[CONTRIBUTION FROM THE CHEMICAL CORPS, FORT DETRICK]

The Reaction of Botulinum Toxin Type A with Ketene[1]

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When botulinum toxin, type A, was exposed to ketene a rapid inactivation took place which followed first-order kinetics. The inactivation was accompanied by a small but continuous acetylation of free amino groups. Some O-acetylation was evident but it was irregular and was not directly correlated with the decrease in toxicity. Toxin, completely inactivated by ketene, was ineffective in immunizing mice against the toxin.

The toxin produced by *Clostridium botulinum*, type A, has been isolated as a crystalline protein and found to be homogeneous in electrophoretic and ultracentrifugal analysis.[2,3] A study of the composition of the toxin[4] showed it to be a simple protein made up of nineteen L-amino acids linked through normal peptide bonds with no evidence of a prosthetic group. Presumably, therefore, the toxicity must be due to the structural configuration within the molecule.

The reaction of ketene with many proteins has been studied by a number of investigators,[5] and information has been obtained on the relation of certain reactive groups within protein molecules to biological activity. Most work has centered on the reaction of ketene with free amino groups and with phenolic hydroxyl groups; the reaction with sulfhydryl groups has been followed to a lesser extent. This report presents evidence that ketene inactivates botulinum toxin by a reaction with a relatively small number of free amino groups.

Results

Exposure of the toxin to ketene brought about a rapid fall in toxicity. The results of a typical experiment are shown in Fig. 1. A plot of the logarithm of the toxicity against the time of ketene treatment pointed out a straight line relationship for almost a three log or 99.8% loss in toxicity. The reaction therefore appears to be pseudo-unimolecular. The change in rate of inactivation during the last part of the exposure period may be attributed to a drop in *p*H which occurred at the same time, since the rate of acetylation decreases as the *p*H is lowered.[6] In these experiments it was necessary to introduce the ketene above the surface of the liquid, instead of bubbling it through the solution as is usually done, in order to prevent surface denaturation of the toxin. Bubbling air or even an inert gas such as nitrogen through a solution of the toxin caused a rapid inactivation accompanied by the appearance of a large amount of insoluble protein.[7] The first-order kinetics observed here were best obtained with small volumes of solution in which the surface could be effectively renewed.

The decrease in toxicity and free amino nitrogen brought about by acetylation with ketene is shown in Table I. It is evident that relatively small changes in amino nitrogen occurred as the toxin was inactivated; thus at 2.5 minutes 43% of the activity was lost when 5% of the amino groups had reacted, and at 5 minutes when 98% of the activity had disappeared, 19% of the amino groups had reacted. A plot of the logarithm of the free amino nitrogen against time yielded a straight line demonstrating a continuous and constant acetylation throughout the reaction; in addition, plotting the fall in toxicity against the fall in free amino nitrogen demonstrated a direct correlation between the two effects. The rate of decrease of toxicity with time of exposure would appear to be much slower than in the experiment shown in Fig. 1. The difference is due to the much greater volume of toxin reacting in this experiment (48-fold) with equal rates of ketene supply. The greater volumes were necessitated in order to have sufficient material for the amino nitrogen determinations.

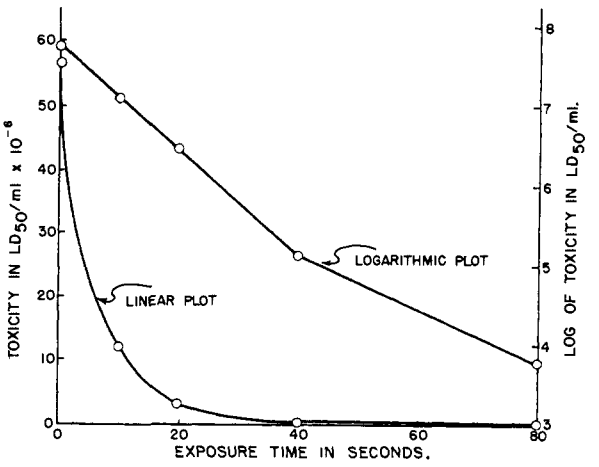

Fig. 1.—The effect of ketene on the toxicity of botulinum toxin, type A. The gas was introduced over the surface of 0.25-ml. samples of toxin in 2 cm. diameter vials.

The extent of O-acetylation brought about by ketene inactivation of the toxin is shown in Table II. The data indicated no reaction during the disappearance of one-third of the toxicity but between a disappearance of one-third and two-thirds some O-acetylation became evident. The tryptophan equivalents in micrograms per mg. of toxin

(1) Presented, in part, before the Division of Biological Chemistry, 128th Meeting, American Chemical Society, Minneapolis, Minn., September, 1955.

(2) C. Lamanna, O. E. McElroy and H. W. Eklund, *Science*, **103**, 613 (1946).

(3) A. Abrams, G. Kegeles and G. A. Hottle, *J. Biol. Chem.*, **164**, 63 (1946).

(4) H. J. Buehler, E. J. Schantz and C. Lamanna, *ibid.*, **169**, 295 (1947).

(5) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Revs.*, **41**, 151 (1947); R. M. Herriott, *Advances in Protein Chem.*, **3**, 169 (1947).

(6) R. M. Herriott and J. H. Northrop, *J. Gen. Physiol.*, **18**, 35 (1934).

(7) L. Spero and E. J. Schantz, unpublished.

TABLE I

THE DECREASE IN TOXICITY AND AMINO NITROGEN OF BOTULINUM TOXIN TREATED WITH KETENE[a]

Exposure time, min.	Toxicity, LD_{50}/ml.	Free amino N.[b] γ/mg. toxin
0	44×10^6	8.33
1.0	40×10^6	8.48
2.5	25×10^6	7.91
5.0	12×10^6	7.44
10.0	1.05×10^6	6.75

[a] In this experiment, 12-ml. samples were exposed to ketene gas. [b] A subsequent determination of the true value of free amino nitrogen (H. A. Rutherford, M. Harris and A. L. Smith, *J. Research Natl. Bur. Standards*, 19, 467 (1937)), that is α-NH_2 and the ε-NH_2 of lysine, gave a figure of 7.78 γ/mg. of toxin, so that the values in the table are a little high. The error is due to the partial reaction of the guanidino group of arginine, but it should have been constant for all the determinations and, therefore, should not affect the interpretation of the data.

dropped from about 44 to 37 and remained there throughout the experiment. The results of several other experiments showed considerable variation in the amount and time of O-acetylation, but as in the experiment cited here there was no evidence of a direct correlation with the decrease in toxicity.

TABLE II

THE O-ACETYLATION AND ACCOMPANYING LOSS IN TOXICITY OF BOTULINUM TOXIN BROUGHT ABOUT BY KETENE[a]

Exposure time, min.	Toxicity, LD_{50}/ml.	Tryptophan equiv., γ/mg. toxin
0	60×10^6	43.5
0.5	$>50 \times 10^6$	44.6
1.0	40×10^6	44.5
1.5	20×10^6	37.0
2.0	13×10^6	37.7
3.0	$<2.5 \times 10^6$	37.4

[a] In this experiment, 4-ml. samples were exposed to ketene gas.

An independent measure of whether the sulfhydryl groups were involved in the ketene inactivation of the toxin was made by observing the effect of *p*-chloromercuribenzoic acid upon the toxicity of botulinum toxin. In eight experiments in four separate runs, an average reduction in toxicity of 32% was found in the presence of at least 100 moles of reagent per mole of toxin. This effect was significant statistically but the magnitude was far less than that observed in the inactivation with ketene. The inactivation with *p*-chloromercuribenzoic acid may not have been specific since it was only partially prevented by cysteine. Furthermore, another SH reagent, iodosobenzoic acid, had no effect whatever on the toxicity.

The combining power of ketene-treated toxin with specific antiserum is presented in Table III. A loss of two-thirds of the toxicity caused no change in flocculation with the antitoxin and even a loss of 99% of the toxicity did not decrease the number of flocculating units although it did retard the rate of flocculation. This suggests that ketene might be useful as a toxoid-producing reagent, but it is necessary for safety purposes that an immunizing antigen be non-toxic; the 99% inactivated material still had 1.1×10^5 LD_{50} per ml. Accordingly, an almost completely inactivated sample was prepared by repeated ketene treatment. This preparation did not flocculate with antitoxin and was ineffective as a toxoid. It provided no protection against as small a challenge dose as 10 LD_{50}. A formalinized toxoid of the same concentration protected against 4×10^4 LD_{50}.[8]

TABLE III

THE EFFECT OF KETENE ON THE COMBINING POWER OF BOTULINUM TOXIN WITH ANTITOXIN

Sample of toxin	Toxicity, LD_{50}/ml.	Flocculation Lf/ml.	Flocculation Kf, min.
Control	11×10^6	27	14
Ketene treated	4.0×10^6	26–33	12
Control	11×10^5	21[a]	10
Ketene treated	1.1×10^5	21[a]	18

[a] A different batch of antitoxin.

Discussion

The groups in proteins with which ketene usually reacts significantly are the free amino, phenolic hydroxyl and sulfhydryl groups.[9] The relatively minor loss in toxicity of botulinum toxin treated with *p*-chloromercuribenzoic acid serves to eliminate sulfhydryl groups as a primary reacting group in the inactivation induced by ketene. Staudinger[10] states that primary amines react faster than phenols and this observation has been generally found to apply to proteins[5]; furthermore, in those proteins in which phenolic hydroxyl groups were necessary for activity there was a considerable time lag during exposure to ketene before inactivation became apparent.

The extremely rapid inactivation of botulinum toxin by ketene suggests, therefore, a primary reaction with amino groups; the amino nitrogen and phenol determinations support this interpretation. The reduction in free amino groups was continuous throughout the inactivation, whereas there was no measurable O-acetylation until a considerable detoxification had taken place. It is hypothesized that the essential groups are the most reactive to ketene, that they are few in number and that either the α-amino groups or a few of the lysine ε-amino groups are involved.

One factor, however, prompts a cautious use of the analytical data. If a very small number of phenolic hydroxyl groups are essential in each molecule, O-acetylation of these groups might go undetected in the chemical determination. The accuracy of the analytical method restricts the number of undetectable groups to a maximum of two in a toxic sub-unit of 70,000 molecular weight[11] containing about fifty tyrosine residues.

(8) G. A. Hottle, C. Nigg and J. A. Lichty, *J. Immunol.*, **55**, 255 (1947).

(9) It should be observed at this point that the inactivation reaction of ketene may involve some other chemical function in the protein. The reactions followed in this work are certainly the primary ones in most cases but the possibility always exists that there is present in the toxin an essential grouping, usually unreactive or slowly reactive to ketene in most proteins but highly reactive in this case.

(10) H. Staudinger, "Die Ketene," F. Enke, Stuttgart, 1912, p. 34.

(11) The toxin as normally isolated has a molecular weight of approximately one million, and it has been calculated that the molecule contains 672 tyrosine residues (*cf.* ref. 4). Recent work (J. Wagman and J. B. Bateman, *Arch. Biochem. Biophys.*, **45**, 375 (1953); J. Wagman, *ibid.*, **50**, 104 (1954)) indicates that the toxic sub-unit of this molecule has a molecular weight of about 70,000, which would contain about fifty tyrosine residues.

Experimental

Materials.—The preparations of botulinum toxin, type A, used in this work were isolated by either the method of Abrams, *et al.*,[3] or by a method based on differential ultracentrifugation.[7] Only crystalline preparations homogeneous in the ultracentrifuge at *p*H 3.8, with a specific activity, when isolated, of at least 200×10^6 LD_{50} per mg. N, were used. The toxin was stored in acetate buffer at 5° and adjusted immediately before use with an appropriate phosphate buffer.

The ketene was produced by the pyrolysis of acetone in an apparatus similar to that designed by Herriott.[12] The rate of production was measured by the neutralization of standard alkali under conditions of the actual acetylations.

Toxicity.—The number of LD_{50} per ml. was determined by injecting 16–20 gram mice intraperitoneally with 0.5 ml. of toxin diluted with a sterile 1% disodium phosphate solution containing 0.2% gelatin and adjusted to *p*H 6.8. Six mice were used in each group and deaths were recorded for 96 hr. The per cent. kill was plotted against the dose on probit-log dose paper with the probit for 100% killed taken as the probit for five out of six killed plus $1/2$ probit unit and the probit of 0% killed taken as the probit of one out of six killed minus $1/2$ probit unit.[13] The best straight line was fitted by inspection and the dose corresponding to probit 5 was read off the graph.

Amino Nitrogen.—The extent of N-acetylation was ascertained by the nitrous acid amino nitrogen method of Van Slyke.[14] The reaction was carried out at 30–32° for 20 minutes in a reaction mixture containing 5 ml. of sample, 2 ml. of glacial acetic acid–saturated sodium acetate (1:1) and 2 ml. of sodium nitrite (saturated at 5°). The use of a buffered reaction, first suggested by Rutherford, *et al.*,[15] serves to decrease the volume of gas evolved and the magnitude of the blank analysis.

Phenolic Hydroxyl.—The extent of tyrosine O-acetylation was determined by Herriott's modification of the Folin phenol method at *p*H 8. The solutions were read at 750 mμ in a Beckman model DU spectrophotometer. The standard curve was made with tryptophan.

Nitrogen.—Nitrogen in protein samples was determined by micro-Kjeldahl using a mercuric oxide catalyst and a 4-hr. digestion period.

Flocculation.—One Lf unit represents the amount of toxin which gives the most rapid flocculation with one standard unit of antitoxin.[16] The Kf is the time interval for the earliest flocculation. The determination was carried out at 40° in a water-bath with 1 ml. of diluted toxin and volumes of antitoxin (100 units per ml.) varying from 0.10 to 0.41 ml. using a dilution factor of 1.25.

The Rate of Inactivation of Toxin by Ketene.—Samples of 0.25 ml. of toxin dissolved in 0.5 *M* potassium phosphate buffer at *p*H 6.6 were placed in a 2 cm. diameter vial. The outlet tube of the ketene generator was placed about $1/2$ cm. above the surface of the liquid and the sample was swirled slowly. After exposure for a predetermined period, the reaction was stopped by adding 4.75 ml. of gelatin–phosphate diluent. Under these conditions, the absorption rate of ketene into standard alkali was found to be 0.36 mmole per minute.

The Reduction in Amino Nitrogen Due to Ketene Treatment of the Toxin.—Twelve-ml. samples of toxin dissolved in 0.5 *M* potassium phosphate buffer at *p*H 6.4 were placed in 30-ml. beakers and exposed to ketene. The solutions were stirred slowly with a magnetic stirrer. Samples were withdrawn for toxicity tests immediately after exposure and amino nitrogen was determined in duplicate analyses the same day.

The Reduction in Phenolic Hydroxyl Due to Ketene Treatment of the Toxin.—Four-ml. samples of toxin dissolved in 0.5 *M* phosphate buffer at *p*H 6.5 were placed in 30-ml. beakers and exposed to ketene. The samples were swirled during exposure. Samples were withdrawn for toxicity tests and phenol determinations immediately after exposure.

The Effect of Sulfhydryl Reagents on the Activity of the Toxin.—Samples of 0.1 ml. of toxin and varying small volumes (0.025 to 0.20 ml.) of dilute solutions (1×10^{-3} to 1×10^{-5} *M*) of the reagent to be tested, usually *p*-chloromercuribenzoic acid, were added to enough 0.1 *M* phosphate buffer at *p*H 7.0 to make a final volume of 2.0 ml. The mixtures had a *p*H of 6.8–6.9. The reaction was allowed to proceed for 15 minutes at room temperature when samples were withdrawn for toxicity determinations.

The Antigenicity of Ketene Treated Botulinum Toxin.—A 2.5-ml. sample of toxin dissolved in 0.5 *M* phosphate buffer at *p*H 6.5 was exposed to ketene for two minutes. The *p*H was then adjusted back to *p*H 6.5 with 0.03 ml. of 10 *N* NaOH. Four exposures were made on the same sample with *p*H adjustment after each. The final solution had less than 40 LD_{50} per ml. compared to an original activity of 35×10^6 LD_{50} per ml. The solution was diluted to a concentration of 1×10^6 LD_{50} per ml. of original unitage and an alum precipitated toxoid was prepared according to the method of Nigg, *et al.*[17] The toxoid was tested by the constant toxoid method of Hottle, *et al.*,[8] in mice.

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(12) R. M. Herriott, *J. Gen. Physiol.*, **18**, 69 (1934).
(13) E. S. Weiss, *Am. J. Public Health*, **38**, 22 (1948).
(14) D. D. Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).
(15) *Cf.* footnote *b*, Table I.
(16) Globulin modified antitoxin, Lederle.
(17) C. Nigg, G. A. Hottle, L. L. Coriell, A. S. Rosenwald and G. W. Beveridge, *J. Immunol.*, **55**, 245 (1947).

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The Reaction of Botulinum Toxin Type A with Nitrous Acid[1]

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Botulinum toxin, type A, was inactivated rapidly by treatment with nitrous acid in the presence of excess nitrite. This reaction, presumably deamination, followed first-order kinetics. It is shown that the deamination of alanine under these conditions is also first order and that this is to be expected from the rate equation for the reaction. These results provide additional evidence for the essential nature of the free amino groups in the toxicity of this protein.

The reaction of botulinum toxin, type A, with ketene was described in the preceding paper,[2] and it was proposed on the basis of the rate of reaction and analysis of reacting groups that the free amino groups, rather than the phenolic hydroxyl groups, were involved in the inactivation. In order to obtain more definitive information on this point, the reaction of the toxin with nitrous acid has been studied. This reaction, when carried out in the presence of excess nitrite, has been proposed[3] as a means of distinguishing between these two reactive

(1) Presented, in part, before the Division of Biological Chemistry, 128th meeting, American Chemical Society, Minneapolis, Minn., September, 1955.
(2) E. J. Schantz and L. Spero, THIS JOURNAL, **79**, 1623 (1957.)
(3) J. E. Little and M. L. Caldwell, *J. Biol. Chem.*, **147**, 229 (1943).