4 4 9 7 2 8

TECHNICAL MANUSCRIPT 150

AMINO ACID COMPOSITION AND TERMINAL AMINO ACIDS OF STAPHYLOCOCCAL ENTEROTOXIN B

AUGUST 1964

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

FOR ERRATA

AD 449728

THE FOLLOWING PAGES ARE CHANGES

TO BASIC DOCUMENT

ERRATA SHEET

SUBJECT REPORT: Technical Manuscript 150
"Amino Acid Composition and Terminal Amino Acids of Staphylococcal Enterotoxin B

DATE: August 1964

CORRECTION: In place of the Project Number on Page 1 please substitute the following:

Project 1C533001D16401

AD 449728

END CHANGE PAGES

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 150

AMINO ACID COMPOSITION AND TERMINAL AMINO ACIDS OF STAPHYLOCOCCAL ENTEROTOXIN B

Leonard Spero

David Stefanye

Peter I. Brecher

Henry M. Jacoby

Edward J. Schantz

Physical Sciences Division
DIRECTOR OF BIOLOGICAL RESEARCH

Project 1C522301A082 August 1964

ABSTRACT

The amino acid composition of purified staphylococcal enterotoxin B was determined by means of an automatic amino acid analyzer. All of the naturally occurring amino acids were found with no indication of any unusual amino acids. Extraordinarily high values of aspartic acid, glutamic acid and lysine were found, but a high amide nitrogen accounted for the alkaline isoelectric point. Isoionic protein with a pH of 8.55 was prepared by passing the toxin through a mixed bed ion exchange column. Glutamic acid was identified as the N-terminal amino acid through the fluorodinitrobenzene procedure. The Edman procedure confirmed this and showed that the residue was present in the protein as the free acid and not as glutamine. The C-terminal amino acid was found by means of hydrazinolysis to be lysine. Quantitative estimates showed 1.10 residue per mole of protein of N-terminal amino acid and 0.73 mole per mole of protein for the C-terminal residue. All results thus far obtained are consistent with the representation of the toxin as a single polypeptide chain.

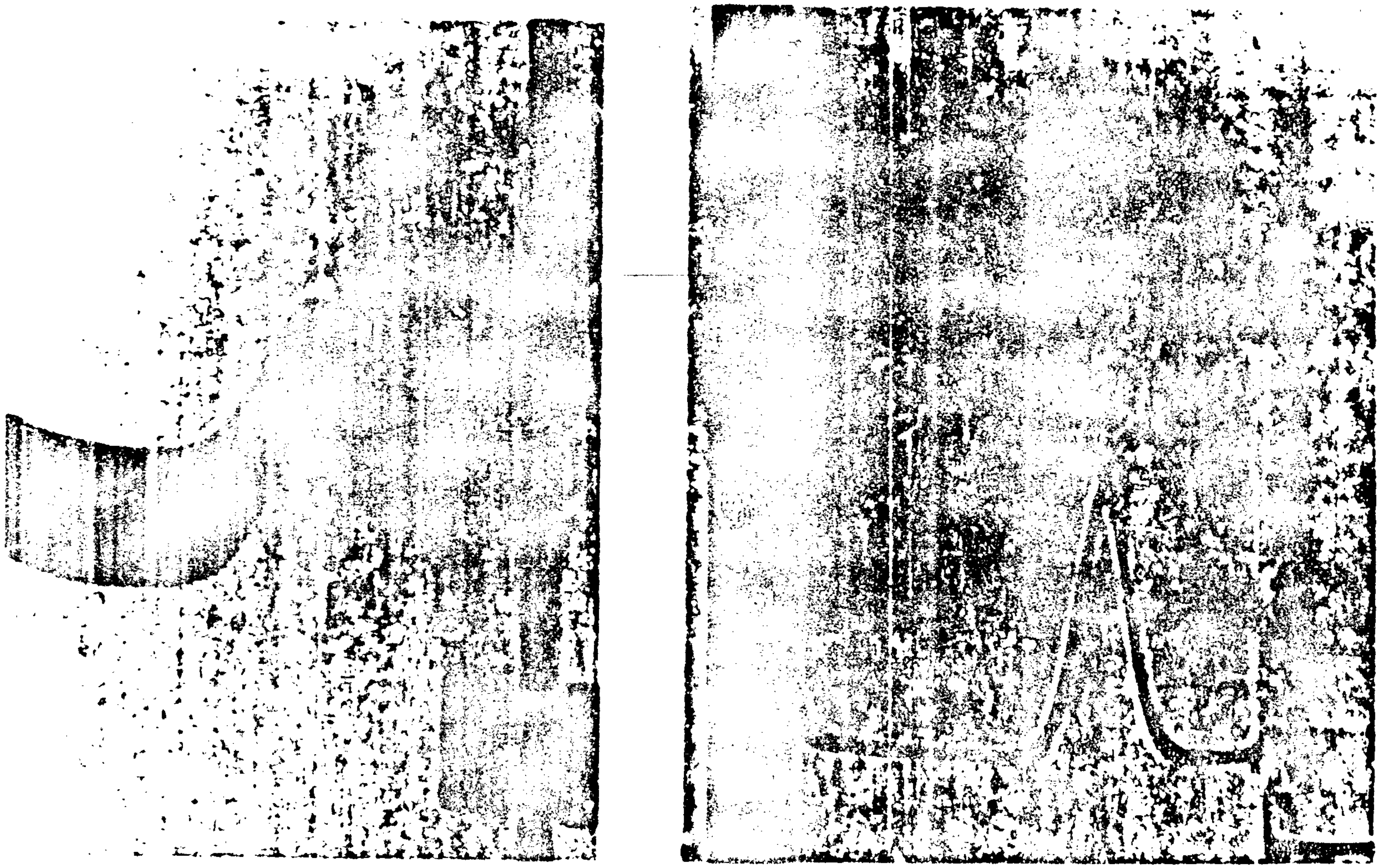
The staphylococci produce, in addition to several hemolytic toxins, a number of other active materials; included in these are the enterotoxins--the substances that cause emesis and diarrhea in food poisoning. In 1959 Bergdoll, Sugiyama, and Dack[1] reported a method for purifying staphylococcal enterotoxin B. Their product contained only one antigenic component and showed a single peak in electrophoresis and in the ultracentrifuge, but the method permitted the isolation of only milligram quantities of the toxin. A method recently developed in our laboratories[2] permits the isolation of larger amounts of this material. Its serological properties are equal to those of the earlier method, and it is more homogeneous. This is demonstrated in Figure 1 which presents a comparison of the sedimentation diagrams of the toxin as purified by Bergdoll and by the present method. The molecular weight of our material has been determined by both sedimentation-diffusion and the Archibald technique by Dr. Wagman[3] of our laboratories and found to be 35,000.

We have subjected this protein to amino acid analyses according to the procedure of Spackman, Stein and Moore[4] on an automatic amino acid analyzer. The sum of the amino acid residues on a weight percentage basis accounted for all the mass of the protein. There was no evidence on the chromatographic traces of any unusual amino acids. In most instances, the analysis is similar to results obtained by Bergdoll[5] on his purified material. His analyses were microbiological and spectrophotometric.

Table I presents the composition of the toxin for all the neutral amino acids except those containing sulfur. Tryptophan was determined by the spectrophotofluorometric method of Duggan and Udenfriend[6] and the N-bromosuccinimide titration method of Peters.[7] Probably the most noteworthy point here is the low level of tryptophan.

TABLE I. NEUTRAL AMINO ACIDS OF STAPHYLOCOCCAL ENTEROTOXIN B

Constituent	Residues per Molecule to Nearest Integer
Alanine	7
Glycine	12
Isoleucine	11
Leucine	20
Phenylalanine	15
Proline	8
Serine	17
Threonine	16
Tryptophan	2
Valine	20



Bergdoll Method

Schantz Method

Figure 1. Comparison of Sedimentation Diagrams of Enterotoxin by Two Different Procedures.

Table II shows the distribution of sulfur containing amino acids. No cysteine was found either as the S-carboxymethyl derivative[8] or by means of the p-chloromercuribenzoate method of Boyer.[9] Cystine was determined as cysteic acid after oxidation by performic acid[10] and as the S-carboxymethyl derivative after reduction with mercaptoethanol.[11] Two residues of half-cystine were found by both methods. All the sulfur in the protein is apparently accounted for by its cystine and methionine content.

TABLE II. SULFUR CONTAINING AMINO ACIDS OF STAPHYLOCOCCAL ENTEROTOXIN B

Constituent	Residues per Molecule to Nearest Integer
Cysteine	0
Half-cystine	2
Methionine	10
Sulfur	12

Table III shows the ionizable amino acids of the enterotoxin and two points should be made here. First, the sum of these amino acids constitutes more than half of the total residues in the molecule. Secondly, despite the high concentration of aspartic and glutamic acids the protein is basic, and this, of course, is due to the high amide value. Because there is an excess of 8 basic groups in the molecule, all the histidine residues and two of the lysine residues must be titrated to achieve electrical neutrality. Assuming a pK of 10[12] for the lysine residues, the isoionic point should be 8.70. This checks very well with the experimental value. We prepared the isoionic protein by passing it through a column of mixed bed Amberlite resin, MB1. This removed the six to seven moles of phosphorus as phosphate that were bound to the purified isolated material. The isoionic pH of a 1.3 per cent solution was 8.55.

The N-terminal amino acid was determined by the fluorodinitrobenzene technique. The dinitrophenyl (DNP) amino acid was separated first by the two-dimensional system of Levy.[13] The resulting chromatogram demonstrated, besides the usual spots of dinitrophenol and dinitroaniline, only one DNP amino acid; it was either DNP-aspartic or DNP-glutamic acid, which coincide in this system. Final identification was made by the Blackburn and Lowther[14] system of t-amyl alcohol on phthalate-buffered

TABLE III. IONIZABLE AMINO ACIDS OF STAPHYLOCOCCAL ENTEROTOXIN B

Constituent	Residues per Molecule to Nearest Integer
Aspartic acid	55
Glutamic acid	26
Tyrosine	24
Amide	35
Arginine	6
Histidine	6
Lysine	42

paper, which demonstrated that the amino acid was glutamic acid. The aqueous layer has been tested for DNP-arginine with negative results. The ether extracts made in the purification of the DNP-protein were examined for premature release of amino acids with negative results, and a short hydrolysis in concentrated hydrochloric acid to test for the presence of DNP-glycine and DNP-proline was also negative. For quantitative estimation of the N-terminal glutamic acid we used the one-dimensional Blackburn and Lowther system. A destruction coefficient was determined by concurrent hydrolyses of the DNP-protein alone and the DNP-protein with added amounts of DNP-glutamic acid.

Table IV shows the results obtained. The recovery of added DNP-glutamic acid was 73.6 per cent and in addition it has been assumed in these calculations that complete dinitrophenylation took place. There is, then, one N-terminal residue per mole.

In order to determine whether the glutamic acid was present in the molecule as the free acid or as glutamine we resorted to Fraenkel-Conrat's paper strip modification of the phenylthiohydantoin (PTH) technique.[15] Separation of the PTHs was carried out on starch-treated paper in the system F of Edman and Sjoquist.[16] Only one spot was present from the unknown and it corresponded exactly with an authentic sample of PTH-glutamic acid.

We employed hydrazinolysis[17] to identify and determine the C-terminal residue of the toxin. Separation and identification were carried out on the automatic amino acid analyzer after reaction with benzaldehyde to remove the amino acid hydrazides. Trace amounts of several amino acids were observed on the 150-centimeter column, but none in sufficient quantity to account for a terminal residue. On the 15-centimeter column, large amounts of an amino

TABLE IV. QUANTITATIVE ESTIMATION OF N-TERMINAL RESIDUE OF STAPHYLOCOCCAL ENTEROTOXIN B BY FDB METHOD

Expt. No.	DNP-Toxin used,[a] μ moles	DNP-Glutamic Acid formed,[b] μ moles	Moles of DNP-Glu per Mole of Toxin
1	0.301	0.333	1.10
2	0.278	0.331	1.19
3	0.295	0.274	0.93
4	0.263	0.310	1.18
		Average	1.1_0

a. Molecular weight of DNP-toxin taken as 46,500.
b. Corrected for destruction by acid hydrolysis.

acid were found in the lysine position. Because arginine is degraded to ornithine in the hydrazinolysis procedure, and ornithine occurs at the identical position with lysine in the chromatographic trace, a run was made on the 50-centimeter column in which a good separation of lysine and ornithine occurs. The results of this analysis showed that only lysine was present. Quantitative estimates of the C-terminal residue were made from results of runs in which the reaction was carried out at 100°C for 10 hours. Determinations were made on the protein alone and on the protein plus added amounts of lysine. The results are shown in Table V.

The recovery of added lysine in the presence of toxin was 67.3 per cent. The average figure, 0.73 moles lysine per mole of toxin, is somewhat low but it seems to be perfectly reasonable to ascribe one C-terminal residue to the molecule along with the single N-terminal residue.

Apparently, then, staphylococcal enterotoxin B has a relatively simple structure -- a single polypeptide chain. This simplicity is emphasized by the cystine analyses. There is no free SH and only one S-S bridge in the molecule. Despite this relative simplicity of structure, the toxin is extremely stable. It is resistant to heat and requires prolonged exposure to 8 M urea before reduction with mercaptoethanol can be complete.

These results have some interesting physiological implications. Classical food poisoning results from eating materials containing pre-existing toxin. That is, it is not the result of bacterial fermentation after

TABLE V. QUANTITATIVE ESTIMATION OF C-TERMINAL RESIDUE OF STAPHYLOCOCCAL ENTEROTOXIN B BY HYDRAZINOLYSIS

Expt. No.	Toxin used, μ moles	Lysine formed,[a] μ moles	Moles of Lys per Mole of Toxin
1	0.632	0.479	0.758
2	0.632	0.533	0.843
3	0.705	0.449	0.637
4	0.705	0.489	0.675
		Average	0.72

a. Corrected for destruction in hydrazinolysis and purification.

ingestion or infection. The toxin, therefore, is absorbed through the intestinal wall. Preliminary data in our laboratories[18] have indicated that the toxin is resistant to the action of pepsin and the pancreatic endopeptidases, although it is attacked by carboxypeptidase B. This suggests that the full molecule is probably responsible for its biological activity and that it passes through the gut wall in its native form as a molecule of 35,000 molecular weight.

One other possible type of structure could explain the present results and circumvent the problem of absorption of a large protein molecule. This would be a small peptide, the toxic moiety, attached to a large peptide by noncovalent bonding, one of which would be cyclic. Physical properties, however, indicate that this is not so, because the material shows no change in sedimentation patterns or electrophoretic properties over a wide pH range.

In summary, amino acid analyses of staphylococcal enterotoxin B have demonstrated the molecule to be a simple protein with only one disulfide linkage. It has one C-terminal residue, lysine, and one N-terminal residue, glutamic acid, per mole.

LITERATURE CITED

1. Bergdoll, M.S.; Sugiyama, H.; and Dack, G.M. "Staphylococcal enterotoxin: I. Purification," Arch. Biochem. Biophys. 85:62-69, 1959.

2. Schantz, E.J.; Roessler, W.G.; Wagman, J.; Spero, L.; Stefanye, D.; Lynch, J.M.; Dunnery, D.A.; Startz, O.C.; Isaac, W.G.; and Grogan, E.W. "Purification and characterization of large amounts of enterotoxin B," Physical Sciences Division, U.S. Army Biological Laboratories, Frederick, Maryland. March 1964. (Technical Report 45).

3. Wagman, J., and Edwards, R. "Studies on staphylococcal enterotoxin (U)," Physical Sciences Division, U.S. Army Biological Laboratories, Frederick, Maryland. Semiannual Technical Summary, 1 October 1962 - 31 March 1963. pp. 18-19. CONFIDENTIAL.

4. Spackman, D.H.; Stein, W.H.; and Moore, S. "Automatic recording apparatus for use in the chromatography of amino acids," Anal. Chem. 30:1190-1206, 1958.

5. Hibnick, H.E., and Bergdoll, M.S. "Staphylococcal enterotoxin: II. Chemistry," Arch. Biochem. Biophys. 85:70-73, 1959.

6. Duggan, D.E., and Udenfriend, S. "The spectrophotofluorometric determination of tryptophan in plasma and of tryptophan and tyrosine in protein hydrolysates," J. Biol. Chem. 223:313-319, 1956.

7. Peters, T., Jr. "Appearance of new N-terminal residues upon treatment of human and bovine serum albumin with N-bromosuccinimide," Comp. rend. trav. lab. Carlsberg 31:227-234, 1959.

8. Gundlach, H.G.; Moore, S.; and Stein, W.G. "The nature of the amino acid residues involved in the inactivation of ribonuclease by iodoacetate," J. Biol. Chem. 234:1754-1760, 1959.

9. Boyer, P.D. "Spectrophotometric study of the reaction of protein sulfhydryl groups with organic mercurials," J. Am. Chem. Soc. 76:4331-4337, 1954.

10. Moore, S. "On the determination of cystine as cysteic acid," J. Biol. Chem. 238:235-237, 1963.

Crestfield, A.M.; Moore, S.; and Stein, W.G. "The preparation and enzymatic hydrolysis of reduced and S-carboxymethylated proteins," J. Biol. Chem. 238:622-627, 1963.

12. Cohn, E.J., and Edsall, J.T. "Proteins, amino acids and peptides," p. 445, Reinhold Publishing Corporation, New York, 1943.

13. Levy, A.L. "Recent developments in techniques for terminal and sequence studies in peptides and proteins," In: Glick, D. ed. "Methods of biochemical analysis," Vol. 2, pp. 360-383, Interscience Publishers, New York, 1955.

14. Blackburn, S., and Lowther, A.G. "Separation of N-2, 4-dinitrophenyl amino acids on paper chromatograms," Biochem. J. 48:126-128, 1951.

15. Fraenkel-Conrat, H., "Recent developments in techniques for terminal and sequence studies in peptides and proteins," In: Glick, D. ed. "Methods of biochemical analysis," Vol. 2, pp. 383-397, Interscience Publishers, New York, 1955.

16. Edman, P., and Sjoquist, J. "Identification and semi-quantitative determination of 3-phenyl-2-thiohydantoins," Acta. Chem. Scand. 10: 1507-1509, 1956.

17. Niu, C-I., and Fraenkel-Conrat, H. "Determination of C-terminal amino acids and peptides by hydrazinolysis," J. Am. Chem. Soc. 77:5882-5885, 1955.

18. Roessler, W.G. "Enzyme action on toxin," Medical Bacteriology Division, U.S. Army Biological Laboratories, Frederick, Maryland. Tri-Annual Technical Report, 1 March - 30 June 1963, p. 17.