Amino Acid Composition and Terminal Amino Acids of Staphylococcal Enterotoxin B*

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ABSTRACT: A complete amino acid analysis is presented for staphylococcal enterotoxin B. The protein is composed solely of amino acids and is particularly rich in aspartic acid and lysine. It has no free sulfhydryl groups and only one disulfide bridge. On the basis of the amino acid composition the molecular weight is 35,380, the isoionic point is 8.70, and the partial specific volume is 0.731. These values are in excellent agreement with those determined experimentally.

Glutamic acid was identified as the N-terminal residue and lysine as the C-terminal residue. Quantitative estimation showed one N-terminal and one C-terminal residue per mole of protein, indicating that the structure is a single polypeptide chain.

Staphylococcal enterotoxin B is the emetic material elaborated by the S-6 strain of *Staphylococcus aureus*. A purification procedure permitting the isolation of large amounts of an immunologically and physically homogeneous material is described in an accompanying paper (Schantz, *et al.*, 1965). The toxin is devoid of any lipid, carbohydrate, or nucleic acid. This report presents a complete amino acid analysis of the protein, using the automatic amino acid analyzer, and a comparison with an earlier, largely microbiological analysis (Hibnick and Bergdoll, 1959). It also describes the identification of the amino- (N-) and carboxyl- (C-) terminal amino acids of the toxin, and quantitation of these residues. The implications these analyses have on the structure and conformation of the protein are discussed.

Materials and Methods

Materials. The enterotoxin was prepared according to the method of Schantz *et al.* (1965). It was stored as the lyophilized powder in a deep freeze. All the solvents used in chromatography were reagent grade. Anhydrous hydrazine was prepared by distillation under reduced pressure in a nitrogen atmosphere from sodium hydroxide pellets and was stored in a desiccator in foil-covered tubes. Benzaldehyde was freshly distilled before use. Dioxane was distilled over sodium, frozen, and stored in a deep freeze. Phenyl isothiocyanate was purified by distillation *in vacuo*.

Amino Acid Analysis

Preparation of Samples. Samples containing approximately 2 mg/ml protein were pipetted into 40-ml Pyrex freeze-drying ampoules. An equal volume of concentrated HCl from a freshly opened bottle was added. The mixture was frozen in a dry ice–acetone bath, evacuated to 50–100 μ Hg, thawed carefully, and agitated to remove dissolved air. Careful performance of this operation is essential to prevent oxidation of methionine, tyrosine, and carboxymethylcysteine when present during hydrolysis (Crestfield *et al.*, 1963). After being sealed under vacuum the ampoule was wrapped in aluminum foil, weighted to maintain a vertical position, and introduced into a flask of boiling toluene. The temperature of the toluene was maintained with an open-air reflux system at 110° ± 1°.

After hydrolysis the samples were dried without transfer on a lyophilization apparatus. As the hydrolysates did not remain frozen under conditions usually sufficient for the lyophilization of aqueous solutions, the evaporation process was carried out on the liquid sample. Control experiments indicated that no material was lost by entrainment, and that there was no bumping or boiling of the supercooled acid. The process required from 4 to 6 hours for a 4-ml sample. The crystalline colorless residue was stored in a desiccator over KOH pellets. For analysis, the samples were dissolved in 5.00 ml of 0.2 N sodium citrate buffer at *p*H 2.2, and 2.00-ml samples were applied to the columns.

Calibration of the Analyzer. Protein hydrolysates were analyzed on a Phoenix automatic amino acid analyzer (Model 5000) according to the methods of Spackman *et al.* (1958). Recently it was reported (Mahowald *et al.*, 1962) that the color yield of the reaction between ninhydrin and an amino acid in the chromatographic eluate decreased progressively but not identically over time for all amino acids, and equipment modifications were recommended to minimize these fluctuations. Consequently, the ninhydrin reservoir in the analyzer was water-cooled by winding a coil about it; a bottle containing Fieser's solution, a potent oxygen scavenger, was interposed between the oil reservoir and the ninhydrin solution. These precautions retarded the rate of

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oxidation of the ninhydrin reagent, as evidenced by the constancy of the integrated chromatographic peak areas of a calibration mixture of amino acids passed through the analytical column before and after the run. As an extra precaution, the same column was used for calibration mixture analyses as well as for the analysis of protein hydrolysates. An average deviation of $\pm 2\%$ was observed in the amino acid analyses of calibration mixtures.

Calculations. Three separate analyses were performed. The nitrogen content of the protein was uncertain at the time of the first two analyses. Initially, therefore, the weight per cents of the individual amino acid residues, based on the best available dry weight of protein, were summed and the total was divided into 100 to obtain a correction factor. Each of the amino acid percentages was then multiplied by this factor and the amino acid composition of a molecule of 35,000, the molecular weight of the protein as determined by physical methods (Wagman *et al.*, 1965), was calculated. Based on the excellent internal precision in the two runs and the fact that it calculated so closely to a unitary value, glutamic acid was taken as a reference standard. All the values were then recalculated relative to glutamic acid, assuming 26 residues per mole. A similar procedure has been followed by other investigators (Bargetzi *et al.*, 1963).

At the time of the last analysis, the per cent nitrogen was known more accurately and was used to determine the size of the sample used in the analysis. Minimal molecular weights were first calculated from the average weight percentages of the individual amino acid residues, and nearest integer numbers for the amino acid residues were obtained for 35,000 g of protein. A new molecular weight was then computed as a first approximation from these numbers and their respective residue molecular weights. These results in turn yielded integral numbers of residues for the recalculated molecular weight, as a second approximation. Summation of the products of these integral numbers multiplied by their respective residue molecular weights yielded a final molecular weight. Further approximations did not change the values for the integral numbers of residues.

Other Methods. Cystine was determined (a) as cysteic acid after performic acid oxidation of the protein followed by acid hydrolysis (Moore, 1963); (b) as *S*-carboxymethylcysteine after reduction with mercaptoethanol, passage through a Sephadex column, alkylation with iodoacetamide, purification on CM-cellulose, and acid hydrolysis; and (c) spectrophotometrically with the reagent developed by Ellman (1959) after reduction with mercaptoethanol and passage through a column of Sephadex G-25. Cysteine was measured by titration with *p*-mercuribenzoate under a variety of conditions (Boyer, 1954) and as *S*-carboxymethylcysteine after alkylation in 8 M urea followed by acid hydrolysis. Tryptophan was determined (a) colorimetrically by the method of Spies and Chambers (1949) and (b) by titration with *N*-bromosuccinimide (Peters, 1959).

Amide nitrogen was calculated from the ammonia content of the hydrolysates of varying duration after linear extrapolation to zero time. The threonine and serine values were calculated in a similar manner. Nitrogen was determined by micro-Kjeldahl, sulfur by the method of Alicino (1958), and phosphorus by the method of King (1932).

End-Group Analysis

Preparation and Hydrolysis of DNP-enterotoxin. The DNP-protein was prepared in a *p*H-stat (Radiometer TTT1b with recorder SBR2c) at *p*H 9.0 and 40° according to procedure (3) of Levy (1955). It was hydrolyzed in constant-boiling HCl (5.7 N) in a constant-temperature oil bath at 105° for 16 hours. Ether-soluble DNP-amino acids were extracted after dilution of the hydrolysates to approximately 1 N with respect to the acid.

Paper Chromatography of DNP-amino Acids. Both the two-dimensional system of Levy (1955) and the *t*-amyl alcohol unidimensional descending system of Blackburn and Lowther (1951) were employed.

Identification of Terminal PTH-amino Acid.[1] The paper strip modification of Fraenkel-Conrat (1955) was used for the preparation of the PTH of the N-terminal amino acid. Toxin (25.8 mg) was applied to five paper strips as a 5% solution. The derivative was identified in system F of Edman and Sjoquist (1956) (*n*-heptane, 75% aqueous formic acid, and ethylene chloride, 1:2:2) on starch-treated paper. Descending chromatograms were developed for 1.5 hours and the spots were visualized by spraying with iodine-azide.

Hydrazinolysis of the Enterotoxin. Samples of toxin solution were introduced into glass ampoules and dried first by lyophilization and then in an oven at 50°. Anhydrous hydrazine (0.5 ml) was added in a drybox and the tubes were sealed under vacuum. Hydrazinolysis was carried out at 100° for 10 hours in a constant-temperature oil bath. The solutions were cooled, transferred to evaporating dishes, and dried over H_2SO_4 *in vacuo*. The residue was dissolved in 1.0 ml of water in a small centrifuge tube, 0.25 ml of benzaldehyde was added, and the mixture was shaken for 2 hours. The tube was centrifuged, the aqueous supernatant was withdrawn, and the benzaldehyde treatment was repeated. The combined aqueous layers containing the C-terminal amino acid and some residual hydrazides were then dried. Prior to analysis on the automatic amino acid analyzer the material was dissolved in the appropriate citrate buffer.

Results

Amino Acid Composition. The elemental composition of the deionized protein was: nitrogen 16.15%; sulfur 1.04%; phosphorus, 0.023%. Table I shows the amino acid composition of staphylococcal enterotoxin B and the calculation of the integral values of the amino acids in the molecule. The results represent the average of three analyses. Each analysis was performed on 24-, 48-, 72-, and 96-hour hydrolysates run singly.

[1] Abbreviations used in this work: PTH, phenylthiohydantoin; FDNB, 1-fluoro-2,4-dinitrofluorobenzene; PTC, phenylthiocarbamyl-.

TABLE I: Amino Acid Composition, Integral Residues, and Molecular Weight of Staphylococcal Enterotoxin B.

Amino Acid	Amino Acid Residues (g/100 g dry protein)[a]	Nitrogen (g/100 g protein)	Nearest Integral Number of Amino Acid Residues for 35,380 g Protein	Integral Number of Residues × Respective Residue Molecular Weights	Nearest Integral Number of Residues (lit.)[b]
Lysine	15.25 ± 0.36[c]	3.33	42	5,384	47
Histidine	2.45 ± 0.13	0.75	6	826	8
Amide ammonia	1.58[d]	1.38	35[d]	(−34.61, +18.01)[e]	31[d]
Arginine	2.67 ± 0.12	0.96	6	936	6
Aspartic acid	17.93 ± 0.30	2.18	55	6,331	59
Threonine	4.69 ± 0.06	0.65	16	1,618	17
Serine	4.23 ● 0.09	0.68	17	1,481	25
Glutamic acid	9.55 ± 0.06	1.04	26	3,357	25
Proline	2.10 ± 0.18	0.30	8	777	6
Glycine	1.90 ± 0.06	0.47	12	685	17
Alanine	1.37 ± 0.02	0.27	7	498	6
Half-cystine	0.58	0.08	2	204	
Valine	5.49 ± 0.14	0.78	20	1,982	21
Methionine	3.70 ± 0.01	0.40	10	1,312	7
Isoleucine	3.45 ± 0.04	0.43	11	1,245	12
Leucine	6.37 ± 0.04	0.79	20	2,264	23
Tyrosine	11.20 ± 0.34	0.96	24	3,917	23
Phenylalanine	6.12 ± 0.01	0.58	15	2,208	14
Tryptophan	1.05	0.16	2	372	2
Total	100.10%	16.19%	299	35,380	318

[a] To avoid computational errors due to rounding off, two decimal places are retained throughout the columns. The average deviation is not better than 2%. [b] Calculated from column 1 of Table I in Hibnick and Bergdoll (1959) for a molecular weight of 35,380. [c] Average deviation. [d] Omitted from the total. [e] To correct for the molecular weight difference between OH and NH_2, 0.989 is subtracted per amide residue. To correct for the mole of water on the terminal amino acids, the molecular weight of water is added.

The destruction of serine and threonine appeared to follow zero-order kinetics and the extrapolation to zero time was accordingly made from the least-squares solution of these data. The amide value was also calculated from an extrapolation of the least-squares straight line but in this case all the data were combined into one solution because of the greater scatter encountered. The other amino acids were essentially constant during the time of hydrolysis except valine and isoleucine, which appeared to increase after the shortest hydrolysis.

The Spies and Chambers (1949) method gave a satisfactory value for tryptophan. It was necessary, however, to apply the enzymatic digestion technique of Harrison and Hofmann (1961) in order to obtain a true tryptophan color with an absorption maximum at 590 mμ. Optimum results were attained with a 5-hour digestion period with trypsin-chymotrypsin. The titration with *N*-bromosuccinimide gave an essentially identical value, but this method has the undesirable feature of requiring the use of a large empirical correction factor. Several other methods for tryptophan were tried (Duggan and Udenfriend, 1956; Beaven and Holiday, 1952; Noltmann *et al.*, 1962) and the results from all of them indicated 2 residues per molecule. None of the results of these latter methods was satisfactory, however, as an analytical value.

From the point of view of reliability, the analytical average deviation of ±2% introduces an uncertainty into the aspartic acid and lysine residue numbers because these substances are present to the extent of more than 40 residues per molecule. Consequently, they can be accepted as reliable to ±1 residue. In some instances even a much smaller analytical error may lead to the selection of the wrong integral value. An error of but 1.0% in the tyrosine analyses, for example, increases the calculated number of residues from 24.2_8 to 25.5_2 and, therefore, the integral number from 24 to 25.

Distribution of Sulfur. Table II shows the distribution of sulfur-containing amino acids in the protein. The analyses by various methods are consistent with 2 residues of half-cystine and 10 residues of methionine.

TABLE II: Distribution of Sulfur in Staphylococcal Enterotoxin B.

Method	Residues per 35,380 g Protein
Sulfur	
(a) From amino acid analysis	12.0
(b) From elemental analysis	11.5
Performic acid oxidation	
(a) Cystine as cysteic acid	2.01
(b) Methionine as sulfone	8.20
Alkylation after reduction with mercaptoethanol	
As *S*-carboxymethylcysteine	2.06
Spectrophotometry after reduction with mercaptoethanol	
As cysteine	1.99
Reaction with *p*-mercuribenzoate	
As free cysteine	<0.1
Direct alkylation with iodoacetate	
As *S*-carboxymethylcysteine	<0.01

No cysteine was found by either of the two methods used. The elemental sulfur is entirely accounted for by two sulfur-containing acids.

The number of methionine residues as determined from the performic acid oxidation is low compared with those values based on amino acid analysis. This is owing to the inaccurate integration of the methionine sulfone on the chromatographic traces because the relatively large amount of aspartic acid prevented complete resolution of the sulfone peak.

Molecular Weight. A molecular weight for the protein was derived from the data in Table I. The number of residues in the protein is 299, and the corresponding molecular weight, 35,380, is in excellent agreement with the value of 35,300 derived by sedimentation-diffusion analysis (Wagman *et al.*, 1965).

The molecular weight of the protein was also calculated from the minimal molecular weight derived from the percentage of each of the individual amino acids. The average from all the amino acids was 35,355. This permits an estimation of the error of the analysis. The standard deviation was 651, the standard error $651/\sqrt{18}$ or 154, and 95% confidence limits ± 324. The coefficient of variation is 1.8%.

Partial Specific Volume. The analytical data are accurate enough to permit a theoretical calculation of the partial specific volume of the enterotoxin from the weight percentages and partial specific volume of each amino acid (Cohn and Edsall, 1943). A value of 0.731 ml/g is attained, which is in good agreement with the value of 0.743 obtained by physical measurements (Wagman *et al.*, 1965).

Acid-Base Balance. There is an apparent excess of 8 basic groups in the molecule. This requires the titration of all the histidine residues and 2 of the lysine residues to achieve electrical neutrality. Assuming a *pK* value of 10 (Cohn and Edsall, 1943) for the lysine residues, the isoionic point should be 8.70. This agrees very well with the experimental values of 8.6 for the isoelectric point and 8.55 for the isoionic *p*H (Schantz *et al.*, 1965).

Comparison with Previous Analysis. Table I presents a comparison of the amino acid composition of the enterotoxin as calculated here and as derived from the analytical figures of Hibnick *et al.* (1959). In these latter calculations a sum of 318 residues was obtained, not including the 2 residues of half-cystine found in our present analysis. This difference of 21 residues reflects the total recovery of 104.7% in the older analysis.

The two analyses are similar for most of the amino acids. Noteworthy differences are apparent only for serine, glycine, and methionine. The variation for the other amino acids is almost certainly owing to the inherent limitation in accuracy in the microbiological methods that were available to these workers when their analysis was performed.

Identification of N-Terminal Amino Acid. The method used for identification of the N-terminal amino acid was the FDNB technique of Sanger (1945). Chromatographic examination of the ether extracts of the hydrolysate of the DNP-toxin by means of the two-dimensional Levy (1955) system yielded a remarkably clean paper. In addition to the usual spots of dinitrophenol and dinitroaniline, only one spot was found. This corresponded to either DNP-glutamic acid or DNP-aspartic acid, which coincide in this system. An excellent separation of these two derivatives is readily achieved in the Blackburn and Lowther (1951) system, and it was possible therefore to demonstrate very quickly that the terminal residue was glutamic acid.

The FDNB procedure cannot distinguish between terminal glutamic acid and its amide because acid hydrolysis cleaves the amide linkage. We resorted, therefore, to the Edman (1949) PTH procedure because under the conditions of the Fraenkel-Conrat (1955) modification the amide is stable. The PTH-terminal amino acid from staphylococcal enterotoxin B was compared chromatographically with authentic samples of PTH-glutamic acid and PTH-glutamine. It corresponded exactly with the derivative of the free acid.

Although the toxin appeared to contain only glutamic acid as an N-terminal residue, other verifying experiments were carried out. The aqueous phase from the hydrolysis was examined for DNP-arginine with negative results. The ether extracts from the isolation of the DNP-protein were examined with negative results. A short hydrolysis in concentrated HCl was performed to test for DNP-proline or DNP-glycine with negative results.

The presence of only one detectable spot on the two-dimensional chromatogram provides an additional means of estimating the purity of the enterotoxin. Less than 0.005 μmole of DNP-amino acid is visually detectable on the paper, and since approximately 0.2 μmole of DNP-toxin was used in the experiments, the maximum impurity is readily calculated. Assuming a 50%

destruction during hydrolysis, it may be safely said that the toxin is >95% pure.

Quantitative Estimation of N-*Terminal Glutamic Acid.* The unidimensional system of Blackburn and Lowther (1951) was used for the quantitative estimation of the N-terminal glutamic acid in the enterotoxin. Quadruplicate determinations were made on DNP-toxin and on DNP-toxin with added quantities of DNP-glutamic acid. This procedure permits the calculation of both the recovery of DNP-glutamic acid and a destruction coefficient for DNP-glutamic acid during the hydrolytic process. The following equation was employed:

$$\frac{A - B(C/D)}{E} \times 100 = \%\text{ recovery of added DNP-glutamic acid}$$

where A = μmoles of DNP-glutamic acid found in samples with added DNP-glutamic acid, B = μmoles of DNP-glutamic acid found in samples without added DNP-glutamic acid, C = μmoles of DNP-toxin in samples with added DNP-glutamic acid, D = μmoles of DNP-toxin in samples without added DNP-glutamic acid, and E = μmoles of added DNP-glutamic acid.

The results are presented in Table III. The recovery of added DNP-glutamic acid was 73.6%, and the observed recoveries of this derivative from the protein have been coreected for this. The number of μmoles of DNP-toxin was calculated assuming complete dinitrophenylation of the molecule, i.e., substitution of 42 lysyl, 24 tyrosyl, 6 histidyl, and 1 amino-terminal residue, with an increase of the molecular weight of the toxin from 35,380 to 47,500.

It is apparent that there is one N-terminal residue per mole. The standard deviation of the set was 0.12 and the 95% confidence limits were 0.94–1.32.

TABLE III: Quantitative Estimation of N-Terminal Residue of Staphylococcal Enterotoxin B by FDNB Method.

Expt	DNP-toxin Used[a] (μmole)	DNP-glutamic Acid Formed[b] (μmole)	Moles DNP-glutamic Acid per Mole of Toxin
1	0.295	0.333	1.13
2	0.272	0.331	1.22
3	0.287	0.274	0.96
4	0.257	0.310	1.21
		Average	1.13

[a] Based on a molecular weight of DNP-toxin as 47,500. [b] Corrected for destruction by acid hydrolysis.

Identification of C-*Terminal Amino Acid.* Hydrazinolysis was employed to identify the C-terminal residue of the enterotoxin. In first attempts the method of Niu and Fraenkel-Conrat (1955) was followed. The hydrazides were removed by treatment with benzaldehyde and the free amino acid was treated with FDNB to form the DNP derivative. Separation and identification was carried out in the Levy (1955) chromatographic system. A residual streak of hydrazides was always found in the first dimension and this interfered with the lysine-tryptophan area with which we were concerned here. We resorted, therefore, to the automatic amino acid analyzer after the reaction with benzaldehyde. Trace amounts of several amino acids (glycine, alanine, leucine, tyrosine, and phenylalanine) were observed on the 150-cm column, but none in sufficient quantity to account for a terminal residue. These traces are customary artifacts in hydrazinolysis.

On the 15-cm column, large amounts of an amino acid were found in the lysine position. Because arginine is degraded to ornithine by hydrazinolysis and ornithine occurs at the identical position with lysine on the chromatographic trace, a run was made on the 50-cm column, in which a good separation of lysine and ornithine occurs. It was clearly evident that only lysine was present in this analysis.

TABLE IV: Quantitative Estimation of C-Terminal Residue of Staphylococcal Enterotoxin B by Hydrazinolysis.

Expt	Toxin Used (μmole)	Lysine Formed[a] (μmole)	Moles of Lysine per Mole of Toxin
1	0.625	0.479	0.766
2	0.625	0.533	0.853
3	0.697	0.449	0.644
4	0.697	0.489	0.702
		Average	0.741

[a] Corrected for destruction in hydrazinolysis and purification.

Quantitative Estimation of C-*Terminal Lysine.* The quantitative estimation of C-terminal lysine was carried out by hydrazinolysis as described above. Quadruplicate determinations were made on the protein alone and on the protein plus added amounts of lysine. An equation identical in form with that used for the N-terminal residue was used to determine the destruction coefficient of lysine in the procedure. The results are presented in Table IV. The recovery of added lysine in the presence

of toxin was 67.3% and the observed lysine recovery from the toxin was corrected for this.[2]

The precision of these results is of the same order obtained in the dinitrophenylation technique. The standard deviation was 0.09 and the 95% confidence limits were 0.59–0.88. The average figure, 0.74 mole of lysine per mole of toxin, appears to be significantly lower statistically than one C-terminal residue per molecule, but it is likely that the 10-hour reaction period was insufficient for this protein. There is abundant documentation in the literature that other proteins require a more extended period for completion of hydrazinolysis (Niu and Fraenkel-Conrat, 1955). In any event it seems to be perfectly reasonable to ascribe one C-terminal residue to the molecule.

Discussion

It is evident from the analyses presented in this paper that staphylococcal enterotoxin B is a simple protein composed solely of amino acids. There are no free SH groups and only one disulfide bridge. It has an unusually high aspartic acid and lysine content. The two comprise one-third of the total number of residues and weight of the protein. Glutamic acid and tyrosine are also present in abundance; indeed ionizable amino acids comprise more than half the residues in the molecule. The total number of polar residues is 209.

The ratio of polar to nonpolar groups, 209:90, invites conjecture on the conformation of the enterotoxin. This ratio in myoglobin is 78:75 (Edmundson and Hirs, 1962), where the molecular weight is half that of the toxin. Myoglobin is a compact molecule with all the polar groups at the surface and nonpolar groups buried in the interior (Kendrew, 1962). If the enterotoxin were equally compact there would be neither sufficient surface area to accommodate all of its polar groups nor enough nonpolar groups to fill the interior. There are two ways in which this representation of the enterotoxin molecule may be altered to accommodate this ratio of polar to nonpolar residues: (a) it may remain compact with at least 50 of the polar residues buried, or (b) it may be extended to provide a sufficiently increased surface area to take all the polar groups.

One amino-terminal residue and 1 carboxyl-terminal residue were detected in the enterotoxin molecule. The simplest and most probable structure satisfying these conditions is a single polypeptide chain. Other structural possibilities must be entertained, however. These may be divided into two categories: structures based on the assumption that certain terminal residues are present but were not determined, or structures based on the assumption of single N and C termini in the molecule.

In the first category the possibility of a cystinyl or an acylated N-terminal residue (which would not be detected by conventional FDNB procedures), and an asparaginyl, glutaminyl, or cystinyl C-terminal residue (which would be lost in hydrazinolysis) must be considered. If a second polypeptide chain existed, it could be bound to the first by either a disulfide bridge or by noncovalent forces. We feel that both these possibilities are quite remote. The enterotoxin has been reduced and alkylated with iodoacetic acid and iodoacetamide without any change in physical, immunological, or biological properties (Dalidowicz *et al.*, 1965). Furthermore, there is no evidence that the molecule can dissociate. It is extremely stable to heat and shows no change in sedimentation patterns or electrophoretic properties over a wide *p*H range.

In the second category two types of structures should be considered: (a) A two-chained molecule, one of which is cyclic, joined by noncovalent bonding. This structure has the attractive feature that dissociation in the intestinal tract might free a small toxic unit that could pass through the gut wall intact. Because preliminary data indicate that the toxin is resistant to the action of the pancreatic endopeptidases, this dissociation would circumvent the problem of adsorption of a large protein molecule. The evidence cited against a molecule of two simple polypeptide chains also applies here. (b) A single polypeptide chain with an internal loop. This would necessitate two unusual linkages in the molecule, e.g., a γ-linked glutamyl peptide and a carboxyl-tyrosyl ester. There is at present no substantive evidence either for or against this type of structure, and although it cannot be rejected offhand there is no reason to assume its existence.

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[2] The recovery for pure lysine was 74.4%. This recovery is significantly higher than the 47% found by Niu and Fraenkel-Conrat (1955). It is also noteworthy that the lysine recovery was reduced in the presence of the toxin in contrast with their findings for other proteins.

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Reactions of Cyanate with Functional Groups of Proteins. III. Reactions with Amino and Carboxyl Groups*

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ABSTRACT: The velocity at which an amino acid or peptide is carbamylated can be estimated if its pK_A is known, since logarithms of the rate constants for reaction of KNCO with a series of such compounds are related linearly to the pK_A values of the amino groups. At *p*H 7 or below, the α-NH_2 groups of peptides and proteins (pK_A about 8) can be expected to react with cyanate about 100 times faster than the ϵ-NH_2 groups of lysine residues (pK_A about 10.7). As a consequence, α-NH_2 groups can be modified selectively under controlled conditions.

The conclusion that the reaction of amines with cyanate occurs by a mechanism involving the uncharged species and not the ions is supported strongly by present evidence that the relative rates of carbamylation by cyanate or ethyl isocyanate in water are the same for a series of ω-NH_2 acids. In addition to the expected carbamyl amino acids, 5-, 6-, and 7-membered lactams are formed by reaction of cyanate or ethyl isocyanate with γ-, δ-, or ϵ-NH_2 acids. These cyclic amides form from intermediate carbamylcarboxylates. Upon exposure of proteins to cyanate, amides, or esters might be formed similarly from favorably situated nucleophiles and the activated carboxyl group. Some general properties of carbamyl compounds are discussed, particularly those of carbamylphosphate that bear on the mechanism of action of ornithine and aspartate carbamyl transferases.

The work reported here began as a systematic attempt to extend our previous observation that glycylalanine reacts much more rapidly than alanine with cyanate at *p*H 8.0 (Stark and Smyth, 1963). Johncock *et al.* (1958) found little correlation between pK_A and rate of carbamylation for a series of 1° and 2° amines but, as shown in the present work, an excellent correlation can be obtained if consideration is limited to the relatively unhindered 1° amino groups of peptides and amino acids. The present results provide a basis for selective modification with cyanate of those protein and peptide amino groups that have low pK_A values. They also provide a way of examining the mechanism of the reaction.

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Experimental Procedures

Materials. Reagent grade KNCO was recrystallized from ethanol-water at a maximum temperature of 50°.