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The Isolation and Characterization of D-Serine Ethanolamine Phosphodiester from Earthworms

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It has been suggested that the biological precursor of lombricine (2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate, I) is serine ethanolamine phosphodiester (2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate, II) and that this may give rise to lombricine by a transamidination reaction involving arginine (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959).

This suggestion received some support since it was shown that serine ethanolamine phosphodiester did in fact exist in small amounts in earthworm tissue (Rosenberg & Ennor, 1959). The evidence for its presence was based on chromatographic data obtained with the compound itself, its hydrolysis products and its guanylated derivative, for it was not possible, at that time, to obtain it in amounts large enough to permit identification by more conventional means. However, the report of the occurrence of D-serine in lombricine (Beatty, Magrath & Ennor, 1959) suggested that it would be of interest to isolate larger amounts of serine ethanolamine phosphodiester and to determine the configuration of the serine present in this molecule.

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This paper describes a procedure for the isolation of serine ethanolamine phosphodiester, and provides both a complete characterization of the compound and a comparison with synthetic D-serine ethanolamine phosphodiester. The specific rotation of the natural compound was found to be the same as that of the synthetic one, indicating that the serine moiety has the D-configuration. The preparation

procedure has also yielded lombricine in amounts considerably greater than those reported previously (Rosenberg & Ennor, 1959).

MATERIALS AND METHODS

All reagents were analytical grade and solvents used for chromatography were purified by fractional distillation. Synthetic serine ethanolamine phosphodiester (D-SEP) was prepared by Beatty & Magrath (1959) from D-serine.

The earthworms used were as previously described (Rosenberg & Ennor, 1959) and were removed from cold storage (-70°) just before use.

Ion-exchange resins and chromatography. The following ion-exchange resins were used: Zeo-Karb 225 (mesh 20–50, H^+ form); Dowex-50 (mesh 200–400, NH_4^+ form); Amberlite CG-120 (mesh 100–200, H^+ form). All resins before use were converted into the H^+ and NH_4^+ forms several times by treatment with 2N-HCl or aq. 2.5N- NH_3 soln. respectively, and when finally in the form required were

washed with water until the effluent was free from either Cl^- ion or NH_4^+ ion.

Gradient elution. The apparatus used for gradient elution was essentially similar to that described by Hurlbert, Schmitz, Brumm & Potter (1954); the reservoir (5 l.) was initially charged with 4 l. of 1.5N-perchloric acid and the mixing vessel (2 l.) with 1.5 l. of water. When 2 l. of the mixture had passed through the column the reservoir was replenished with a similar volume of 2N-perchloric acid and again replenished with 3N-perchloric acid when a further 3 l. had emerged.

Determination of amino acid. Ninhydrin-reacting components of eluates were determined in a semi-quantitative manner by taking 0.05 ml. of the various fractions and heating at 100° for 5 min. in the presence of one drop of 5N-HCl, 0.5 ml. of water and 0.5 ml. of 1% (w/v) ninhydrin in pyridine. When cool the mixtures were diluted to 10 ml. with water and the extinctions measured in a Hilger-Spekker photometer with a spectrum-yellow filter.

Chromatographic solvent systems. Ascending paper chromatography was carried out on Whatman no. 3 papers in ethanol-formic acid-water (70:10:20, by vol.).

RESULTS

Isolation of serine ethanolamine phosphodiester

The extraction procedure was similar to that described for lombricine (Rosenberg & Ennor, 1959), except that the acid extract was not heat-treated. The perchloric acid extract (8 l. from 3.2 kg. of frozen worms) was treated with an equal volume of ethanol and the precipitate (glycogen and fine clay) was removed by centrifuging in a Sharples centrifuge. Potassium hydroxide (10N) was added to adjust the supernatant to pH 7.0 and the mixture was cooled to 0° . After standing for several hours in the cold room the precipitated KClO_4 was removed by filtration and washed with cold 50% (v/v) ethanol. The combined filtrate and washings (17 l.) were passed through a column (6 cm. $\times$ 70 cm.) of Zeo-Karb 225 ion-exchange resin (H^+ form) at the rate of 40 ml./min. The effluent contained a small amount of ninhydrin-reacting material which was found to be free from serine ethanolamine phosphodiester (SEP) and was discarded. The column was then washed with water until the effluent was substantially free from acid (pH 6.0) and the absorbed material eluted with aq. 2.5N- NH_3 soln. at the rate of 15 ml./min. The eluate was collected automatically in 25 ml. fractions and those containing ninhydrin-reacting material were pooled. The NH_3 was removed under reduced pressure at 40° and the solution (500 ml., pH 7.0–8.0) was applied to a column (78 cm. $\times$ 4 cm.) of Amberlite CG-120 ion-exchange resin (H^+ form) at the rate of 20 ml./min. The resin was washed with 200 ml. of water, the column was eluted with perchloric acid by the gradient-elution technique (see Materials and Methods section) at the rate of 5 ml./min. and the eluate collected

automatically in 50 ml. fractions. Samples of these were then taken for the colorimetric estimation of ninhydrin-positive material. The elution pattern (Fig. 1) indicated the presence of three major peaks, and samples from the appropriate fractions were taken for paper-chromatographic examination. SEP was found to be present in those fractions at the beginning of peak B (Fig. 1) and to be spread over fractions 50–56 (Fig. 2).

The fractions containing SEP were combined, neutralized (pH 7.0) by the addition of 10N-KOH, cooled to 0° and allowed to stand for 2 hr. The precipitated KClO_4 was removed by filtration and washed with 50 ml. of water at 0° . The combined filtrate and washings (500 ml.) were passed through a column (32 cm. $\times$ 3.5 cm.) of Zeo-Karb 225 ion-exchange resin (H^+ form, rate 20 ml./min.) and

Fig. 1. Elution pattern of ninhydrin-positive material obtained by gradient elution. Details of gradient-elution technique and methods of estimation are given in the text.

Fig. 2. Ninhydrin-treated paper chromatogram of samples of fractions from peaks A and B (Fig. 1). Solvent system: ethanol-formic acid-water (70:10:20). Tube nos. were as in Fig. 1; SEP denotes reference standard (synthetic D-SEP). Areas outlined by broken lines indicate faint spots.

eluted as before. Those fractions containing ninhydrin-positive material were combined and evaporated to dryness under reduced pressure at 40°. Water (10 ml.) was added to the dry material (0.9 g.) and a small amount of insoluble residue removed by centrifuging and discarded. The solution was transferred to a column (4 cm. × 20 cm.) of Dowex-50 ion-exchange resin (NH_4^+ form) and allowed to pass through at the rate of 1 ml./min. Water was then passed through the resin at the same rate and the effluent collected in 5 ml. fractions. A sample of each fraction containing ninhydrin-reacting material was taken for paper chromatography, which revealed (Fig. 3) a complete separation of SEP from contaminating ninhydrin-reacting materials. Those fractions containing SEP were combined and evaporated to dryness under reduced pressure at 40°. The dry residue (120 mg.) was combined with the yields of two similar preparations and the combined material (400 mg.) was crystallized from aqueous methanol to yield 0.139 g. of crystalline material. A further yield (0.159 g.) was obtained by successive crystallizations from the mother liquor.

Fig. 3. Ninhydrin-treated paper chromatogram of samples from fractions collected from column of Dowex-50 ion-exchange resin (NH_4^+ form). Solvent system was as in Fig. 2. SEP denotes reference standard (synthetic D-SEP). Areas outlined by broken lines indicate faint spots.

Some physical properties of the isolated SEP are compared with those of synthetic D-SEP. These together with the analytical data are given in Table 1. The infrared spectrum (potassium bromide disk) (Fig. 4) was identical with that of the synthetic SEP and showed peaks at 3430, 2913, 2080, 1682, 1640, 1530, 1462, 1412, 1368, 1345, 1305, 1230, 1075, 1038, 1023, 994, 912, 896, 833 and 779 cm^{-1} .

The results provide proof of the presence of D-serine in SEP.

Isolation of lombricine

Chromatographic examination of the fractions obtained in the gradient-elution step showed the presence of lombricine in peak C (Fig. 1) which, between tubes 70 and 83, contained only a minor contamination of ninhydrin-reacting material. The contents of these tubes were combined, neutralized (pH 7.0) by the addition of 10N-KOH, cooled to 0° and the KClO_4 was removed by filtration. The filtrate was freed from KClO_4 by passage through Zeo-Karb 225-H and eluted as described above as for the initial extract. The eluate (500 ml.) was reduced in volume to 20 ml. by distillation under reduced pressure and ethanol (3 vol.) was added. Immediate crystallization occurred and, after

Fig. 4. Infrared spectra. Upper and lower curves represent the spectra of synthetic D-SEP and isolated SEP respectively. Spectra were obtained by the use of a KBr disk in a Perkin-Elmer model 21 double-beam infrared spectrometer.

Table 1. Analytical data and physical properties of D-serine ethanolamine phosphodiester

	Isolated SEP	Synthetic SEP*	Theoretical for $\text{C}_5\text{H}_{13}\text{O}_6\text{N}_4\text{P}_2\text{CH}_2\cdot\text{OH}$
Analyses (%)			
C	26.73	—	27.05
H	6.32	—	6.19
N	11.17	—	11.48
P	12.50	—	12.69
M.p. (corr.)	144–145°	144°	—
α_D^{25}	+18.0°	+18.2°	—
	(C = 0.71 in water)	(C = 0.66 in water)	—
Crystalline form	Microcrystalline powder, very hygroscopic	—	—

* From Beatty & Magrath (1959).

standing for 2 hr. at 0°, the crystalline material was recovered by filtration, recrystallized from aqueous ethanol and dried *in vacuo* over P₂O₅. Yield was 3.5 g. This material was chromatographically pure and on analysis gave data identical with that previously reported (Rosenberg & Ennor, 1959).

DISCUSSION

Evidence for the presence of serine and ethanolamine in SEP isolated from earthworms was presented previously (Rosenberg & Ennor, 1959).

The present isolation of SEP in amounts sufficient to permit crystallization and chemical and physical analysis has also permitted comparison with D-SEP obtained by synthetic routes involving the use of D-serine. The results leave no doubt about the structure of the isolated material and provide proof of the presence of serine with the D-configuration in the molecule.

The yield of SEP (300 mg. from 9.6 kg. of earthworms), although small, is nevertheless believed to represent the bulk of that present in the starting material, for no loss of ninhydrin-reacting components containing SEP was detected throughout the fractionation procedures. The yield corresponds to 3.1 mg./100 g. and contrasts with the minimal content of 50 mg./100 g. reported by Roberts & Lowe (1954) in river-turtle tissue.

It has been suggested that SEP may be the precursor of lombricine (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959), and the present results indicating the presence of D-serine in SEP (also present in lombricine: Beatty *et al.* 1959) are consistent with this hypothesis.

The procedure developed for the isolation of SEP has yielded lombricine as a 'by-product' in amounts (1.1 g./kg.) appreciably larger than those reported earlier and represents almost a complete recovery of this compound from earthworm tissue.

SUMMARY

1. Serine ethanolamine phosphodiester has been isolated from earthworm tissue in amounts sufficient to permit positive chemical and physical identification.

2. The serine moiety of serine ethanolamine phosphodiester has been shown to possess the D-configuration.

3. The presence of D-serine in the compound is consistent with the thesis that serine ethanolamine phosphodiester is the precursor of lombricine.

4. A simple and improved method for the isolation of lombricine is described.

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Further Evidence of Tautomerism in Chromatograms of Indolyl-3-Pyruvic Acid

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Indolyl-3-pyruvic acid is a very unstable substance and with the usual methods and solvents cannot be detected in chromatograms, either because it is completely destroyed (Jepson, 1958*a*; Bentley, Farrar, Housley, Smith & Taylor, 1956; Kaper & Veldstra, 1958) or because the solvents used do not separate it from its decomposition products.

With the method of 'double chromatography' (in which the same solvent is used in succession in two

directions: Schwarz & Bitancourt, 1957*a*) we have shown that in chromatograms of indolyl-3-pyruvic acid two rows of spots of decomposition products are formed, in the second development, originating from two spots that had been separated in the first development. Both rows reproduce the row of spots of the first development, including the two original spots. We have considered this as evidence of the keto-enol equilibrium of indolyl-3-pyruvic