nmol of trichloroacetic acid–precipitable, cell-associated ^{82}Br (mean ± SEM, *n* = 3).
25. S. H. Ingbar and K. Woeber, in *Harrison's Principles of Internal Medicine*, R. G. Petersdorf *et al.*, Eds. (McGraw-Hill, New York, 1983), pp. 614–633.
26. J. Geigert, S. L. Neidleman, D. J. Dalietos, *J. Biol. Chem.* **258**, 2273 (1983); R. P. Magnusson, A. Taurog, M. L. Dorris, *ibid.* **259**, 13783 (1984); B. W. Griffin, R. Haddox, *Arch. Biochem. Biophys.* **239**, 305 (1985).
27. M. G. Ch. Carlson, G. B. Peterson, P. Venge, *J. Immunol.* **134**, 1875 (1985).
28. A. J. Downs, C. J. Adams, in *Comprehensive Inorganic Chemistry*, J. C. Bailar, H. J. Emeleus, R. Nyholm, A. F. Trotman-Dickenson, Eds. (Pergamon, Oxford, 1973), pp. 1399–1412.
29. M. Nieder and L. Hager, *Arch. Biochem. Biophys.* **240**, 121 (1985).
30. J. R. Kanofsky, *J. Biol. Chem.* **258**, 5991 (1983); A. V. Khan, P. Gebauer, L. P. Hager, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5596 (1983); J. R. Kanofsky, J. Wright, G. E. Miles-Richardson, A. Tauber, *J. Clin. Invest.* **74**, 1489 (1984).
31. R. Theiler *et al.*, *Science* **202**, 1094 (1978); W. Fenical, *ibid.* **215**, 923 (1982); K.-H. Van Pee and F. Lingens, *J. Bacteriol.* **161**, 1171 (1985).
32. S. T. Test, M. B. Lampert, P. J. Ossanna, J. G. Thomas, S. J. Weiss, *J. Clin. Invest.* **74**, 1341 (1984).
33. M. Market, P. C. Andrews, B. M. Babior, *Methods Enzymol.* **105**, 358 (1984).
34. M. A. Vadas, J. R. David, A. Butterworth, N. T. Pisani, T. A. Siongok, *J. Immunol.* **122**, 1228 (1979).
35. E. L. Thomas, M. B. Grisham, M. M. Jefferson, *J. Clin. Invest.* 72, 441 (1983).
36. The incorporation of ^{82}Br into albumin is an underestimate of the total amount of HOBr generated. Bromination products of sulfhydryls, thioethers, or amines will not accumulate as stable adducts. For example, 25 nmol of $HO^{82}Br$ [generated by incubating 25 nmol of HOCl with an excess of $^{82}Br^-$; (*29*)] incorporated 4.6 ± 0.6 nmol of ^{82}Br into albumin (2 mg/ml; *n* = 5) under conditions identical to those used for the eosinophil.
37. We thank J. Gallin and C. J. White (National Institutes of Health) and G. Higashi (University of Michigan) for helpful discussions and J. Jones (Phoenix Laboratories, University of Michigan) for performing the bromide analyses. This work was supported by PHS grant R01 AI 21301 and by grant 900-512-057 from the Foundation for Medical Research (FUNGO) which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO).

27 May 1986; accepted 11 August 1986

Detoxification of Bacterial Lipopolysaccharides (Endotoxins) by a Human Neutrophil Enzyme

ROBERT S. MUNFORD AND CATHERINE L. HALL

Lipopolysaccharides in the cell walls of Gram-negative bacteria elicit toxic as well as potentially beneficial inflammatory responses in animals. It is now reported that tissue toxicity caused by lipopolysaccharides is preferentially reduced by an enzymatic activity in human neutrophils. Acyloxyacyl hydrolysis removes fatty acyl chains that are linked to the hydroxyl groups of 3-hydroxytetradecanoyl residues in the bioactive lipid A moiety of the lipopolysaccharides. Maximal acyloxyacyl hydrolysis reduced lipopolysaccharide tissue toxicity, as measured in the dermal Shwartzman reaction, by a factor of 100 or more. In contrast, the ability of the deacylated lipopolysaccharides to stimulate B lymphocytes to divide was decreased only by a factor of 12. It is suggested that during tissue invasion by Gram-negative bacteria acyloxyacyl hydrolysis may be a defense mechanism that reduces the toxicity of lipopolysaccharides while preserving some of their potentially beneficial inflammatory and immune stimuli.

ANIMALS MOUNT A COMPLEX ARRAY of inflammatory responses to tissue invasion by Gram-negative bacteria. Many of these responses are provoked by lipopolysaccharides (LPS) in the bacterial outer membrane. Much evidence suggests that the lipid A moiety of LPS stimulates various animal cells that, in turn, mediate the inflammatory changes (*1*). Some responses to LPS are toxic (hypotension, coagulation disturbances, death), while others may benefit the infected host (enhancement of antibody synthesis, mobilization of phagocytes, or acute-phase protein synthesis, for example). Although both humoral and cellular processes for detoxifying LPS have been proposed (*2*), no specific enzymatic mechanism has been described. We report here that partial deacylation of LPS by an enzymatic activity in human neutrophils greatly reduces the tissue toxicity of the molecules while preserving some of their immunostimulatory potency.

In LPS from enteric bacteria (such as *Salmonella* and *Escherichia*), lipid A is a glucosamine disaccharide that is phosphorylated at positions 1 and 4′ and has six or seven covalently linked fatty acids (Fig. 1). Four molecules of 3-hydroxytetradecanoate (3-OH-14:0) are attached to the glucosamine backbone at positions 2, 3, 2′, and 3′; the hydroxyl groups of the 3-OH-14:0 residues at positions 2′ and 3′ (and sometimes 2) are substituted with nonhydroxylated fatty acids (NFA) (dodecanoate, tetradecanoate, and hexadecanoate) to form acyloxyacyl groups (*3*). It was previously reported that human peripheral blood neutrophils have an enzymatic activity that selectively removes the nonhydroxylated acyl chains from the lipid A moiety of *Salmonella typhimurium* LPS (acyloxyacyl hydrolysis) (*4*). Partial purification of large amounts of enzyme has now enabled us to determine the effects of acyloxyacyl hydrolysis on some of the bioactivities of LPS.

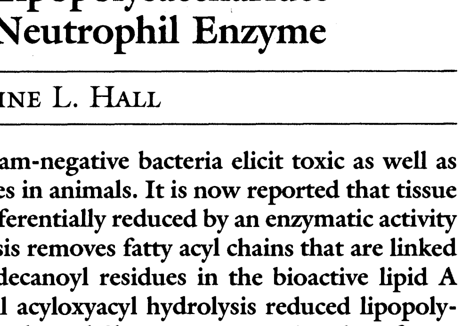


Fig. 1. Structure of *S. typhimurium* lipid A (*3*). R labels the site of attachment of the polysaccharide chain. We used three LPS that had different numbers of saccharides attached to lipid A. Rc (rough), SR (one O-repeat unit in the polysaccharide chain), and smooth (O-chains of different lengths) LPS had approximately 7, 15, and 11 to 151 or more saccharides, respectively. Aminoarabinose and phosphoethanolamine may be substituted to the phosphates at positions 1 and 4′, respectively (*18*). The arrows show the sites of cleavage by acyloxyacyl hydrolases.

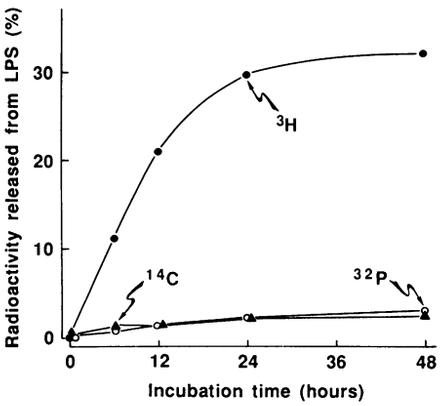
Acyloxyacyl hydrolase activity was partially purified (*5*) (approximately 1500-fold increase in specific activity) from HL-60 human promyelocytes (*6*). Biosynthetically radiolabeled LPS were prepared from *S. typhimurium* cells grown with [^3H]acetate and *N*-acetyl-1-[^{14}C]glucosamine (incorporated into fatty acids and the glucosamine backbone, respectively, of lipid A) as described (*4*).

The enzyme (or enzymes) released ^3H-labeled fatty acids, but not ^{14}C-labeled glucosamine, from the double-labeled LPS (Fig. 2). The reaction reached an apparent maximum when approximately 32% of the ^3H radioactivity was cleaved from the LPS; since 32% of the ^3H radioactivity in this preparation was in NFA, 32% deacylation was consistent with nearly complete removal of these residues. This conclusion was supported by analysis of the fatty acid composition of the substrate LPS and the reaction products: 68% of the radioactivity from the ^3H-labeled fatty acids in the LPS was in 3-hydroxytetradecanoate and 32% was in NFA, whereas the ^3H radioactivity that was enzymatically released from LPS was almost entirely (94%) in NFA, and 95% of the ^3H radioactivity in the partially deacylated LPS

Departments of Internal Medicine and Microbiology, Southwestern Medical School, University of Texas Health Science Center, Dallas, TX 75235.

was in 3-hydroxytetradecanoate (*7*). When ^{32}P-labeled *S. typhimurium* LPS were treated with enzyme in parallel with the double-labeled LPS, less than 5% of the ^{32}P was released from the LPS, indicating that the enzyme preparation contained negligible phosphatase activity.

The migration of the LPS in SDS–polyacrylamide gel electrophoresis (SDS-PAGE), was increased by enzymatic deacylation, which suggests that the size of the molecules was reduced (Fig. 3). This increase in migration was less than that observed for LPS treated with alkali, which removes all four of the ester-linked fatty acids (compare lanes 5 and 6 and 9 and 10). Preservation of the original ladder pattern [which reflects the presence of molecules with different numbers of O-repeat units (*8*)] in the deacylated smooth LPS (lanes 1, 2, and 11) further indicates that the polysaccharide chain was not removed by enzymatic treatment. Thus the only evident modification in the enzymatically treated LPS was the loss of NFA.



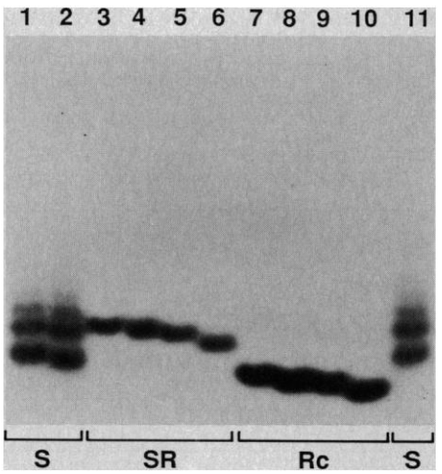


Fig. 2 (left). Time course of LPS deacylation by neutrophil acyloxyacyl hydrolase. Double-labeled ($^{3}H/^{14}C$) LPS (5 μg) were incubated at 37°C with enzyme in a reaction mixture (0.5 ml) that contained fatty acid–free bovine serum albumin (BSA) (1 mg/ml) (Sigma), 5 m*M* $CaCl_2$, 0.5% (v/v) Triton X-100, and 20 m*M* tris-citrate, *p*H 4.8. The reaction was stopped at the indicated times by precipitating the LPS and BSA with 1.2 ml of 95% ethanol. The precipitates were collected by centrifugation (12,000*g*, 10 minutes, 4°C), washed once with 1.0 ml of 80% ethanol, suspended in 0.5 ml of normal saline, and stored at −20°C. Aliquots of the precipitates and supernatants were counted, and the proportion of each radiolabel that appeared in the supernatant was calculated. ^{32}P-labeled *S. typhimurium* Rc LPS (5 μg), prepared by growing the bacteria in a low-phosphate medium (*19*) that contained $^{32}PO_4$ (orthophosphate, New England Nuclear), were incubated in parallel and precipitated in the same way; $^{32}PO_4$, when incubated and processed identically, was completely recovered in the ethanol supernatant. Values are the means of two determinations. Fig. 3 (right). Analysis of intact and deacylated LPS by SDS-PAGE. Samples of LPS were solubilized in sample buffer and subjected to electrophoresis as described (*8*). The gel was treated with Fluoro-Hance (Research Products International) and exposed on Kodak X-Omat XAR-5 film at −70°C. The lanes contained the following LPS preparations (percent loss of ^{3}H radioactivity): 1 and 11, S LPS (1%); 2, S LPS (25%); 3, SR LPS (0.6%); 4, SR LPS (15%); 5, SR LPS (28%); 6, SR LPS (65%); 7, Rc LPS (1%); 8, Rc LPS (20%); 9, Rc LPS (32%); and 10, Rc LPS (65%). LPS were enzymatically deacylated except for those in lanes 6 and 10, which were treated with 1*M* NaOH at 25°C for 18 hours (*19*).

Experiments to test the effects of acyloxyacyl hydrolysis on the bioactivities of LPS were guided by the reported bioactivities of chemically synthesized lipid A analogs (*9*) and biosynthetic precursors of lipid A (*10*). Lipid A analogs and precursors that lack acyloxyacyl groups are less potent, by a factor of 500 or more, than complete lipid A in killing chick embryos, are nontoxic in the dermal Shwartzman reaction, and are approximately 10 to 100 times less pyrogenic than complete lipid A, yet they are almost as potent (within tenfold) as lipid A in several assays for immunostimulatory activity (for example, B-cell mitogenicity, adjuvant activity, and macrophage stimulation) (*9, 10*). Acyloxyacyl hydrolysis was thus expected to reduce the toxicity of LPS more than the immunostimulatory activity. Accordingly, we tested the effects of deacylation using the dermal Shwartzman reaction, an assay for tissue toxicity, and the B-cell mitogenicity assay, a test that has correlated well with other assays of lipid A immunostimulation (*9, 10*).

When a rabbit is injected intradermally with LPS and then, 20 to 24 hours later, is injected intravenously with LPS, hemorrhagic necrosis of the skin occurs at the intradermal injection site [dermal Shwartzman reaction (*11*)]. Enzyme-treated and untreated LPS were tested simultaneously in the same New Zealand White rabbits; untreated LPS were used for the intravenous dose. Maximally deacylated LPS (32% loss of ^{3}H radioactivity) produced no reaction in two animals, one of which was given an intradermal dose of 10 μg and the other, 10.9 μg, and a third animal had a barely positive reaction at a dose of 9.6 μg. In contrast, LPS that had been incubated in the same reaction mixture without enzyme produced areas of hemorrhage (3 mm or greater in diameter) at doses of 0.1 μg or more. The reduction in tissue toxicity was thus at least by a factor of 100. On the other hand, the ability of the same deacylated LPS to stimulate murine B-cell division (Fig. 4) was reduced only by a factor of 6 to 20 (the

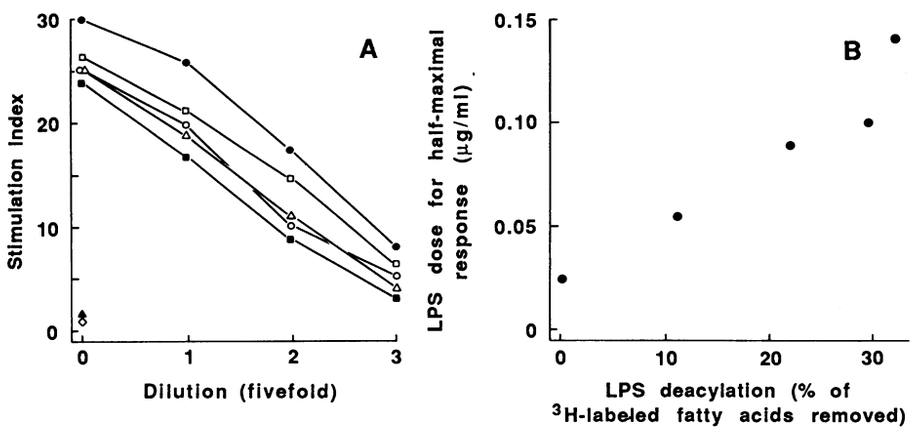


Fig. 4. Mitogenic activity of intact and deacylated LPS. (A) Rc LPS deacylated as described in Fig. 2 and added to single-cell suspensions of C3H/HeN mouse spleen in 96-well microtiter plates (*20*); cultures (0.2 ml) contained 300,000 cells in RPMI 1640 with antibiotics and 5% fetal bovine serum. After 24 hours, [*methyl*-^{3}H]thymidine (0.5 μCi) was added, and the amount of radioactivity incorporated into the cells in each well was measured 18 hours later. Each LPS was tested in three fivefold dilutions from an initial concentration of 1.0 μg/ml. Symbols indicate the percent loss of ^{3}H-labeled fatty acids from the LPS: ●, 0%; □, 11%; ○, 22%; △, 30%; ■, 32%; ▲, no added test sample; and ◇, deacylation incubation mixture with enzyme but no LPS. Stimulation index = (^{3}H counts in stimulated cells)/(^{3}H counts in cells incubated without LPS). (B) Probit analysis (*21*) of the data in (A) indicated the concentration of each preparation that produced half-maximal stimulation of B-cell proliferation. The relation between LPS deacylation and loss of activity was essentially linear.

median reduction was by a factor of 12, n = 5 experiments). Acyloxyacyl hydrolysis thus appeared to produce a greater reduction, by approximately a factor of 10, in LPS tissue toxicity than in immunostimulatory activity.

These results confirm that acyloxyacyl groups are important for tissue toxicity, as expressed in the Shwartzman test; presumably, they provide the necessary stimulus to critical target cells. Although acyloxyacyl groups also add to the immunostimulatory activity of LPS, as measured by the B-cell mitogenicity test, overall this contribution seems quantitatively less important than their contribution to toxicity. In previous studies of the biological role of acyloxyacyl groups, isolated lipid A and its analogs were used. The present results suggest that acyloxyacyl groups are also critical determinants of the bioactivity of the lipid A that occurs in nature, linked to a saccharide chain in LPS (*12*).

To our knowledge, this is the first description of an enzymatic activity that modifies the bioactivities of LPS. Antibody-opsonized LPS undergo deacylation by neutrophils and macrophages (*4, 13*); a plausible hypothesis places acyloxyacyl hydrolases in an acid intracellular compartment (for example, lysosomes), where deacylation of bacterial LPS may accompany the digestion of other bacterial components (*14*). Enzymatically deacylated LPS, released from phagocytic cells by exocytosis (*15*) or cell death, might then interact with target cells such as B lymphocytes. However, the complete intracellular fate of LPS is not known; our studies have all dealt with purified LPS, not the LPS in bacteria per se; and other enzymes may also process LPS. For example, murine macrophages can remove 3-hydroxytetradecanoyl residues from LPS (*13*); this deacylation may completely inactivate the molecules.

We suggest that a major function of neutrophil acyloxyacyl hydrolases is to detoxify LPS. Maneuvers that increase enzyme activity in vivo thus might prevent or ameliorate some of the toxic manifestations of Gram-negative bacterial diseases. It is also possible that enzymatically detoxified LPS may stimulate potentially beneficial components of the inflammatory response. Preservation of some of the immunostimulatory potency of detoxified LPS might allow animals to derive immune priming (*16*) during health and enhancement of antibody formation during infections. Acyloxyacyl hydrolases thus may have a biological role that parallels one proposed function of lysozyme, another enzyme in phagocytic cells that cleaves a toxic bacterial cell wall polymer (peptidoglycan) into products (muramyl peptides) that may have immunostimulatory activities (*17*).

REFERENCES AND NOTES

1. D. C. Morrison and R. J. Ulevitch, *Am. J. Pathol.* **93**, 527 (1978).
2. R. J. Ulevitch, A. R. Johnston, D. B. Weinstein, *J. Clin. Invest.* **64**, 1516 (1979); S. H. Rutenburg, F. B. Schweinburg, J. Fine, *J. Exp. Med.* **112**, 801 (1960); J. P. Filkins, *Proc. Soc. Exp. Biol. Med.* **137**, 1396 (1971); P. E. Gimber and G. W. Rafter, *Arch. Biochem. Biophys.* **135**, 14 (1969).
3. K. Takayama, N. Qureshi, P. Mascagni, *J. Biol. Chem.* **258**, 12801 (1983).
4. C. L. Hall and R. S. Munford, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6671 (1983). Radiolabeled smooth (S) and rough (Rc) LPS were prepared from *S. typhimurium* strain PRX20 (provided by P. Rick) and LPS with one O-repeat unit in the polysaccharide chain (SR) were prepared from strain SL1054 (provided by H. Nikaido). The Rc LPS that were used for most of the experiments contained 107,000 dpm of ^{3}H-labeled fatty acid and 3750 dpm of ^{14}C-labeled glucosamine per microgram.
5. ———, unpublished results.
6. S. J. Collins, R. C. Gallo, R. E. Gallagher, *Nature (London)* **270**, 347 (1977).
7. Lipopolysaccharides were deacylated and precipitated with ethanol (see legend to Fig. 2), the ethanol-water supernatant was dried under N_2, and the ^{3}H-labeled fatty acids were extracted into a mixture of chloroform and methanol (2:1 by volume). Unincubated LPS and the deacylated LPS in the ethanol precipitate were hydrolyzed (*4*), and the fatty acids were extracted into chloroform. The samples were analyzed by one-dimensional thin-layer chromatography with silica gel-G plates (Analtech) (*4*). The spots that contained NFA and 3-OH-14:0 were scraped from the plates and counted. Recovery of radioactivity at each step was 84% or greater. Values are the means of two determinations.
8. R. S. Munford *et al.*, *J. Bacteriol.* **144**, 630 (1980).
9. H. Takada *et al.*, *Infect. Immun.* **48**, 219 (1985); S. Kotani *et al.*, *ibid.* **49**, 225 (1985).
10. C. Galanos *et al.*, *Eur. J. Biochem.* **140**, 221 (1984).
11. G. Shwartzman, *Phenomenon of Local Tissue Reactivity and Its Immunological, Pathological, and Clinical Significance* (Hoeber, New York, 1937); H. Z. Movat, *Surv. Synth. Pathol. Res.* **1**, 241 (1983).
12. When optimal in vitro conditions for deacylating Rc or smooth LPS are used, isolated *Salmonella* lipid A is a relatively poor substrate for acyloxyacyl hydrolysis (R. S. Munford and C. L. Hall, unpublished results).
13. ———, *Infect. Immun.* **48**, 464 (1985).
14. P. Elsbach, *Rev. Infect. Dis.* **2**, 106 (1980).
15. I. W. Devoe, *J. Bacteriol.* **125**, 258 (1976); R. D. Berlin, J. P. Fera, J. R. Pfeiffer, *J. Clin. Invest.* **63**, 1137 (1979); R. L. Duncan and D. C. Morrison, *J. Immunol.* **132**, 1416 (1984).
16. M. J. Pabst, H. B. Hedegaard, R. B. Johnston, *J. Immunol.* **128**, 123 (1982); H. Goris, R. de Boer, D. van der Waaij, *Infect. Immun.* **50**, 437 (1985); R. P. Cornell, *Diabetes* **34**, 1253 (1985).
17. L. Chedid, *Microbiol. Immunol.* **27**, 723 (1983).
18. S. M. Strain *et al.*, *J. Biol. Chem.* **260**, 16089 (1985).
19. M. R. Rosner, J. Tang, I. Barzilay, H. G. Khorana, *ibid.* **254**, 5906 (1979).
20. M. J. Wannemuehler *et al.*, *J. Immunol.* **133**, 299 (1984).
21. S. Gillis, M. M. Fern, W. Ou, K. A. Smith, *ibid.* **120**, 2027 (1978).

22. We thank M. F. Griffith for technical support, A. Erwin for help with the Shwartzman testing, and J. M. Lipton, W. B. Baine, J. R. Kettman, J. W. Uhr, J. P. Luby, and L. Eidels for critical review of the manuscript. This work was funded by grant AI 18188 from the National Institute of Allergy and Infectious Diseases.

20 May 1986; accepted 28 July 1986

Inhibin-Mediated Feedback Control of Follicle-Stimulating Hormone Secretion in the Female Rat

CATHERINE RIVIER, JEAN RIVIER, WYLIE VALE

The secretion of follicle-stimulating hormone (FSH) by the anterior pituitary gland is regulated by the interaction of hypothalamic and gonadal hormones. Recently, proteins termed inhibins that selectively suppress FSH secretion have been purified and characterized from the gonadal fluids of several species. Antibodies to a synthetic peptide encompassing the amino terminal 25 residues of the recently characterized porcine inhibin were used to develop a specific radioimmunoassay (RIA) for inhibin and to neutralize endogenous inhibin during the estrous cycle of the rat. The administration of 20 international units of pregnant mare serum gonadotropin (PMSG) stimulated the secretion of inhibin in intact immature female rats, whereas ovariectomy caused an abrupt decrease in plasma inhibin concentrations that were not prevented by the injection of PMSG. The infusion of a polyclonal antiserum to inhibin, from 12 noon on proestrus to 1 a.m. on the morning of estrus, as well as its acute intravenous injection during diestrus I or II, caused an increase in plasma FSH (but not luteinizing hormone) concentrations. These results support the hypothesis of a feedback loop between the release of ovarian inhibin and FSH in the female rat.

ALTHOUGH GONADOTROPIN-RELEASING hormone (GnRH) and sex steroids play a major role in controlling the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the anterior pituitary gland (*1, 2*), data from many studies indicate the presence of an additional regulatory protein referred to as an inhibin. This water-soluble, nonsteroidal protein is of gonadal origin and selectively inhibits the release of FSH (*3, 4*). Several groups (*5–10*) have reported the isolation of proteins that may account for inhibin activity from porcine and bovine follicular fluid and have described partial amino terminal sequences of these proteins, which have two

The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037.