glandular hyperplasia was marked. In two females the cervical epithelium showed proliferation, downgrowth, and glandular invasion of the stroma. The ovaries seemed normal, but the oviducts were distended with fluid and contained cysts without epithelium. One of nine male pups had testicular tubules in which spermatogenesis did not proceed beyond secondary spermatocytes. Interstitial cells were abundant. The epididymis was filled only with fluid and cell fragments and showed local cysts with inflammatory reaction. The remaining male offspring appeared normal.

The abnormalities observed in this report may arise from the early and intense estrogenic stimulation of developing fetal tissues by Clomid. Continuous exposure to high concentrations of estrogen during fetal or neonatal life is known to increase the incidence of preneoplastic and neoplastic changes in the reproductive tract (*5*, *6*). Clomid belongs to a class of compounds that has the ability to cause long-term estrogenic stimulation of certain cell types. Clomid causes long-term retention of the estrogen receptor in the nucleus of uterine cells, and this is accompanied by a sustained stimulation of uterotropic activity (*7*). This stimulation is primarily due to the ability of Clomid to stimulate the epithelium of the uterine lumen, whereas, estradiol, a physiological estrogen, causes all tissues of the uterus to grow (*8*). Therefore, Clomid can be a long-acting estrogen in some cell types and may cause hyperestrogenization of fetal tissues which ultimately leads to the expression of abnormal growth.

The abnormalities that were observed in the maternal tissues may also result from a similar hyperestrogenization. We have observed extensive epithelial cell stimulation in adult cycling rats (*9*) and assume that this takes place in the pregnant rat.

The long-acting effects of triphenylethylene derivatives and their ability to cause differential cell stimulation have obvious implications for the use of these drugs in humans. Clomid has been widely used for the past 15 years to induce ovulation in anovulatory women (*1*). For treatment a dose of 50 to 100 mg of Clomid is taken orally for 5 days. If the initial trial fails and no signs of pregnancy are present, the treatment is resumed the following month, about 40 days later. This treatment regimen is often continued for many months, hence the exposure of a woman to Clomid can be extensive. Under these circumstances, Clomid may be stimulating some cell types while acting as an antagonist in others. The eventual effects of such stimulation may remain unknown for many years. It is possible that Clomid and other triphenylethylene derivatives could cause hyperestrogenization of certain cell types in humans and hence great caution should be applied when these drugs are used in humans.

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References and Notes

1. R. B. Greenblatt, W. E. Barfield, E. C. Jungck, A. W. Ray, *J. Am. Med. Assoc.* **178**, 101 (1961).
2. J. H. Clark and S. A. McCormack, *Science* **197**, 164 (1977).
3. Clomid, triethylamine, 2[*p*-(chloro-1,2-diphenylvinyl)-phenoxy]-citrate, was injected as a suspension in either water or oil. Both methods were equally effective. Clomid is a mixture of *cis* and *trans* isomers and was used as such because this is the form which is administered to women. Clomid was a gift from Merrell-National Laboratories.
4. O. W. Davidson, K. Wada, E. B. Schuchner, *Fertil. Steril.* **16**, 495 (1965); M. R. N. Prasad, S. J. Segal, S. P. Kalra, *ibid.*, p. 101; P. E. Staples, *Endocrinology* **78**, 82 (1966).
5. A. L. Herbst, H. Ulfelder, D. C. Poskanzer, *N. Engl. J. Med.* **284**, 878 (1971).
6. N. Takasugi and H. A. Bern, *J. Natl. Cancer Inst.* **33**, 855 (1964); J. Furth, G. Ueda, K. H. Clifton, in *Methods in Cancer Research*, H. Busch, Ed. (Academic Press, New York, 1973) vol. 10, p. 201; J. G. Forsberg, *Am. J. Obstet. Gynecol.* **121**, 101 (1975).
7. J. H. Clark, E. J. Peck, J. N. Anderson, *Nature (London)* **251**, 446 (1974); J. H. Clark, Z. Paszko, E. J. Peck, *Endocrinology* **100**, 91 (1977); T. S. Ruh and L. J. Baudendistel, *ibid.*, p. 420.
8. J. H. Clark, J. W. Hardin, H. H. Padykula, C. A. Cardasis, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2781 (1978); J. H. Clark, J. W. Hardin, S. A. McCormack, H. A. Padykula, in *Hormones and Receptors in Breast Cancer*, W. L. McGuire, Ed. (Raven, New York, in press).
9. S. McCormack and J. H. Clark, unpublished data.

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Inhibition of Mast Cell Histamine Secretion by *N*-Substituted Derivatives of Phosphatidylserine

Abstract. *The structural basis for the highly specific action of phosphatidylserine in enhancing mast cell histamine secretion induced by concanavalin A was investigated by studying the activities of three* N*-substituted derivatives:* N*-acetyl phosphatidylserine,* N*-1-dimethylaminonaphthalene-5-sulfonyl phosphatidylserine, and* N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylserine. None of the derivatives was capable of activating concanavalin A–induced histamine secretion at concentrations two to three times that required for maximal activation by phosphatidylserine. Instead, the derivatives were found to inhibit the secretory response of mast cells to the calcium ionophore A23187 as well as to concanavalin A. The inhibition was noncytotoxic, partially reversible by washing, and associated with binding of* N*-substituted phosphatidylserine to the mast cell.*

Upon stimulation of a mast cell with an appropriate secretagogue, its cytoplasmic granules are exocytosed in an energy-dependent process that involves fusion of the cell surface membrane with underlying perigranule membranes (*1*). As a result of mast cell degranulation, histamine stored in the secretory granules is released and is capable of increasing vascular permeability and stimulating smooth muscle contraction (*2*). Phosphatidylserine (PS) potentiates histamine release from mast cells exposed to dextran (*3*), antigen (*4*), or concanavalin A (Con A) (*5*). Other phospholipids—phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidic acid, and phosphatidylethanolamine (PE)—have no effect on mast cell secretion (*6*, *7*).

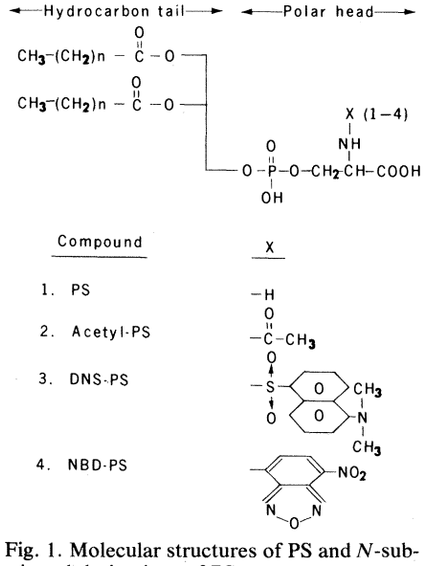

Fig. 1. Molecular structures of PS and *N*-substituted derivatives of PS.

We sought further information on the chemical specificity of the effect of PS on mast cell secretion by examining the properties of *N*-substituted derivatives of PS. Three such compounds were synthesized (*8*) and studied for their effects on mast cell secretion: *N*-acetyl phosphatidylserine (acetyl-PS), *N*-1-dimethylaminonaphthalene-5-sulfonyl phosphatidylserine (DNS-PS), and *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylserine

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(NBD-PS). The structures of these three compounds as well as that of PS are given in Fig. 1.

The failure of *N*-substituted derivatives of PS to potentiate histamine secretion from rat mast cells stimulated with Con A is illustrated in Table 1. These data provide evidence that the unblocked amino group of PS is required for its ability to potentiate mast cell secretion and lend further support to the notion that the effect of PS on mast cell secretion is highly specific. The carboxyl group of PS is also required for its stimulatory effect on secretion since PE is not capable of potentiating secretion (*3*).

The three derivatives of PS were tested for their ability to block the effect of PS on mast cell secretion. In a preliminary series of experiments with Con A and PS, it was observed that both NBD-PS and acetyl-PS were potent inhibitors of secretion, while DNS-PS was inhibitory to a lesser extent. We have subsequently concentrated our studies on the inhibitory properties of NBD-PS and in selected instances compared the effects of acetyl-PS.

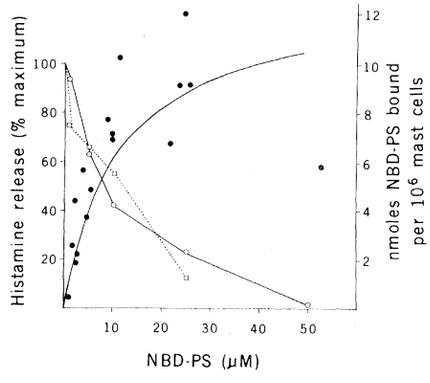
The dose-response curves presented in Fig. 2 demonstrate the inhibitory effects of NBD-PS on mast cell secretion induced by Con A and the calcium ionophore A23187. The NBD-PS effectively inhibited secretion induced by A23187 and Con A. The dose-inhibition curves were very similar and inversely related to the binding of NBD-PS to the mast cell. Half-maximal binding of NBD-PS occurred at the concentration (10 μM) required to inhibit secretion by 50 percent. With Con A and PS as the secretagogue, 20 μM acetyl-PS was required for 50 percent inhibition of histamine release; in the case of A23187, 30 μM acetyl-PS was required to obtain this degree of inhibition. Half-maximal binding of acetyl-PS to mast cells occurred at approximately 10 μM.

Table 1. Effect of *N*-substitution on the ability of PS to potentiate Con A–induced histamine secretion from mast cells. Peritoneal cells containing 2×10^5 mast cells were obtained from adult male rats and incubated in 1.0 ml of BSS (*13*) with the indicated combinations of PS (30 μM), DNS-PS (60 μM), acetyl-PS (60 μM), or NBD-PS (50 μM) and Con A (100 μg/ml) and Ca^{2+} (0.68 mM) for 15 minutes at 37°C. Histamine release is expressed as a mean percentage of total cell histamine after direct assay of supernatants and perchloric acid extracts of cell pellets by a modification of the *o*-phthalaldehyde fluorometric method (*14*). The derivatives do not interfere with the histamine assay. Duplicate determinations were performed in each experiment; the number of individual experiments is shown in parentheses.

Treatment	Histamine release (%)
Con A + Ca^{2+} (N = 6)	1.5 ± 0.8
NBD-PS + Con A + Ca^{2+} (N = 3)	1.5 ± 0.4
DNS-PS + Con A + Ca^{2+} (N = 6)	3.4 ± 1.2
Acetyl-PS + Con A + Ca^{2+} (N = 4)	4.8 ± 2.4
PS + Con A + Ca^{2+} (N = 11)	47.3 ± 4.0

Since histamine secretion from rat mast cells exposed to Con A requires exogenous PS (Table 1), the inhibitory action of NBD-PS and acetyl-PS on secretion induced by Con A and PS might be explicable in terms of competition between the inactive structural analogs of PS and PS for binding sites on the mast cell. Competitive binding experiments were carried out to test this hypothesis. The data presented in Fig. 3 demonstrate the ability of NBD-PS to block binding of ^{3}H-labeled dipalmitoyl PS ([^{3}H]DPPS) to mast cells. The K_I for NBD-PS determined from these competitive binding experiments was 13 μM (inset, Fig. 3), a value within experimental error of the independently determined dissociation constant for binding of NBD-PS to the mast cell (Fig. 2).

Competitive inhibition by the *N*-substituted PS derivatives of exogenous PS binding cannot account for the inhibition of secretion observed with A23187, since secretion elicited by this secretagogue is unaffected by exogenous PS at concentrations between 1 and 50 μM. It is pos-



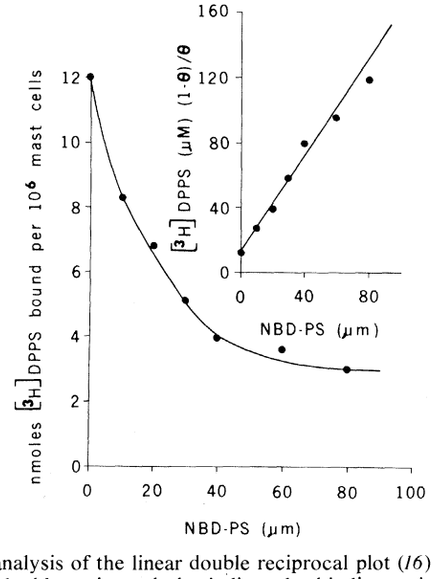


Fig. 2 (middle). Inhibition of histamine secretion from mast cells by NBD-PS. Rat peritoneal cells containing 2×10^5 mast cells were incubated in 1.0 ml of BSS with Ca^{2+} (0.68 mM) for 15 minutes at 37°C in the presence of increasing concentrations of NBD-PS and a constant concentration of secretagogue. Histamine release was assayed as described in Table 1, and the inhibition dose-response curves presented as percentage of maximum histamine release. The secretagogues were (○) A23187 (1 μM) and (□) Con A (100 μg/ml) plus PS (30 μM). The means of the maximum histamine release (± standard errors) were 56.8 ± 12.8 and 47.3 ± 4.0, respectively. Binding experiments were performed with mast cells purified from peritoneal cells by centrifugation through albumin (*15*). Cells (2×10^5) were incubated with NBD-PS in 1.0 ml of BSS for 1 hour at 22°C. The cells were centrifuged at 800*g* for 6 minutes and 0.5 ml of the supernatant was removed. The cell pellet was solubilized by the addition of 0.5 ml of 1 percent Triton X-100 and brief sonication. After addition of 2 ml of ethanol to the solubilized pellet and supernatant fractions, fluorescence emission at 525 nm was measured with excitation at 474 nm. The amount of NBD-PS bound by the cells was calculated from a standard curve after correcting for the amount of unbound NBD-PS carried over from the supernatant. The curve drawn through the data points (●) is a transformation of the line obtained by a least-squares analysis of the linear double reciprocal plot (*16*) of the same data. Each point shown represents the average of duplicate determinations. The double reciprocal plot indicated a binding stoichiometry of 12.7 nmole of NBD-PS per 10^6 mast cells and an apparent dissociation constant of 9 μM. Fig. 3 (right). Inhibition of [^{3}H]DPPS binding to mast cells by NBD-PS. Purified mast cells were obtained as described in Fig. 2 and incubated at a concentration of 4×10^5 cells per milliliter at 22°C for 1 hour in 0.2 ml of BSS containing a fixed concentration of [^{3}H]DPPS (12 μM) and increasing concentrations of NBD-PS. Cells were centrifuged at 800*g* for 6 minutes and samples from the supernatant and pellet were taken for radioactivity assay. The amount of [^{3}H]DPPS bound per 10^6 cells was calculated from the known specific activity of [^{3}H]DPPS. The K_I is defined as the dissociation constant for NBD-PS binding to mast cells determined in this competitive binding experiment by the linear plot shown in the inset (*17*). The intercept on the ordinate is K_D, the dissociation constant for the binding of [^{3}H]DPPS to mast cells, and the slope is equal to K_D/K_I. The symbol θ refers to the fractional saturation of [^{3}H]DPPS binding sites on the mast cell and is calculated by assuming that saturation binding is 28 nmole of [^{3}H]DPPS per 10^6 mast cells (unpublished data).

sible that NBD-PS and acetyl-PS either displace or otherwise perturb essential endogenous PS.

Three lines of evidence make it unlikely that NBD-PS acts to inhibit mast cell secretion by a more or less nonspecific cytotoxic effect. First, treatment of mast cells with NBD-PS at a concentration (25 μM) sufficient to inhibit secretion by 70 to 80 percent does not significantly decrease intracellular adenosine triphosphate (ATP) levels (*9*). Second, electron microscopy of mast cells treated with this concentration of NBD-PS indicates no evidence of cytotoxicity. Third, the inhibitory action of NBD-PS on secretion induced by A23187 is partially reversible by removing the medium containing NBD-PS and resuspending the cells in fresh balanced salt solution (BSS) (*10*). Significant NBD-PS dissociates from the cells under these conditions. Reversibility argues against displacement of endogenous PS as a mechanism of action of NBD-PS.

A possible trivial alternative explanation of the observed inhibition is complexing of A23187 by *N*-substituted PS aggregates in solution, as proposed for 48/80 and polymyxin B by Read *et al.* (*11*). Arguing against this hypothesis is the failure of PS itself to significantly inhibit secretion by A23187 at concentrations five times those required for half-maximal inhibition by the *N*-substituted PS derivatives.

Although the evidence that is available cannot establish the mode of action of the inhibitory *N*-substituted PS derivatives, it is evident that *N*-substitution of PS converts this phospholipid from a potent activator of a specific class of secretagogues to a potent inhibitor of two different secretory stimuli. *N*-Substituted derivatives of PS may prove useful for the investigation of stimulus-secretion coupling in the mast cell and perhaps in other cells as well (*12*).

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References and Notes

1. D. Lagunoff, *J. Cell Biol.* **57**, 252 (1973).
2. W. W. Douglas, in *The Pharmacological Basis of Therapeutics*, L. S. Goodman and A. Gilman, Eds. (Macmillan, New York, ed. 5, 1975), p. 590.
3. A. Goth, H. R. Adams, M. Knoohuizen, *Science* **173**, 1034 (1971).
4. D. J. Stechschulte and K. F. Austen, *J. Immunol.* **112**, 970 (1974).
5. T. J. Sullivan, W. C. Greene, C. W. Parker, *ibid.* **115**, 278 (1975).
6. J. L. Mongar and P. Svec, *Br. J. Pharmacol.* **46**, 741 (1972).
7. J. H. Baxter and R. Adamik, *Proc. Soc. Exp. Biol. Med.* **152**, 266 (1976).
8. The DNS-PS and NBD-PS were originally synthesized for use as fluorescent probes to study the mode of the interaction of PS with mast cells. Since these *N*-substituted derivatives were found to be inactive, a requisite function of the unblocked amino group of PS in its action was indicated. As a further test of this hypothesis, we chose the acetyl substituent to block the amino group of PS with a minimal alteration in the structure of PS. The three *N*-substituted derivatives of PS were synthesized from PS isolated from bovine brain by the method of H. Sanders [*Biochim. Biophys. Acta* **144**, 485 (1967)]. The reaction conditions and the isolation of the products were virtually identical for the three derivatives. The PS (180 μmole) was reacted for 4 hours at 22°C under N_2 with 270 μmole of dimethylaminonaphthalene 5-sulfonyl chloride, ^{3}H-labeled acetic anhydride, or 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole in a final volume of 4 ml chloroform-triethylamine (3:1 by volume). The reaction mixture was then made up to 20 ml with chloroform and extracted three times with 50-ml portions of methanol-H_2O (1:1 by volume, *p*H 4.0). The extracted chloroform phase was taken to dryness in vacuo; the residue was dissolved in 10 ml of chloroform and applied to a 50-g column of silicic acid. The column was eluted with five column volumes each of chloroform, chloroform-methanol (95:5 by volume), and finally chloroform-methanol (6:4 by volume). The last fraction contained the desired product, which was quantitatively separated from unreacted PS by chromatography on DEAE-cellulose (Whatman DE-52), using the solvent system chloroform-methanol-H_2O (2:3:1 by volume). The PS eluted from the column with this solvent containing 0.018*M* ammonium acetate. The *N*-substituted derivatives were eluted in 0.05 to 0.06*M* ammonium acetate. In some preparations a contaminant that comigrated on thin-layer plates with the corresponding *N*-substituted lyso-PS derivative remained after DEAE-cellulose chromatography. The purified *N*-substituted lyso-PS derivatives were found to be extremely cytolytic and were removed by preparative thin-layer chromatography on 1-mm silica gel G plates (Analtech) developed in diisobutylketone-CH_3COOH-H_2O (40:30:7 by volume). The final products migrated as single spots on silica gel H thin-layer plates developed in this solvent; R_F = 0.46.
9. Mast cells (10^6 per milliliter) in a final volume of 1 ml were treated with 25 μM NBD-PS for 10 minutes at 22°C, and intracellular ATP was assayed in parallel with untreated control cells [L. Ko and D. Lagunoff, *Exp. Cell Res.* **100**, 313 (1976)]. The ATP content of the cells (0.769 μg per 10^6 cells in untreated controls) was not decreased by treatment with 25 μM NBD-PS (0.858 μg per 10^6 cells) under these conditions.
10. Although 25 to 50 μM NBD-PS presented to the cells with A23187 results in 80 to 95 percent inhibition of secretion, secretion is inhibited by only 30 percent when the cells are washed and resuspended in fresh medium after exposure to NBD-PS but before challenge with the ionophore.
11. G. W. Read, M. Knoohuizen, A. Goth, *Eur. J. Pharmacol.* **42**, 1971 (1977).
12. W. E. Harris and W. L. Stahl [*Mol. Pharmacol.* **12**, 115 (1976)] used DNS-PS incorporated in isolated synaptosome membranes to examine the interaction of cations with the membrane.
13. Balanced salt solution: 4 m*M* Na_2HPO_4, 2.7 m*M* KH_2PO_4, 150 m*M* NaCl, and 2.7 m*M* KCl, *p*H 7.2.
14. L. T. Kremzner and I. B. Wilson, *Biochim. Biophys. Acta* **50**, 364 (1961).
15. D. Lagunoff, in *Techniques of Biochemical and Biophysical Morphology*, D. Glick and R. Rosenbaum, Eds. (Wiley, New York, 1975), vol. 2, p. 283.
16. J. T. Edsall and J. Wyman, *Biophys. Chem.* **1**, 591 (1958).
17. C. Tanford, *Physical Chemistry of Macromolecules* (Wiley, New York, 1963), p. 574.
18. Supported in part by NIH grants HL-03174, HL-23593, and HL-07312.

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Neurotoxic Fragrance Produces Ceroid and Myelin Disease

Abstract. *Acetyl ethyl tetramethyl tetralin (AETT), a component of soaps, deodorants, and cosmetics, produces hyperirritability and limb weakness in rats repeatedly exposed to the compound. Brain, spinal cord, and peripheral nerves are discolored blue, show progressive neuronal ceroid degeneration, and develop spectacular myelin bubbling. These neurotoxic properties of AETT provide the basis for industry's decision to withdraw the compound from consumer products. In addition, AETT offers the experimentalist a new probe to explore the etiology and pathogeneses of human ceroid and myelin diseases.*

Acetyl ethyl tetramethyl tetralin (*1*) (Fig. 1) was widely used as a musk fragrance in soaps and cosmetics (*2*) until industry scientists discovered that repeated application of the compound to the skin of experimental animals produced a blue discoloration of internal organs and a vacuolar degeneration of the brain (*3*). By the early part of 1978, the fragrance industry had voluntarily withdrawn AETT from all cosmetic formulations (*4*). On this basis, the U.S. Food and Drug Administration (FDA) denied a request from the Environmental Defense Fund to ban the use of AETT (*5*). Recall of the existing inventory of contaminated products was considered unnecessary, so that some of these may remain available in the United States until existing stocks have been exhausted.

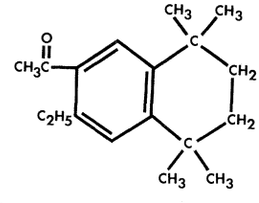

Fig. 1. Structure of acetyl ethyl tetramethyl tetralin.

We have studied the clinical and neuropathological changes in rats repeatedly exposed to AETT (*6*) and have found that this compound causes progressive ceroid-like pigmentation and widespread demyelination throughout the central and peripheral nervous systems. These degenerative changes are associated with hyperirritability, limb weakness, and ataxia.

Behavioral changes, developing as a function of dose and time, were noted within the first 2 to 3 weeks of treatment (*6*) in rats receiving 25 or 50 mg of AETT per kilogram of body weight per day. Animals became overtly aggressive and were easily startled. Rats receiving the

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