Edeine. IV. Structures of the Antibiotic Peptides Edeines A_1 and B_1*

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ABSTRACT: Edeine A has been shown to be a mixture of the antibiotic peptide edeine A_1 and its inactive isomer, edeine A_2. Edeine B is likewise a similar mixture of active edeine B_1 and inactive edeine B_2. The amino acid sequence of edeine A_1, determined by examination of 30 fragments obtained by partial acid hydrolysis, is *N*-(β-tyrosyl)isoseryl-(α→α)-2,3-diaminopropionyl-(α→α)-2,6-diamino-7-hydroxyazelayl-(ω→α)-glycyl-$NH(CH_2)_3NH(CH_2)_4NH_2$. In edeine B_1, the sequence of the fragments is identical, except that the terminal amino group of the spermidine residue is replaced by a guanidino group. Edeines A_1 and B_1 undergo reversible intramolecular isomerization reactions to give, respectively, edeines A_2 and B_2, in which the isoseryl-2,3-diaminopropionyl linkage is α → β instead of α → α. Partial acid hydrolysates of edeine A contained a number of fragments arising from secondary reactions, among which were γ lactams of peptides containing 2,6-diamino-7-hydroxyazelaic acid, and diketopiperazines of fragments containing 2,3-diaminopropionic acid and 2,6-diamino-7-hydroxyazelaic acid. Edeines A_2 and B_2 are cleaved by carboxypeptidase B at the 2,3-diaminopropionyl-(α→α)-2,6-diamino-7-hydroxyazelayl bond to give a tripeptide and a dipeptide amide, neither of which has antibiotic activity.

Ten years ago Kurylo-Borowska (1959) reported the isolation from soil of a strain of *Bacillus brevis* called Vm4 which had inhibitory effects on the growth of a number of microorganisms. Partial purification of the antibiotic principle indicated that the activity was associated with a strongly basic peptide fraction which was given the name "edeine" Subsequent studies (Borowski *et al.*, 1966; Kurylo-Borowska, 1967) showed that edeine was not a single substance, but a mixture of closely related compounds. We have recently described the isolation and partial characterization of the two major components of the edeine complex, edeines A and B (Roncari *et al.*, 1966; Hettinger and Craig, 1968; Hettinger *et al.*, 1968).

Edeines A and B are peptides which contain five amino acid residues and a basic component joined together by peptide linkages (Roncari *et al.*, 1966; Hettinger and Craig, 1968; Hettinger *et al.*, 1968). The five amino acid fragments, which are the same for both antibiotics, are glycine, isoserine, 2,6-diamino-7-hydroxyazelaic acid, β-tyrosine, and α,β-diaminopropionic acid. The basic constituent of edeine A is spermidine, and in edeine B, it is *N*-guanyl-*N'*-(3-aminopropyl)-1,4-diaminobutane (guanylspermidine).

There are in edeines A and B a total of five free basic groups and one free carboxyl group (Hettinger *et al.*, 1968). The free amino groups of edeine A were found to be the two nitrogens of the C-4 unit of spermidine, one amino group of DAHAA,[1] one amino group of DAPA, and probably the amino group of β-tyrosine. The same end groups were obtained for edeine B, except that the terminal amino group of the C-4 unit of spermidine is replaced by a guanidino group.

In the present paper, the results of further structural studies on edeines A and B are reported. It will be shown that edeines A and B are themselves isomeric mixtures, edeine A being a mixture of edeine A_1 and edeine A_2, and edeine B a mixture of edeine B_1 and edeine B_2. Since these isomers differ only in the nature of the DAPA linkage, the original preparations of edeine A and edeine B were found to be suitable for most of the sequence studies.

The standard stepwise sequential methods of protein chemistry could not be used because of the unusual nature of the amino acid fragments. Therefore the complete sequences of edeines A_1 and A_2 were deduced largely by identification of peptides from partial acid hydrolysates. The structures of edeines B_1 and B_2 were then inferred from these results and from the data on the enzymic digestion of edeines A and B.

Initial attempts to specifically hydrolyze edeines A and B by means of proteolytic enzymes were unsuccessful. No cleavage resulted from incubation of edeine A or edeine B with trypsin, chymotrypsin, Nagarse, aminopeptidase M, pepsin, or carboxypeptidase A. However, it was subsequently found that edeines A and B could be slowly hydrolyzed by carboxypeptidase B, a result which proved to be very useful in structural studies.

* From The Rockefeller University, New York, New York 10021. *Received November 10, 1969.* This work was supported in part by U. S. Public Health Service Grant AM 02493.

[1] Abbreviations used are: DAHAA, 2,6-diamino-7-hydroxyazelaic acid; DANDA, 2,6-diamino-7-nonenedioic acid; DAPA, α,β-diaminopropionic acid; Ise, isoserine (β-aminolactic acid); Spe, spermidine; Speguan, guanylspermidine (*N*-guanyl-*N'*-(3-aminopropyl)-1,4-diaminobutane); β-Tyr, β-tyrosine (β-(*p*-hydroxyphenyl)-β-alanine). All linkages of fragments by means of peptide bonds are shown by the usual notation, where a hyphen indicates a normal CO–NH bond, irrespective of whether the linkage is α or otherwise, as in β-Tyr-Iser. In DAPA peptides, the structures Ise-α-DAPA and Ise-β-DAPA indicate that the α or β amino group, respectively, of DAPA is joined in peptide linkage, while the other is free. In the sequence DAPA-DAHAA, the peptide bond involves the α amino group of DAHAA, and in the sequence DAHAA-Gly it involves the ω carboxyl group of DAHAA. DAPA-DAHAA and DAPA-DAHAA indicate a diketopiperazine of DAPA and DAHAA in the first case and a lactam linkage of the ε amino group and ω carboxyl group of DAHAA in the second. In Gly-Spe and Gly-Speguan, the amide bond is formed from the glycine carboxyl group and the terminal amino group of the C-3 unit of the base.

Experimental Section

Materials. The tetraacetates of edeines A and B were isolated as previously described (Hettinger *et al.*, 1968). The lyophilized preparations containing some residual ammonium acetate were used without preliminary desalting. DFP-treated porcine carboxypeptidase B was obtained from Worthington Biochemical Corp.

Paper Chromatography and Electrophoresis. High-voltage paper electrophoresis of amino acids and peptides was carried out with a cooled-plate apparatus at 0° on Macherey–Nagel 214 paper. For analytical separations, electrophoresis was run at pH 6.4 [pyridine–acetic acid–water (100:4:896)] at 40 V/cm for 1.5 hr or at pH 3.5 [pyridine–acetic acid–water (10:100:890)] at 35 V/cm for 2 hr. For preparative isolation of peptides, 1–4 μmoles of peptide mixture, spread over 5–10 cm of paper, was resolved by electrophoresis at the more favorable pH for 2 hr. Ascending chromatography of amino acids and peptides was carried out on Whatman No. 3MM paper in the system 1-butanol–acetic acid–pyridine–water (6:3:2:3) for 30 hr. For peptide maps of partial hydrolysates, electrophoresis was performed with Macherey–Nagel 214 paper at pH 3.5 for 1.5 hr in the first direction, followed by chromatography in the second direction.

Determination of Antibiotic Activity. Assays were carried out on agar plates inoculated with *B. subtilis*. Inhibition zones for duplicate samples were measured after incubation for 30 hr at 25°. Antibiotic activity was estimated from the diameter of the inhibition zone, which was found to increase 7 mm for a tenfold increase in antibiotic concentration in the range of 15–25 mm. This relationship held for different plates, although the diameter of the inhibition zone for a given sample varied from plate to plate. All comparisons were made with a standard solution of 20 μg of edeine A in 5 μl, which gave an inhibition zone of about 20 mm. The probable error of a single measurement is estimated to be ±0.5 mm, which corresponds to a relative error of ±15%.

Partial Acid Hydrolysis of Edeine A. Edeine A tetraacetate (200 mg) was dissolved in 10 ml of 6 N HCl and heated at 100° for 10 min. The acid was removed by evaporation under reduced pressure at 35°. Evaporation was repeated several times after addition of water.

Isolation of Peptides from Partial Acid Hydrolysates. The hydrolysate was dissolved in 5 ml of buffer 1[2] and put into a column (2.2 × 26 cm) of Bio-Rad AG50-X4(200–400 mesh) equilibrated with the same buffer. The column was operated at 50° with a flow rate of 60 ml/hr maintained by means of a Milton-Roy "miniPump." Fractions of 5 ml were collected with the following elution schedule: buffer 1, 100 fractions; then a linear gradient of increasing pH and buffer concentration starting with 1000 ml of buffer 1 and 1000 ml of buffer 2, 300 fractions; and finally, buffer 3, 100 fractions. For analysis by the ninhydrin method (Moore and Stein, 1954), 0.5-ml samples were treated with 1 ml of ninhydrin reagent, heated at 100° for 15 min, and diluted with 5 ml of 50% ethanol, and the absorbance read at 570 mμ. The results are shown in Figure 1.

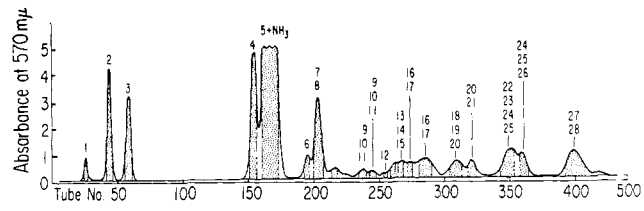


FIGURE 1: Chromatography of the partial acid hydrolysate of edeine A on Bio-Rad AG 50-X4 with pyridine acetate buffers at 50°. Fractions were pooled as indicated by the shaded areas. The numbers correspond to the isolated fragments listed in Table I.

The peptide fractions were pooled as indicated and evaporated to dryness under reduced pressure at 35°. Water was added and the evaporation was repeated several times. Where necessary, the pooled fractions were further purified by paper electrophoresis at pH 6.4 or pH 3.5. The zones were then eluted from the paper with water and the resulting solutions were evaporated to dryness. Purity of the isolated peptides was checked by paper chromatography. Less than 1 μmole of peptide was required for characterization. The strongly basic fractions, designated **29** and **30**, were not eluted from the column but were isolated directly from a 10-min hydrolysate of 3 mg of edeine A by electrophoresis for 1.5 hr at pH 3.5.

Identification of the Products of Partial Acid Hydrolysis. Peptide compositions were determined by hydrolyzing about 0.2 μmole of peptide in 40 μl of 6 N HCl at 110° for 8 hr and examining the hydrolysate by means of paper electrophoresis at pH 3.5 and by paper chromatography. This combination permits identification of all of the components of edeine A (Hettinger and Craig, 1968).

Free amino groups were determined by the DNP method (Fraenkel-Conrat *et al.*, 1955). About 0.2 μmole of peptide was dissolved in 10 μl of 4% $NaHCO_3$ and treated with 20 μl of 2% (v/v) 2,4-dinitrofluorobenzene in 95% ethanol with occasional shaking for 3 hr. After several extractions with ether, the mixture was dried and hydrolyzed in 50 μl of 6 N HCl for 5 hr at 100°. Water- and ether-soluble compounds were separated and identified as previously described (Hettinger *et al.*, 1968).

Digestion of Edeines A and B with Carboxypeptidase B. Reaction mixtures contained 2.8 mg of edeine A or B and 0.24 mg of carboxypeptidase B in 100 μl of 0.1 M ammonium bicarbonate (pH 8.1). Samples containing edeine A were incubated with enzyme at 25° for 9, 24, and 105 hr; edeine B was reacted for 105 hr only. Blanks without enzyme were also prepared and incubated for 0 and 105 hr. The samples were assayed directly for antibiotic activity and resolved into the various fractions by paper electrophoresis at pH 6.4 for 2 hr at 40 V/cm. The ninhydrin-positive bands were cut out, eluted with 1 N acetic acid, evaporated, and subjected to amino acid and end-group analyses. For quantitative determination of edeine A_1, edeine A_2, β-Tyr-Ise-α-DAPA, and β-Tyr-Ise-β-DAPA, their DNP derivatives were hydrolyzed and the products separated by electrophoresis at pH 1.9 (Hettinger *et al.*, 1968). The α-DNP-DAPA and β-DNP-DAPA were eluted from the paper with 1 N acetic acid and the amount of each was calculated from the absorbance at 350 mμ, assuming a molar absorbancy index of 15,000. The relative amount of each pep-

[2] For ion-exchange chromatography of the peptides, the following deaerated buffers were used: buffer 1, 0.2 M pyridine–acetic acid, pH 3.1 (32.2 ml of pyridine, 558 ml of acetic acid, diluted to 2000 ml); buffer 2, 2.0 M pyridine–acetic acid, pH 5.0 (322 ml of pyridine, 286 ml of acetic acid, diluted to 2000 ml); and buffer 3, 3.0 M pyridine–acetic acid, pH 5.0 (242 ml of pyridine, 215 ml of acetic acid, diluted to 1000 ml).

TABLE I: Structures and Properties of Fragments Isolated from the Partial Acid Hydrolysate of Edeine A.

Fragment No.	Structure	Mobility (mm) pH 6.4[a]	Mobility (mm) pH 3.5[b]	R_F[c]	Initial Ninhydrin Color[d]
1	DAHAA	11	23	0.27	Blue
2	DAHAA-Gly	13	37	0.26	Blue–grey
3	Ise	14	53	0.26	Blue[g]
4	β-Tyr-Ise	14	58	0.41	Yellow–grey[g]
5	DAHAA-Gly	12	58[e]	0.13	Blue
6	DAHAA	13	95	0.16	Blue
7	β-Tyr	15	80[e]	0.57	Yellow–grey[g]
8	DAPA	142	215[e]	0.22	Red–blue
9	DAPA-DAHAA	90[e]	125	0.30	Yellow
10	Ise-α-DAPA-DAHAA	75[e]	115	0.17	Blue
11	Ise-β-DAPA-DAHAA	61[e]	115	0.17	Blue
12	DAPA-DAHAA	18[e]	115	0.24	Red–brown
13	β-Tyr-Ise-α-DAPA	83	109[e]	0.27	Blue
14	β-Tyr-Ise-β-DAPA				
15	DAPA-DAHAA-Gly	78	136[e]	0.15	Yellow
16	β-Tyr-Ise-α-DAPA-DAHAA	61[e]	93	0.24	Blue
17	β-Tyr-Ise-β-DAPA-DAHAA	52[e]	93	0.24	Blue
18	Ise-α-DAPA-DAHAA-Gly	68[e]	121	0.08	Blue
19	Ise-β-DAPA-DAHAA-Gly	57[e]	121	0.08	Blue
20	DAPA-DAHAA	87[e]	172	0.19	Yellow
21	DAPA-DAHAA-Gly	16	117[e]	0.09	Red–brown
22	Ise-α-DAPA-DAHAA	73[e]		0.08	Blue
23	Ise-β-DAPA-DAHAA	62[e]		0.09	Blue
24	β-Tyr-Ise-α-DAPA-DAHAA-Gly	54[e]	93	0.11	Blue
25	β-Tyr-Ise-β-DAPA-DAHAA-Gly	47[e]	93	0.11	Blue
26	DAPA-DAHAA	18	148[e]	0.14	Red–brown
27	β-Tyr-Ise-α-DAPA-DAHAA	60[e]	122	0.16	Blue
28	β-Tyr-Ise-β-DAPA-DAHAA	52[e]	122	0.16	Blue
29	Gly-Spe		338[e,f]	0.24	Yellow–grey
30	Spe	267	372[e,f]	0.28	Blue

[a] Pyridine–acetic acid–water (100:4:896); 1.5 hr at 40 V/cm. [b] Pyridine–acetic acid–water (10:100:890); 2.0 hr at 35 V/cm. [c] Whatman No. 3 MM paper; 1-butanol–acetic acid–pyridine–water (6:3:2:3). [d] On electrophoretograms developed at room temperature after dipping into 0.25% ninhydrin in acetone. Colors obtained on paper chromatograms were in general similar. A notable exception was β-tyrosine, which appeared pink on chromatograms. [e] Method used for purification of fragment. [f] Calculated from data for 1.5-hr electrophoresis. [g] After heating for 1 min at 100°.

tide was assumed to be proportional to the amount of mono-DNP-DAPA derived therefrom.

Separation of Edeine A_1 and Edeine A_2. Edeine A was resolved into edeine A_1 and edeine A_2 by electrophoresis on cellulose acetate strips at pH 6.4 (40 V/cm, 65 min). Separation was critically dependent on the amount applied, the wetness of the strips, and the temperature, and was somewhat lacking in reproducibility. The best results were obtained at 0° with a load of 25 μg of edeine A/cm. Marginal separation of the two cationic compounds was revealed by spraying with ninhydrin: edeine A_1, 23.2–24.0 cm; edeine A_2, 22.0–23.0 cm. They appeared to be present in approximately equal amounts. Both gave the same products on hydrolysis of their DNP derivatives, except that edeine A_1 gave largely β-DNP-DAPA, while edeine A_2 gave largely α-DNP-DAPA. Only edeine A_1 had antibiotic activity.

Results

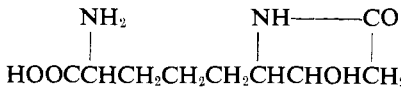
A peptide map of the 10-min hydrolysate of edeine A indicated the presence of at least 30 distinct spots. Hydrolysis for longer times gave simpler patterns, but resulted in extensive conversion of edeine A into amino acids, and there was an insufficient number of peptides remaining for sequence determination.

Chromatography of the 10-min hydrolysate on Bio-Rad

AG 50-X4 gave the results shown in Figure 1. Of the 20 pooled fractions obtained, only those containing fragments **1, 2, 3, 4,** and **6** were essentially pure. The others required further purification by paper electrophoresis. In some cases the same peptides appeared in well-separated fractions, possibly due to transformation during work-up. In the results given below, peptides are listed in the fractions in which they predominantly occur. From these fractions a total of 28 different fragments (**1–28**) were ultimately isolated and identified. Two additional fragments (**29** and **30**) were obtained directly by electrophoresis of an edeine A hydrolysate.

In Table I are given the structures of the fragments isolated from the 10-min hydrolysate. The structures were determined from the amino acid compositions and end-group analyses. In all cases, the results were consistent with those expected for the structures given, even though the actual products obtained are not enumerated in each case where they are largely self-evident. To some degree, chromatographic and electrophoretic properties, as well as ninhydrin colors, could be correlated with structure. The yields given below are rough estimates based on ninhydrin colors. Where not mentioned, yields were generally in the range of 1–5%. The numbers of the fragments correspond to those of Figure 1.

1. DAHAA. This fragment gave only DAHAA[3] on hydrolysis, and its DNP derivative gave mono-DNP-DAHAA[4] on hydrolysis. These facts, along with the evidence that **1** is neutral and has a higher R_F than DAHAA, indicate a lactam structure. The electrophoretic mobility of only 23 mm at pH 3.5 suggests an α amino acid function. The most likely structure of **1** is therefore



The yield of **1** was about 1%.

2. Gly. The yield was about 10%.

3. Ise. The yield was about 20%.

4. β-Tyr-Ise. Hydrolysis of the DNP derivative gave free Ise.[5] The electrophoretic mobility of **4** at pH 3.5 suggests that the carboxyl group is more acidic than in β-Tyr, which also indicates that the Ise residue is C terminal. The yield of this dipeptide was about 20%.

5. DAHAA-Gly. The DNP derivative gave Gly and bis-DNP-DAHAA[6] on hydrolysis. The acidity of the carboxyl groups on the average appears to be greater than in DAHAA, which suggests that Gly is attached to the ω carboxyl group of DAHAA. Most of the ninhydrin color of the band containing **5** is due to ammonia, which arises from residual ammonium acetate present in the edeine A sample. Edeine A itself does not yield ammonia, except by decomposition of the amino acids after long periods of hydrolysis (Hettinger *et al.*, 1968).

6. DAHAA. The yield was about 2%.

7. β-Tyr. This was the major component of the band containing **7** and **8.** Its yield was estimated to be approximately 20%. Since β-Tyr is thought to be N terminal in edeine A, its yield should exceed that of the next amino acid, Ise. However, the fact that β-Tyr is not completely stable may explain the relatively low recovery of this fragment.

8. DAPA. The approximate yield was 2%.

9. DAPA-DAHAA. The DNP derivative on hydrolysis gave β-DNP-DAPA and free DAHAA.[3] The presence of only a single free amino group in fragment **9** indicates a structure containing two rings. It is reasonable to assume that the DAHAA residue has a lactam structure like that of fragment **1.** The second ring is readily accounted for by a diketopiperazine structure. The existence of fragments **12, 20,** and **26** supports these conclusions. The yellow ninhydrin color of peptide **9** appears to be general for DAPA diketopiperazines, as it is given also for peptides **15** and **20,** which also have this type of structure.

10. Ise-α-DAPA-DAHAA. Hydrolysis of the DNP derivative gave β-DNP-DAPA, free DAHAA,[3] and no Ise.[5] DAPA must be attached to the α amino group of DAHAA, since the latter exists in the lactam form.

11. Ise-β-DAPA-DAHAA. The DNP derivative on hydrolysis gave α-DNP-DAPA, free DAHAA,[3] and no Ise.[5] The separation of isomers **10** and **11,** as well as other isomeric DAPA peptides (see below), is apparently due to differences between the dissociation constants of the α and β ammonium groups.[7]

12. DAPA-DAHAA. This compound gave bis-DNP-DAPA and free DAHAA[3] on hydrolysis of its DNP derivative. This fragment, as well as fragments **21** and **26,** had a very low mobility at pH 6.4 and gave a red-brown ninhydrin color,

[3] All fragments containing DAHAA gave on hydrolysis some DANDA in addition to DAHAA. DANDA is an artifact which arises from DAHAA by β elimination of 1 mole of water (Hettinger and Craig, 1968). It is assumed that no peptide isolated contained DANDA instead of DAHAA, since no free DANDA was detected in the partial acid hydrolysate.

[4] The mono-α- and ϵ-DNP derivatives of DAHAA were indistinguishable under the conditions used for separation. In general, it was possible to place the point of substitution through other considerations.

[5] The DNP derivatives of β-Tyr and Ise were not obtained from the hydrolysate of any dinitrophenylated peptide since in peptide linkage they do not survive acid hydrolysis. Identification of β-Tyr as an end group in peptides is based on the failure to observe β-Tyr or *O*-DNP-β-Tyr in the hydrolysates of their DNP derivatives. In the same way, if Ise is the amino terminal residue of a peptide, no Ise is found in the hydrolysate of its DNP derivative. When in peptide linkage, DNP-Ise appears to be less stable than free DNP-Ise for the latter does survive acid hydrolysis to an extent sufficient for identification.

[6] The bis-DNP derivatives of DAHAA and DANDA could be distinguished by thin-layer chromatography. Bis-DNP-DANDA had an R_F equal to that of DNP-Gly, but this did not prove to be a problem, as only traces of bis-DNP-DANDA are formed from bis-DNP-DAHAA under the conditions used for hydrolysis of the DNP derivatives.

[7] The pK value of the free α amino group of DAPA in the sequence Ise-β-DAPA-DAHAA is within the buffering region of the buffer (pH 6.4) used for electrophoresis. Assuming Ise-α-DAPA-DAHAA to have a net charge of +1 at pH 6.4 and that a compound with zero net charge would migrate 12 mm due to electroendosmotic flow, Ise-β-DAPA-DAHAA is calculated to have a net charge of $(61 - 12)/(75 - 12) \times 1$, or +0.78. The p$K$ of the α amino group is therefore estimated to be 6.4 + log (0.78/0.22), or approximately 6.9. Calculations based on other isomeric DAPA peptides give similar results. This value is reasonable, since the pK should be less than that for Ala-Ala (8.1) due to the strong inductive effect of the β-acylamido group. An approximate measure of this effect can be determined from the difference between the pK values of ethylamine (10.7) and β-acetamidoethylamine (9.2). All things being equal, the pK of the α amino group of internal DAPA would then be about 6.6. The pK of the β amino group of internal DAPA would be at least one unit higher.

TABLE II: Structures and Properties of Edeines A and B and Products of Carboxypeptidase B Digests.[a]

Peptide	Structure	Mobility at pH 6.4 (mm)	R_F
Edeine A_1	β-Tyr-Ise-α-DAPA-DAHAA-Gly-Spe	127	0.08
Edeine A_2	β-Tyr-Ise-β-DAPA-DAHAA-Gly-Spe		
A1	β-Tyr-Ise-α-DAPA	80	0.27
A2	β-Tyr-Ise-β-DAPA		
A3	DAHAA-Gly-Spe	163	0.09
Edeine B_1	β-Tyr-Ise-α-DAPA-DAHAA-Gly-Speguan	124	0.11
Edeine B_2	β-Tyr-Ise-β-DAPA-DAHAA-Gly-Speguan		
B1	β-Tyr-Ise-α-DAPA	79	0.28
B2	β-Tyr-Ise-β-DAPA		
B3	DAHAA-Gly-Speguan	157	0.13

[a] The electrophoretic data were calculated from 2-hr runs and normalized to 1.5 hr to conform to the conditions of Table I. The R_F values also refer to the chromatographic system of Table I. All peptides in this table gave blue colors with ninhydrin.

due to the fact that both amino groups of DAPA are free.[8,9]

13 and **14**. β-Tyr-Ise-α-DAPA and β-Tyr-Ise-β-DAPA. Hydrolysis of the DNP derivative gave free Ise[5] and a mixture of α-DNP-DAPA and β-DNP-DAPA. This was the only pair of isomeric α- and β-DAPA peptides which could not be resolved by electrophoresis at pH 6.4. The lack of separation is due to the fact that the ammonium groups of C-terminal DAPA have higher pK values[10] than those of internal DAPA.

15. DAPA-DAHAA-Gly. Free Gly, mono-DNP-DAHAA,[4] and β-DNP-DAPA were obtained from the hydrolysate of the DNP derivative.

16. β-Tyr-Ise-α-DAPA-DAHAA. The DNP derivative gave DAHAA,[3] Ise,[5] and β-DNP-DAPA on hydrolysis.

17. β-Tyr-Ise-β-DAPA-DAHAA. Isomer of **16**.

18. Ise-α-DAPA-DAHAA-Gly. The DNP derivative on hydrolysis gave no Ise.[5]

19. Ise-β-DAPA-DAHAA-Gly. Isomer of **18**.

20. DAPA-DAHAA. The DNP derivative on hydrolysis gave β-DNP-DAPA and mono-DNP-DAHAA.[4] Like **9** and **15**, **20** gave a yellow ninhydrin reaction, due to the diketopiperazine structure.

21. DAPA-DAHAA-Gly.

22. Ise-α-DAPA-DAHAA. The mobility of this compound is greater than that of **10** at pH 3.5 due to the presence of protonated ω ammonium and carboxyl groups.

23. Ise-β-DAPA-DAHAA. Isomer of **22**.

24. β-Tyr-Ise-α-DAPA-DAHAA-Gly. Of all peptides containing α-linked DAPA, **24** had the lowest mobility at pH 3.5, owing to its large size. The R_F of **24** is slightly greater than that of **18**, which lacks β-Tyr.

25. β-Tyr-Ise-β-DAPA-DAHAA-Gly. Isomer of **24**.

26. DAPA-DAHAA. Like **12** and **21**, this peptide gave a red–brown color with ninhydrin, had a very low mobility at pH 6.4, and gave bis-DNP-DAPA on hydrolysis of its DNP derivative.

27. β-Tyr-Ise-α-DAPA-DAHAA. This fragment had a higher R_F than **24**, which contains Gly, and a lower R_F than **16**, in which the DAHAA residue has the lactam structure.

28. β-Tyr-Ise-β-DAPA-DAHAA. Isomer of **27**.

29. Gly-Spe. This compound was obtained directly in about 50% yield by electrophoresis of the total digest. The structure must be Gly-NH$(CH_2)_3$NH$(CH_2)_4NH_2$, since the two nitrogens of the C-4 portion of Spe are not involved in amide linkages (Hettinger *et al.*, 1968).

30. Spe. The yield of this fragment, which was isolated from the total digest in the same way as **29**, was about 20%.

The above results indicate the sequence β-Tyr-Ise-DAPA-DAHAA-Gly-Spe for edeine A, but do not demonstrate whether the Ise-DAPA linkage is α or β. In our previous paper (Hettinger *et al.*, 1968) we reported the suspicion that edeines A and B were themselves mixtures in that the isolated preparations contained both α- and β-linked DAPA. Since the results with smaller DAPA peptides showed clear separation of α and β DAPA peptides by electrophoresis at pH 6.4, it was thought that the two edeine A isomers could be resolved by this method. However, it was not possible to achieve separation on

[8] DAPA-DAHAA migrates only 6 mm beyond the neutral zone, while the peptide DAPA-DAHAA (**20**), of equivalent size, migrates 75 mm. If the latter is taken to have a net charge of +1 at pH 6.4, it follows that DAPA-DAHAA should have a net charge of only +0.08. Therefore, the pK of the α amino group would be 6.4 − log (0.92/0.08), or about 5.3. A crude estimate based on the pK values of the α amino group of DAPA (6.6), Ala (9.7), and Ala-Ala (8.1) gives a value of 5.0 for the pK of the α amino group of N-terminal DAPA.

[9] An unusual reaction of free DAPA with ninhydrin may be responsible for the abnormally low color value obtained on the amino acid analyzer (Hettinger *et al.*, 1968).

[10] Of the two isomers, the pK of the free DAPA amino group of **14** would be lower than that of **13**. If an acylamido group in the β position lowers the pK of an amine by 1.5 units,[6] the pK of the α amino group of C-terminal DAPA in RCO-β-DAPA would be reduced from that of Ala (9.7) to approximately 8.2. The pK of the free β amino group of RCO-α-DAPA would be even higher. At pH 6.4, both **13** and **14** would thus have essentially the same net charge (+1), and no separation would be observed. On the other hand, the presence of powerful electron withdrawing groups in both the α and β positions of **13** and **14** makes the carboxyl group very acidic. This is reflected in the fact that, of all peptides containing DAPA, **13** and **14** showed the least difference in electrophoretic mobility between pH 6.4 and 3.5.

TABLE III: Antibiotic Activity and Isomeric Composition of Edeine A and Carboxypeptidase B Digest of Edeine A.

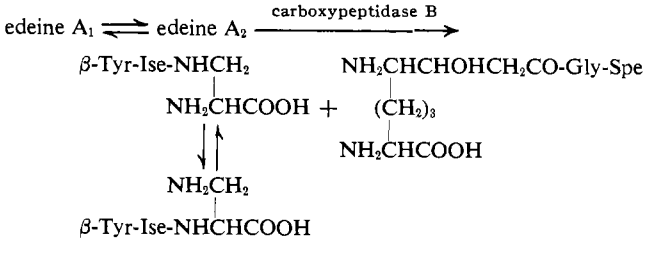
	Initial Mixture % of All Products	105-hr Control % of All Products	105-hr Control % of Initial Value	105-hr Reaction % of All Products	105-hr Reaction % of Control Value
Activity			45		55
Edeine A_1	55	41	75	27	66
Edeine A_2	45	59	131	3	5
Total edeine A	100	100	100	30	30

paper due to streaking, which probably results from adsorption to the paper. Marginal separation of edeine A_1 and edeine A_2 was obtained by running the electrophoresis on cellulose acetate strips at pH 6.4. Amino acid and end-group analyses indicated that the faster-moving active edeine A_1 had a free β amino group, while in the inactive edeine A_2 the α amino group of DAPA was free.[11]

The enzymic hydrolysis of edeines A and B was achieved by digestion with carboxypeptidase B for 105 hr. Qualitative analysis of the products gave the results shown in Table II. For comparison, the properties of edeines A and B are also given. The major products of the cleavage were the peptides DAHAA-Gly-Spe (A3, from edeine A) or DAHAA-Gly-Speguan (B3, from edeine B), and β-Tyr-Ise-β-DAPA (A2, from edeine A, or B2, from edeine B). Small amounts of β-Tyr-Ise-α-DAPA (A1, from edeine A, or B1, from edeine B) were also found.

The course of the enzymic digestion of edeine A was followed kinetically in order to establish the nature of the reaction more clearly (Figure 2). It is apparent from the data that edeine A_2 is attacked readily by carboxypeptidase B, whereas edeine A_1 is cleaved very slowly or not at all. The rate of disappearance of edeine A_2 follows approximately first-order kinetics, while the decrease in edeine A_1 is biphasic. These results can be explained by a mechanism in which edeine A_1 is first reversibly converted into edeine A_2, which is then acted upon by the enzyme (Scheme I).

SCHEME 1



[11] The separation of the isomeric edeine A components appears to depend on a difference between the pK values of the free ammonium group of their DAPA residues. Edeine A_1 and edeine A_2 migrated 24 and 23 cm, respectively, during electrophoresis on cellulose acetate strips at pH 6.4 (see Experimental Section). If edeine A_1 is taken to have a net charge of +4.00 at this pH, the net charge of edeine A_2 is calculated to be +3.83. From this, the pK of the free α amino group of edeine A_2 is estimated to be 6.4 + log (0.83/0.17), or approximately 7.1. Similar results are obtained for smaller DAPA peptides.[6]

The distribution of the products of the 105-hr carboxypeptidase B digest of edeine A is given in Table III. The amount of residual edeine A_2 was only 5% of the control value, while 66% of edeine A_1 still remained. The antibiotic activity of the digest was 55% of the control value, which, in view of the relatively large errors of the measurements, corresponds reasonably well with the amount of edeine A_1 remaining. There was poor agreement between the activity and the amount of edeine A_2 or total edeine A remaining. In the control experiment in which no enzyme was added, no cleavage of the peptide chain occurred, but there was an increase in the amount of edeine A_2 at the expense of edeine A_1. Initially, edeine A_1 constituted 55% of the total, and after 105 hr, it was 41%. This demonstrates that $\alpha \rightleftharpoons \beta$ acyl migration can occur, but this is a relatively slow process at pH 8.1. Calculations based on the results of enzymic digestion indicate that the rate constants for the interconversion cannot exceed 0.01 hr^{-1}. The activity in the control experiment was only 45% of the initial value, while the amount of edeine A_1 was estimated to be 75% that present at zero time.

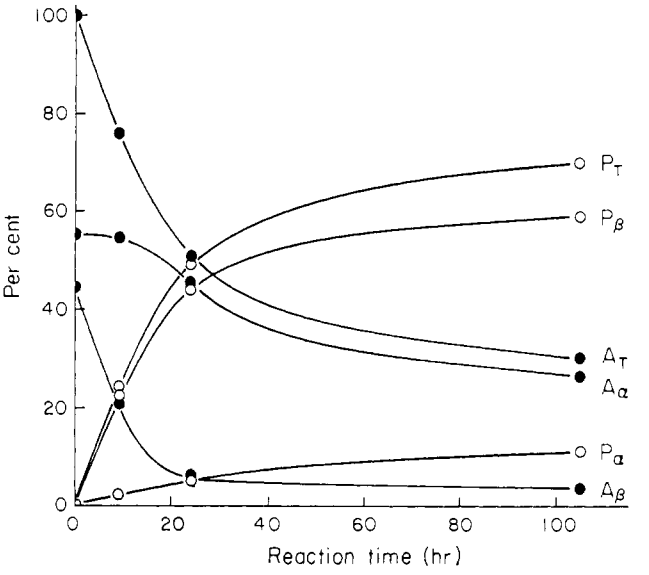


FIGURE 2: Kinetics of hydrolysis of edeine A by carboxypeptidase B in 0.1 M NH_4HCO_3 buffer, pH 8.1, at 25°. The initial edeine A concentration was 2.8%, and carboxypeptidase B, 0.24%. A_α = edeine A_1; A_β = edeine A_2; $A_T = A_\alpha + A_\beta$ = total edeine A; P_α = β-Tyr-Ise-α-DAPA; P_β = β-Tyr-Ise-β-DAPA; $P_T = P_\alpha + P_\beta$ = total tripeptide.

It appears that there is some inactivation of the antibiotic which cannot be explained by $\alpha \rightleftharpoons \beta$ acyl migration, but the cause of this inactivation is as yet unknown.

Discussion

In our previous paper (Hettinger *et al.*, 1968), results were obtained which indicated that edeines A and B could be described as acyclic pentapeptide amides. This follows from the fact that, of the six carboxyl groups of the six fragments, one exists in the free state, while only five remain to join the six fragments together. Furthermore, β-Tyr can be considered to be the amino terminus insofar as all the other constituents contain an amino group bound in peptide linkage. From this amino terminus the peptide chain proceeds until it reaches DAHAA, at which point branching occurs, with one arm leading to the free carboxyl group, and the other terminating in an amide linkage with the base.

Since the identification of as few as five peptides could be sufficient to establish the sequential arrangement of the fragments in such a structure, it was hoped that partial acid hydrolysis would be a convenient method of fragmentation. A chromatography–electrophoresis peptide map (see Experimental Section) of the 10-min hydrolysate of edeine A, however, proved to be very complex, and it became necessary to analyze nearly all of the products in order to explain how as many as 30 fragments could be obtained from a peptide from which a maximum of 21 normally could be expected.

A number of peptides were isolated which had different properties but identical amino acid compositions (Table I). For example, peptides with the compositions DAPA, DAHAA; Ise, DAPA, DAHAA; and β-Tyr, Ise, DAPA, DAHAA each appeared no fewer than four times, and several other peptides occurred twice. Dinitrophenylation of these peptides revealed differences in the amino terminal groups, and it was possible to derive reasonable structures for the multiple forms of most of the fragments. Seven isomeric pairs of peptides were obtained which differed in the Ise-DAPA linkage, which could involve either the α or β amino group of DAPA: **10** and **11, 13** and **14, 16** and **17, 18** and **19, 22** and **23, 24** and **25,** and **27** and **28**. Peptides of DAPA were isolated in which DAPA was the amino terminal residue (**12, 21,** and **26**) or part of the corresponding diketopiperazine (**9, 15,** and **20**). Fragments containing carboxyl terminal DAHAA were found to exist in either the open (**6, 20, 22, 23, 26, 27,** and **28**) or corresponding lactam (**1, 9, 10, 11, 12, 16,** and **17**) forms. All three types of duplications can be explained in terms of well-known intramolecular aminolysis reactions. The isomerization of α- and β-DAPA peptides is known to occur in strong acid solution (Poduška *et al.*, 1965), although it is not necessary to invoke such a transformation in this case, since α- and β-DAPA isomers were shown to exist preformed in unhydrolyzed edeine A. Diketopiperazines are known to be products of partial acid hydrolysis of certain α peptides (Greenstein and Winitz, 1961), and peptides containing amino terminal glutamine can be transformed into pyrrolidone derivatives under acidic conditions (Hill, 1965).

The 30 fragments included in Table I represent nearly all of the theoretically possible peptides and transformation products obtainable from edeine A, except for the fragments containing spermidine, of which only Gly-Spe (**29**) and Spe (**30**) were isolated. Spermidine peptides would not be expected in any of the fractions from ion-exchange chromatography, as they are too basic to be eluted from the column. The dipeptide pair Ise-α-DAPA and Ise-β-DAPA also was not found, and it is possible that these products were transformed under the conditions of hydrolysis. In particular, the peptide Ise-α-DAPA could readily cyclize to a diketomorpholine derivative, which would be strongly adsorbed by the Bio-Rad column. Possible structures of DAPA peptides which were not found are those in which both DAPA amino groups were blocked, either in the form of an imidazoline or as a diketopiperazine in which the β amino group was bound in peptide linkage, as in Ise-β-DAPA-DAHAA. The failure to observe the latter type of compound suggests that formation of DAPA diketopiperazines requires a free β ammonium group. It is possible that small amounts of these missing peptides may be found in the two Bio-Rad 50 bands from which no peptides could be obtained in pure form and in sufficient yield for characterization. The peptide map does not distinguish between α- and β-DAPA peptides, so that the 30 isolated fragments do not represent all of the fragments produced by partial acid hydrolysis of edeine A. They do, however, include all of the major products in the hydrolysate, and all of these were ultimately explainable in terms of the complete structure of edeine A.

The overall sequence of the residues of edeine A can be seen from inspection of Table I, which indicates the structure β-Tyr-Ise-DAPA-DAHAA-Gly-Spe. Since the nature of each linkage is ambiguous when presented in this form, we shall consider briefly each in turn, and some of the evidence which establishes the identity of the individual peptide bonds.

The Linkage β-Tyr-Ise. Evidence for β-Tyr as the amino terminal residue in edeine A has already been presented (Hettinger *et al.*, 1968), and the combination of overlapping sequences of the peptides in Table I allows no other choice. Free Ise was obtained from the hydrolysate of the DNP derivative of edeine A and the dipeptide **4** (β-Tyr-Ise), which indicates that the carboxyl group of β-Tyr is linked to the amino group of Ise and not to the hydroxyl group. There was obtained a fraction not given in Table I, which also contained only β-Tyr and Ise, but it was not obtained in pure form, and may be a mixture of β-Tyr-Ise and β-Tyr-*O*-Ise. The latter compound could be formed during acid hydrolysis by an N → O acyl migration. In any case it does not exist as such in edeine A, since an ester linkage would be at variance with the results of dinitrophenylation and acid–base titrations (Hettinger *et al.*, 1968). The failure to titrate a weakly basic function in edeine A also appears to rule out an oxazoline structure for the β-Tyr-Ise linkage.

The Linkage Ise-DAPA. The choice here is between the α linkage, Ise-α-DAPA, and the isomeric β peptide structure. The results of electrophoresis of edeine A on cellulose acetate strips indicate that the active edeine A_1 has the α linkage and that the corresponding edeine A_2 with the β linkage is inactive. The native antibiotic is therefore considered to have the α peptide structure, while the β isomer is thought to be an artifact of isolation resulting from $\alpha \rightleftharpoons \beta$ acyl migration (Poduška *et al.*, 1965).

The Linkage DAPA-DAHAA. The DAPA residue must be connected to the α amino group of DAHAA, otherwise the diketopiperazine structure of peptides **9, 15,** and **20** would not be possible. Three normal peptides, **12, 21,** and **26,** contained amino terminal DAPA and not the inverted sequence

DAHAA-DAPA. Furthermore, DAPA is the carboxyl terminal residue of fragments **13** and **14**.

The Linkage DAHAA-Gly. In the peptide DAPA-DAHAA-Gly (**15**), the α carboxyl group of DAHAA is bound in the diketopiperazine. Therefore, the ω carboxyl group of DAHAA must be connected to the amino group of glycine. End-group analyses indicate that the diketopiperazine structure does not exist in edeine A itself, so that the free carboxyl group of edeine A must be the α carboxyl group of the DAHAA residue. The pK of 2.9 found for the free carboxyl group (Hettinger *et al.*, 1968) is reasonable for this structure. The occurrence of the lactam structure in some peptides of DAHAA is interesting, since free DAHAA does not appear to give DAHAA on hydrolysis. This suggests that the ω peptide structure facilitates cyclization.

The Linkage Gly-Spe. The Gly residue must be attached to the terminal amino group of the C-3 unit of the Spe residue, as the other two nitrogens of Spe are free (Hettinger *et al.*, 1968).

The structures of edeines A_1 and A_2 deduced from the results of partial acid hydrolysis were fully confirmed by examination of the products of enzymic digestion of edeine A. Cleavage of edeine A by carboxypeptidase B gave the fragment DAHAA-Gly-Spe (A3) and the isomeric pair of peptides β-Tyr-Ise-α-DAPA (A1) and β-Tyr-Ise-β-DAPA (A2), which accounted for all the constituents of edeine A. Since carboxypeptidase B cleaves peptides only at carboxyl terminal residues containing an onium function at the δ or ϵ position (Folk and Gladner, 1958), it follows that in A3 both amino groups and the α carboxyl group of DAHAA must be free. Therefore, the ω carboxyl group must be attached to the Gly-Spe moiety. The peptide pair A1 and A2 appeared to be identical with the fragments **13** and **14** obtained from the partial acid hydrolysate. In unhydrolyzed edeine A, the α amino group of DAHAA is substituted by the β-Tyr-Ise-DAPA unit. The specific cleavage of edeine A_2 at the DAPA-DAHAA bond, in contrast to the failure to hydrolyze the corresponding bond of edeine A_1, could not have been predicted on the basis of the available specificity data for carboxypeptidase B. The isomer which is cleaved is analogous to an ordinary dipeptide in having free α amino and α carboxyl groups. The rate of cleavage of edeine A_2 by carboxypeptidase B is similar to that for glycyl-L-lysine.

Hydrolysis of edeine B by carboxypeptidase B gave results which were entirely analogous. The products β-Tyr-Ise-α-DAPA (B1) and β-Tyr-Ise-β-DAPA (B2) were indistinguishable from the corresponding peptides A1 and A2 obtained from edeine A. Furthermore, the strongly basic fragment B3 (DAHAA-Gly-Speguan) differed from A3 only in that it gave guanylspermidine on hydrolysis instead of spermidine. These results establish that edeines A_1 and B_1 cannot be cyclic and that they are most likely described by the structures shown in Figure 3.

These structures do not appear to be similar to those of any other well-characterized peptides. The amino acids isoserine, β-tyrosine, and 2,6-diamino-7-hydroxyazelaic acid have not previously been found to be components of any naturally occurring peptide. There have been few reports of the existence in nature of peptides containing spermidine (Tabor and Tabor, 1964) or diaminopropionic acid (Haskell *et al.*, 1952; Takita *et al.*, 1968). The β-Tyr-Ise-DAPA portion of edeines A and B only vaguely resembles the partial sequence β-Lys-Ser-DAPA which may occur in the antibiotic viomycin (Kitagawa *et al.*, 1968; Bycroft *et al.*, 1968).

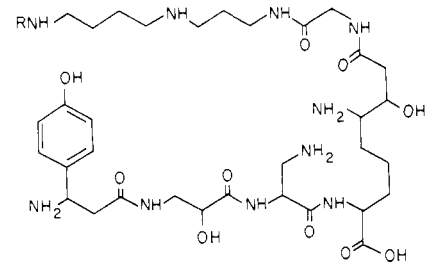


FIGURE 3: Proposed structural formulas of the edeine antibiotics: edeine A_1, R = H; edeine B_1, R = C(=NH)NH_2.

There are in edeines A and B four optically active amino acids, which contain a total of six asymmetric carbon atoms. Since isoserine and diaminopropionic acid have negative and positive $[\alpha]_D$ values, respectively (Roncari *et al.*, 1966), the configurations of both are L (*S*) (Fischer and Jacobs, 1907; Freudenberg, 1914; Karrer and Schlosser, 1923). The α carbon atom of DAHAA presumably has the L (*S*) configuration, by virtue of the fact that it can be acted upon by carboxypeptidase B. Preliminary results suggest that the two amino groups of DAHAA have the same relative configuration as in *meso*-diaminopimelic acid, and that the relative configuration of the C-6 and C-7 amino and hydroxyl groups is *erythro*. If this is true, the C-6 and C-7 carbon atoms would have the *R* and *S* configurations, respectively; in the Fischer projection, the two amino groups and the hydroxyl group would then be written to the left. The configuration of the β-tyrosine residue is unknown.

It is not possible at the present time to judge what specific structural features of edeines A_1 and B_1 are responsible for their antibiotic activity. The edeine antibiotics are known to inhibit DNA and protein synthesis (Kurylo-Borowska, 1967), but detailed mechanisms of their action are not known. The spermidine residue of the edeines is probably a necessary feature, in view of the fact that spermidine itself has weak antibiotic activity (Tabor and Tabor, 1964). It cannot be said that the edeines function as polycations in a nonspecific manner, as the activity is dependent on the structural integrity of nearly the whole molecule. The isomerization of the Ise-α-DAPA linkage to Ise-β-DAPA causes inactivation, and so does replacement of the β amino group by a guanidino group (Hettinger *et al.*, 1968). Cleavage of the DAPA-DAHAA bond by carboxypeptidase B likewise inactivates edeines A and B. Since edeines A and B have approximately equal antibiotic activity, the substitution of the terminal amino group of the spermidine residue by a guanidino group appears to produce only a nominal change in the structural and functional characteristics of the antibiotics.

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Effect of pH on the Binding of *N*-Alkyl Sulfates to Bovine Serum Albumin*

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ABSTRACT: The binding of octyl and dodecyl sulfates to the sites of highest affinity of native bovine serum albumin is independent of solution pH (and therefore protein charge) over the range 4.8–6.8. This result is consistent with the current view that the energy of interaction is primarily hydrophobic rather than coulombic. The free energy of interaction between decyl sulfate and native bovine serum albumin, however, increases with decreasing pH, possibly as the result of an alteration in the interaction of the anionic end group of this ligand with a charged group on the protein, an interaction which is restricted to this particular ligand for steric reasons. The observed effects of pH are interpreted in terms of the effects on numbers of binding sites, and the binding constants, of native and denatured bovine serum albumin.

The effect of pH on the initial unfolding transition of bovine serum albumin, induced by the binding of certain long-chain detergents, such as dodecyl sulfate, at molar ratios slightly greater than 10–15 is to require progressively higher values of free ligand concentration as the pH is increased. This phenomenon cannot be accounted for entirely by assuming a *single* pH-dependent equilibrium constant between native and unfolded bovine serum albumin in the absence of ligand, but it is unlikely that the affinity of unfolded protein to these ligands depends on pH. At pH 6.8 and above the first unfolding transition can be observed only at free ligand concentrations above those at which a second, more extensive transition occurs.

It has been shown that the free energy of binding of a number of anions to serum albumin involves other than coulombic forces, and is primarily entropic in origin (see, for example, Kauzmann, 1959; Ray *et al.*, 1966; Reynolds *et al.*, 1967, 1968). Thus, native bovine serum albumin binds 4–10 moles of alkyl sulfates, sulfonates, carboxylates, and alcohols (the number depending on the chain length) with negative free energies which exceed 5 kcal/mole of ligand—far larger than would be expected from ionic interactions alone. Markus *et al.* (1964) have implicated cationic sites in the binding of dodecyl sulfate to human serum albumin, but again the free energy of interaction is large and must contain a major contribution from the nonionic entropic term.

Nevertheless, there is a substantial effect of solution pH on certain characteristics of the interaction between detergent-like molecules and bovine serum albumin (*e.g.*, Decker and Foster, 1966). The present work deals with these pH effects when two nonunfolding ligands (octyl and decyl sulfate; Reynolds *et al.*, 1967) and one unfolding ligand (dodecyl sulfate) are bound to bovine serum albumin.

The purpose of this study has been twofold: first, to elucidate the mechanism of large pH effects on binding–unfolding equilibria which are determined only slightly by charge interactions; and second, perhaps more important, to further test the ability of the model equilibria proposed in an earlier paper (Reynolds *et al.*, 1967) to predict or account for the effects of pH described here. In order to accomplish the latter, an effort is made to express the pH effects in terms of the

* From the Department of Chemistry, Georgetown University, Washington, D. C. 20007. *Received October 23, 1969.* This work was supported by Grant GM 12085 (NIH) to one of us (J. S.).

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