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TECHNICAL MANUSCRIPT 242

AMINO ACID COMPOSITION  
OF CRYSTALLINE BOTULINUM TOXIN, TYPE A

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BOTULINUM TOXIN, TYPE A

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August 1965

In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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#### ABSTRACT

The amino acid composition of botulinum toxin, type A, was determined with the aid of the automatic amino acid analyzer. The results are compared with an earlier, largely microbiological analysis.

## I. INTRODUCTION

The isolation of botulinum toxin, one of the most toxic substances known, in crystalline form<sup>1,2</sup> has led to an interest in its chemical composition. An amino acid analysis of this protein was reported by this laboratory in 1947.<sup>3</sup> Since that time, improvements and innovations in the techniques of amino acid analysis have enabled more accurate and precise determinations of the amino acid compositions of proteins to be made. This work presents the amino acid composition of botulinum toxin, type A, and affords a re-examination of earlier analyses on this substance.

## II. EXPERIMENTAL PROCEDURE

### A. MATERIALS

A single batch of toxins was used for all analyses. This preparation was homogeneous in the ultracentrifuge and exhibited a specific activity by mouse intraperitoneal injection of  $2.3 \times 10^8$  LD<sub>50</sub>/mg nitroge . It was isolated by the method Duff et al.<sup>4</sup> and recrystallized twice from ammonium sulfate. The toxin was dissolved in 0.05 M sodium acetate, pH 4.2, and dialyzed exhaustively against this buffer at 5 C, until the Nessler's test for ammonium ion was negative. Solutions of the toxin in this buffer were stored at 5 C. Under these conditions the toxin is stable to prolonged storage.

### B. PREPARATION OF SAMPLE AND CALIBRATION OF THE AMINO ACID ANALYZER

The methods used to prepare the sample and calibrate the amino acid analyzer are described in a previous publication.<sup>5</sup>

### C. OTHER METHODS

Total cystine and cysteine were determined as cysteic acid after performic acid oxidation of the protein at 0 C according to the method of Moore<sup>6</sup> followed by acid hydrolysis. Cysteine was determined (i) spectrophotometrically in 10 M urea and 6 M guanidine hydrochloride with the reagent developed by Ellman,<sup>7</sup> and (ii) by alkylation with iodoacetate in 8 M urea. Tryptophan was determined by the method of Spies and Chambers.<sup>8</sup> Ammonia was determined by (i) extrapolation of the ammonia liberated to zero hydrolysis time, and (ii) by the microdiffusion method of Stegemann.<sup>9</sup>



Total nitrogen was determined by micro-Kjeldahl. The analyses were conducted both on lyophilized preparations and on the stock solution from which samples were drawn for hydrolysis. Sulfur was determined by the method of Alicino.<sup>10</sup>

#### D. CALCULATIONS

Three separate analyses were performed. Nearest decimal numbers for the amino acid residues were obtained for 100,000 g, or approximately one-ninth of the molecular weight of the crystalline protein as determined by physical methods.<sup>11</sup> These values in most cases are obviously beyond the accuracy of the method and are reported in this manner for computational reference.

### III. RESULTS

The nitrogen content of the toxin was redetermined and found to be 16.08%. The protein was dissolved in phosphate buffer on the alkaline side of its isoelectric point and was dialyzed exhaustively against water. The solution was lyophilized and then dried to constant weight in vacuo at 25 C over P<sub>2</sub>O<sub>5</sub>. Attempts to determine nitrogen content from solutions in acetate buffer gave consistently lower values, suggesting that acetate is bound to the molecule firmly enough to resist removal by dialysis. The higher N previously reported is probably due to the difficulty of obtaining a completely satisfactory determination of the dry weight. Drying at 100 C may have led to some decomposition.

The sulfur content was 0.55% and phosphorus was 0.05%. The  $E_1^{1\%}$  at 278 m $\mu$  was found to be 16.6.

The results of the amino acid analyses are presented in Table 1. Each figure represents the average of three analyses. Each analysis was performed on 24-, 48-, 72-, and 96-hour hydrolyzates run singly.

Serine and threonine were calculated by extrapolation to zero time. The data gave a superior fit to the first-order decay when plotted by the method of least squares, and this was used in preference to zero order.

Tyrosine values also decreased with time, but the data were too scattered to permit an extrapolation and the figures for 24-hour hydrolyzates were therefore selected. The valine and isoleucine values increased with hydrolysis and peak values were selected for these two amino acids. The values for all other amino acids were essentially constant.

TABLE 1. AMINO ACID COMPOSITION OF BOTULINUM TOXIN

Amino Acid	Amino Acid Residues, g/100 g protein	Nitrogen, g/100 g protein	Number of Residues per 10 <sup>6</sup> g protein	Amino Acid Residues, g/100 g protein (lit. value) <sup>a/</sup>	Number of Residues per 10 <sup>5</sup> g protein (lit. value) <sup>a/</sup>
Lysine	6.65±0.12	1.45	51.9	6.78	52.9
Histidine	0.89±0.02	0.27	6.5	0.91	6.6
Arginine	3.69±0.04	1.32	23.6	4.14	26.5
Aspartic Acid	18.41±0.13	2.24	159.9	17.34	150.7
Threonine	5.27±0.12	0.73	52.1	7.19	71.1
Serine	5.42±0.12	0.87	62.2	3.60	41.3
Glutamic Acid	9.81±0.10	1.06	76.0	13.67	105.9
Proline	2.58±0.10	0.37	26.6	2.19	27.1
Glycine	1.97±0.03	0.48	34.5	1.05	18.4
Alanine	2.35±0.03	0.46	33.0	3.12	43.9
Half-cystine	0.74 - b/	0.10	7.3	0.45	4.5
Cysteine	0.39 - c/	0.05	3.8	0.23	2.2
Valine	4.34±0.04	0.61	43.8	4.45	44.9
Methionine	1.14±0.01	0.12	8.7	0.93	7.1
Isoleucine	8.98±0.04	1.25	79.3	10.33	91.3
Leucine	8.40±0.07	1.04	74.2	8.91	78.7
Tyrosine	8.87±0.17	0.76	54.4	12.18	74.6
Phenylalanine	5.59±0.12	0.53	38.0	1.04	7.1
Tryptophan	2.46	0.37	13.2	1.69	9.1
Amide groups	2.03±0.09d/	1.78	126.8d/	2.43	152.1
TOTAL	97.95	15.86	849.0		

a. Calculated from column 5 of Table 1 of Buehler, et al.<sup>3</sup>  
 b. Difference between total cystine and cysteine determined as cysteic acid and cysteine determined directly.  
 c. Determined by alkylation with iodoacetate in guanidine.  
 d. Not included in total.

The content of tryptophan is significantly higher than that found by Boroff et al.<sup>1,2</sup> by the same method. The discrepancy between the two laboratories has not yet been resolved.

Excellent agreement was attained between the two methods used to determine ammonia. Linear extrapolation to zero time by the method of least squares of the ammonium liberated during hydrolysis gave 126 amide residues per  $10^5$  g of protein, and the diffusion method gave 127 residues.

#### IV. DISCUSSION

Botulinum toxin, type A, was crystallized by two groups working independently in 1945.<sup>1,2</sup> The material was homogeneous in the ultracentrifuge and in electrophoresis and had a molecular weight of approximately 900,000.<sup>11</sup> A complete amino acid analysis was carried out at that time by Buehler et al.<sup>3</sup> by the techniques then available. These were largely microbiological, with a few of the analyses being chemical and spectrophotometric. The present analysis has been carried out on crystalline material prepared by a different method<sup>4</sup> but with essentially the same physical, biological, and immunological properties. The results tend to confirm the earlier analysis. Some of the amino acids, particularly phenylalanine and glycine, show striking differences, but for the most part the errors may be attributed to inherent inaccuracies in the older methodology. In addition crystalline toxin isolated from a nonsporulating strain has been analyzed by present techniques with no differences detectable.

It was quickly recognized, however, that this high molecular weight material did not represent a single, simple molecule. Lamanna showed in 1948<sup>13</sup> that the material possessed hemagglutinating activity, and he was later able to demonstrate<sup>14,15</sup> that the hemagglutinating and neurotoxic activities were separable. He also stated that adsorption of the hemagglutinin caused the rest of the molecule to dissociate into fragments of low molecular weight, as evidenced by an increased diffusion coefficient.

The first physical evidence of dissociation of the toxin was obtained by Wagman and Bateman.<sup>16-18</sup> They found that at a pH 7.5 some of the protein separated as a component with a sedimentation constant of 7.0. At ionic strength 0.13 the fraction formed was quite polydisperse, with an estimated molecular weight range of 40,000 to 100,000. One isolated fraction (4.7S) was estimated by sedimentation and diffusion to have a molecular weight of 71,000. These dissociated components had about the same specific activity as the starting material, but a low molecular weight fraction formed at ionic strength 1.0 (7.0S) was reported to have a specific toxicity 2 or 3 times that of the original sample. All of these fractions reaggregated to high molecular weight, often insoluble, substances when the pH was lowered to the acid side of the isoelectric point.

In a later study, Wagman<sup>19</sup> demonstrated that exposure to more strenuous conditions — pH 9.2 at an ionic strength of 1.0 — led to the formation of a material of 158,000 molecular weight (7.0S). The 158,000 fragment's specific activity was slightly reduced from the original but it had the attractive property of not reaggregating when the pH was lowered to 3.8, thus making it more amenable to physicochemical characterization.

The possibility of even smaller subunits in the crystalline material was evoked by diffusion studies in agar gel.<sup>20</sup> A diffusion coefficient of  $7.5 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$  was obtained in phosphate buffer, pH 6.8, containing 1% gelatin, and it was suggested by one of us (E.J.S.)<sup>21</sup> that a fragment in the 10,000 to 20,000 range was involved. Very recently a report from Dolman's laboratory<sup>22</sup> described the isolation in good percentage yield of an active botulinum toxin fragment with a molecular weight of 12,200. It was prepared from the original culture fluid by a procedure that is said to "avoid or inhibit the marked polymerizing potentialities of the molecule." It is not yet clear whether this small material is actually formed at this molecular level in the culture or is a dissociated product formed by the experimental techniques used in its purification.

It is abundantly clear, however, that the crystalline toxin is an aggregate of subunits of considerably smaller molecular size. The size of these subunits and the extent of dissociation depends upon the conditions employed for their formation. The smallest unit present may well be a 12,200 component, but it is quite likely that there are other moieties present in the aggregated macromolecule. Evidence for this comes from at least three avenues of investigation: (i) the presence of a hemagglutinating material in the crystalline toxin; (ii) the increased specific activity obtained in one of the isolated fragments; and (iii) the present amino acid analysis. The smallest repeating unit containing histidine is 15,400, the smallest containing cystine is 27,400, and the smallest containing cysteine is 26,700. No amino acid analyses have been reported on any of the fractions obtained, neither Wagman's 7.0S or Dolman's 0.93S material. A comparison of these with the present analysis should give a definitive answer to this question and provide information on the make-up of the high molecular weight aggregate. Present studies in this laboratory center around the characterization of the 158,000 molecular weight material, and attempts to prepare the smallest fragment contained in the aggregated material without rupture of covalent bonds.

LITERATURE CITED

1. Lamanna, C.; Eklund, H.W.; McElroy, D.E. 1946. The purification and crystallization of Clostridium botulinum type A toxin. *Science* 103:613-614.
2. Abrams, A.; Kegeles, G.; Hottle, G.A. 1946. The purification of toxin from Clostridium botulinum type A. *Biol. Chem.* 164:63-79.
3. Buehler, H.J.; Schantz, E.J.; Lamanna, C. 1947. The elemental and amino acid composition of crystalline Clostridium botulinum type A toxin. *Biol. Chem.* 169:295-302.
4. Duff, J.T.; Wright, G.G.; Klerer, J.; Moore, D.E.; Bibler, R.H. 1957. Studies on immunity to toxins of Clostridium botulinum: I. A simplified procedure for isolation of type A toxin. *J. Bacteriol.* 73:42-47.
5. Spero, L.; Stefanye, D.; Brecher, P.I.; Jacoby, H.M.; Dalidowicz, J.E.; Schantz, E.J. 1965. Amino acid composition and terminal amino acids of staphylococcal enterotoxin B. *Biochemistry* 4:1024-1030.
6. Moore, S. 1963. On the determination of cystine as cysteic acid. *J. Biol. Chem.* 238:235-237.
7. Ellman, G.L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82:70-77.
8. Spies, J.R.; Chambers, D.C. 1949. Chemical determination of tryptophan in proteins. *Anal. Chem.* 21:1249-1266.
9. Stegemann, H. 1958. Eine Mikrobestimmung von Amid-Stickstoff, speziell in Proteinen. *Z. Physiol. Chem.* 312:255-263.
10. Alicino, J.F. 1958. The determination of sulfur in organic compounds. *Microchem. J.* 2:83-90.
11. Putman, F.W.; Lamanna, C.; Sharp, P.G. 1948. Physicochemical properties of crystalline Clostridium botulinum type A toxin. *J. Biol. Chem.* 176:401-412.
12. Boroff, D.A.; DasGupta, B.R. 1964. Study of the toxin of Clostridium botulinum: VII. Relation of tryptophan to the biological activity of the toxin. *J. Biol. Chem.* 239:3694-3698.

13. Lamanna, C. 1948. Hemagglutination by botulinal toxin. Proc. Soc. Exptl. Biol. Med. 69:332-336.
14. Lamanna, C.; Lowenthal, J.P. 1951. The lack of identity between hemagglutinin and the toxin of type A botulinal organism. J. Bacteriol. 61:751-752.
15. Lowenthal, J.P.; Lamanna, C. 1951. Factors affecting the botulinal hemagglutination reaction, and the relationship between hemagglutinating activity and toxicity of toxin preparations. J. Hyg. 54:342-353.
16. Wagman, J.; Bateman, J.B. 1951. The behavior of the botulinus toxin in the ultracentrifuge. Arch. Biochem. Biophys. 31:424-430.
17. Wagman, J.; Bateman, J.B. 1953. Botulinum type A toxin: Properties of a toxic dissociation product. Arch. Biochem. Biophys. 45:375-383.
18. Wagman, J. 1954. Isolation and sedimentation study of low molecular weight forms of type A botulinus toxin. Arch. Biochem. Biophys. 50:104-112.
19. Wagman, J. 1963. Low molecular weight forms of type A botulinum toxin: II. Action of pepsin on intact and dissociated toxin. Arch. Biochem. Biophys. 100:414-421.
20. Schantz, E.J.; Lauffer, M.A. 1962. Diffusion measurements in agar gel. Biochemistry 1:658-663.
21. Schantz, E.J. 1964. Purification and characterization of C. botulinum toxins, p. 91-102. In K.H. Lewis and K. Cassel, Jr. (Ed.) Botulism, Proceedings of a Symposium. Public Health Service Publication 999-FP-1, Public Health Service, Cincinnati.
22. Gerwing, J.; Dolman, C.E.; Bains, H.S. 1965. Isolation and characterization of a toxic moiety of low molecular weight from Clostridium botulinum type A. J. Bacteriol. 89:1383-1386.

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