Butylated Hydroxytoluene Protects Chickens Exposed to Newcastle Disease Virus

Abstract. *Dietary butylated hydroxytoluene, an antioxidant widely used in food and feed processing, prevents mortality of chickens exposed to virulent Newcastle disease virus and prevents the serological response of chickens exposed to avirulent Newcastle disease virus. This chemoprophylactic effect is evident when chickens are fed diets containing concentrations of butylated hydroxytoluene normally used for antioxidant purposes (100 to 200 parts per million of total diet).*

Newcastle disease virus (NDV) causes a highly contagious and sometimes devastating disease of domestic poultry and other avian species. The disease of poultry is controlled primarily through immunization with live virus vaccines composed of naturally occurring avirulent NDV strains. Yet, for diverse and often unexplained reasons, these vaccines sometimes fail to immunize recipient birds (*1*). We report here that a widely used poultry feed additive, butylated hydroxytoluene (BHT), protects chickens exposed to either vaccine or virulent strains of NDV.

Butylated hydroxytoluene is one of the antioxidants added to human and animal food products to delay degradation of the labile lipid components, and the concentration generally used ranges from 50 to 200 parts per million (ppm) of the total food weight (*2*). Dietary BHT is considered innocuous in these concentrations, but in high concentrations, ranging from 2×10^3 to 10^4 ppm, it produces diverse biological effects (*3*), some possibly harmful and others beneficial. For example, high dietary levels of BHT produce cytological and metabolic alterations (*4*), inhibit the toxic and carcinogenic activity of certain chemicals (*5*), and increase the life-span of mice (*6*). Furthermore, a recent study has revealed that the infectivity of certain lipid-containing viruses—that is, human herpes simplex virus (HSV) and two bacterial viruses—is markedly reduced in vitro after exposure to low levels (20 to 100 ppm) of BHT (*7*). This observation plus the extensive use of BHT in poultry feed provide the basis for the study reported here.

Uncloned strains of NDV (*8*) were propagated in embryonated chicken eggs and stored undiluted at −70°C; unpurified virus diluted in balanced salt solution was used in both in vitro and in vivo experiments. In tests to determine the in vitro effect of BHT on NDV infectivity, 1 volume of BHT dissolved in ethanol was added to 100 volumes of virus at 37°C, and control virus was mixed with ethanol alone. Treatment was ended by immersion of test samples in ice, dilution in iced diluent, and inoculation on cell culture. Figure 1 shows that exposure of NDV to low concentrations (11 to 22 μg/ml) of BHT for 30 minutes at 37°C causes a marked reduction in viral infectivity, and this effect is more pronounced with avirulent NDV LaSota than with virulent NDV Texas-GB. Three other NDV strains were also inactivated in vitro by low BHT concentrations (22 μg/ml). The time and temperature dependence of the inactivation was determined by measuring viral infectivity at intervals after addition of BHT (22 μg/ml); the infectivity of NDV LaSota was reduced 98 percent and that of NDV Texas-GB was reduced 94 percent after treatment for 5 minutes at 37°C. However, after treatment for 5 minutes at 25°C, the infectivity of NDV LaSota was reduced 50 percent and that of NDV Texas-GB was reduced 25 percent. The action of BHT on these two viruses was essentially complete after 10 to 15 minutes at 37°C or after 30 minutes at 25°C. Thus, the in vitro inactivation of NDV by BHT is dependent on the concentration of the chemical, exposure time and temperature, and strain of virus tested.

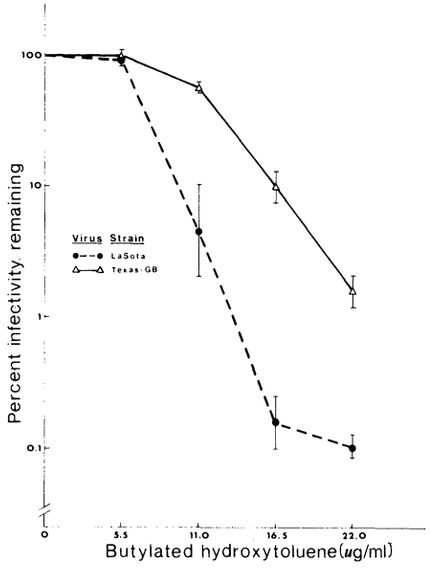

Fig. 1. Inactivation of Newcastle disease virus by butylated hydroxytoluene. Fifty microliters of BHT dissolved in absolute ethanol was mixed with 5 ml of virus stock (containing approximately 10^6 PFU/ml) in Earle's balanced salt solution, *p*H 7.2, without $NaHCO_3$ and containing 10 percent tryptose phosphate broth; virus controls were mixed with ethanol alone. The BHT-NDV mixtures and controls were incubated for 30 minutes at 37°C and agitated at 5-minute intervals, then chilled in ice, diluted, and assayed in cell culture by the plaque method (*12*). Each point represents the mean ± standard error of three determinations. Infectivity of the control virus is shown as 100 percent, and virus infectivity of BHT-treated preparations is shown as the percentage deviation from this level.

Because NDV is bound by a membrane that contains hydrophobic structural elements (*9*), inactivation could result from membrane alterations caused by interaction of the virus with the hydrophobic BHT molecule (*7, 10*). However, the ability of NDV LaSota to agglutinate chicken erythrocytes, a normal membrane function of the virion (*9*), was not altered by BHT treatment (≤ 44 μg/ml for 30 minutes at 37°C).

In tests to assess the in vivo effects of BHT on NDV, immature susceptible chickens were fed nutritionally adequate diets containing up to 2000 ppm of BHT and were then exposed to terminal dilutions of virus. All groups of chickens in each experiment (five or six birds per group) were maintained separately; BHT-supplemented and control diets were fed ad libitum throughout the test period, beginning 7 or 14 days before virus exposure. The first in vivo parameter of infection studied was the serological response following exposure to NDV LaSota (seroconversion). With this virus, a widely used vaccine strain, seroconversion is the expected sequel of infection (*1*). Figure 2, A and B, shows that seroconversion was maximum in chickens fed BHT-free diets, and the seroconversion percentages of BHT-treated groups were inversely related to BHT concentrations in the diet. Results were similar at 7, 14, and 21 days after exposure to virus. The seroconversion differences were highly dependent on the dose of virus administered; for example, with a virus dose of approximately 30 plaque-forming units (PFU), cumulative seroconversion percentages in the groups fed 0, 100, and 1000 ppm of BHT were 100 (11 of 11), 36 (4 of 11), and 0 (0 of 11), respectively; however, when the virus dose was increased to approximately 3000 PFU, seroconversion was 100 (11 of 11) percent regardless of BHT level. The serum hemagglutination-inhibiting (HI) antibody levels in the BHT-treated and control groups with 100 percent seroconversion were not significantly different ($P > .05$) at 7, 14, or 21 days after infection. Furthermore, dietary BHT (100 and 1000 ppm) did not significantly alter ($P > .05$) the HI antibody response of chickens to parenterally ad-

ministered inactivated NDV LaSota (10^9 PFU adsorbed to aluminum hydroxide). The similar HI antibody responses in treated and control groups support the possibility that the decreased seroconversion resulted from in vivo antiviral activity of BHT and not from BHT impairment of immune function.

The second in vivo parameter of infection studied was the development of overt disease after exposure of chickens to virulent NDV Texas-GB. In three of four experiments (Fig. 2, C, E, and F), mortality was maximum in untreated controls, and survival of BHT-treated chickens generally was proportional to BHT concentration. As with seroconversion, the mortality differences were most apparent when the virus dose was minimal (25 PFU). Cumulative mortalities (Fig. 2, C to F) at 14 days after exposure to approximately 25 PFU of virus were 96 (23 of 24), 58 (14 of 24), and 33 (8 of 24) percent with BHT levels of 0, 100, and 1000 ppm, respectively; in the same experiments, cumulative morbidities (marked by muscular tremors, paralysis, or clonic spasms) at 14 days were 100 (24 of 24), 67 (16 of 24), and 46 (11 of 24) percent. The aberrant response seen in one of four experiments (Fig. 2D) is unexplained but could result from experimental error or from the influence of unidentified factors on the test system.

It is of interest to consider possible mechanisms through which BHT could exert in vivo antiviral activity. Because tissue residues of up to 60 ppm of BHT and its metabolites are found in chickens fed BHT (*11*), the in vivo effect of BHT could result from a direct virus-chemical interaction in the body tissues and fluids. This possibility is appealing because only about 10 ppm of BHT is necessary in vitro for 50 percent inactivation of NDV (Fig. 1). Alternatively, a direct virus-chemical interaction could occur at the surface of the anterior digestive tract as a result of contact between the virus inoculum and BHT-laden ingesta soon after virus inoculation. The following observations provide possible support for this mechanism: (i) chickens fed BHT-supplemented and control diets as described above were exposed to varying concentrations of NDV LaSota deposited in the respiratory tract as a fine-particle aerosol, and the resulting seroconversion percentages did not differ between the BHT-treated and control groups; and similarly (ii) dietary BHT administered therapeutically after exposure to the virus by conjunctival sac instillation did not alter seroconversion.

The possibility cannot be excluded that BHT exerts in vivo and in vitro antiviral activity through separate mechanisms. For example, the in vivo activity need not be directly antiviral but could be the consequence of some nonspecific BHT effect on host physiology or metabolism. Also we have not excluded the possible antiviral activity of BHT metabolites.

It is significant that an antioxidant classified "generally recognized as safe" by the Food and Drug Administration (*2*) is effective experimentally in the chemoprophylaxis of a viral disease, particularly when one considers that BHT is one of the extensively used antioxidants in both human food and animal feeds, and that it is generally considered to lack in vivo toxicity when fed to animals in additive concentrations (up to 200 ppm)

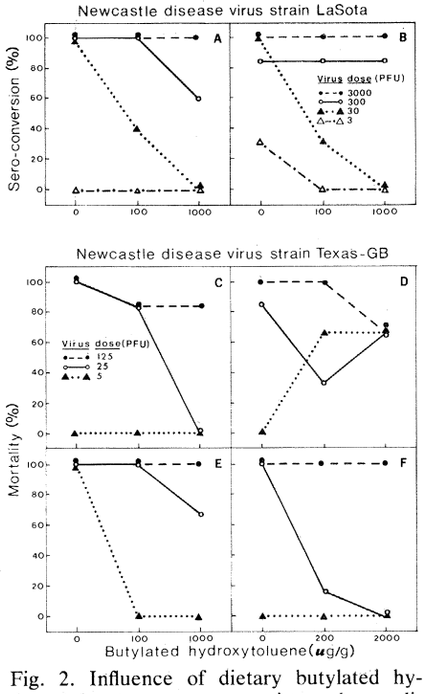

Fig. 2. Influence of dietary butylated hydroxytoluene on seroconversion and mortality of chickens exposed to Newcastle disease virus. Groups of five (A) or six (B to F) chickens (White Plymouth Rock, 2 to 5 weeks old) maintained in separate isolation units (*13*) were exposed to NDV by conjunctival sac instillation. The chickens were fed BHT-supplemented diets ad libitum throughout the test period, beginning 7 (A, E, and F) or 14 (B, C, and D) days before virus exposure. Ordinate values show (A and B) the percentage of birds with a positive serological response (*14*) (hemagglutination-inhibition titer $\geq 2^{-3.3}$) at 21 days after exposure to avirulent NDV LaSota or (C to F) the percentage of birds dead 14 days after exposure to virulent NDV Texas-GB. The BHT-supplemented diets were prepared by dropwise addition of a BHT solution (400 to 500 ml) in acetone to 45 kg of constantly stirred BHT-free commercial poultry-growing ration; acetone was likewise mixed with the negative control diets. The feed then was air-dried for 18 to 24 hours at ambient temperature and stored no more than 4 weeks before feeding.

(*3*, *11*). Nevertheless, to seriously consider immediate clinical use of BHT for control of virulent Newcastle disease is premature. Also of significance is the fact that feed-additive levels of BHT can prevent the normal serological response to an avirulent strain of NDV. When this result is considered in light of the extensive use of BHT in poultry feed, it suggests a possible explanation for vaccination failure sometimes encountered with live NDV vaccines.

This in vivo effect of BHT on NDV in chickens may indicate a new direction in the development of antiviral therapy. Of particular interest is the possible broad spectrum of the BHT antiviral activity. While most antiviral compounds are active against either RNA or DNA viruses, BHT is active in vitro against both an RNA virus (NDV) and a DNA virus (HSV) (*7*). Of importance now is the need to define the mechanism of the apparent antiviral action of BHT and to extend these observations to other viruses, using both BHT and related compounds.

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References and Notes

1. R. P. Hanson, *Adv. Vet. Sci. Comp. Med.* **18**, 213 (1974); H. P. Chu and J. Rizk, *World Anim. Rev. No. 2* (1972), p. 33; W. H. Allan, *Nature (London)* **234**, 129 (1971).
2. Butylated hydroxytoluene is classified "generally recognized as safe" by the Food and Drug Administration. For further information see B. N. Stuckey, in *Handbook of Food Additives*, T. E. Furia, Ed. (Chemical Rubber Co., Cleveland, Ohio, 1972), p. 185.
3. A. L. Branen, *J. Am. Oil Chem. Soc.* **52**, 59 (1975).
4. B. E. March, V. Coates, J. Biely, *Science* **164**, 1398 (1969); C. M. Botham, D. M. Conning, J. Hayes, M. H. Litchfield, T. F. McElligott, *Food Cosmet. Toxicol.* **8**, 1 (1970); N. K. Clapp, R. L. Tyndall, R. B. Cumming, *ibid.* **11**, 847 (1973).
5. L. W. Wattenberg, *J. Natl. Cancer Inst.* **48**, 1425 (19 → B. M. Ulland, J. H. Weisburger, R. S. Yammamoto, E. K. Weisburger, *Food Cosmet. Toxicol.* **11**, 199 (1973); R. B. Cumming and M. F. Walton, *ibid.* **11**, 547 (1973).
6. D. Harman, *J. Gerontol.* **23**, 476 (1968).
7. W. Snipes, S. Person, A. Keith, J. Cupp, *Science* **188**, 64 (1975).
8. The NVD LaSota strain was propagated from a commercial vaccine stock, and all other strains were obtained from the NDV Repository, University of Wisconsin, Madison.
9. A. P. Waterson, in *Newcastle Disease Virus—An Evolving Pathogen*, R. P. Hanson, Ed. (Univ. of Wisconsin Press, Madison, 1964), p. 119.
→ S. Eletr, M. A. Williams, T. Watkins, A. D. Keith, *Biochim. Biophys. Acta* **339**, 190 (1974).
11. J. P. Frawley, J. H. Kay, J. C. Calandra, *Food Cosmet. Toxicol.* **3**, 471 (1965); P. G. C. van Stratum and H. J. Vos, *ibid.*, p. 475; A. C. Francois and A. Pihet, *Ann. Zootech.* **9**, 195 (1960).
12. M. Brugh, *J. Clin. Microbiol.*, **5**, 410 (1977).
→ L. N. Drury, C. W. Beard, S. R. Hopkins, *Avian Dis.* **13**, 400 (1969).
14. C. W. Beard and W. J. Wilkes, in *Proceedings, 77th Annual Meeting of the United States Animal Health Association* (Spencer Printing Co., Richmond, Va., 1973), pp. 596–600.
15. I thank C. W. Beard and H. S. Siegel for helpful discussions and W. J. Wilkes and L. Dillard for technical assistance.

28 March 1977; revised 20 May 1977