Chemical Modification of Amino Groups in Staphylococcal Enterotoxin B*

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ABSTRACT: Chemical modification of amino groups in staphylococcal enterotoxin B with acetic anhydride, succinic anhydride, 2-methylisourea, and potassium cyanate has been studied. Neither the emetic activity nor the combining power of the antigen–antibody reaction was altered when 90% of lysine residues were guanidinated. The combining power of the acetylated, succinylated, and carbamylated enterotoxin B with antibody, however, was reduced. The decrease in the combining power of enterotoxin B after modification with acetic anhydride or succinic anhydride is directly proportional to the amino groups modified. Monkey feeding tests of the acetylated and succinylated enterotoxin B reveal that the loss of toxicity is closely related to decrease of the antigen–antibody reaction. Physicochemical studies on the modified enterotoxin B indicates that enterotoxin B undergoes a molecular expansion after acetylation and succinylation. Since guanidination did not alter the biological activity of the toxin, it is concluded that the reduction of the biological activity after acetylation and succinylation is due to a decrease of the net positive charges in the protein. The role of the positive charges on the conformation and biological activity of enterotoxin B is discussed.

The chemistry of staphylococcal enterotoxin B produced by *Staphylococcus aureus* strain S-6 has been studied extensively since this simple protein, which causes emesis and diarrhea when ingested, was first purified in 1959 (Bergdoll *et al.*, 1959, 1965b; Hibnick and Bergdoll, 1959; Schantz *et al.*, 1965). Subsequent work on other strains of *Staphylococcus aureus* has led to the isolation and characterization of enterotoxin A (Chu *et al.*, 1966) and enterotoxin C (Borja and Bergdoll, 1967; Avena and Bergdoll, 1967; Huang *et al.*, 1967). Studies on the amino acid composition (Bergdoll *et al.*, 1965b; Spero *et al.*, 1965) and the hydrogen ion equilibria of enterotoxin B (Chu, 1968) have provided the following information on the structural features of this toxin: (1) a single polypeptide chain containing only one disulfide bond and a large number of polar amino acids, especially lysine, aspartic acid, and glutamic acid; (2) only two tryptophan residues per mole of toxin; (3) most of the lysyl and carboxyl groups evenly distributed on the surface of the molecule; and (4) only one-third of the tyrosyl residues considered as "free tyrosine."

The effect of selective chemical modification of enterotoxin B on its biochemical and biological properties has appeared in two recent communications. Dalidowicz *et al.* (1966) reported that neither the biological activity nor the protein conformation has been altered after the toxin was reduced and alkylated. The tyrosyl residues are considered to be essential for maintance of the protein conformation (Chu, 1968). No loss of biological activity was observed when the free tyrosyl residues were chemically modified; however, biological activity was lost after modification of the abnormal tyrosyl residues. In the present study, the amino groups in the toxin were modified with acetic anhydride, succinic anhydride, 2-methylisourea, and potassium cyanate. The effects of acetylation, succinylation, guanidination, and carbamylation on the biological activity and biochemical properties are presented in this communication.

Experimental Section

Materials. Staphylococcal enterotoxin B was prepared according to a method described previously (Bergdoll *et al.*, 1965b). Acetic anhydride, ethyl acetate, boric acid, and sodium acetate were obtained from the Baker Chemical Co.; succinic anhydride, potassium cyanate, and hydroxylamine hydrochloride from Eastman Organic Chemicals; ninhydrin from Pierce Chemical Co.; trypsin and pepsin from Worthington Biochemical Corp.; cytochrome *c* from Nutritional Biochemical Corp.; Blue Dextrin 2000 from Pharmacia (Uppsala, Sweden); EDTA from Bersworth Chemical Co. (Framingham, Mass.); Amido Black (Buffalo Black, NBR) from Allied Chemical Co.; and *O*-methylisourea hydrogen sulfate from Aldrich Chemical Co. (Milwaukee, Wis.). The DEAE-cellulose was Selectacel ion-exchange cellulose, No. 71, type 21 (Carl Schleicher & Schuell Co.). Sephadex G-75 (40–120 μ, Pharmacia Co., Uppsala, Sweden) was hydrated in appropriate buffer at least 24 hr before using. Connaught (Toronto, Canada) hydrolyzed starch was used in the starch gel electrophoresis.

Chemical Modifications. ACETYLATION. The acetylation of enterotoxin B was carried out according to the method described by Fraenkel-Conrat (1957). In a typical experiment 184 mg of enterotoxin B in 10 ml of half-saturated sodium acetate was cooled in an ice bath; 25 μl of acetic anhydride was added to the cooled solution every 10 min over a period of 70 min. A sample containing 50 μl was withdrawn from the reaction mixture every 10 min and immediately diluted to 5 ml with distilled water. Stirring was facilitated by a magnetic stirring bar throughout all the reactions. After 2 hr, the remaining reaction mixture was diluted to 50 ml with distilled

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water and dialyzed overnight. In some experiments the acetylation was carried out for only 1 hr.

SUCCINYLATION. The succinylation was carried out in the presence of 1.0 M sodium bicarbonate buffer at pH 8.0 and at 25° as described by Habeeb *et al.* (1958). In a typical experiment, 180 mg of enterotoxin B was dissolved in 15 ml of 1.0 M sodium carbonate buffer at pH 8.0; 20 mg of solid succinic anhydride was added to the solution every 10 min for 70 min. The reaction mixture was maintained at pH 8.0 with 0.1 N NaOH during the reaction by pH stat titration with a radiometer. Samples (50 μl each) were withdrawn from the mixture every 10 min and diluted as described above. Stirring was also performed throughout all the reactions. The reaction was stopped at either 1 or 2 hr by dilution to 50 ml with distilled water, then dialyzed overnight.

GUANIDINATION. Guanidination followed the method of Chervenka and Wilcox (1956). Enterotoxin B (100 mg) in 45 ml of 2-methylisourea solution prepared from *O*-methylisourea hydrogen sulfate according to the method described by Kassell and Chow (1966) was adjusted to pH 10.3 with 5 N NaOH. The reaction was carried out at 5° for 52 and 98 hr. After the reaction, the solution was dialyzed against 4 l. of 0.001 M HCl for 48 hr with two changes of the outer solution. The precipitate that occurred during the reaction was removed by centrifugation and both the supernatant and precipitate were lyophilized.

CARBAMYLATION. The reaction of potassium cyanate with enterotoxin B was carried out according to the method described by Stark *et al.* (1960) with only slight modification. Enterotoxin B (80 mg) was dissolved in 5 ml of 0.05 M Tris-HCl at pH 8.0; 10 mg of KCNO was added to the mixture every hour for a period of 4 hr. After 6 hr the reaction mixture was diluted to 50 ml and dialyzed overnight. The carbamylation was carried out at 40° and the pH was maintained at pH 8.0 by a radiometer with 0.1 N NaOH as described.

DEAE-cellulose Chromatography. The modified enterotoxin B, after dialysis, was subjected to chromatography on a DEAE-cellulose column which was prepared according to the method described by Peterson and Sober (1956). In general, the DEAE-cellulose column employed was 1.9 × 40 cm equilibrated with 0.01 M sodium phosphate buffer at pH 8.0. The sample of protein (100–170 mg) obtained from the reaction mixture after dialysis was adjusted to pH 8.0 and then transferred into the column. After washing with the initial buffer (0.01 M, pH 8.0), stepwise elution with approximately 240 ml each of 0.1 M sodium phosphate buffer (pH 6.85), 0.1 M sodium phosphate buffer (pH 6.0), 0.1 M NaH_2PO_4, and 0.1 M NaH_2PO_4 plus 1 M NaCl was performed. The samples obtained from each peak were pooled, dialyzed, and lyophilized.

Sephadex Gel Filtration. The lyophilized material obtained from DEAE-cellulose chromatography was dissolved in 5 ml of water, transferred into a Sephadex G-75 column (2.2 × 54 cm), and equilibrated with 0.005 M sodium phosphate buffer at pH 6.85. The modified enterotoxin B was eluted from the column with the same buffer, then lyophilized without dialysis. For analytical purposes the column was equilibrated with 0.2 M sodium phosphate buffer at pH 6.85 and the material eluted with the same buffer (0.2 M sodium phosphate buffer, pH 6.85) in 3-ml fractions. Trypsin, pepsin, cytochrome *c*, and enterotoxin B were used to calibrate the Sephadex column. In general, 10 mg of protein was dissolved in either 3 ml of water (as for cytochrome *c* and enterotoxin B) or 0.001 N HCl (as for trypsin and pepsin) and subjected to Sephadex gel filtration. Blue Dextran and tyrosine were used to determine the void volume, V_0, and the internal volume, V_i, of the column.

Chemical Analysis of Modified Enterotoxin B. The degree of amino group substitution was measured by the ninhydrin procedure of Moore and Stein (1948) using either phenylalanine or unmodified enterotoxin B as a standard. The number of *O*-acyl groups introduced into the enterotoxin B after acetylation or succinylation was measured by the alkaline hydroxylamine method of Uraki *et al.* (1957). Amino acid composition of guanidinated enterotoxin B was determined with a Spinco Model 120 B amino acid analyzer as described previously (Bergdoll *et al.*, 1965b).[1]

Physicochemical Measurements of Modified Enterotoxin B. Viscosity measurements were performed at 20° with a 0.5-ml capillary viscosimeter as described by Schachman (1957). Modified enterotoxin B (2–10 mg/ml) was dialyzed against 0.05 M sodium phosphate buffer, pH 6.85, for at least 48 hr and centrifuged before using. The least squares method was used to compute the viscosity at zero concentration, η_0, and intrinsic viscosity (η_{sp}/C at $C \rightarrow 0$, where C is the concentration of protein).

Starch gel electrophoresis was carried out by a modification of the method described by Smithies (1955) using an E-C pressure plate electrophoresis cell as described previously (Chu *et al.*, 1966). Sodium borate (0.025 M at pH 8.55) was used as the supporting buffer. EDTA, at a concentration of 1×10^{-3} M, was added to the buffer for the gel preparation. Buffalo Black NBR in a methanol–acetic acid–water solution (5:2:5, v/v) was used to locate the protein.

Spectrophotometric titration was performed by using a Radiometer Automatic Titrator, type TTTl (Copenhagen, Denmark), with a Radiometer Titrigraph, type SBR2, and syringe buret, type SBU 1 (Chu, 1968). A micro glass electrode, Model G 2222B, and a calomel reference electrode, type K 4112, were used. Since the time-dependent dissociation of tyrosyl residues began above pH 11.0, the values (greater than pH 11.0) were extrapolated to zero time. Optical density measurements were performed in a Beckman Model DU spectrophotometer with an optical path of 1 cm. An extinction value of 14 ($\epsilon^{1\%}_{1\text{ cm}}$) was used for enterotoxin B. However, the molar extinction of modified enterotoxin was decreased by 1160/mole of tyrosyl residue which had been O acetylated (Simpson *et al.*, 1963).

Biological Assay of Modified Enterotoxin B. A modified Ouchterlony plate gel diffusion method (Bergdoll *et al.*, 1965a) was used to identify any change due to the modification of enterotoxin B. Quantitative precipitin tests were used to determine the combining power of the modified enterotoxin B with the enterotoxin B antisera. These were carried out as follows: 100 μl of enterotoxin B antiserum was added to a series of test solutions containing 10, 20, 30, and 40 μg of enterotoxin B in 1 ml of sodium phosphate buffer (0.02 M, pH 7.4) containing 0.85% NaCl. The mixture was incubated at 37° for 30 min and kept at 5° overnight; 1 ml of 0.15 M NaCl was added to this suspension and centrifuged. The precipitates were washed twice with 2 ml of 0.15 M NaCl and dissolved in 0.5–1.0 ml of 0.1 M NaOH. The protein in the solution was determined by a modification of the Folin method (Lowry *et al.*,

[1] We are indebted to Dr. I-Y. Huang for his help on the amino acid composition analysis.

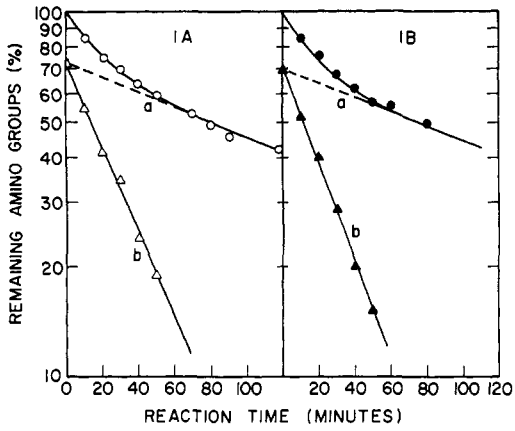

FIGURE 1: Semilogarithmic plot of the loss of amino groups after reaction with acetic anhydride (A) and succinic anhydride (B). The circles represent original data. The triangles represent the difference between the extrapolated values (dotted line) and the original data.

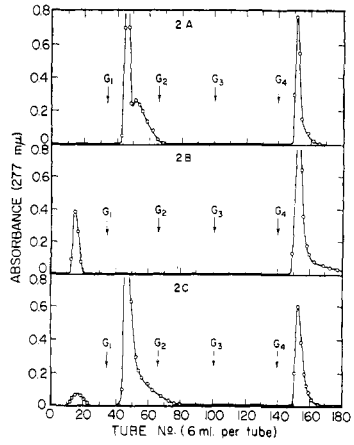

FIGURE 2: Chromatography of modified enterotoxin B on DEAE-cellulose. Elution patterns were obtained from experiments in which the dialyzed reaction mixture was applied to a DEAE-cellulose column (1.6 × 40 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 8.0). Stepwise elution was performed with the following buffers: G_1 = 0.1 M sodium phosphate buffer, pH 6.85; G_2 = 0.1 M sodium phosphate buffer, pH 6.0; G_3 = 0.1 M NaH_2PO_4; G_4 = 0.1 M NaH_2PO_4 plus 1 M NaCl. Parts A and B represent the elution patterns of enterotoxin B which was acetylated and succinylated for 2 hr, respectively. Part C represents the elution pattern of enterotoxin B which has been carbamylated for 6 hr. The flow rate was 3 ml/min.

1951). The loss of antigen–antibody combining power, x, was calculated by using eq 1. The antisera used in these tests were

$$x = \frac{\text{protein precipitated at the equivalent concentration}}{\text{protein precipitated at the equivalent concentration of native enterotoxin B}} \times 100 \quad (1)$$

obtained from rabbits that had been immunized with purified enterotoxin B as described previously (Bergdoll *et al.*, 1965a). The toxicity of modified enterotoxin B was determined by intragastric administration of the enterotoxin solution (usually 50 ml) by catheter to young rhesus monkeys (*Maccaca mulatta*, wt 2–3 kg). Details on the monkey feeding assay have been described elsewhere (Bergdoll *et al.*, 1959).

Results

Reaction of Acetic Anhydride, Succinic Anhydride, and KCNO with Enterotoxin B. Figure 1 shows the remaining or unsubstituted amino groups in enterotoxin B after acetylation and succinylation. A biphasic curve was obtained in both reactions. The experimental data were further analyzed according to the method described by Ray and Koshland (1960). A pseudo-first-order slope was obtained after extrapolating the curve to zero time. The rate constants for acetylation and succinylation were calculated to be 0.01 and 0.008 per min, respectively. Substracting the extrapolated values (curve a, Figure 1A,B) from the experimental data gave curve b (Figure 1A,B). Rate constants for acetylation and succinylation calculated from this curve were 0.03 and 0.04 per min, respectively. The results indicate that 26–30% of the amino groups (9–11 moles/mole of enterotoxin B) were acetylated or succinylated at a faster rate than the remainder. The reaction of KCNO with enterotoxin B is much slower than acetylation or succinylation. Only 25% of the amino groups in enterotoxin B was modified by KCNO after 2-hr reaction. Since precipitation was observed, the kinetics of carbamylation and guanidination have not been analyzed.

Chromatographic Analysis of Chemically Modified Enterotoxin B. Since two different rate constants were obtained during acetylation and succinylation, the reaction mixtures were further subjected to DEAE-cellulose chromatography. The elution patterns of the acetylated, succinylated, and carbamylated enterotoxin B are shown in Figure 2A, B, and C, respectively. Two reaction products were separated with DEAE-cellulose chromatography. Unreacted enterotoxin B, if any, usually passed through the column with the initial buffer (0.01 M sodium phosphate buffer, pH 8.0). Except for the carbamylated product, the material obtained with the first buffer in the stepwise elution (0.1 M sodium phosphate buffer, pH 6.85) contained enterotoxin B in which 30–40% of the amino groups had been modified. When more than 40% of the amino groups were modified by the different methods, the altered enterotoxin B was eluted from the column only with the last buffer in the stepwise elution series (0.1 M NaH_2PO_4 plus 1 M NaCl).

The distribution of the modified enterotoxin B between the peak obtained with buffer G_1 and the peak obtained with buffer G_4 (Figure 2A) from DEAE column chromatography usually depended upon the reaction conditions, and especially the time of reaction. In general, approximately 40–60% of the modified material was recovered from the first peak after 1-hr acetylation or succinylation. The yield of modified enterotoxin B recovered from this peak after 2-hr reaction, however, was found to be less than 15% for the succinylated product or 50% for the acetylated product. By slight alteration of the reaction time or by reacetylation of the partially acetylated enterotoxin B obtained from the first peak, different degrees of acetylated and succinylated enterotoxin B were obtained. Since the rate of carbamylation is much slower than acetylation and succinylation, almost 90% of the altered enterotoxin B after 6-hr carbamylation, in which time approximately only 20% of the amino groups were modified, was eluted from the column by the first buffer.

Gel Filtration of the Modified Enterotoxin B on Sephadex G-75. Different forms of modified enterotoxin B obtained from

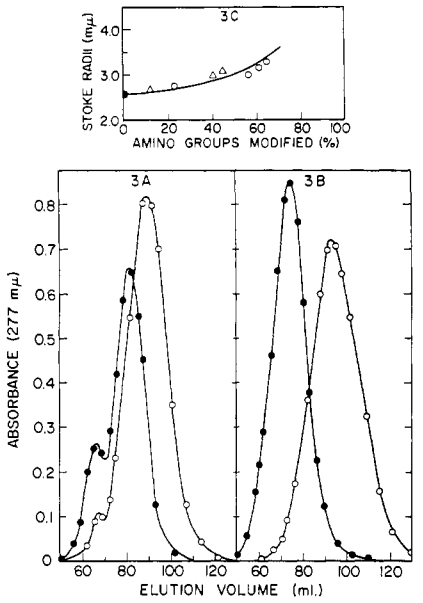

FIGURE 3: Sephadex G-75 gel filtration of the modified enterotoxin B. The elution patterns were obtained from experiments in which 5–10 mg of DEAE-cellulose chromatographed Ac- or Su-enterotoxin in 5 ml of distilled water was applied to a Sephadex G-75 column (2.2 × 50 cm) equilibrated with 0.005 M sodium phosphate buffer (pH 6.85). The elution patterns in part A were obtained from experiments using Ac-(58)-enterotoxin B (●) and Ac-(24)-enterotoxin (○). The elution patterns in part B were obtained from experiments using Su-(43)-enterotoxin B (●) and Su-(12)-enterotoxin B (○). The insert, C, represents the Stokes radii of enterotoxin B after chemical modification (○—○, acetylation, and △—△, succinylation). The results were obtained from an analytical Sephadex G-75 column (2.2 × 54) equilibrated and eluted with 0.2 M sodium phosphate buffer (pH 6.85). The flow rate was 24 ml/hr.

DEAE-cellulose chromatography were subjected to gel filtration on a Sephadex G-75 column using 0.005 M sodium phosphate at pH 6.85 as an eluent. The elution patterns are shown in 3A,B. Only one peak was obtained when Su-enterotoxin B[2] was passed through the column (Figure 3B); however, small amounts of higher molecular weight species were separated from the Ac-enterotoxin B (Figure 3A). Since the peak volume of the modified enterotoxin B on Sephadex G-75 shifted to a lower value, all the preparations were further analyzed in a Sephadex G-75 column by elution with 0.2 M sodium phosphate buffer at pH 6.85.

The column was calibrated with cytochrome *c*, trypsin, pepsin, and enterotoxin B. The effective pore radius of the gel was calculated to be 10.07, according to the method described by Ackers (1964). Stokes radii of 1.74 2.30, 2.75, and 2.56 mμ for cytochrome *c*, trypsin, pepsin, and enterotoxin B, respectively, were used in this calculation. The Stokes radius of modified enterotoxin was, therefore, estimated according to the parameter as described by Ackers (1964). Figure 3C shows the relationship between the degree of amino group modification and the size of the Stokes radius. At a high degree of succinylation or acetylation the Stokes radius of enterotoxin B is increased from 2.56 to 3.17 or 3.27 mμ.

[2] Abbreviations used are: Ac-(*x*)-enterotoxin B, Su-(*x*)-enterotoxin B, Ca-(*x*)-enterotoxin B (enterotoxin B with *x*% of amino groups acetylated, succinylated, or carbamylated); Gu-(*x*)-enterotoxin B (enterotoxin B with *x*% of lysine residues guanidinated).

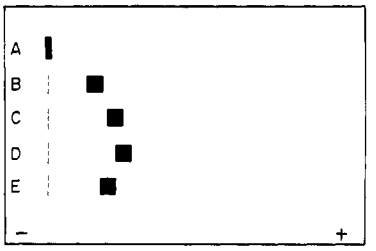

FIGURE 4: Starch gel electrophoretic patterns of modified enterotoxin B. The starting positions are indicated by dotted lines. Electrophoresis was carried out in sodium borate buffer (0.025 M, pH 8.65) at 20 V/cm for 5 hr. A = native enterotoxin B; B = Ac-(21)-enterotoxin B; C = Ac-(61)-enterotoxin B; D = Su-(43)-enterotoxin B; E = Su-(12)-enterotoxin B.

Physicochemical Analysis of Ac- and Su-enterotoxin B. Figure 4 shows the degree of migration of modified enterotoxin B in starch gel after electrophoresis for 6 hr at pH 8.55 and a voltage gradient of 20 V/cm. Each of the preparations migrated as a single spot. The native enterotoxin B remained at the origin while both the Ac- and Su-enterotoxin B moved toward the anode. The rate of migration was correlated with the number of amino groups modified. Su-enterotoxin B migrated faster than Ac-enterotoxin B.

The number of *O*-acyl groups introduced in the enterotoxin B after modification is presented in Table I. Approximately six to eight *O*-acyl residues were detected after acetylation. However, less than one *O*-succinyl residue was found in the succinylated product.

The effect of chemical modification on the intrinsic viscosity of enterotoxin B is shown in Figure 5. The intrinsic viscosity for the modified enterotoxin B was not increased significantly when less than 40% of the amino groups were chemically modified, but was noticeably increased when more than 50% were acetylated.

Spectrophotometric titration of tyrosyl residues in proteins has been used as one of the sensitive methods for detecting any conformational changes. The spectrophotometric titration of tyrosyl residues of the native enterotoxin B is compared with that of Ac- or Su-enterotoxin B in Figure 6. Neither acetylation nor succinylation altered the titration curve significantly.

TABLE I: *O*-Acyl Group Content of Modified Enterotoxin B.

Method of Modification	Amino Groups Modified (%)[a]	No. of Substituted Groups (moles/mole of Protein)[b]
Acetylation	25	5.43
	36	6.20
	58	7.67
Succinylation	40 ~ 43	0.45 ~ 0.55

[a] Determined by the ninhydrin method of Moore and Stein (1948). [b] Determined by the alkaline hydroxylamine method of Uraki *et al.* (1957).

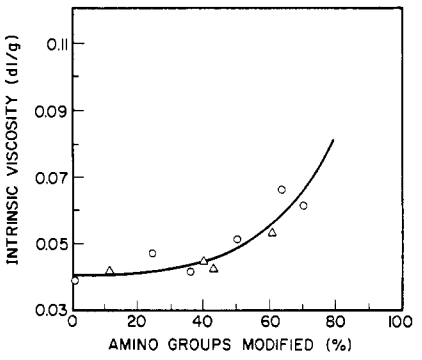

FIGURE 5: Intrinsic viscosities of modified enterotoxin B. The circles indicate the acetylated products and the triangles represent the succinylated products. Viscosities were determined at 20° in 0.05 M sodium phosphate buffer at pH 6.85. The intrinsic viscosities were calculated as described by Schachman (1957).

Although the titration curves of the modified enterotoxin B were shifted slightly to the acidic pH, the abnormal tyrosyl residues, which dissociate above pH 12.5, were not disturbed. Curve C (Figure 6), which was obtained by substracting the original titration curve (curve A, Figure 6) from the curve obtained from the Ac- and Su-enterotoxin B (curve B, Figure 6), indicates that approximately four tyrosyl residues became more readily accessible to the solvent for titration at pH 11.9 as a result of acetylation or succinylation.

Since precipitation was observed after guanidination, the solution was centrifuged before chemical and biological analysis. Both the supernatant and precipitates were analyzed. Table II summarizes the basic amino acid composition of the guanidinated product. The results indicated that more than 95% of the lysine in enterotoxin B was modified after 98-hr reaction.

Biological Reactivity of Modified Enterotoxin B. Figure 7 shows the reactivity of acetylated, succinylated, and carbamylated toxin with the antisera for native enterotoxin B in an Ouchterlony double diffusion plate. Reaction of the modified enterotoxin B with antibody occurred when less than 40% of the amino groups had been chemically modified. The reactivity, however, was either decreased as in the case of Su-enterotoxin B (well 4 of Figure 7) and Ca-enterotoxin B (well 2), or altered as in the case of Ac-enterotoxin B (well 6) when more than 40% of the amino groups were chemically modified. Quantitative precipitin tests indicated a direct correlation between the number of amino groups modified by acetylation or succinylation and the loss of combining power of the altered enterotoxin B with the native toxin antibody. The results are given in Figure 8. Except for the carbamylation and guanidination products, the decrease in the combining power to enterotoxin B antibody after modification of the amino groups can be approximated from the following formula: $x = y/1.75$, where x and y represent the loss of combining power (per cent) and the extent of amino groups unaltered (per cent), respectively. No loss of combining power of enterotoxin B with antibody was observed when 96% of the lysyl residues in the

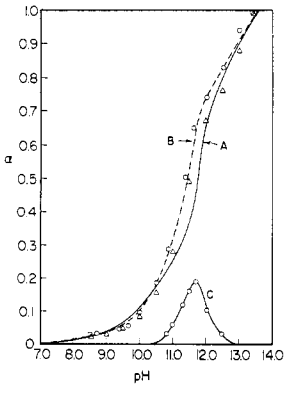

FIGURE 6: Spectrophotometric titration of modified enterotoxin B. α represents the degree of dissociation. Curve A represents the titration curve of native enterotoxin B. Curve B represents the titration curve of Ac-(64-)enterotoxin B (△) and Su-(40)-enterotoxin B (○). Curve C is the difference of curves A and B. Spectrophotometric titration was performed at 295 mμ at 25° in the presence of 0.16 M KCl.

TABLE II: Basic Amino Acid Composition of Guanidinated Enterotoxin B.

Reaction Time (hr)	Fraction[a]	Amino Acid Composition[b] (moles/mole of protein)			
		Lysine	Histidine	Arginine	Homoarginine[c]
52	S (30)	4.0	4.58	5.0	30.5
	P (70)	1.50	5	5	33.27
98	S (10)	3.33	5	5	31.70
	P (90)	1.28	5	5	32.91

[a] Since precipitation was observed after the reaction mixture had been dialyzed, the solution was centrifuged. The amino acid composition of supernatant, S, and precipitates, P, were determined. The number in parentheses indicates percentage of the total protein. [b] Five moles of histidine and five moles of arginine per mole of protein were used as a standard to calculate the lysine and homoarginine content. [c] The average integration constant of lysine, histidine, and arginine was used to estimate the homoarginine residues.

TABLE III: Effect of Chemical Modification on the Emetic Activity of Enterotoxin B.

Method of Modification	Amino Groups Modified (%)	Enterotoxin B (μg/Monkey)[a]	Result[b]
None	0	20	(4–5)/6
Acetylation	31	50	4/6
	70	50	1/6
Succinylation	34	50	2/6
Guanidination	89	20	3/6

[a] All the tests were carried out by intragastric administration of 50 ml of the test solution by catheter to young rhesus monkeys. [b] Number of vomiting *vs.* number challenged.

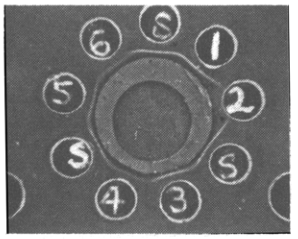

FIGURE 7: Ouchterlony immunodiffusion of native and modified enterotoxin B. The enterotoxin B antisera (1:4 dilution) was placed in the center well. Wells 1 and 2 represent Ca-(11)- and Ca-(57)-enterotoxin B; wells 3 and 4 represent Su-(12)- and Su-(43)-enterotoxin B; wells 5 and 6 represent Ac-(24)- and Ac-(55)-enterotoxin B. The native enterotoxin B was placed in the wells indicated by letter S. All the tests were performed at a concentration of 50 μg of protein/ml in the isotonic saline–phosphate buffer (0.02 M, pH 7.2) at room temperature.

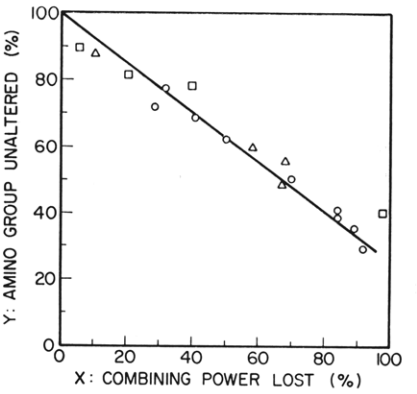

FIGURE 8: Effect of acetylation and succinylation on the combining power of enterotoxin B with antibody. The individual points were obtained from quantitative precipitin tests and were calculated according to eq 1 as described in the text.

toxin were guanidinated. The inhibitory effect of carbamylation on the combining power of the modified enterotoxin indicated KCNO interaction with other amino acids (Stevens and Feeney, 1963); hence, carbamylation was not studied in detail.

The effect of chemical modification of amino groups in enterotoxin B on the emetic activity is presented in Table III. Here again, the biological activity is decreased after acetylation and succinylation. No loss of emetic activity was observed after guanidination. The loss of emetic activity in the Ac- or Su-enterotoxin B is directly related to the number of amino groups modified.

Discussion

Recent studies from our laboratory (Bergdoll, 1966) and others (Silverman *et al.*, 1966) have indicated that the emetic activity of enterotoxin B is greatly reduced or destroyed after treatment of the toxin with formalin. However, due to the nonspecific nature of this reagent, its application to the modification of amino groups has been limited (Putman, 1953), even though earlier studies with diphtheria toxin showed the loss of toxicity after formalin treatment accompanied loss of amino groups in the toxin (Hewitt, 1930; Pappenheimer, 1938). Acetic anhydride, succinic anhydride, 2-methylisourea, and potassium cyanate, which are specific for amino groups under certain conditions, were selected for the present study. Acetylation of an amino group in the protein converts the positively charged ammonium ion into a neutral amino group while succinylation converts the positively charged amino group into a negatively charged group. On the other hand, guanidination of the lysine residues yields a substituted group that maintains the positive charge. Our experimental results indicate that the effect of these reagents on the biochemical and biological properties of enterotoxin B are different, and thus give a clearer picture of the role of the amino groups in the enterotoxin B molecule.

In addition to the amino groups, six to eight tyrosyl residues were acetylated in enterotoxin B. The possibility of the tyrosyl groups affecting the biological activity of the toxin was ruled out by the fact that the decrease in the combining power of Ac-enterotoxin B with the antibody is identical or only slightly less than that of Su-enterotoxin B (Figure 8), where less than one *O*-succinyl group was detected. Furthermore, modification of six free tyrosyl residues in enterotoxin B with *N*-acetylimidazole or with nitromethane (Chu, 1968) revealed no alteration of the biological activity. The tyrosyl residues which were acetylated with acetic anhydride may be identical with those acetylated with *N*-acetylimidazole or nitrated with nitromethane, and are probably the free tyrosyl groups. The failure to detect *O*-succinyl groups in Su-enterotoxin B is probably due to spontaneous deacylation similar to that reported for succinylcarboxylpeptidase A (Riordan and Vallee, 1964).

Since enterotoxin B became more acidic after acetylation and succinylation, the increase of the net negative charges may create a repulsive force, thus resulting in both the expansion of the molecule and the decrease of antigen–antibody combining power (Habeeb *et al.*, 1958; Habeeb, 1967). The loss of hydrogen bonding between the ammonium and carboxyl groups in the modified enterotoxin B may also play an important role in the conformation. Because the decrease of combining power is generally proportional to the number of amino groups modified, and also because no significant change on the antigen–antibody reaction has been observed with guanidinated enterotoxin B, it is concluded that the positive charges contributed by the amino groups in enterotoxin B play an important role in the antigen–antibody reaction. Recent experiments on the modification of carboxyl groups in enterotoxin B[3] further support the importance of the charges on the antigen–antibody reaction. The enterotoxin no longer reacted with its antibody when all of the carboxyl groups of the toxin were chemically modified.

Investigations on the importance of amino groups in *Clostridium botulinum* type A toxin (Schantz and Spero, 1957; Spero and Schantz, 1957) and in the ϵ-toxin of *Clostridium perfringens* type D (Habeeb, 1963) have been reported. In the first case, more than 98% of activity was lost when only 19% of the amino groups in the botulinum toxin had been chemically modified. Most of the biological activity of the perfringens toxin was lost when 50% of the amino groups in the toxin were modified. The role of amino groups in enterotoxin B appears different from those in the botulinum and perfringens toxins. We feel that it may be due to the positive charges

[3] F. S. Chu, manuscript in preparation.

of the amino groups playing an important role in the emetic activity of enterotoxin B. Similar results have been obtained by Schantz[4] when he deaminated the amino groups in enterotoxin B with nitrous acid. In conclusion, the function of the positive charges on the enterotoxin B molecule may be twofold: (1) to maintain certain conformations which are essential for the biological activity; and (2) they are essential for binding with the homologous antibody or receptor sites of the target tissue through the electrostatic forces as discussed since they may locate near or around the active center.

References

Ackers, G. K. (1964), *Biochemistry 3*, 723.

Avena, R. M., and Bergdoll, M. S. (1967), *Biochemistry 6*, 1474.

Bergdoll, M. S. (1966), *J. Infect. Dis. 116*, 191.

Bergdoll, M. S., Borja, C. R., and Avena, R. M. (1965a), *J. Bacteriol. 90*, 1481.

Bergdoll, M. S., Chu, F. S., Huang, I-Y., Rowe, C., and Shih, T. (1965b), *Arch. Biochem. Biophys. 112*, 104.

Bergdoll, M. S., Sugiyama, H., and Dack, G. M. (1959), *Arch. Biochem. Biophys. 85*, 62.

Borja, C. R., and Bergdoll, M. S. (1967), *Biochemistry 6*, 1467.

Chervenka, C. H., and Wilcox, P. E. (1956), *J. Biol. Chem. 222*, 635.

Chu, F. S. (1968), *J. Biol. Chem. 243*, 4342.

Chu, F. S., Thadhani, K., Schantz, E. J., and Bergdoll, M. S. (1966), *Biochemistry 5*, 3281.

Dalidowicz, J. E., Silverman, S. J., Schantz, E. J., Stefanye, D., and Spero, L. (1966), *Biochemistry 5*, 2375.

Fraenkel-Conrat, H. (1957), *Methods Enzymol. 4*, 247.

Habeeb, A. F. S. A. (1963), *Biochim. Biophys. Acta 74*, 113.

Habeeb, A. F. S. A. (1967), *Arch. Biochem. Biophys. 121*, 652.

Habeeb, A. F. S. A., Cassidy, H. G., and Singer, S. J. (1958), *Biochim. Biophys. Acta 29*, 587.

Hewitt, L. F. (1930), *Biochem. J. 24*, 983.

Hibnick, H. E., and Bergdoll, M. S. (1959), *Arch. Biochem. Biophys. 85*, 70.

Huang, I-Y., Shih, T., Borja, C. R., Avena, R., and Bergdoll, M. S. (1967), *Biochemistry 6*, 1480.

Kassell, B., and Chow, R. B. (1966), *Biochemistry 5*, 3449.

Lowry, O. H., Rosebrough, N. J., Farr, A. C., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.

Pappenheimer, A. M. (1938), *J. Biol. Chem. 125*, 201.

Peterson, E. A., and Sober, H. A. (1956), *J. Am. Chem. Soc. 78*, 751.

Putnam, F. W. (1953), *Proteins 1*, 924.

Ray, W. J., Jr., and Koshland, D. E., Jr. (1960), *Brookhaven Symp. Biol. 13*, 135.

Riordan, J. F., and Vallee, B. L. (1964), *Biochemistry 3*, 1768.

Schachman, H. K. (1957), *Methods Enzymol. 4*, 95.

Schantz, E. J., Roessler, W. G., Wagman, J., Spero, L., Dunnery, D. A., and Bergdoll, M. S. (1965), *Biochemistry 4*, 1011.

Schantz, E. J., and Spero, L. (1957), *J. Am. Chem. Soc. 79*, 1623.

Silverman, S. J., Schantz, E. J., Espeseth, D. A., and Roessler, W. G. (1966), *Bacteriol. Proc.*, 43.

Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.

Smithies, O. (1955), *Biochem. J. 61*, 629.

Spero, L., and Schantz, E. J. (1957), *J. Am. Chem. Soc. 79*, 1625.

Spero, L., Stefanye, D., Brecher, P. I., Jacoby, H. M., Dalidowicz, J. E., and Schantz, E. J. (1965), *Biochemistry 4*, 1024.

Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.

Stevens, F. C., and Feeney, R. E. (1963), *Biochemistry 2*, 1346.

Uraki, Z., Terminiello, L., Bier, M., and Nord, F. F. (1957), *Arch. Biochem. Biophys. 69*, 644.

[4] Personnel communication from Dr. E. J. Schantz Biological Laboratory, Fort Detrick, Frederick, Md.