The Configuration of the Serine Moiety of Lombricine and Reptilian Serine Ethanolamine Phosphodiester

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In the course of investigations concerned with the synthesis of lombricine (Equation 1), it was found that the serine component possessed the D configuration. This result was reported in preliminary communications (1, 2) in which the opinion was proffered that if serine ethanolamine phosphodiester were the precursor of lombricine in the earthworm, as had been suggested earlier (3), it might contain D-serine also. Subsequently it was shown (4) that this is in fact the case.

It was also suggested (1) that Roberts and Lowe (5) may have wrongly ascribed an L configuration to the serine component of serine-EP (1) which was first isolated by these workers from river turtle (Pseudemys elegans) muscle. The present communication describes the isolation of D-serine from lombricine obtained from earthworms, and provides evidence confirmatory to that of Roberts and Lowe (5) for the presence of L-serine in serine-EP isolated from reptilian muscle.

EXPERIMENTAL PROCEDURE

The earthworms used were as previously reported (6) and were removed from cold storage (70°) just before use. Crocodile muscle was taken from a large crocodile immediately after capture in the area of Darwin, Australia, frozen within 6 hours of dissection, and transported to Canberra in packages containing solid carbon dioxide.

River turtles (Chelodina longicollis) were obtained from Southern Queensland and were kept in an aquarium until required. The animals were killed by decapitation, and the skeletal muscle was removed and immediately homogenized in ice-cold 1.5 N perchloric acid (1.5 volumes).

The D-amino acid oxidase used was prepared according to Negelein and Brümel (7), and the procedure taken to the first ammonium sulfate fractionation stage. The preparation was stored as a suspension in 50% saturated aqueous ammonium sulfate. Portions of the suspension were taken as required, centrifuged, and the precipitate was dissolved in water and dialyzed overnight against two changes (each 100 volumes) of water. The dialyzed solution was centrifuged to remove a small amount of insoluble material and was kept frozen. The concentration of the solution was so adjusted that 1.0 ml was equivalent to 1 g of the sheep kidney acetone powder from which the preparation was initially made. Catalase was not added to this preparation, since it was found that 0.1 ml decomposed 10 μmoles of H₂O₂ in 30 seconds when tested under the conditions described by Herbert (8).

Reference samples of D- and L-serine-EP and 2-guanidinoethyl phosphate were synthetic (9).

Lombricine was isolated from earthworms, and serine-EP from both earthworms and reptilian muscle, by the method previously described (4).

D-, L-, and DL-Serine (obtained from British Drug Houses, Ltd., Poole, England; California Corporation for Biochemical Research, Los Angeles, California, Cfp grade; and Schwarz Laboratories, Inc., Mt. Vernon, New York, optically standardized grade, respectively) were recrystallized from aqueous ethanol. Melting points (decomposition points) were 221–222°, 220–221°, and 242–243°, respectively, with discoloration at about 210°.

For the D isomer, [α]D° = +7.4 (c, 2.02 g, H₂O), and for the L isomer, −6.94 (c, 4.04 g, H₂O).

Samples of serine-EP were prepared for analysis by drying at 80° for 3 hours at 0.1 to 0.5 mm over P₂O₅, and subsequent sampling was carried out in a dry-box because of the hygroscopic nature of the crystals.

Nitrogen was determined by the Kjeldahl method, and phosphorus was determined colorimetrically (10) after wet ashing.

All melting points quoted are corrected, and since all the substances melted with decomposition at temperatures which varied with the rate of heating and the state of subdivision, comparison was made by heating the substances in a metal block type of apparatus which enabled simultaneous determinations to be made. The rate of heating was about 2° per minute.

Optical rotations were determined in 0.5- or 1.0-dm small bore tubes in a polarimeter reading to ±0.01°.

Infrared spectra were obtained on potassium bromide disks with a Perkin-Elmer model 12C (serine) or a Perkin-Elmer model 21 double beam (serine-EP) spectrophotometer, both being equipped with sodium chloride optics.

All paper chromatography was carried out with Whatman 3MM paper, and the following solvent systems were used: I. H₂O-NH₄OH (20:1), II. Benzene-ethyl acetate (2:1), III. Benzene-ethyl acetate (3:2), IV. Chloroform-ethyl acetate (2:1), and V. Chloroform-ethyl acetate (3:2).

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1 The abbreviation used is: serine-EP, serine ethanolamine phosphodiester.
methyl ethyl ketone, methyl Cellosolve, acetic acid, water (40:15:6:24); II., n-propanol, water, ammonia (specific gravity, 0.91) (73:7:20); III., n-butanol, acetic acid, water (50:20:30).

Compounds containing amino or guanidino groups were detected on paper by spraying with 0.2% ninhydrin in acetone or with an alkaline α-naphthol-diacetyl reagent (11), respectively. Phosphates were detected by the Hanes and Isherwood (12) reagent.

RESULTS

Enzymatic Studies—The capacity of lombricine and d-serine-EP, together with the products of acid hydrolysis of lombricine, to act as substrate for d-amino acid oxidase was tested.

The acid hydrolysate of lombricine was prepared by heating 27 mg in a sealed tube in 1.0 ml of 6 N HCl in 110° for 24 hours. The contents of the tube were removed, evaporated to dryness under reduced pressure, and placed in a desiccator over solid KOH for 24 hours to remove the bulk of the residual HCl. The material was then dissolved in 0.5 ml of pyrophosphate buffer (0.1 M, pH 8.3).

Assuming complete hydrolysis and no destruction of the serine released, the theoretical amount of serine present in the hydrolysate should have amounted to approximately 100 μmoles and thus to approximately 40 μmoles in the 0.2-ml sample taken for enzymatic assay. This amount of d-serine is readily detectable under the conditions employed (cf. Table I). The enzyme was without action on lombricine or serine EP. The unfraccionated hydrolysate of lombricine produced some inhibition of the enzyme, as measured both in the presence and absence of added d-serine; in earlier experiments (1), in which n-amino acid oxidase toward the effect of the first two of these compounds on the activity of d-serine oxidase toward d-serine has also been tested. The results (Table I) suggest that none of the inhibition by the hydrolysate could have been due to these products.

Isolation and Characterization of Serine and 2-Guanidinoethanol Phosphate in Lombricine Hydrolysate—A solution of lombricine (580 mg) in 6 N sulfuric acid (12 ml) was heated in a sealed tube at 110° for 9 hours. After cooling, sulfuric acid was removed as barium sulfate which was collected by centrifugation and thoroughly washed with water. Washings and original supernatant solution were combined, concentrated to approximately 10 ml and applied to a column (3.5 by 24 cm) of Dowex 50 (NH₄⁺) resin. Washing with water and examination of fractions (5 ml) of the eluate by paper chromatography indicated the successive elution of 2-guanidinoethanol phosphate, traces of a ninhydrin-positive material (probably alanine (9)), serine, and lombricine, with some overlap occurring between serine and the “alanine”- and lombricine-containing fractions. Fractions containing serine were combined, concentrated to small volume, and applied as a narrow band to two sheets (18 by 42 cm) of washed paper which were then developed in solvent system II (descending). The papers were dried, and the serine was located and eluted with water. The crystalline residue obtained on evaporation of the eluate was recrystallized from aqueous ethanol, yielding colorless bladelike crystals (22 mg). An additional 20 mg were obtained by a similar hydrolysis and isolation procedure, and this material was combined with the former lot. All analyses were carried out on this material. The melting point was 220–221° decomposition; the mixed melting points (by solution and recrystallization) with authentic d- and L-serine were 220–221° decomposition and 243–244° decomposition, respectively; [α]D²⁴ +7.4 (c, 3.92, H₂O).

The infrared spectrum of the solid showed peaks at 3427, 3030, 2920, 855, and 805 cm⁻¹, and this spectrum was identical with that of d-serine.

The dinitrophenyl derivative was prepared by the method of Sanger (13). This derivative alone, and when mixed with the dinitrophenyl derivative of authentic d-serine, melted at the same temperature as did authentic dinitrophenyl serine, i.e. at 174–175° decomposition.

A sample of the isolated serine was tested for its ability to act as a substrate for d-amino acid oxidase. Within experimental error, the oxygen uptake was close to that of an equivalent amount of authentic d-serine (Table I), and to that theoretically expected.

The fractions from the two ion exchange columns which contained 2-guanidinoethanol phosphate were evaporated to dryness, and the residue (75 mg) was redissolved in water (5 ml) and applied to a column (1 by 2.5 cm) of Dowex 50-X4 ion exchange resin (200 to 400 mesh, H⁺ form). The column was washed with water, and those fractions containing 2-guanidinoethanol phosphate were evaporated to dryness, giving a crystalline residue. This was twice recrystallized from aqueous methanol and yielded 44 mg of colorless rod-shaped crystals, melting point 208° decomposition. Authentic 2-guanidinoethanol phosphate had a melting point of 207–208° decomposition. The infrared spec-

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<p>| Table I |</p>
<table>
<thead>
<tr>
<th>Compound added</th>
<th>Amount</th>
<th>Oxygen consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>atoms</td>
<td>moles</td>
</tr>
<tr>
<td>Lombricine</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Lombricine hydrolysate</td>
<td>40*</td>
<td>7.1</td>
</tr>
<tr>
<td>Serine (isolated from lombricine hydrolysate)</td>
<td>21</td>
<td>0.7</td>
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<tr>
<td>d-Serine (authentic)</td>
<td>10</td>
<td>0.7</td>
</tr>
<tr>
<td>d-Serine + lombricine hydrolysate</td>
<td>40*</td>
<td>7.1</td>
</tr>
<tr>
<td>d-Serine + 2-guanidinoethanol phosphate</td>
<td>40</td>
<td>0.8</td>
</tr>
<tr>
<td>d-Serine + 2-guanidinoethanol</td>
<td>40</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* These values refer to the original amount of the material hydrolyzed; lombricine was hydrolyzed for 24 hours in 6 N HCl at 110° in a sealed tube.
Characterization of Serine-EP from Reptilian Muscle

1. Crocodile Muscle—The isolated material (332 mg from 2 kg of muscle) was recrystallized from aqueous methanol to yield colorless, hygroscopic microcrystals, melting point 144-145° decomposition. Authentic L-serine-EP had a melting point of 142-143° decomposition (cf. 139-141° uncorrected decomposition (14)). \([\alpha]_D^{25} -16.6 (c, 0.78, \text{H}_2\text{O});\text{ authentic L-serine-EP, }[\alpha]_D^{25} +14.8\) \((c, 0.31, \text{H}_2\text{O})\).

The infrared spectrum was identical with that of synthetic D or L diester (9, 14).

The present work is presumably due to slight variations in the conditions of recrystallization.

-14.8 \((c, 0.31, \text{H}_2\text{O})\) (cf. \([\alpha]_D^{25} -15.0 (c, 2.2, \text{H}_2\text{O})\). (14)).

The infrared spectrum was identical with that of synthetic D- or L-serine-EP (9, 14).

2. Turtle Muscle—The isolated material (560 mg) was free from other ninhydrin-positive compounds and separated from aqueous methanol as colorless, hygroscopic microcrystals, melting point 144-145° decomposition; \([\alpha]_D^{25} +12.1 (c, 0.58, \text{H}_2\text{O})\).

Serine-EP is also being investigated and will be reported.

DISCUSSION

Although the occurrence of D-amino acids in microorganisms is now well established, their presence in animal tissues is much less certain. Apart from several reports of their isolation in small amounts from acid hydrolysates of various animal proteins (e.g. see Neuberger (15), Greenstein (16), and Meister (17)), there may be mentioned the isolation of octopine from scallop (9, 14), D-alanine and D-glutamic acid from goose and insect tissues, respectively (35), and finally the identification of D-alanine in the blood of the milkweed bug Oncopeltus fasciatus (26). Each of these reports, however, is subject to reservation (15, 17) on grounds of inadequate structural characterization, doubts as to whether racemization might not have occurred during isolation, or the possibility that the compound may have originated from contaminating microorganisms. It is also noteworthy that no functional importance has been ascribed to such amino acids.

The present results show clearly that on acid hydrolysis, lombricine, isolated from earthworms and shown to be identical with synthetic D-2-amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate (9), yields D-serine. In this case, the D-amino acid is functional in the sense that it is a component part of lombricine which in its N-phosphorylated form is known (27, 28) to act as a phosphagen in the earthworm. Thus it would seem that this is the first occasion on which a D-amino acid of fully defined structure has been unequivocally shown to exist in animal tissue.

It should be noted that D-serine has since been shown to occur as a constituent part of serine-EP isolated from earthworms (4), as well as in the free form in the tissues of this animal (29). The only other reports on the occurrence of D-serine with which we are familiar concern its isolation from acid hydrolysates of polyaminos (30), cycloserine (31-33), and O-carbamyl D-serine (34), all of which are derived from microorganisms.

The earlier conclusion of Roberts and Lowe (5) that L-serine is present in serine-EP isolated from river turles has been fully substantiated by a study of the material isolated from the muscle of river turtles. L-Serine-EP has also been shown to be present in the muscles of crocodiles. There are thus two naturally occurring isomeric forms of serine-EP, and these appear to have little in common in the biological sense. Experiments at present in progress in this laboratory indicate that L-serine-EP, known to exist in river turtles (5), is present in other reptiles and also in birds. Serine-EP of a configuration not yet determined, but presumably \(L\), has also been found in fish and amphibians (35). Its function is not known at present, but experiments on its mode of biosynthesis and distribution will be reported shortly.

The position with respect to D-serine-EP is somewhat clearer. This compound was first detected in the earthworm (6) in which it is known (36) to be the biological precursor of lombricine. The possibility remains, however, that it may have additional functions, and these may be similar to those of the L isomer in the species referred to above. The mode of synthesis of D-serine-EP is also being investigated and will be reported.

SUMMARY

1. The serine moiety of lombricine has been isolated after acid hydrolysis and characterized as D-serine.

2. Serine ethanolamine phosphodiester from two reptilian sources has been shown to contain L-serine.

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