components of complement [for example, C5a chemotactic factor (*16*)] may damage or otherwise interact with endothelial cells to induce intravascular thrombosis. We studied only endothelial cells of bovine pulmonary artery; it remains to be determined whether endothelial cells of other vascular beds or of other species do or do not possess receptors for C3b or the Fc portion of IgG. However, we believe that it is significant that Shin *et al.* (*17*), using human renal biopsy tissue, found that C3b-coated bacteria were bound by glomerular epithelial cells but not by endothelial cells.

Over the past 12 years it has become evident that endothelial cells have far more complex functions than previously envisioned. These cells, previously believed to be little more than a mechanical barrier between blood and parenchyma, possess peptidase enzymes, cyclooxygenase, phosphatase enzymes, protease inhibitors, hemostatic factors, and receptors for polypeptide hormones such as insulin and angiotensin II (*5, 6, 18*). Efforts to determine the subcellular distribution of these various components will be facilitated by the use of immunocytochemical techniques, which have already proved useful in locating angiotensin-converting enzyme (*5, 6*). We conclude that the absence of Fc receptors on pulmonary endothelial cells is of major practical importance. If, indeed, Fc receptors occurred in close juxtaposition to the components (antigens) of interest, one could not use specific IgG to locate them immunocytochemically without using Fab (antibody-binding fragments) or without adding rigorous controls to minimize nonspecific binding. Our results indicate that the binding of antibody to Fc receptors need not be a concern. Thus, it appears that the rate-limiting step in developing an improved understanding of the role of endothelium in hemostasis, the processing of hormones, and the transport of metabolites is that of obtaining the relevant monospecific antibodies.

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References and Notes

1. S. O. Freedman, *Clinical Immunology* (Harper & Row, New York, 1971).
2. J. W. Eagen, J. T. Borune, M. M. Schwartz, J. V. Jones, E. J. Lewis, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 1204 (1979).
3. W. E. Chapman and P. A. Ward, *ibid.*, p. 1204.
4. P. J. Lachmann and R. R. A. Coombs, in *Ciba Foundation Symposium: Complement*, G. E. W. Wolstenholme and J. Knight, Eds. (Little, Brown, Boston, Mass., 1965), pp. 242-280.
5. U. S. Ryan, E. Clements, D. Habliston, J. W. Ryan, *Tissue Cell* **10**, 535 (1978).
6. U. S. Ryan, J. W. Ryan, C. Whitaker, A. Chiu, *ibid.* **8**, 125 (1976).
7. U. S. Ryan, J. W. Ryan, A. Chiu, *Adv. Exp. Med. Biol.* **70**, 217 (1976).
8. J. W. Ryan, A. R. Day, U. S. Ryan, A. Chung, D. I. Marlborough, F. E. Dorer, *Tissue Cell* **8**, 111 (1976).
9. A. T. Chiu, J. W. Ryan, J. M. Stewart, F. E. Dorer, *Biochem. J.* **155**, 189 (1976).
10. J. W. Ryan, A. Chung, L. C. Martin, U. S. Ryan, *Tissue Cell* **10**, 555 (1978).
11. R. J. Winchester and G. Ross, in *Manual of Clinical Immunology*, N. R. Rose and H. Friedman, Eds. (American Society for Microbiology, Washington, D.C., 1976), pp. 64-76.
12. J. H. Hanks and R. E. Wallace, *Proc. Soc. Exp. Biol. Med.* **71**, 196 (1949).
13. R. A. Nelson, J. Jensen, I. Gigli, N. Tamura, *Immunochemistry* **3**, 111 (1966).
14. D. H. Vroon, D. R. Schultz, R. M. Zarco, *ibid.* **7**, 43 (1970).
15. M. C. Gelfand, M. L. Shin, R. B. Nagle, I. Green, M. M. Frank, *N. Engl. J. Med.* **295**, 10 (1976).
16. T. W. Muhlfelder, J. Niemetz, D. Kreutzer, D. Beebe, P. A. Ward, S. I. Rosenfeld, *J. Clin. Invest.* **63**, 147 (1979).
17. M. L. Shin, M. C. Gelfand, R. B. Nagle, J. R. Carlo, I. Green, M. M. Frank, *J. Immunol.* **118**, 869 (1979).
18. J. W. Ryan, U. S. Ryan, D. R. Schultz, C. Whitaker, A. Chung, F. E. Dorer, *Biochem. J.* **146**, 497 (1975); J. W. Ryan and U. Smith, *Trans. Assoc. Am. Physicians* **84**, 297 (1971); J. W. Ryan, U. Smith, R. S. Neimeyer, *Science* **176**, 64 (1972); J. W. Ryan, U. S. Ryan, D. Habliston, L. Martin, *Trans. Assoc. Am. Physicians* **91**, 343 (1978); B. B. Weksler, C. W. Ley, E. A. Jaffe, *J. Clin. Invest.* **62**, 923 (1978); C. G. Becker and P. C. Harpel, *J. Exp. Med.* **144**, 1 (1976); E. A. Jaffe, D. W. Hoyer, R. L. Nachman, *J. Clin. Invest.* **52**, 2757 (1973); L. W. Hoyer, R. P. De Los Santos, J. R. Hoyer, *ibid.*, p. 2737; E. A. Jaffe and D. F. Mosher, *J. Exp. Med.* **147**, 1779 (1978); A. S. Todd, *J. Pathol. Bact.* **78**, 281 (1959); D. J. Loskutoff and T. S. Edgington, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3903 (1977); V. Buonassisi and J. C. Venter, *ibid.* **73**, 1612 (1976); M. A. Gimbrone and R. W. Alexander, *Circulation* **56**, III-209 (1977); R. S. Bar, J. C. Hoak, M. L. Peacocck, *J. Clin. Endocrinol. Metab.* **47**, 699 (1978).
19. We thank C. Whitaker and P. Arnold for technical assistance. Supported by PHS grants HL 21568, HL 22896, HL 22087, the Council for Tobacco Research—U.S.A., Inc., and the John A. Hartford Foundation, Inc.

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Nitrous Oxide from Soil Denitrification: Factors Controlling Its Biological Production

Abstract. *Increasing concentrations of nitrate, nitrite, and molecular oxygen enhanced production of nitrous oxide relative to molecular nitrogen during denitrification in soils. Soil acidity interacted with nitrate to increase the ratio of nitrous oxide to molecular nitrogen. In response to anoxic conditions, nitrous oxide production initially increased but nitrous oxide was then consumed, a pattern which resulted from the sequential synthesis of nitrogenous oxide reductases.*

The role of nitrous oxide (N_2O) in stratospheric chemical reactions (*1*) has led to concern that increased terrestrial production of N_2O may cause depletion of the earth's ozone shield (*2*). Nitrous oxide may also contribute to a warming of the earth's surface by influencing the radiative budget of the troposphere (*3*). As the amounts of industrially or biologically fixed nitrogen used for crop production increase, the quantity of N_2O produced by microbial nitrogen metabolism in soil may increase (*4*).

Nitrous oxide is a product of denitrification, but it is also produced during nitrification (*5*) and during reduction of NO_3^- to NH_4^+ (*6*). Denitrification in soil produces both N_2O and N_2; hence this bacterial process may serve either as a major source of N_2O or as a sink for N_2O, through the reduction of N_2O to N_2. Little is known about the environmental parameters that control the relative production of N_2O and N_2 during biological denitrification.

Using soils and bacterial isolates from soils, we have attempted to identify factors that control the proportion of N_2O and N_2 produced by denitrification. To focus on the biological control of N_2O evolution, we used well-mixed soil slurries, thus minimizing the influence of diffusion through the soil matrix. To directly and sensitively quantify N_2O and N_2 production, we added to the soils $^{13}NO_3^-$ produced at the Michigan State University cyclotron (*7*). We also used the inhibition of N_2O reduction by acetylene (*8*) coupled with gas chromatographic analysis of the N_2O, using thermal conductivity or ^{63}Ni electron-capture detectors (*9*). The ratio of N_2O to $(N_2O + N_2)$ can be determined by quantifying the N_2O produced in the presence and absence of acetylene (*9*). The data that we report here summarize the results from a Brookston loam soil. Similar results from other soils and bacterial cultures are described elsewhere (*10*).

To determine if N_2O is a free intermediate during the reduction of NO_3^- to N_2 in the heterogeneous denitrifying microflora of soils (*11*), we added $^{13}NO_3^-$ to two soils and three bacterial cultures, thus labeling the N_2O produced during denitrification (*7, 10, 12*). The exchange of the $[^{13}N]N_2O$ with pools of added nonlabeled N_2O ($^{14}N_2O$) was quantified after a 10-minute incubation in the Brookston soil (Fig. 1A). The labeled N_2O mixed rapidly and quantitatively with the nonlabeled N_2O pool in the soils and soil isolates (*10*). This freedom of exchange indicates that operationally N_2O exists as a

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free intermediate able to diffuse away from the site of active reduction. Hence, any factor that produces a change in the relative rates of N_2O reduction and production can alter the proportion of N_2O and N_2.

By varying the amount of nonlabeled NO_3^- or NO_2^- added with the ^{13}N-labeled substrate (*13*), we found that as the concentration of NO_3^- or NO_2^- increased the proportion of product as N_2O increased (Fig. 1, B and C). The effect with NO_2^- was stronger than with NO_3^-; thus the influence of NO_3^- may have resulted from its reduction to and the subsequent effect of NO_2^-.

Recently Blackmer and Bremner suggested that soil acidity enhances the influence of NO_3^- on the composition of the gaseous products of denitrification (*14*). It has been observed that soils with an acid *p*H produce proportionately more N_2O than soils with a neutral or alkaline *p*H (*15*). But in the previously reported investigations relatively high concentrations of NO_3^- were required before the gaseous products could be quantitated. Thus, it had been impossible to isolate the effect of *p*H from the influence of NO_3^- concentration. Using the ^{13}N substrate, we have been able to investigate the influence of soil *p*H at extremely low NO_3^- concentrations (*16*). In the absence of measurable quantities of NO_3^-, soil acidity had very little influence on the N_2O/N_2 ratio (Fig. 1D). However, in the presence of 10 parts per million (ppm) of NO_3^--N, much more N_2O was produced at *p*H 4.9 than at *p*H 6.5. The influence of soil acidity appears to be exerted through or to be interactive with the effect of NO_3^- or NO_2^- concentration.

Analysis by a predictive kinetic model (*17*) has suggested that the aeration status of soil may also influence the composition of the gaseous products. In short-term experiments (10 minutes in duration), varying quantities of O_2 were added to vigorously agitated soil slurries (*18*). By analyzing the gases produced from the ^{13}N-labeled substrate, we found that the proportion of N_2O produced during denitrification increased with increasing O_2 concentrations (Fig. 1E). As expected (*19*), the denitrification activity declined sharply in the presence of increasing quantities of O_2 (*10*). Hence, O_2 influenced the distribution as well as the quantity of the gaseous products of denitrification.

Using the acetylene inhibition technique, we examined three mineral soils to determine if repeatable temporal patterns occurred in the composition of the gaseous products of denitrification after the onset of anaerobic conditions (*20*). A distinct pattern of gas production was found to result from changing activities within the reduction sequence after the imposition of anaerobiosis (Fig. 1F). During the early period of anaerobiosis (between 0 and 1 to 3 hours), N_2 was the dominant product of denitrification. The NO_3^- to N_2O reducing activity then increased but was not accompanied by a corresponding increase in the N_2O reducing activity. This resulted in a relatively extended period (between 1 to 3 hours and 16 to 33 hours) during which N_2O was an important or dominant product. Eventually (after 16 to 33 hours) an increase in the N_2O reducing activity occurred without a comparable increase in the N_2O producing activity. This increase in the rate of N_2O reduction did not occur in the presence of chloramphenicol (an inhibitor of protein synthesis) and required the presence of N_2O or NO_3^- during the preceding anaerobic incubation (*10*). During the final period (between 16 to 33 hours and 48 hours), N_2 was generally the sole product of denitrification, because the capacity of the soil to reduce N_2O exceeded the N_2O producing activity. This pattern was independent of NO_3^- concentration (*10*). Apparently the temporal pattern is a result of a sequential synthesis of denitrification enzymes which occurs in response to anoxic conditions.

The production of N_2O relative to N_2 during denitrification in soils is strongly influenced by the physical environment and by the physiological characteristics of the microbial community. Environmental parameters other than those discussed here, such as carbon availability (*21*), H_2S concentration (*22*), and soil structural characteristics, should also be expected to influence N_2O production or consumption, or both. Because of the extreme variability of the N_2O/N_2 ratio, valid estimates of total soil N_2O production cannot be obtained by assuming (*4*) that the N_2O component of denitrification is a constant fraction of the total gaseous product. It would also be expected that the control of N_2O production by other microbial processes (*5*, *6*) is quite

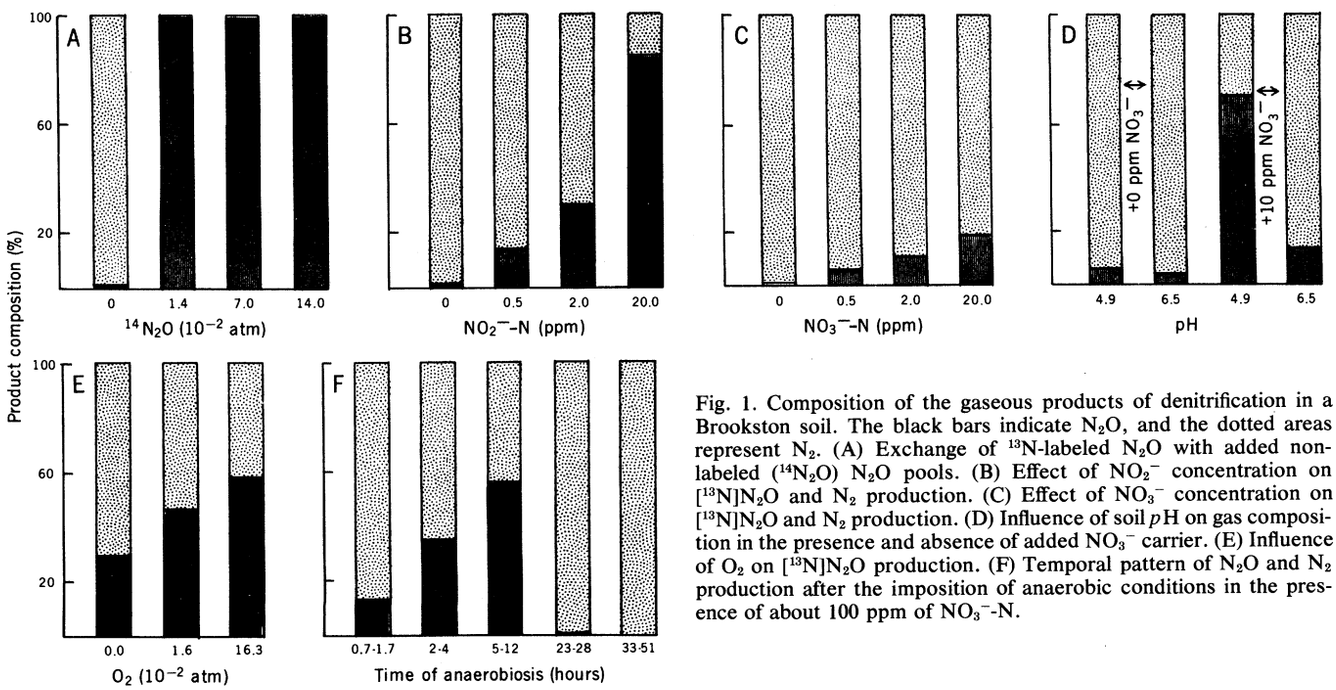

Fig. 1. Composition of the gaseous products of denitrification in a Brookston soil. The black bars indicate N_2O, and the dotted areas represent N_2. (A) Exchange of ^{13}N-labeled N_2O with added nonlabeled ($^{14}N_2O$) N_2O pools. (B) Effect of NO_2^- concentration on $[^{13}N]N_2O$ and N_2 production. (C) Effect of NO_3^- concentration on $[^{13}N]N_2O$ and N_2 production. (D) Influence of soil *p*H on gas composition in the presence and absence of added NO_3^- carrier. (E) Influence of O_2 on $[^{13}N]N_2O$ production. (F) Temporal pattern of N_2O and N_2 production after the imposition of anaerobic conditions in the presence of about 100 ppm of NO_3^--N.

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.

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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.

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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

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