The novel 2,6-diamino-7-hydroxyazelaic acid is in certain respects homologous with lysine and 2,6-diaminopimelic acid. This suggests several possible biosynthetic and functional relationships between these compounds. Considered in a different way, the new amino acid is also a β-hydroxy acid. By analogy with well-known pathways for fatty acid biosynthesis, it may be conjectured that I could be synthesized from a derivative of diaminopimelic acid and a two-carbon fragment.

As I has three asymmetric carbon atoms, the natural compound could exist as any one of eight different optical isomers. There is a suggestion that the material isolated can be a mixture of stereoisomers. While it emerges as a single band on the amino acid analyzer, the band is often asymmetrical. The nuclear magnetic resonance spectra of I show resonances assigned to the C-6, C-7, and C-8 protons which appear too complex for one isomer only. The epimerization of I can be expected to occur in more than one way, the most apparent of which is due to the reversible dehydration reaction. The directive influence of C-6 of II on the stereospecificity of hydration of the double bond would be expected to be small, and would lead to a mixture of *threo* and *erythro* modifications of I. Moreover, inversions at the other optical centers cannot be ruled out. Conditions which cause minimum steric alterations of I during its isolation are presently being sought.

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Edeine. III. The Composition of the Antibiotic Peptide Edeine B*

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ABSTRACT: A new antibiotic peptide, edeine B, has been isolated from strain Vm4 of *Bacillus brevis*. Edeine B is composed of one residue each of glycine, isoserine, β-tyrosine, α,β-diaminopropionic acid, 2,6-diamino-7-hydroxyazelaic acid, and a novel base, *N*-guanyl-*N'*-(3-aminopropyl)-1,4-diaminobutane (guanylspermidine). Edeine B differs from edeine A in the replacement of an amino group on the spermidine residue by a guanidino group. Both antibiotics contain five free basic groups and one free carboxyl group.

Cultures of *Bacillus brevis* Vm4 have been shown to produce at least two distinct antibiotic substances (Borowski *et al.*, 1966; Kurylo-Borowska, 1967). The first of these, edeine A, has been partially characterized and found to contain equimolar amounts of glycine, isoserine, α,β-diaminopropionic acid, β-tyrosine, 2,6-diamino-7-hydroxyazelaic acid, and spermidine (Roncari *et al.*, 1966; Hettinger and Craig, 1968). A second antibiotic peptide, edeine B, whose presence had been indicated by paper chromatography (Borowski *et al.*, 1966), has now been isolated and its composition determined.

The previous isolation method, although capable of giving pure edeine A (Roncari *et al.*, 1966), is not suit-

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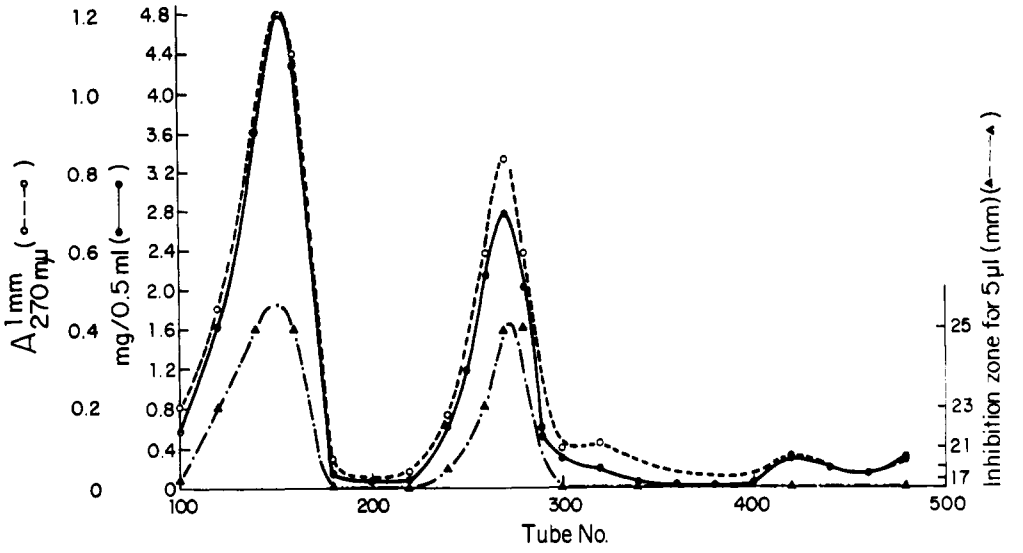


FIGURE 1: Isolation of edeines A and B by countercurrent distribution. Tubes 240–290, edeine A; tubes 120–170, edeine B.

able for the purification of edeine B. Edeine B is very soluble in phenol and is lost during the step in which edeine A is extracted from phenol into aqueous acid. We have therefore developed a modified procedure for the purification of both edeines A and B which eliminates the initial extraction with phenol.

Experimental Section

Isolation of Edeines A and B. After the cultures had grown as described (Roncari *et al.*, 1966), they were shaken with Dowex 50-X4 H^+ (50–100 mesh) (50 ml/l. of culture), which adsorbed most of the edeines. The resin was allowed to settle and the bacterial suspension was removed by decantation. After several washings with water, the resin was suspended in water, the pH was adjusted to 7 with K_2HPO_4, and the resin was again washed several times with water. The resin was then poured into a column and washed with three volumes of 0.1 N NH_4OH. Edeines A and B were then eluted with two volumes of 1 N NH_4OH. The antibiotic fraction was concentrated under reduced pressure and adjusted to pH 7 with acetic acid. The neutralized solution derived from 30 l. of culture was concentrated to 30 ml and applied to a column of Sephadex G-25 (4 × 105 cm). The column was eluted with water at a rate of 80 ml/hr. The antibiotic fraction was eluted between 600 and 1000 ml. On lyophilization this gave 5 g of solids.

The residue was then dissolved in the system 88% phenol–(0.15 M ammonium acetate–0.30 M acetic acid) (1:1), distributed over the first 30 tubes of a 500-tube countercurrent distribution machine (5/5 ml volumes), and the distribution carried to 600 transfers. The lower phases were analyzed for antibiotic activity and ultraviolet absorption after the phenol was removed by several extractions with ether. Total solids in the lower phases were also determined by residue weight analysis. The results are shown in Figure 1. The appropriate fractions were pooled and extracted with 3 volumes of ether and then three times with 1 volume of ether. The aqueous phase was concentrated on a rotary evaporator and lyophilized for several days to remove the ammonium acetate. The yields of edeine A (tubes 240–290) and edeine B (tubes 120–170) were about 1.5 g each.

Hydrolysis of Edeine B and Isolation of the Products. Edeine B acetate (0.70 g) was dissolved in 200 ml of 6 N HCl and the solution was refluxed for 30 hr. The hydrolysate was evaporated to dryness several times with the addition of water. The residue was loaded into tubes 0–6 of a 60-tube countercurrent distribution machine (4/3 ml volumes) and distributed for 60 transfers in the system 1-butanol–1.5 N HCl (1:1). Tubes 0–16 contained all of the fragments except for β-tyrosine, which was found in tubes 17–36.

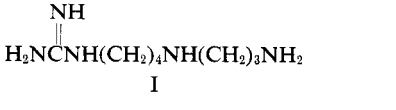
Tubes 0–16 were pooled and evaporated to dryness. The residue was dissolved in 5 ml of water and chromatographed on a column of Bio-Rad AG50-X2 H^+ (200–400 mesh) (2.2 × 24 cm) equilibrated with 1 N HCl. The column was eluted with 1 N HCl for 100 fractions, after which it was eluted with 3 N HCl. The flow rate was 26 ml/hr and the volume of each fraction was 6.5 ml. Analysis with ninhydrin (50 μl of sample plus 1 ml of ninhydrin plus 5 ml of diluent) gave the results shown in Figure 2. The appropriate fractions were pooled and evaporated to give the fragments as their hydrochlorides.

Unknown Base. Fractions 157–179 (Figure 2) contained a strongly basic compound which could not be crystallized in the form of its hydrochloride. However, the substance gave a crystalline picrate (mp 204–206° dec) from water and a crystalline chloroplatinate (mp 212–213° dec) from aqueous methanol. *Anal.* Calcd for $(C_8H_{21}N_5)_2 \cdot (H_2PtCl_6)_3$: C, 11.98; H, 3.02; N, 8.73; Pt, 36.5. Found: C, 12.14; H, 3.39; N, 8.84; Pt, 36.4.

Synthesis of N-Guanyl-1,4-diaminobutane (Agmatine). To a solution of 18.0 g (204 mmoles) of 1,4-diaminobutane (Aldrich) in 50 ml of methanol was added with stirring over a period of 10 min 11.0 g (100 mmoles) of *O*-methylisourea hydrochloride (Kur-

zer and Lawson, 1954) in 50 ml of methanol. After 1.5 hr at room temperature, 50 ml of concentrated HCl in 500 ml of methanol was added. On cooling to 5°, the bulk of unreacted 1,4-diaminobutane·2HCl crystallized and was removed by filtration. The filtrate, after evaporation and drying over P_2O_5 and KOH, gave 26.0 g of solid, which was extracted with 200 ml of boiling isopropyl alcohol and then with 200 ml of boiling absolute ethanol. The ethanol extract on evaporation gave 12.7 g of crude product, which on crystallization from 50 ml of boiling methanol gave 10.1 g (50 mmoles) of agmatine·2HCl. After two recrystallizations from methanol, the substance had a melting point of 176–178°. *Anal.* Calcd for $C_5H_{14}N_4 \cdot 2HCl$: C, 29.56; H, 7.94; N, 27.59. Found: C, 29.37; H, 7.96; N, 27.36.

Synthesis of N-Guanyl-N'-(3-aminopropyl)-1,4-diaminobutane (Guanylspermidine) (I). To a suspension of agmatine·2HCl (284 mg; 1.4 mmoles) in 1.5 ml of methanol was added with stirring 0.28 ml of 5 N NaOH followed by 100 μl (1.5 mmoles) of acrylonitrile (Aldrich). After standing at room temperature for 2 hr, the reaction mixture was refluxed on a steam bath for 1 hr, and then adjusted to pH 3 with HCl. The solvent was removed by evaporation under reduced pressure and the residue was extracted with 8 ml of hot absolute ethanol. Evaporation of the extract yielded the crude syrupy nitrile, which was not purified further before proceeding. It was dissolved in 3 ml of absolute ethanol, and 200 μl of concentrated HCl and 50 mg of platinic oxide were added. Hydrogenation was carried out with a pressure of 2.5 atm for 3 hr at 30°. Water was then added, the catalyst filtered, and the acidic solution evaporated. The residue was dissolved in 5 ml of water and chromatographed on a 2.2 × 20 cm column of Bio-Rad AG50-X2 (200–400 mesh), equilibrated with 3 N HCl. On elution with the same solvent, guanylspermidine emerged at 340–460 ml. The yield of glassy guanylspermidine·3HCl on evaporation was 175 mg (42% based on agmatine). The compound was characterized as the picrate and chloroplatinate. The picrate was prepared by adding excess saturated aqueous picric acid to the hydrochloride. After two recrystallizations from water, the guanylspermidine picrate had a melting point of 204–205° dec. The chloroplatinate was prepared by adding excess aqueous 0.5 M chloroplatinic acid to the hydrochloride and precipitating with ethanol. After two recrystallizations from aqueous methanol, the guanylspermidine chloroplatinate melted at 211–212° dec. *Anal.* Calcd for $(C_8H_{21}N_5)_2 \cdot (H_2PtCl_6)_3$: C, 11.98; H, 3.02; N, 8.73; Pt, 36.5. Found: C, 11.86; H, 3.00; N, 8.63; Pt, 36.5.



I

Analysis of the Edeine Fragments on the Amino Acid Analyzer. Edeines A and B were desalted by passage through a column of Sephadex G-10 equilibrated with 1 N acetic acid. After elution with the same solvent, the solutions were evaporated on a rotary evaporator and finally dried *in vacuo* over P_2O_5 for 1 day. The residues were weighed and dissolved in water to give a concentration of 2 mg/ml. Using ε 1310 at 270 mμ (Roncari *et al.*, 1966), the concentrations of edeines A and B were found to be 1.84 and 1.73 mM, respectively. The minimum molecular weights determined from these values are 1087 and 1156, respectively (calculated for edeine A and B tetraacetate, 995 and 1037).

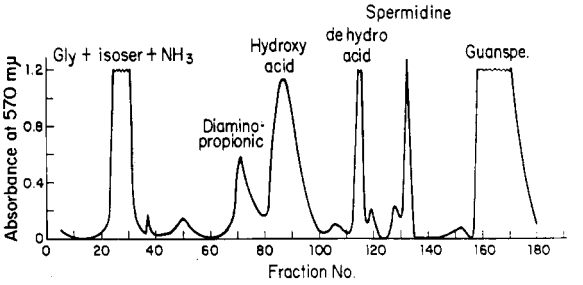


FIGURE 2: Chromatography of edeine B fragments on Bio-Rad AG50-X2 H^+. Gly = glycine; isoser = isoserine; hydroxy acid = 2,6-diamino-7-hydroxyazelaic acid; dehydro acid = 2,6-diamino-7-nonenedioic acid; guanspe = guanylspermidine.

For amino acid analysis, 1-ml samples (1.84 μmoles of edeine A or 1.73 μmoles of edeine B) were treated with 1 ml of concentrated HCl and hydrolyzed in sealed, evacuated tubes at 110° for periods of time up to 64 hr. The samples were then evaporated to dryness *in vacuo* over KOH pellets and dissolved in 4 ml of pH 2.2 buffer. Samples of unhydrolyzed edeines A and B were also prepared for ammonia analysis. The samples (1 ml, equivalent to 0.460 μmole of edeine A or 0.432 μmole of edeine B) were analyzed on each column of a Beckman–Spinco Model 120 amino acid analyzer. The areas of the peaks were converted into micromoles of amino acid by using the following individually determined micromolar color values at 570 mμ: glycine, 15.26; isoserine, 5.86; DAHAA, 17.20;[1] DANDA, 14.98; β-tyrosine, 11.12; DAPA, 4.20; and ammonia, 15.58. The elution positions of the various fragments have been given elsewhere (Hettinger and Craig, 1968). Spermidine and guanylspermidine are not eluted from either column.

Partial Guanylation of Edeines A and B. Edeine A or B (10 mg; 10 μmoles) was dissolved in 0.5 ml of water and treated with 27.5 mg (250 μmoles) of *O*-methylisourea hydrochloride and 0.125 ml of 1 N sodium hydroxide. After the reaction mixtures had stood at 25° for various times (0.5, 2, and 8 hr for edeine A; 8 hr for edeine B), the reaction was terminated by the addition of 1 ml of 1 N acetic acid. The products were desalted by passage through a 100-ml column of Sephadex G-10 equilibrated with 1 N acetic acid. For the separation of the products according to the degree of guanylation, they were chromatographed on Whatman No. 3MM paper in the system isopropyl alcohol–concentrated ammonia–water (4:1:1) for 20 hr. In

[1] Abbreviations used: DABA, α,γ-diaminobutyric acid; DAHAA, 2,6-diamino-7-hydroxyazelaic acid; DANDA, 2,6-diamino-7-nonenedioic acid; DAPA, α,β-diaminopropionic acid.

this system edeines A and B had R_F values of 0.19 and 0.11, respectively.

In order to determine the structure of the guanylspermidine produced on reaction of edeine A with *O*-methylisourea, edeine A (90 mg) was guanylated for 4 hr, and the guanylspermidine was isolated after acid hydrolysis and chromatography on Bio-Rad AG50-X2 H^+. The yield was 17 mg of the hydrochloride, whose behavior on paper electrophoresis and chromatography was indistinguishable from that of guanylspermidine isolated from edeine B. The melting points of its picrate and chloroplatinate were likewise identical with those of the same salts of guanylspermidine from edeine B and synthetic *N*-guanyl-*N'*-(3-aminopropyl)-1,4-diaminobutane. Mixture melting points showed no depression.

Dinitrophenylation. Edeine A or B (1 mg) was dissolved in 100 μl of 4% w/v sodium bicarbonate and treated with 200 μl of 2% v/v 2,4-dinitrofluorobenzene in 95% ethanol. The mixture was allowed to stand at 25° with occasional shaking for 2 hr. The yellow precipitate was washed with water and ether several times, dried, and dissolved in 1 ml of concentrated HCl. To this was added 1 ml of water. Hydrolysis was carried out at 100° for periods of time from 1 to 10 hr. These relatively mild hydrolytic conditions were used because of the pronounced tendency of certain of the DNP derivatives to undergo decomposition.

After hydrolysis, the acid was removed by evaporation *in vacuo* over KOH pellets, and the residue was taken up in water and extracted with ether. The ether-soluble DNP fraction was examined by thin-layer chromatography, and the water-soluble compounds by paper electrophoresis.

All the ether-soluble DNP compounds of interest were studied by chromatography on silica gel G in the solvent system chloroform–1-butanol–acetic acid (70:30:3) for 90 min. The following R_F values were obtained: DNP-isoserine, 0.21; bis-DNP-DAHAA, 0.35; bis-DNP-DAPA, 0.48; DNP-glycine, 0.65; DNP-NH_2, 0.90; and bis-DNP-β-tyrosine, 0.98.

The water-soluble fraction was initially subjected to electrophoresis at pH 6.4 (pyridine–acetic acid–water, 100:4:896), 40 V/cm, 80 min). This separated the cationic mono-DNP-guanylspermidine (−12.7 cm) and its decomposition products from mono-DNP-DAHAA (+5.9 cm) and neutral compounds. In this system, DAPA migrated 13.3 cm toward the cathode. The neutral components were eluted from the paper and subjected to electrophoresis at pH 1.9 (acetic acid–88% formic acid–water, 150:50:800), 40 V/cm, 90 min). Reference compounds gave the following distances of migration toward the cathode: glycine, 15.7 cm; isoserine, 14.5 cm; DANDA, 14.5 cm; DAHAA, 14.0 cm; β-tyrosine, 11.0 cm; *O*-DNP-β-tyrosine, 7.9 cm; α-DNP-DAPA, 5.9 cm; and β-DNP-DAPA, 4.9 cm.

Acidimetric Titration of Edeines A and B. For titration the acetates were first converted to the isoelectric forms. Edeine A or B acetate (100 mg) was dissolved in 5 ml of water and adsorbed on a 3-ml column of Bio-Rad AG50-X2 NH_4^+ (200–400 mesh). The column was washed with 10 ml of water and then the edeine was eluted with 8 ml of 3 N NH_4OH. The solution was immediately evaporated to a syrup on a rotary evaporator, the syrup taken up in water, and the evaporation repeated. The edeine residue (*ca.* 80 μmoles) was dissolved in 4 ml of water. The pH of this solution was about 9.6–9.7, which is somewhat lower than the isoelectric point, probably due to absorption of CO_2 from the air. The solution was titrated at 25° with standardized 6 N HCl to pH 1.5 and then back-titrated with standardized NaOH to pH 12.0. Only the titration with NaOH was used in the calculations, since some bicarbonate was present during the titration with HCl. Appropriate corrections for the titration of water were applied to the results. After completion of the titration, the solution was adjusted to pH 6 with 6 N HCl and diluted to a known volume with water. The amount of edeine in the solution was then determined from its ultraviolet spectrum, using 1310 l. mole^{-1} cm^{-1} as the molar absorbancy index at 270 mμ (Roncari *et al.*, 1966).

Results and Discussion

Earlier reports (Borowski *et al.* 1966; Kurylo-Borowska, 1967) on the nature of the antibiotic principles elaborated by *B. brevis* Vm4 indicated that two major antibiotic substances were produced. We succeeded in isolating one of these, edeine A, by a procedure which involved extraction of the bacterial cultures with phenol, followed by gel filtration and countercurrent distribution (Roncari *et al.*, 1966). The other major component, edeine B, was not satisfactorily isolated by this procedure. By elimination of the phenol extraction and introduction of an ion-exchange procedure, we have been able to obtain both edeines A and B in purified form. Since edeines A and B are very basic substances, they are readily adsorbed preferentially on Dowex 50 from dilute aqueous solutions. If a coarse resin is used, it can be added directly to the culture without prior removal of the bacteria, which sediment more slowly than the resin. The antibiotics could be obtained largely free of inorganic salts by elution from the resin with dilute ammonia.

The basic character of both edeines A and B was apparent from their behavior on paper electrophoresis. At pH 6.4 each had a cathodic mobility nearly equal to that of α,β-diaminopropionic acid and they were not resolved from each other by this procedure. They could be distinguished, however, by paper chromatography. In the system 1-butanol–acetic acid–pyridine–water (6:3:2:3) and on Whatman No. 3MM paper R_F values of 0.08 and 0.11 for edeines A and B, respectively, were found, and their R_F values in the system isopropyl alcohol–concentrated ammonia–water (4:1:1) were 0.19 and 0.11, respectively. Each gave a blue color with ninhydrin and reacted with diazotized sulfanilic acid (Pauly reaction) to give a yellow color, apparently due to coupling with the β-tyrosine residue. Unusual colored products were formed with the Sakaguchi reagent (8-hydroxyquinoline followed by alkaline hypobromite). Edeine A gave a blue color, which was found to arise

from the reaction with β-tyrosine and which appears to be fairly specific for this amino acid, since no reaction is observed with tyrosine or any other of the simple phenols tested. In this test edeine B gave a maroon color which appears to be a composite of the blue color due to reaction with β-tyrosine and the normal red color given by substituted guanidines, for which the test is generally employed.

When hydrolysates of edeines A and B were examined by paper chromatography and electrophoresis, it was apparent that the two antibiotics had a similar composition. The amino acids glycine, isoserine, β-tyrosine, α,β-diaminopropionic acid, 2,6-diamino-7-hydroxyazelaic acid, and 2,6-diamino-7-nonenedioic acid (Hettinger and Craig, 1968) were all found in hydrolysates of both edeines A and B. However, the remaining constituent of edeine A, spermidine, appeared to be missing in edeine B, as only a very weak spot corresponding to spermidine was found in its hydrolysate. Furthermore, edeine B hydrolysates contained a component which was not found in hydrolysates of edeine A. This was a basic substance having a cationic mobility 0.89 times that of spermidine at pH 3.5 and which had by paper chromatography (Whatman No. 3MM paper) in the system 1-butanol–acetic acid–pyridine–water (6:3:2:3) an R_F of 0.33 (R_F of spermidine = 0.27).

Chromatography of the fragments of edeine B on Bio-Rad AG50-X2 (Figure 2) confirmed that all of the same components of edeine A were present except for spermidine, which was obtained in a very low yield. There was, in addition, another peak beyond the normal elution position of spermidine. It contained a very basic substance which corresponded to the additional component detected by paper chromatography and electrophoresis.

The unknown base had the formula $C_8H_{21}N_5$ and gave a blue color with ninhydrin and a normal red color with the Sakaguchi reagent. The base was converted by hydrolysis in saturated $Ba(OH)_2$ at 100° for 2 hr into a compound which behaved the same as spermidine on paper chromatography and electrophoresis. The hydrolysis product gave a crystalline picrate, which had a melting point of 212–213° dec, the same as spermidine picrate. The melting point was not depressed by admixture with authentic spermidine picrate.

These results suggested that the unknown base was one of three possible isomers of monoguanylspermidine. At this point, synthesis of the most likely isomer, *N*-guanyl-*N'*-(3-aminopropyl)-1,4-diaminobutane (I), seemed advisable. This choice was based on the analogy between I and spermidine and the known pathway (Tabor *et al.*, 1958) for the biosynthesis of spermidine. As the C_4 unit of spermidine is derived from putrescine, a simple extrapolation allows the C_4 unit of I to originate from agmatine. Whether this is correct remains to be established, but in any case, the unknown base proved to have structure I by comparison of the natural and synthetic materials. Thus the chloroplatinate and picrate of the unknown base had the same melting points as the corresponding salts of synthetic I, and mixture melting points showed no depression. Furthermore, the infrared spectra of the two salts of I were identical with the spectra of the same salts of the base isolated from edeine B. The natural and synthetic I were likewise indistinguishable by paper chromatography and electrophoresis.

The yield of guanylspermidine from the hydrolysate of edeine B was 0.84 mole/mole of edeine B, assuming a molecular weight of 1037 for edeine B tetraacetate. The amount of spermidine isolated corresponded to 0.06 mole/mole of edeine B. Since guanylspermidine is stable to acid hydrolysis, the small amount of spermidine obtained probably reflects some minor edeine component. The amounts of the other fragments determined on the amino acid analyzer were the same as those obtained from edeine A, *i.e.*, one residue each of β-tyrosine, α,β-diaminopropionic acid, glycine, isoserine, and 2,6-diamino-7-hydroxyazelaic acid (Table I). By analysis of edeine which had been desalted by passage through Sephadex G-10, it was ascertained that, as in the case of edeine A, the ammonia found in the hydrolysates of edeine B arises from decomposition of the several fragments.

Thus, the only difference in the compositions of edeines A and B resides in the nature of the basic component. Whereas the base spermidine is a constituent of edeine A, it is replaced by *N*-guanyl-*N'*-(3-aminopropyl)-1,4-diaminobutane in edeine B. Spermidine itself has wide natural occurrence, but the guanylspermidine described here has not previously been shown to exist in nature. However an isomer, *N*-(3-guanidinopropyl)-1,4-diaminobutane, and a diguanyl derivative, *N*-guanyl-*N'*-(3-guanidinopropyl)-1,4-diaminobutane, have been reported to be constituents of leeches (Robin and van Thoai, 1961; Robin *et al.*, 1967).

The dialysis studies on edeine A reported in an earlier paper (Roncari *et al.*, 1966) indicated that the diffusional size of the antibiotic corresponded to a molecular weight in the neighborhood of 1500. However, the results were not conclusive enough for a decision to be made on whether edeine A had a monomeric or dimeric structure. Partial substitution (Battersby and Craig, 1951) of edeines A and B was performed in order to resolve this point. With 2,4-dinitrofluorobenzene as the modifying reagent, edeine A gave a complex mixture of products that were not easily resolved. We turned to *O*-methylisourea as the substituting reagent partly because it is more selective (Klee and Richards, 1957) and because it was thought that it might establish more clearly the relationship between edeines A and B.

A chromatographic analysis of the products of partial guanylation of edeines A and B (Figure 3) showed that only three zones had to be considered. These had R_F values of 0.19, 0.11, and 0.04, and corresponded to edeine A, guanyledeine A (edeine B), and diguanyledeine A (guanyledeine B), respectively. In 1 hr edeine A was partially converted into a compound with an R_F of 0.11 which was indistinguishable from edeine B and had strong antibiotic activity. This was converted more slowly into a substance which had an R_F of 0.04 and which was almost completely inactive. Edeine B was also partially converted into the latter product in

TABLE I: Amino Acid Analysis of Edeines A and B.

Amino Acid	Micromoles of Amino Acid per Micromole of Edeine: Hours of Hydrolysis[a] 0	2	4	8	16	32	64	Av or Extrapolated Value	Cor Value[d]
				Edeine A					
Glycine		0.99	0.98	0.99	1.00	0.99	0.97	0.99[b]	0.99
Isoserine		1.01	0.94	0.91	0.94	1.07	0.87	0.96[b]	0.96
DAHAA		0.56	0.65	0.63	0.60	0.45	0.19	0.80[c]	1.08
DANDA		0.08	0.16	0.23	0.21	0.16	0.07	0.28[c]	(0.00)
β-Tyrosine		0.77	0.76	0.54	0.66	0.49	0.39	0.81[c]	0.81
DAPA		0.65	0.87	0.97	0.97	0.97	0.93	0.96[b]	0.96
Ammonia	0.05	0.15	0.16	0.23	0.33	0.54	0.83	0.12[c]	(0.07)
				Edeine B					
Glycine		1.01	0.99	1.00	1.00	0.96	1.06	1.00[b]	1.00
Isoserine		1.11	0.97	0.96	0.94	0.95	1.07	1.00[b]	1.00
DAHAA		0.57	0.64	0.66	0.60	0.46	0.19	0.78[c]	1.07
DANDA		0.09	0.14	0.23	0.21	0.15	0.07	0.29[c]	(0.00)
β-Tyrosine		0.60	0.48	0.67	0.58	0.48	0.52	0.77[c]	0.77
DAPA		0.66	0.83	0.96	0.94	0.95	1.02	0.97[b]	0.97
Ammonia	0.07	0.13	0.16	0.23	0.30	0.49	1.01	0.11[c]	(0.04)

[a] In 6 N HCl at 110°. [b] Average value; for DAPA the values for the 2- and 4-hr hydrolysates were excluded. [c] Value obtained by extrapolation to zero time; for β-tyrosine a nonsystematic variation, probably caused by traces of oxygen, made extrapolation difficult. [d] The extrapolated ammonia value was corrected by the amount of ammonia found in unhydrolyzed edeine; the corrected value of DAHAA was obtained by addition of the amount of the transformation product DANDA (Hettinger and Craig, 1968).

8 hr. The progressive change in the color produced on development with the Sakaguchi reagent from blue to red-orange as the R_F values decreased was in agreement with the suggestion that the components of R_F 0.19, 0.11, and 0.04 were substitution products of edeine A having 0, 1, and 2 guanyl groups, respectively. In order for this to be the case, both edeines A and B must have the minimum molecular weight corresponding to the monomeric structure.

The compounds of R_F 0.11 and 0.04 derived from edeine A which had been guanylated for 8 hr were eluted from the paper with 1 N acetic acid, and the hydrolysates (6 N HCl, 110°, 20 hr) of these compounds were examined by paper chromatography and electrophoresis. The compound of R_F 0.11 gave on hydrolysis guanylspermidine and all the other components of edeine B. Spermidine was absent and no additional components were detected. This indicated that the guanyl group was located exclusively on the spermidine portion of the molecule. Hydrolysis of the substance having an R_F of 0.04 gave the same pattern as edeine B. except that the DAPA spot was much weaker and a strong spot corresponding to β-guanidinoalanine was seen. In addition, a weak spot which is thought to be diguanylspermidine (*N,N'*-diguanyl-*N'*-(3-aminopropyl)-1,4-diaminobutane) was observed. This was a ninhydrin- and Sakaguchi-positive substance having a mobility 0.93 times that of guanylspermidine at pH 3.5. No other new Sakaguchi-positive compounds were seen. The results suggest that the second guanyl group could have gone largely on the β-amino group of DAPA but partly on the imino group of spermidine. Quantitative analysis on the amino acid analyzer indicated that 70% of the DAPA had been converted to β-guanidinoalanine.

The components of edeines A and B contain a total of ten basic groups and six carboxyl groups. If all of the fragments are connected by means of peptide bonds, four or five amino functions may be free, but there can be no more than one free carboxyl group. For determination of the amino-terminal groups, the usual conversion of the free amino groups to their DNP derivatives (Fraenkel-Conrat *et al.*, 1955) was employed.

Hydrolysis of dinitrophenylated edeines A and B yielded the same components in each case except for the DNP derivatives of their respective bases. Of the amino acids only glycine and isoserine were obtained unmodified. It was expected, therefore, that the remaining fragments had been converted to their DNP derivatives. No yellow ether-soluble DNP compounds aside from DNP-NH_2 (a decomposition product) were found, so the products were looked for in the water-soluble fraction.

DAHAA appeared in the hydrolysate as its mono-DNP derivative, which migrated in electrophoresis at the same rate as authentic mono-DNP-DAHAA and

gave a positive ninhydrin reaction. The location of the DNP group has not yet been established. Since it may be contaminated to a variable and unknown extent with mono-DNP-DANDA depending upon the hydrolytic conditions, a more efficient method has to be developed for the separation of this group of compounds.

The guanylspermidine from edeine B was isolated as its mono-DNP derivative. The fact that the compound gave positive ninhydrin and Sakaguchi reactions suggested that the DNP group was located on the secondary amine function of the molecule. This was supported by examination of the ultraviolet spectrum, which had a λ_{max} at a long wavelength (387 mμ in 1 N acetic acid) characteristic of DNP derivatives of secondary amines (Dubin, 1960). DNP-guanylspermidine was not very stable to hydrolysis and was always accompanied by several yellow artifacts which migrated more slowly than the parent DNP derivative. A hydrolysis time of about 5 hr appeared to be optimal. The decomposition of DNP-guanylspermidine is probably analogous to the destruction of DNP-proline which is observed during acid hydrolysis (Scanes and Tozer, 1956). The DNP derivative of the spermidine portion of edeine A has not been characterized, but the free groups could be identified in an indirect fashion. Guanyledeine A, after reaction with 2,4-dinitrofluorobenzene and hydrolysis, gave DNP-guanylspermidine, whose properties were identical with those described above for the compound derived from edeine B. This means that both nitrogens of the C_4 unit of spermidine are free in intact edeine A.

The neutral water-soluble fraction contained mono-DNP-DAPA, which when subjected to electrophoresis at pH 1.9 showed the presence of both α-DNP-DAPA and β-DNP-DAPA. These were identified by comparison with the mixed isomers prepared by reacting DAPA with 1 equiv of 2,4-dinitrofluorobenzene. The assignments were made on the basis that the β-DNP isomer, having a more acidic carboxyl group, migrated more slowly than the α-DNP isomer, and by the fact that blue and brown colors, respectively, were produced on reaction with ninhydrin. Since edeines A and B are monomeric, the DNP derivatives must reflect substitution of the same DAPA residue in the sequence. They cannot arise from bis-DNP-DAPA, since this compound is relatively stable to hydrolysis and none was found in the ether-soluble fraction. It is possible that DAPA exists as a cyclic amidine structure in the native antibiotic, although it would have to be readily convertible to some other form, since amidines themselves do not react with 2,4-dinitrofluorobenzene (Hunter and Ludwig, 1962). A more plausible explanation would be that DAPA is linked through one of its amino groups in the native structure, and that during isolation a mixed population of α and β linkages is produced as a result of $\alpha \rightleftharpoons \omega$ acyl migration. Such acyl migration has been demonstrated for synthetic peptides of DAPA and DABA (Poduška *et al.*, 1965) and has been found to occur in the antibiotic peptide polymixin M (Silaev *et al.*, 1966). It does not seem likely that the results could be accounted for by an ambiguity in the biosynthetic mechanism.

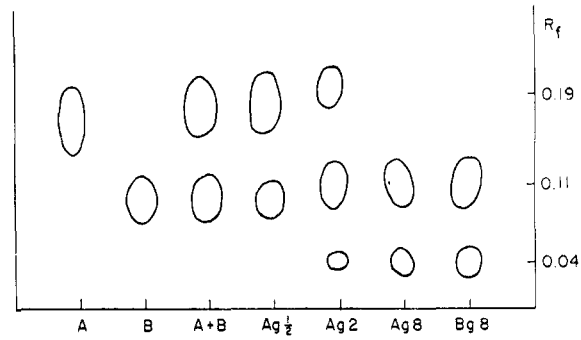


FIGURE 3: Paper chromatography of edeines A and B and products of partial guanylation. A = edeine A; B = edeine B; Ag½, Ag2, and Ag8 = edeine A guanylated for 0.5, 2, and 8 hr, respectively; Bg8 = edeine B guanylated for 8 hr.

The nature of the product of dinitrophenylation of the β-tyrosine residue has not yet been established. This residue has not been accounted for in the hydrolysate either as β-tyrosine, *O*-DNP-β-tyrosine or *N*,*O*-bis-DNP-β-tyrosine. However, the bis-DNP derivative, if formed, would not be observed, as it is very unstable and does not survive even 2-hr hydrolysis. A possible product would be *O*-DNP-*p*-hydroxycinnamic acid, which could arise through β elimination of the bis-DNP derivative. This has not been checked. A suggestion that the amino group of β-tyrosine is free is given by the fact that edeine A produces a blue color with the Sakaguchi reagent. Edeine A in which all of the amino groups had been acetylated, but in which the phenolic hydroxyl group is free, no longer gives a positive reaction. Although the specificity of this reaction has not been fully established, it appears that a free *p*-hydroxybenzylamine structure is probably essential.

The DNP derivatives of glycine and isoserine are sufficiently stable to allow identification if these had been end groups. Since they were found in the hydrolysates as free amino acids, their amino groups must be bound in peptide linkages. One amino group of spermidine (or guanylspermidine), DAHAA, and DAPA also appears to be blocked, leaving a total of five free basic groups. Two of these are on spermidine (or guanylspermidine) and one each on DAHAA, DAPA, and probably β-tyrosine. As five of the six carboxyl groups must be used for peptide linkages, one should be free unless blocked in some other way.

Acidimetric titration of edeine A at 25° indicated that a total of seven groups were titrated between pH 1.5 and 12.0. One group was titrated in the acid region and had a p*K* of 2.9. The remaining six groups were titrated in the pH range 6–12. Of these, five could be accounted for as the free amino groups determined by dinitrophenylation, and the sixth as the phenolic hydroxyl group of β-tyrosine. With edeine B, the same group with a p*K* of 2.9 was present, but only five groups were titrated between pH 6 and 12. In this case, all of the groups could be accounted for except the guanidino group, whose p*K* is probably greater than 12. The group having a p*K* of 2.9 in both edeines A and B is probably a free carboxyl group, but it has not yet been

identified. The presence of a free carboxyl group is a further indication that there should be five free basic groups. Further experiments on the structures of edeines A and B are in progress.

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The Photolability of Co-alkylcobinamides*

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ABSTRACT: The spectral properties and photodecomposition of N^5-methyltetrahydrofolate–homocysteine transmethylase suggest that in the enzyme-bound cobalamin the coordinate linkage between cobalt and the 5,6-dimethylbenzimidazole moiety is broken. Thus it would be expected that enzyme-bound cobalamin should react like a cobinamide rather than a cobalamin. Methylcobinamide–ligand complexes were used as model systems to investigate the influence of ligands on the spectrum of methylcobinamide and on the photodecomposition of the carbon–cobalt bond. In general the formation constants of the alkylcobinamide–ligand complexes are very small compared with those of the cyanocobinamide–ligand complexes as a result of the strong electron-donating influence of the alkyl ligands. The formation constant of the *n*-propylcobinamide–imidazole complex was found to be two orders of magnitude smaller than the formation constant of the methylcobinamide–imidazole complex, reflecting the positive inductive effect of the extra ethyl group. Whereas these and earlier results suggest that displacement of water coordinated to cobalt by other bases renders the carbon–cobalt bond more susceptible to photolytic cleavage, the displacement of water by imidazole, 1-methylimidazole, and ammonia were found to greatly decrease the rate of photolysis.

These results have been used to explain the spectral properties and the unexpected light stability of the carbon–cobalt bond of methylated N^5-methyltetrahydrofolate–homocysteine transmethylase, as well as the spectral properties and photolability of propylated transmethylase.

N^5-Methyltetrahydrofolate–homocysteine transmethylase has been highly purified from *Escherichia coli* B and has been shown to contain aquocobalamin as a prosthetic group (Taylor and Weissbach, 1967b). When this purified transmethylase is incubated with [^{14}C]methyltetrahydrofolate in the presence of $FMNH_2$, dithiothreitol, and a catalytic amount of *S*-adenosyl-L-methionine, ^{14}C-labeled holoenzyme is formed (Taylor and Weissbach, 1967a). Subsequent incubation of this labeled enzyme with homocysteine yields ^{14}C-labeled methionine. Taylor and Weissbach (1968) showed conclusively that in the methylated holoenzyme the methyl group is linked to the cobalt atom of the cob-

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