

Effect of Alkyl-Dimethyl-Benzyl-Ammonium Chlorides (Zephiran) Upon Tetanus Toxin.

ERWIN NETER.

From the Laboratories of the Children's Hospital and the Department of Bacteriology and Immunology, University of Buffalo, School of Medicine.

Numerous organic and inorganic compounds destroy the toxic properties of bacterial exotoxins. An excellent review on this subject was presented by Eaton.¹ Certain substances reduce the toxicity without affecting the antigenicity of the toxins, whereas others destroy both of these properties. An investigation² on the possible detoxifying effects of antimicrobial substances of biological origin revealed that some of them, such as pyocyanase, detoxify tetanus toxin and others, such as tyrothricin and actinomycin A, fail to do so. In the following communication are presented the results of experiments on the action upon tetanus toxin of Zephiran chloride, a mixture of high molecular alkyl-dimethyl-benzyl-ammonium chlorides. This substance contains alkyl radicals ranging from C_8H_{17} to $C_{13}H_{27}$ whose source is a mixture of fatty acids of coconut oil.

Material and Methods. Tetanus toxin was obtained through the courtesy of Dr. Edwin F. Voigt, Director, Human Biological Division, Lederle Laboratories. This toxin, in a dilution of 1:1,000 (volume 0.2 ml), when injected subcutaneously into the hind leg of white mice, caused local tetanus within 18 hr and death within 24 to 48 hr. In a dilution of 1:10,000 it produced local tetanus after 24 to 48 hr and usually death after several days. In a dilution of 1:100,000 it caused no noticeable toxic effects.

An aqueous solution of Zephiran chloride was used. According to Mr. Charles G. Marshall, Alba Pharmaceutical Company, Inc., New York, New York (personal communication) this solution has a pH of approximately 7.1.

White mice weighing between 18 and 22 g were used.

Results. In the first experiment the effects of Zephiran chloride upon tetanus toxin in various concentrations was studied. The final concentration of Zephiran chloride was 1:2,000 and those of tetanus toxin 1:100, 1:1,000, and 1:10,000, respectively. The mixtures (volume 0.2 ml), immediately after they were prepared, were injected subcutaneously into the hind leg of mice. The animals were observed daily for 14 days for the appearance of local tetanus. The results of this experiment are presented in Table I.

It may be seen from Table I that Zephiran chloride (1:2,000) completely prevented the toxic effects of tetanus toxin in dilutions of 1:10,000 and 1:1,000. Tetanus toxin in a dilution of 1:100 mixed with Zephiran chloride (1:2,000) produced local tetanus and caused death after 14 days; however, Zephiran chloride markedly delayed the lethal effect of tetanus toxin. Essentially the same results were obtained in repeated experiments. With this particular toxin at least 10 minimal lethal doses were detoxified by 0.1 mg of Zephiran chloride. It may be mentioned that some of the mice injected with a mixture of tetanus toxin and Zephiran chloride died several days or even 2 weeks after the injection without having developed any signs of local or generalized tetanus.

A second series of experiments was carried out to determine the smallest amount of Zephiran chloride capable of abolishing the toxic effects of tetanus toxin. It was found that Zephiran chloride in a dilution of 1:2,000 completely abolished the toxic effects of 10 minimal lethal doses of tetanus toxin in mice, but in dilutions of 1:20,000 and higher it failed to affect its activity.

In order to determine whether or not this effect of Zephiran chloride upon tetanus toxin is due to the pH of the Zephiran chloride solution, the following experiments

¹ Eaton, M. D., *Bact. Rev.*, 1938, **2**, 3.

² Neter, E., *Science*, 1942, **96**, 209.

TABLE I.
Effect of Alkyl-dimethyl-benzyl-ammonium Chlorides (Zephiran) upon Tetanus Toxin Injected into Mice.

Days of observation	Zephiran (1/2000) plus tetanus toxin in dilutions of			Physiological salt solution plus tetanus toxin in dilutions of		
	1/102	1/103	1/104	1/102	1/103	1/104
1	—	—	—	D	++++	—
2	+	—	—		D	++++
3	++++	—	—			++++
4	++++	—	—			++++
5	++++	—	—			++++
8	++++	—	—			++++
10	++++	—	—			D
14	D	—	—			

— = No signs of local tetanus.

+ to ++++ = Various degrees of local tetanus.

D = Death.

were performed. Zephiran chloride was dissolved in disodium phosphate-citric acid buffer solution of pH 7.0 with phenol red as indicator. The resulting pH ranged from approximately 7.0 to approximately 7.4, depending upon the concentration of Zephiran chloride. Controls revealed that tetanus toxin dissolved in buffer solutions of corresponding pH (7.0 to 7.4) produced toxic effects in mice. No marked difference in the time of appearance and severity of local tetanus were noted at this pH range. Furthermore, the experiments revealed that Zephiran chloride markedly reduced the toxic effects of tetanus toxin in buffer solutions of pH 7.0 to 7.4. It may be concluded, therefore, that under the conditions of the experiment the effects of Zephiran chloride upon tetanus toxin are independent of any changes due to influences of the pH of the solution.

Tissue fluids interfere with the activity of many antimicrobial agents. It is of interest, therefore, to see whether serum prevents the detoxifying effects of Zephiran chloride. Human serum (0.1 ml) was mixed with Zephiran chloride prior to the addition of tetanus toxin. This mixture caused local tetanus and death of the mice, indicating that serum inhibited the detoxifying action of Zephiran chloride. Quantitative experiments showed that human serum in dilutions up to 1:100 almost completely prevented the toxin inhibiting activity of Zephiran chloride. Serum in a dilution of 1:1,000 failed to do so.

On the other hand, it is interesting to note that human serum added to a mixture of tetanus toxin and Zephiran chloride did not interfere to any great extent with the detoxifying effects of this compound. The results of this experiment indicate, furthermore, that Zephiran chloride rather rapidly detoxifies tetanus toxin and that this change is not readily reversible.

Discussion. High molecular alkyl-dimethyl-benzyl-ammonium chlorides (Zephiran chloride) possess marked germicidal and bacteriostatic potency. This antimicrobial agent also prevents the toxic effects of tetanus toxin in mice. Ten minimal lethal doses of tetanus toxin were detoxified by 0.1 mg of Zephiran chloride. It has not as yet been determined whether Zephiran chloride interferes solely with the toxic properties of the toxin or affects also its antigenic pattern. Furthermore, the mode of action of this substance upon tetanus toxin remains to be elucidated, particularly in regard to the question whether it causes strictly chemical changes or primarily physical effects. Formaldehyde, for instance, detoxifies by chemical action, whereas lanolin probably acts on the basis of adsorption on colloidal particles (Ramon and associates).³

It is of interest to mention that the salts of fatty acids, beginning with those containing 8 carbon atoms in the chain, cause a de-

³ Ramon, G., Lematayer, E., Richou, R., and Nicol, L., *Rev. Immunol.*, 1937, **3**, 285.

struction of diphtheria toxin. Larson and associates⁴⁻⁶ found that the sodium salt of ricinoleic acid destroys the toxic properties of diphtheria toxin and Schmidt⁷ reported that this substance changes its antigenic properties as well. According to Schmidt⁷ not all unsaturated fatty acids destroy bacterial exotoxins; for instance, fumaric acid and maleic acid do not have this effect.

From a practical point of view it seems desirable to have available for the prevention or treatment of infections caused by toxin-producing bacteria antimicrobial compounds of marked effectiveness and low toxicity which, moreover, may be destructive to tox-

ins. Zephiran chloride in concentrations which are bacteriostatic or bactericidal *in vitro* and *in vivo* inhibits the toxic effects of tetanus toxin. The inactivation of this toxin takes place almost immediately. However, it must be emphasized that presence of serum definitely interferes with this inactivation. The possible effects of antimicrobial agents upon the activity of toxins deserves further study.

Summary. (1) Alkyl-dimethyl-benzyl-ammonium chlorides (Zephiran chloride) in a concentration of 1:2,000 inactivates tetanus toxin. This effect takes place almost immediately. Zephiran chloride in a dilution of 1:20,000 fails to do so. (2) Human serum mixed with Zephiran chloride prior to the addition of tetanus toxin markedly interferes with the inactivation of toxin by this compound. Addition of serum to a mixture of Zephiran chloride and tetanus toxin does not reactivate the toxin.

⁴ Larson, W. P., and Nelson, E., *PROC. SOC. EXP. BIOL. AND MED.*, 1923-24, **21**, 278.

⁵ Larson, W. P., Evans, R. D., and Nelson, E., *PROC. SOC. EXP. BIOL. AND MED.*, 1924-25, **22**, 194.

⁶ Larson, W. P., and Halvorson, H. O., *PROC. SOC. EXP. BIOL. AND MED.*, 1924-25, **22**, 550.

⁷ Schmidt, S., *Bioch. Z.*, 1932, **256**, 158.

13924

Effects of Alkyl-Dimethyl-Benzyl-Ammonium Chlorides upon Plasma-Coagulation by Staphylococcus and Fibrinolysis by Streptococcus.

ERWIN NETER.

From the Laboratories of the Children's Hospital and the Department of Bacteriology and Immunology, University of Buffalo, School of Medicine.

Certain surgical antiseptics and chemotherapeutic substances inhibit *in vitro* streptococcal fibrinolysis and coagulation of plasma by staphylococcus. Madison and Snow¹ showed that tincture of iodine (1:3,000) renders fibrin but one-eighth its normal susceptibility to liquefaction by streptococci. Other substances such as merthiolate (1%), phenol (2%), and mercuric chloride (1%) did not affect fibrinolysis, nor did sulfanilamide (10%) and pron-tosil (2.5%). Spink and Vivino² observed that sulfadiazine inhibits the plasma-clotting

effect of staphylocoagulase; in contrast, sulfathiazole and, to a slightly lesser extent, sulfanilamide accelerated clot formation. Recently it was shown³ that two antimicrobial agents of biological origin, namely, tyrothricin and actinomycin A, inhibit both coagulation of plasma by staphylococcus and lysis of plasma clots by group A *beta hemolytic streptococcus*. Alkyl-dimethyl-benzyl-ammonium chlorides (Zephiran chloride), a disinfectant of marked antimicrobial activity, inhibits the toxic effects of tetanus toxin.⁴ It seemed of interest to determine what

¹ Madison, R. R., and Snow, J. E., *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **36**, 522.

² Spink, W. W., and Vivino, J. J., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **50**, 37.

³ Neter, E., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **49**, 163, 167.

⁴ Neter, E., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **51**, 254.