholic mothers (*13*). Even after the tremors ceased, the animals remained hyperresponsive to innocuous stimuli (for example, the pups squeaked when touched lightly). Further, they appeared uncoordinated

in their movements—especially movements involving the hind limbs—throughout the study.

Whereas the administration of ethanol for only 4 days during the brain growth spurt did not interfere with body growth, it had a marked effect on brain growth, particularly that of the cerebellum. The cerebellum grows at a faster rate than the rest of the brain during the spurt; cerebellar development consequently tends to be particularly vulnerable to insults during this period (*14*). [Our results confirm those of another experiment, in which ethanol was administered to rats by inhalation throughout the preweaning period (*15*).] However, the fact that ethanol administration produces substantial growth deficits in other brain areas during the brain growth spurt should not be ignored. This is especially important when accounting for the observed behavioral deficits.

Whether or not the developing CNS would compensate, given time, for the brain growth and behavioral deficits seen by GD 40 remains to be determined. Although there was gradual improvement of motor coordination in the experimental animals, the hyperreactivity showed no indication of change.

Blood ethanol levels induced in these rats were high, but still within the range seen in human female chronic alcoholics (*16*). Since alcohol passes the placenta easily, and since fetal blood ethanol levels approximate those of the mother (*17*), it is likely that the fetus of a drinking mother would experience these levels of exposure.

It is clear that while ethanol may act as a teratogen early in an organism's development (*18*), there are critical periods later in development that may also be vulnerable to the influence of ethanol. Although it is difficult to make direct comparisons between species, the present data suggest that ethanol could have a toxic effect on the development of the human CNS relatively late in gestation, particularly during the third trimester. In fact, clinical studies have reported that when mothers who are heavy drinkers abstain or reduce their alcohol intake during the third trimester, fewer abnormalities occur in their offspring than in those of mothers who continue heavy alcohol consumption (*19*). The present study has added to the evidence by demonstrating that ethanol exposure relatively late in development can cause microcephaly, one of the three major components of FAS.

JAIME DIAZ
HERMAN H. SAMSON
Department of Psychology, University of Washington, Seattle 98195

References and Notes

1. C. Ulleland, R. P. Wennberg, R. P. Igo, N. J. Smith, *Pediatr. Res.* **4**, 474 (1970); K. L. Jones and D. W. Smith, *Lancet* **1973-II**, 999 (1973); ———, J. W. Hanson, *Ann. N.Y. Acad. Sci.* **273**, 130 (1976); L. Erb and B. D. Andresen, *Clin. Pediatr. (Philadelphia)* **17**, 644 (1978); S. Clarren and D. Smith, *N. Engl. J. Med.* **298**, 1063 (1978).
2. K. L. Jones, D. W. Smith, A. P. Streissguth, N. C. Myrianthopoulous, *Lancet* **1974-I**, 1267 (1974); A. P. Streissguth, *Ann. N.Y. Acad. Sci.* **273**, 140 (1976); ———, C. S. Herman, D. W. Smith, *Pediatrics* **92**, 363 (1978); S. K. Clarren, E. C. Alvord, Jr., S. M. Sumi, A. P. Streissguth, D. W. Smith, *ibid.*, p. 64.
3. J. Dobbing and J. Sands, *Arch. Dis. Child.* **48**, 757 (1973); J. Dobbing, in *Scientific Foundations of Paediatrics*, J. Davis and J. Dobbing, Eds. (Heinemann, London, 1974), p. 565.
4. A. Davison, in *Biochemical Correlates of Brain Structure and Function*, A. Davison, Ed. (Academic Press, New York, 1977); J. Dobbing, in *Applied Neurochemistry*, A. Davison and J. Dobbing, Eds. (Blackwell, Oxford, 1968), p. 287.
5. A. R. Fuchs, *Acta Endocrinol. (Copenhagen)* **62**, 546 (1969); E. Cobo, *Am. J. Obstet. Gynecol.* **107**, 1195 (1970).
6. W. Hall, *Science* **190**, 1313 (1975).
7. M. Messer, E. Thoman, A. Teresa, P. Dallman, *J. Nutr.* **98**, 404 (1969).
8. J. Diaz and R. Schain, *Biol. Neonate* **32**, 77 (1977).
9. The milk formula was basically the same as the one used by Hall (*6*) and Messer *et al.* (*7*).
10. The 3 percent formula given on the first day provided a mean total (for 24 hours) of 4.8 g of ethanol per kilogram of body weight; the 5 percent formula (day 2), 7.2 g; the 1 percent formula (day 3), 2.0 g; and the 5 percent formula (day 4), 9.8 g. This graded dosage regimen minimizes death from overdose.
11. N. G. Brink, R. Bonnichsen, H. Theorell, *Acta Pharmacol. Toxicol.* **10**, 223 (1954).
12. W. Fox, *Anim. Behav.* **13**, 234 (1965); J. Smart and J. Dobbing, *Brain Res.* **28**, 85 (1971).
13. O. Schaefer, *Can. Med. Assoc. J.* **87**, 1333 (1962); M. M. Nichols, *Am. J. Dis. Child.* **113**, 714 (1967); S. Pierog, O. Chandavasu, I. Wexler, *Pediatrics* **90**, 630 (1977); E. M. Ouellette, H. L. Rosett, N. P. Rosman, L. Weiner, *N. Engl. J. Med.* **297**, 528 (1977); S. Landesman-Dwyer, L. S. Keller, A. P. Streissguth, *Alcohol. Clin. Exp. Res.* **2**, 171 (1978).
14. J. Dobbing, *Pediatrics* **53**, 2 (1974); ———, J. Hopewell, A. Lynch, *Exp. Neurol.* **32**, 439 (1971).
15. C. Bauer-Moffet and J. Altman, *Exp. Neurol.* **48**, 378 (1975); *Brain Res.* **119**, 249 (1977).
16. B. M. Jones and M. K. Jones, in *Alcoholism Problems in Women and Children*, M. Greenblatt and M. A. Schuckit, Eds. (Grune & Stratton, New York, 1976), p. 103; P. Dehaene, C. Samaille-Vellette, P.-P. Samaille, G. Crepin, R. Walbaum, P. Deroubaix, A.-P. Blanc-Garin, *Rev. Alcool.* **23**, 145 (1977).
17. M. Nicloux, *Obstetrique* **5**, 97 (1900); P. V. Dilts, Jr., *Am. J. Obstet. Gynecol.* **107**, 1195 (1970); Y. A. Kesaniemi and H. W. Sippel, *Acta Pharmacol. Toxicol.* **37**, 43 (1975); L. N. Cook, R. J. Short, B. F. Andrews, *Am. J. Dis. Child.* **129**, 1075 (1975).
18. G. F. Chernoff, *Teratology* **15**, 223 (1977); C. L. Randall, in *Alcohol and Opiates*, K. Blum, Ed. (Academic Press, New York, 1977), p. 91; K. L. Jones, D. W. Smith, C. N. Ulleland, A. P. Streissguth, *Lancet* **1973-I**, 1267 (1973); E. M. Ouellette, H. L. Rosett, N. P. Rosman, L. Weiner, *N. Engl. J. Med.* **297**, 528 (1977). For studies in which ethanol was not found to have teratogenic effects, see G. I. Henderson and S. Schenker [*Res. Commun. Chem. Pathol. Pharmacol.* **16**, 15 (1977)]; E. L. Abel [*Psychopharmacologia* **57**, 5 (1978)]; J. F. Oisund, A. E. Fjorden, and J. Morland [*Acta Pharmacol. Toxicol.* **43**, 145 (1978)]; B. A. Schwetz, F. A. Smith, and R. E. Staples [*Teratology* **18**, 385 (1978)]; and G. I. Henderson, A. M. Hoyumpa, Jr., C. McClain, and S. Schenker [*Alcohol. Clin. Exp. Res.* **3**, 99 (1979)].
19. H. Rosett, E. Ouellette, L. Weiner, E. Owens, *Obstet. Gynecol.* **51**, 41 (1978); *Food and Drug Administration Bulletin 7* (1977), p. 18.
20. We thank T. Roehrs, E. Robisch, C. Stamper, E. Moore, D. Waterman, and R. Kirby for their assistance. Supported by a grant from the Alcoholism and Drug Abuse Institute of the University of Washington.

19 November 1979; revised 29 January 1980

Flavor-Illness Aversions: The Peculiar Roles of Odor and Taste in Memory for Poison

Abstract. *When either taste or odor alone was followed by poison, rats acquired a strong aversion for the taste but not for odor, especially if poison was delayed. When odor-taste combinations were poisoned, however, odor aversions were potentiated, as if odor could gain the enduring memorial property of taste by associative contiguity.*

Strong associations are formed when consumption of a distinctive flavor is followed by visceral feedback on a single occasion. Flavor aversions have been acquired by human patients eating ice cream before undergoing chemotherapy, by wild predators eating the flesh of their prey tainted with toxic lithium, and by laboratory rats drinking saccharin water before being injected with noxious substances; flavor preferences have been acquired when saccharin water is followed by recuperation from illness or thiamine deficiency (*1*). This associative process resembles classical conditioning, with the flavor acting as the conditioned stimulus (CS) and the visceral feedback acting as the unconditioned stimulus (UCS); it differs in that the flavor CS is selected over other stimuli in the feeding situation and in that long CS-UCS intervals can be spanned with ease.

There are other differences. In classical conditioning, when strong and weak stimuli are combined into a compound CS, the strong component overshadows or blocks conditioning to the weak component. In flavor-visceral conditioning the opposite is true for laboratory rats; strong taste stimuli facilitate conditioning to odors which are weak signals for slow-acting poison when used alone (*2*). Wild predators show a similar effect. Coyotes that feed on the tainted flesh of their prey and become ill quickly learn to avoid live prey without biting, as if the odor were a sufficient signal for poison. Of course, the predators we tested had prior experience with prey, which suggests that prior associations of prey odor with prey taste might facilitate the potentiation of odor by taste at the time of poi-