50. *The Structure of Evolidine.*

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The cyclic heptapeptide nature of evolidine has been confirmed. The sequence of the amino-acid residues has been investigated and evidence obtained for the mode of linkage of the aspartyl residue. The structure proposed for evolidine is

cyclo[Ser.Phe.Leu.Pro.Val.Asp-(β-NH_2).Leu].*

The amino-acids obtained on hydrolysis are shown all to have the L-configuration.

Evolidine was isolated from the leaves of *Evodia xanthoxyloides* and characterised by Hughes, Neill, and Ritchie.[2] It is insoluble in water or dilute aqueous alkali or acid, but readily soluble in acetone or ethanol. It crystallises from ethanol in colourless prisms, m. p. 277—279°, $[\alpha]_D^{16}$ −129° (*c* 0·45 in EtOH). *X*-Ray studies by Curtis [3] gave an estimate of 769 ± 6 for the molecular weight.

The substance did not form an *N*-acetyl derivative, even on vigorous treatment with acetic anhydride, but on hydrolysis gave in high yield a mixture of α-amino-acids and ammonia. Preliminary analysis of this mixture and a consideration of the properties of

* The abbreviations used for the amino-acids are those suggested by Brand and Edsall.[1]

[1] Brand and Edsall, *Ann. Rev. Biochem.*, 1947, **16**, 223.
[2] Hughes, Neill, and Ritchie, *Austral. J. Agric. Res.*, 1952, *A***5**, 401.
[3] Eastwood, Hughes, and Ritchie, with *X*-ray studies by Curtis, *Austral. J. Chem.*, 1955, **8**, 552.

evolidine led Hughes and his co-workers to suggest that the substance is probably a cyclic heptapeptide of composition Val, Leu_2, Pro, Phe, Ser, Asp-NH_2 (required: *M*, 771).

A specimen of evolidine was given to us by Professor A. J. Birch, F.R.S. We have investigated the structure of the substance with regard to (i) its cyclic nature, (ii) its amino-acid composition, (iii) the sequence of the amino-acid residues in the molecule and (iv) the configuration of the amino-acid residues; and a brief account of the findings has been given.[4]

(i) *Cyclic Nature.*—Evolidine does not yield a 2,4-dinitrophenyl derivative when treated with 1-fluoro-2,4-dinitrobenzene in suspension in aqueous sodium hydrogen carbonate at room temperature or when methanol has been added to dissolve the evolidine. This observation and the neutral character of the substance indicate a cyclic peptide structure.

(ii) *Amino-acid Composition.*—The amino-acids in the hydrolysate of evolidine were separated by the ion-exchange method due to Moore and Stein [5] and estimated colorimetrically after reaction with ninhydrin.[6] The composition found (Table 1) agrees with that postulated by Hughes and his co-workers.[2,3]

TABLE 1.

Amino-acid	Amino-acid (g. per 100 g. of peptide)	Residue ·HN·CHR·CO· (g.) per 100 g. of peptide	No. of residues calc. for *M* 771
Aspartic acid *	16·89	14·60	0·98 ≡ 1
Serine	14·25	11·80	1·05 ≡ 1
Proline	14·71	13·16	1·05 ≡ 1
Valine	15·44	13·06	1·02 ≡ 1
Leucine	33·36	28·77	1·96 ≡ 2
Phenylalanine	21·78	19·41	1·02 ≡ 1
Total	116·43	100·80	7·08 ≡ 7

* The aspartic acid is present in the peptide as asparagine.

(iii) *Sequence of Amino-acid Residues.* Specimens of evolidine were partially hydrolysed by concentrated hydrochloric acid at 35° for times ranging from 15 to 72 hours. Similar partial hydrolyses were carried out at 80°, for up to 6 hours. The amino-acids and oligopeptides produced were separated by two-dimensional paper chromatography and by electrophoresis.

The two-dimensional chromatograms (butan-1-ol–acetic acid–water, and phenol–water) varied somewhat. A general and composite diagram of the ninhydrin pattern is illustrated.

The chromatogram of the products of hydrolysis at 35° for 44 hours consisted of the characteristic spots of the six constituent amino-acids and the ninhydrin positive areas A and B_2: with longer hydrolyses, an area B_1 appeared; after hydrolyses for 72 hours, the aspartic acid spot was intensified, area A was missing, and areas B_1 and B_2 were fainter. Hydrolysis in the 15—44 hour range gave an unresolved mixture of ninhydrin-positive material in the valine–phenylalanine–leucine area and also a separate area C.

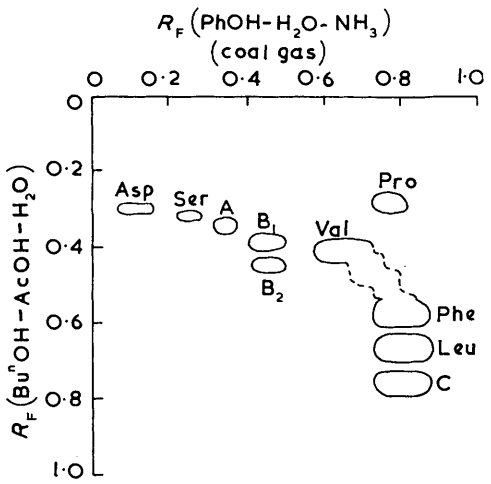
The areas A, B_1, B_2, and C were located on fresh chromatograms by ultraviolet light and were then eluted, and studied individually by a combination of the following techniques: (*a*) identification of the amino-acid components (hydrolysis followed by paper chromatography); (*b*) *N*-terminal-residue sequence studies by Sanger's fluorodinitrobenzene method [7] or Edman's stepwise phenyl isothiocyanate method; [8] (*c*) *C*-terminal-residue sequence studies by the carboxypeptidase method.[9]

[4] Law, Millar, Springall, and Birch, *Proc. Chem. Soc.*, 1958, 198.
[5] Moore and Stein, *J. Biol. Chem.*, 1951, **192**, 663.
[6] Moore and Stein, *J. Biol. Chem.*, 1948, **176**, 367.
[7] Sanger, *Biochem. J.*, 1945, **39**, 507; Sanger and Thompson, *ibid.*, 1953, **53**, 353, 366; Levy, *Methods Biochem. Analysis*, 1955, **2**, 360.
[8] Edman, *Acta Chem. Scand.*, 1950, **4**, 283; 1953, **7**, 700; Fraenkel-Conrat, *Methods Biochem. Analysis*, 1955, **2**, 383.
[9] Harris, *Methods Biochem. Analysis*, 1955, **2**, 397.

Area A (from hydrolysis at 80° for 6 hours) consists of valine and aspartic acid in equimolecular proportions (estimated by the dinitrophenyl technique [10]), all the valine being *N*-terminal; the A-peptide is therefore valylaspartic acid.

Areas B_1 and B_2 (from hydrolysis at 80° for 6 hours) each consist of aspartyl-leucine dipeptides.

Electrophoresis on paper at pH 6·7 of materials B_1 and B_2 showed identical rates of anionic migration; in the pH range 6—3, the rates of anionic migration were in the order, aspartic acid > B_2 > B_1; at pH <3 the rates of cationic migration were in the order,



B_2 > aspartic acid > B_1. These findings show that neither B_1 nor B_2 is an asparaginyl-peptide and are compatible with the identification B_1 = β-Asp.Leu, B_2 = α-Asp.Leu. Material B_1 gives the blue (rather than purple) ninhydrin colour characteristic of β-aspartyl-peptides.

Authentic α-aspartyl-leucine, given to us by Dr. G. T. Young, proved to be chromatographically identical with B_2. A sample of material B_2 was heated at 100° for some hours (under these conditions α-aspartylvaline, α-aspartyltyrosine, and α-aspartylglutamic acid rearrange partially to the β-aspartyl isomers [11]) and was then chromatographed. Two ninhydrin-positive areas appeared, identical with B_1 and B_2 in colour and location. Since (*a*) the same effect was observed when the α-aspartyl-leucine was heated in acid solution under the conditions used for the partial hydrolyses, (*b*) on chromatograms of mixtures obtained by brief hydrolysis of evolidine, area B_2 appeared strongly, while B_1 was only faint or absent, and (*c*) β-aspartylvaline does not rearrange to the α-isomer,[11] it is felt (i) that the identification of materials B_1 and B_2 as β- and α-aspartyl-leucine respectively is established and (ii) that the α-isomer is the original species in evolidine, the β-isomer arising from it during hydrolysis.

Area C was investigated as follows. An evolidine hydrolysate (80° for 1 hour) was subjected to electrophoresis, which separated the hydrolysate mixture into three zones. These were eluted and found to consist respectively of (*a*) aspartic acid, (*b*) materials A, B_1, and B_2, (*c*) the remaining components. Paper chromatography (butanol–acetic acid–water) of the eluate from the last zone separated material C into three regions, C_1, C_2, and C_3, the last having an R_F value very close to that of valine. Material from C_1, when

[10] Levy, *Nature*, 1954, **174**, 126; Levy and Chung, *J. Amer. Chem. Soc.*, 1955, **77**, 2899; Levy, *Methods Biochem. Analysis*, 1955, **2**, 360.

[11] Le Quesne and Young, *J.*, 1952, 24; John and Young, *J.*, 1954, 2870.

treated with fluorodinitrobenzene and then hydrolysed, gave a high yield of *N*-dinitrophenylleucine, while that from C_3 gave *N*-dinitrophenylphenylalanine. (Both regions gave traces of dinitrophenylserine presumably owing to contamination with traces of serine or seryl peptides.) The free acids found in this process from both materials C_1 and C_2 were valine, leucine, proline, and aspartic acid. Application of the Edman technique to materials C_1 and C_2 indicated that the *N*-terminal sequences are Leu.Pro.Val . . . and Phe.Leu.Pro . . . respectively. The action of carboxypeptidase [9] on the mixture C_1–C_2 liberated leucine exclusively.

The findings on these peptides are therefore:

$$C_1 = \text{Leu.Pro.Val.(Asp)Leu}$$
$$C_2 = \text{Phe.Leu.Pro.(Val.Asp)Leu}$$

A hydrolysate of the material eluted from area C_3 contained serine, valine, and phenylalanine and only minute traces of other amino-acids. The fluorodinitrobenzene technique for *N*-terminal-residue identification gave dinitrophenyl-serine and -valine. The only free amino-acid present was phenylalanine. Since valine had been shown to occur in the sequence . . . Val.Asp . . ., material C_3 is probably a mixture of serylphenylalanine and free valine.

The structures of the five peptides described permit the sequence of the amino-acid residues in evolidine to be uniquely assigned:

Asp.Leu	B_1 and B_2
Val. Asp	A
Leu.Pro. Val.(Asp)Leu	C_1
Phe.Leu.Pro.(Val. Asp)Leu	C_2
Ser.Phe	C_3
Ser.Phe.Leu.Pro. Val. Asp.Leu	

(iv) *Configuration of Amino-acid Residues.*—A hydrolysate of evolidine was treated with fluorodinitrobenzene and the resulting mixture of products was separated by partition chromatography on columns of buffered " Hyflo Super Cel ". The following fractions were obtained (all dinitrophenylamino-acids): (*a*) serine and aspartic acid, (*b*) proline, (*c*) valine and some 2,4-dinitrophenol, (*d*) leucine and phenylalanine. Mixture (*d*) was resolved and the compositions of materials (*a*), (*b*), (*c*), and (*d*) were determined quantitatively by one-dimensional paper chromatography.

The directions of optical rotation of the dinitrophenyl derivatives in these fractions all agreed with those reported for the appropriate L-amino-acid derivatives.[12] Those $[M]_D$ values estimated were in agreement with the published values.[12]

The total evidence thus indicates that evolidine has the structure:

cyclo(-L-Ser-L-Phe-L-Leu-L-Pro-L-Val-L-Asp(β-NH_2)-L-Leu-)

Experimental

Hydrolysis of Evolidine.—(a) *Complete.* Specimens (~10 mg.) of evolidine (recrystallised six times from aqueous ethanol) were weighed into tubes of 6 mm. bore. Constant-boiling hydrochloric acid (1 ml.) was added, the mixtures were frozen, and the tubes evacuated, sealed, and heated at 105° for 20 hr. The contents of each tube were then diluted with water (2 ml.) and evaporated to dryness *in vacuo* over sodium hydroxide.

(b) *Partial.* The conditions were similar to those used for the complete hydrolyses, except that the solutions were heated in 5 ml. stoppered flasks at 35° ± 0·25° for 15—72 hr. or 80° ± 0·1° for 2—6 hr.

Amino-acid Analysis.—(a) *Identification.* The amino-acids in mixtures were identified by comparison with authentic specimens (Roche Products Ltd.) on single-dimension paper chromatograms on Whatman No. 1 paper. Various solvent systems were used, especially

[12] Rao and Sober, *J. Amer. Chem. Soc.*, 1954, **76**, 1328.

butan-1-ol–acetic acid–water (4 : 1 : 5) and " mixture P " (butan-1-ol–butan-2-one–water–dicyclohexylamine, 10 : 10 : 5 : 2). Ninhydrin (" AnalaR " grade; 0·025% in acetone) was used to detect the amino-acids, by spraying and heating at 105° for 10 min.

(b) *Estimation.* The amino-acids were estimated by the Moore–Stein procedure of ion-exchange adsorption separation, elution,[5] and eluate analysis by quantitative colorimetry of the ninhydrin reaction.[6]

The ion-exchange column (100 cm. long; bore 0·9 cm.) was packed with Dowex-50-X8 sulphonated polystyrene resin (supplied by Greeff and Co. Ltd.) and was thermostatically controlled to run within ±0·25° in the range 37·5° to 75°.

Each estimation was performed on a weighed specimen of ~5 mg. of the amino-acid mixture (dissolved in citrate buffer of pH 3·41). The elution was carried out by citrate buffer solutions, of pH 3·42 at 37·5° in the initial stages and of pH 4·25 at 75° in the later stages, and was complete after about 125 hr. The eluate (about 500 ml. in all) was collected in an automatic fraction-collector designed at the National Institute for Medical Research, Mill Hill,[13] and supplied by Towers and Co. Ltd. The amino-acid concentration in each fraction was estimated by measuring, in a Unicam S.P. 500 ultraviolet spectrophotometer, the optical density at 570 mμ of the colour produced by reaction with ninhydrin under Moore–Stein standardised conditions.[6]

Analysis of Products of Partial Hydrolysis of Evolidine.—Chromatograms of the partial hydrolysates were prepared on Whatman No. 1 paper, according to Sanger and Tuppy's directions.[14] After development in the first dimension with phenol, the solvent was removed by suspending the paper in a strong draught in a fume cupboard at room temperature for 2 days. The peptides and amino-acids for further investigation were located by fluorescence in ultraviolet light after the paper had been heated at 105° for 20 min.[15] On single-dimension chromatograms the material for separation was applied as a line at the origin. The positions of bands on the chromatograms were located by fluorescence and were confirmed by cutting vertical strips from the paper and treating them with ninhydrin. For two-dimensional chromatograms the second solvent was butanol–acetic acid–water.

Electrophoresis was carried out on Whatman No. 1 paper, supported between strips of plate glass and dipping at each end into the electrode dishes.[16] 0·05M-Ammonium acetate (pH ~6·7) was used as solvent. After 5 hr. with a potential difference of 500 v, aspartic acid moved 10—12 cm. towards the anode; materials B_1, B_2, and A moved 4—6 cm. towards the anode; and the other products of hydrolysis stayed near to the origin. Whatman No. 31 paper gave more even bands but these did not fluoresce well.

Material was removed from particular areas of the chromatogram and electrophoresis papers by the capillary method.[17] It was taken to dryness and stored for further investigation.

Identification of N-*Terminal Residues.*—The *N*-terminal residues of peptides were identified by the fluorodinitrobenzene technique, as described by Levy. The dinitrophenyl derivatives of the constituent amino-acids of evolidine, except for those of leucine and phenylalanine, are separated on paper in the toluene-type solvent described by Levy. *N*-Dinitrophenyl-leucine and -phenylalanine are readily separated in 1·5M-phosphate.

Stepwise Degradations.—The phenyl isothiocyanate method for determination of *N*-terminal-residue sequence was used, virtually as described by Fraenkel-Conrat,[8] the cyclisation of the phenylthiocarbamoyl-peptides to the corresponding phenylthiohydantoins being followed by observing the shift of the ultraviolet absorption maxima from 240 to 268 mμ.

The modifications used were: (i) During the formation of the phenylthiocarbamoyl-peptides, the pH was tested by B.D.H. narrow-range (8·5—10) indicator paper and kept in the range 8·7—9·0 by adding aqueous 0·01N-sodium hydroxide. The solutions were stirred by a stream of nitrogen. (ii) The aqueous solutions of the phenylthiocarbamoyl-peptides remaining after the extraction of excess of phenyl isothiocyanate were freed from dissolved benzene by a stream of nitrogen.

The phenylthiohydrantoins were identified by chromatography, and by chromatography of the amino-acids regenerated from them by acid hydrolysis. Traces of contaminating amino-acids (from non-specific cleavage of the peptides) were present at all stages.

[13] Lister, *Chem. and Ind.*, 1955, 583.
[14] Sanger and Tuppy, *Biochem. J.*, 1951, **49**, 463.
[15] Phillips, *Nature*, 1948, **161**, 53.
[16] Linstead, Elvidge, and Whalley, " Modern Techniques of Organic Chemistry," Butterworths Sci. Publ., London, 1955, p. 33.
[17] Consden, Gordon, and Martin, *Biochem. J.*, 1947, **41**, 590; Sanger and Tuppy, *ibid.*, 1951, **49**, 463.

Identification of C-Terminal Residues.—This was carried out by Harris's enzymic method,[9] using carboxypeptidase (Light and Co. Ltd.) treated with di-isopropyl phosphofluoridate (provided by Dr. B. C. Saunders) to inhibit endopeptidase action.

Rearrangements of Aspartyl Peptides.—α-Aspartyl-leucine (1 mg.) was heated in solution in water (0·1 ml.) in a sealed tube at 100° for 6 hr. Chromatography of the resulting solution showed the presence of α-aspartyl-leucine (strong), β-aspartyl-leucine (strong), aspartic acid (trace), and leucine (trace).

α-Aspartyl-leucine (1 mg.) was heated in solution in concentrated hydrochloric acid (0·1 ml.) in a sealed tube at 35° for 2 days. Electrophoresis of the resulting solution in 10% acetic acid showed the presence of α-aspartyl-leucine (strong), β-aspartyl-leucine (faint), aspartic acid (strong), and leucine (strong). After hydrolysis for 7 days at 35°, appreciable amounts of β-aspartyl-leucine were present as well as the other components.

When a solution of α-aspartyl-leucine in concentrated hydrochloric acid was heated at 80°, β-aspartyl-leucine appeared on chromatograms after 4 hr.

Assignment of Configuration to the Amino-Acids from Evolidine.—The mixture of amino-acids obtained on complete hydrolysis of evolidine (80 mg.) was converted into mixed dinitrophenyl derivatives by Levy's method [10] (treatment in mildly alkaline aqueous solution with fluorodinitrobenzene, extraction of the excess of this with ether, acidification, extraction of the dinitrophenyl derivatives with ether).

The mixture was separated into its components by an adaptation and combination of the methods due to Perrone [18] and to Levy.[10]

Three columns were set up, in series, containing " Hyflo Super Cel " supporting aqueous phases made up of citric acid–phosphate buffers of pH 4, 6·5, and 7 severally. The mixture of dinitrophenylamino-acids in ether was placed at the top of the pH 4 column and the chromatogram developed and eluted with ether. The eluate of the fast-moving band was passed on to the pH 6·5 column: that from the slow-moving band was collected. The process was repeated with the columns of pH 6·5 and pH 7. The fractions thus obtained were the slow bands from (*a*) col. pH 4, (*b*) col. pH 6·5 (*c*) col. pH 7, and (*d*) the fast band from col. pH 7. (A very fast-moving band on col. pH 7 was due to 2,4-dinitroaniline and was discarded.) These fractions were further separated by paper chromatography on Whatman No. 3 paper [ether–aqueous phosphate buffer (NaH_2PO_4, M; $+Na_2HPO_4$, 0·5M). They were identified as dinitrophenyl derivatives of: (*a*) serine and aspartic acid; (*b*) proline; (*c*) valine (and 2,4-dinitrophenol); (*d*) leucine and phenylalanine.

The optical rotations of these systems, in solution in 4% aqueous sodium hydrogen carbonate, were examined in a Bellingham and Stanley micropolarimeter. The concentrations of the solutions of dinitrophenyl derivatives of (i) an equimolecular serine–aspartic acid mixture, (ii) valine, and (iii) leucine were estimated from measurements of the extinction coefficients at 360 mμ, and thence the corresponding $[M]_D^{25}$ values were calculated. The results are given in Table 2.

TABLE 2.

Dinitrophenyl derivatives of	Observed rotation: Sense	Observed rotation: $[M]_D^{25}$	Recorded [12] for L-enantiomorph
Serine + aspartic acid	+	319°	+308°
Proline	—		−2172°
Valine	+	290°	+309°
Leucine	+	155°	+168°
Phenylalanine	—		−261°

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[18] Perrone, *Nature*, 1951, **167**, 513.