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Biochem. J. (1961) **79**, 424

The Occurrence of Free D-Serine in the Earthworm

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The occurrence of D-serine in lombricine (2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate) and serine ethanolamine phosphodiester, isolated from the earthworm, has been reported from this laboratory (Beatty, Magrath & Ennor, 1959; Ennor, Rosenberg, Rossiter, Beatty & Gaffney, 1960; Beatty, Ennor, Rosenberg & Magrath, 1961).

This paper describes the isolation of DL-serine from earthworm tissues and provides conclusive proof that D-serine is present in the tissues immediately before extraction and that it could not arise as a result of racemization of L-serine during the isolation procedure.

A preliminary report of this work has appeared (Rosenberg & Ennor, 1960).

MATERIALS AND METHODS

All reagents were A.R. grade. Ethanol used for chromatography and other purposes was purified by refluxing over KOH and aluminium powder and then by fractional distillation. D-, L- and DL-Serine were purchased from California Corporation for Biochemical Research, Los Angeles, Calif., U.S.A. L-[3-¹⁴C]serine was obtained from Dr R. L. Blakley of this Department and was prepared from glycine and [¹⁴C]formaldehyde with serine transhydroxymethylase (McDougall & Blakley, 1961). The earthworms used in this work were as described by Rosenberg & Ennor (1959).

Isolation of serine. The experimental procedures used for the isolation of serine from the tissue have been described

in detail (Ennor *et al.* 1960), and the various steps will be referred to in general terms only. Such modifications as have been introduced will be described in the text.

D-Amino acid oxidase. This was prepared according to Negelein & Brömel (1939) and was taken to stage III [(NH₄)₂SO₄ precipitation]. The precipitate containing the enzyme was stored at 0° as a suspension in 50% saturated (NH₄)₂SO₄. Portions of this suspension were centrifuged as required and the precipitate was resuspended in 0.01 M-pyrophosphate buffer, pH 8.1, and dialysed against 100 vol. of the same buffer, with two changes, for 24 hr. The dialysed solution was cleared by centrifuging and its volume adjusted with water so that each millilitre of the solution corresponded to 1 g. of the acetone-dried kidney powder used in the preparation. The solution was tested for the presence of catalase according to Herbert (1955) and it was found that 0.1 ml. of the solution decomposed 100 μmoles of H₂O₂ in 30 sec. The solution was kept frozen at -10° and retained activity for at least 3 months. Assays with this enzyme were carried out in Warburg manometers at 37° in air. The vessels contained 2.0 ml. of 0.1 M-sodium pyrophosphate buffer, pH 8.3, the amino acid tested and 0.3 ml. of the enzyme solution, in a total volume of 3 ml.

Ascending paper chromatography. This was carried out on Whatman no. 3 papers and the following solvent systems were employed: (I) ethanol-formic acid-water (70:10:20, by vol.); (II) water-saturated phenol; (III) butan-2-one-methylcellosolve (2-methoxyethanol)-acetic acid-water (40:15:6:24). Amino acids and related compounds were detected by dipping the dried papers into 0.2% solution of ninhydrin in acetone, followed by heating for 10 min. at 80°.

Table 1. *Influence of alkali on the racemization of L-serine as tested with D-amino acid oxidase*

Full experimental details of the method employed and of the treatment with alkali are given in the text.

Substrate ...	D-Serine	L-Serine	L-Serine	L-Serine
Amount present (μ moles)	10.0	238	238	238
Treatment	Nil	Nil	Aq. 2.5 N-NH ₃ (48 hr.)	Satd. Ba(OH) ₂ (48 hr.)
Uptake of O ₂ (μ g.atoms/3 hr.)	10.0	0	0.9	0

Radioactivity determinations. These were carried out as described by Rossiter, Gaffney, Rosenberg & Ennor (1960). Samples of crystalline serine (1–2 mg.) were weighed directly into the counting vessels. Samples (20 μ l.) of aqueous fractions emerging from columns were dried in the counting vessel at 110° but, whenever the solutions contained mineral acid, excess of 2N-ammonia was added before drying to avoid discoloration.

RESULTS

Optical stability of L-serine

Daft & Coghill (1931) isolated DL-serine from acid hydrolysates of protein after treatment with excess of Ba(OH)₂ and assumed that racemization took place whilst the solution was alkaline. Because of these findings it seemed desirable to ensure that the D-serine isolated from perchloric acid extracts of earthworms (Rosenberg & Ennor, 1960) had not arisen as a result of exposure to alkali. The procedure employed in these experiments was similar to that described for the isolation of lombricine and serine ethanolamine phosphodiester (Ennor *et al.* 1960). This involved a desalting step, which consisted in the passage of the neutralized and filtered extract through Zeo-Karb 225 ion-exchange resin (H⁺ form) followed by elution of the adsorbed material with aq. 2.5N-NH₃ soln. This eluate, which was salt-free, was then taken to near-dryness under reduced pressure in a rotary evaporator at 40°, at which stage it was NH₃-free and pH 7–8. The subsequent fractionation and purification did not involve any treatment at pH greater than 7.

The present investigation into the effects of alkaline solutions on the optical homogeneity of L-serine has been carried out with both aq. 2.5N-NH₃ soln. and aq. saturated Ba(OH)₂. A quantity of L-serine (0.5 ml. of 5%, w/v, soln.) was placed in each of two flasks: to one of these was added 2 ml. of aq. 2.5N-NH₃ soln. and to the other 2 ml. of saturated Ba(OH)₂ soln. Both flasks were allowed to stand at room temperature (about 22°) for 48 hr., when the NH₃ was removed by evaporation as before. The Ba(OH)₂ soln. was passed through a column (6 cm. $\times$ 1.2 cm.) of Dowex-50 ion-exchange resin (NH₄⁺ form), which was then washed with water. The combined effluent and washings contained serine in addition to the ammonia displaced by the Ba²⁺ ion. The ammonia was removed by evaporation and both dry residues

Table 2. *Action of D-amino acid oxidase on a serine-containing fraction isolated from rabbit muscle and serine obtained after passage of L-serine through the isolation procedure.*

Substrate ...	D-Serine	L-Serine (authentic)	L-Serine reisolated
Amount present (μ moles)	10.0	238	200
Uptake of O ₂ (μ g.atoms/2 hr.)	9.6	0	0.3

were dissolved in water (0.7 ml.) and quantitatively transferred to the main chamber of Warburg manometric flasks. The ability of the serine present in these flasks to act as a substrate for D-amino acid oxidase was then compared with that of samples of authentic L- and D-serine. The results (Table 1) show that no detectable racemization occurred with the baryta treatment but that some racemization did occur in the presence of ammonia. This, however, was small and on the basis of the uptake of O₂ amounted to only 0.4% of the serine present. This degree of racemization was not considered serious, particularly in view of the fact that the earlier isolation procedure (Rosenberg & Ennor, 1960) involved contact with NH₃ for no longer than 30 min. Moreover, the comparative yields of DL- and L-serine reported would have necessitated an inversion to the extent of at least 25%. Such a change in the optical purity would seem most unlikely in view of the above-mentioned results but it was nevertheless decided to carry out an additional control experiment. The behaviour of an authentic sample of L-serine (50 mg. in 200 ml. of 1.5N-HClO₄) has been checked through the entire isolation procedure and tested enzymically for the presence of D-serine as before. The results (Table 2) show that no detectable degree of racemization of serine occurred. This and the preceding experiments provide conclusive proof of the safety of the extraction and fractionation procedures in so far as the retention of optical homogeneity is concerned. But some doubt may remain when such procedures are applied to earthworm tissue; it seemed possible that the latter might contain some unrecognized factor(s) which would catalyse racemization in acid or alkaline solution. This possibility has been examined by employing a variant of the isolation technique which did not involve the use of an alkaline medium at any stage and which employed

an internal check on racemization through the addition of L-[3-¹⁴C]-serine.

Approximately 5 kg. of frozen earthworms was extracted in a Waring Blendor with 1.5 vol. of 1.5N-HClO₄. Labelled L-serine (20 μmoles) was added to the resultant suspension, which was then treated as described by Ennor *et al.* (1960), except that the NH₃-elution stage was avoided as follows. After adsorption on to a cation-exchange resin (Dowex 50, H⁺ form; mesh 50–100), the material was eluted with 0.2N-HClO₄ instead of NH₃. The eluate was collected in 25 ml. fractions and those containing serine were located by radioactivity determinations. These were pooled, adjusted to pH 7 by the addition of 10N-KOH at 0° and the precipitated KClO₄ was removed by filtration. The filtrate was transferred to a column (3 cm. × 55 cm.) of Amberlite CG-120 (H⁺ form) and the adsorbed material eluted by gradient elution with HClO₄ as described by Ennor *et al.* (1960).

Those fractions containing serine were identified by measurement of radioactivity, combined and the perchloric acid was removed as described above. The resultant solution of serine was evaporated under reduced pressure to about 5 ml. and further purification of the serine was achieved by repeated passage through columns of Dowex 50 (NH₄⁺ form) as described by Ennor *et al.* (1960). After each passage those fractions containing serine were located as described above and then examined by paper chromatography in solvent II. In this way those fractions containing uncontaminated serine were located. They were then combined and stored at –10°. The fractions containing serine contaminated with other amino acids were combined and again passed through Dowex 50 (NH₄⁺ form). After three such passages the bulk of the serine was free from contamination by other amino acids and the combined solutions of uncontaminated serine were evaporated under reduced pressure at 30° to about 7 ml. Ethanol (about 10 ml.) was added to this residue and crystallization commenced immediately. The mixture was allowed to stand overnight at room temperature and filtered to yield crystalline plates (crop I), which corresponded to those referred to in the preliminary communication (Rosenberg & Ennor, 1960) and which were found to contain mainly DL-serine. This was repeatedly recrystallized from aq. ethanol. The filtrate from which crop I was obtained was treated with a further 20 ml. of ethanol and kept at 0° for 24 hr. Long fine needles of L-serine (crop II) were recovered by filtration and the mother liquor was discarded. The crystals were repeatedly recrystallized from aq. ethanol. (Found: crop I, C, 33.97; H, 6.68; N, 13.19. Crop II, C, 33.95; H, 6.69; N, 13.08. C₃H₇O₃N requires C, 34.28; H, 6.72; N, 13.33 %). Comparison of the yields (Table 3) of DL- and L-

serine (crops I and II respectively) indicates that about 20 % of the serine isolated was in the D-form. The results of the radioactivity determinations (Table 3) show that the specific radioactivity of the DL-serine was approximately one-half of that of the L-serine. The small excess of radioactivity of the serine racemate over that demanded by theory could represent a maximum of 5 % conversion of L-serine into the D-enantiomorph. In order to obtain a more precise estimate of any possible conversion of L-serine into the D-isomer 40 mg. of the racemate was dissolved in 2 ml. of water and 70 mg. of authentic D-serine was added. The solution was warmed and mixed with 3 ml. of ethanol and allowed to stand for 18 hr. at room temperature. The crystals which separated were found to have a specific radioactivity of 7700; they were assumed to be predominantly racemate (with D-serine contamination) and were discarded. D-Serine was crystallized from the supernatant after the addition of 20 ml. of ethanol. The crystals were collected and dried, and the radioactivity was assayed in a small sample. The remainder was dissolved in water (2 ml.), ethanol (1.5 vol.) was added and the mixture allowed to stand overnight at room temperature to permit crystallization of DL-serine. This was removed by filtration and discarded, and additional ethanol was added as before to permit crystallization of the D-isomer. This was recovered and the specific radioactivity again measured on a small sample. This second recrystallization resulted in a slight fall in specific radioactivity (Table 4). The remainder of the D-serine was again dissolved in water and about 1 mg. of authentic L-serine added. DL- and D-Serine were crystallized from this solution by a repetition of the procedure described and the procedure was again repeated after a further addition of L-serine. The third and fourth recrystallizations resulted in a sharp fall in the specific radioactivity (Table 4) and indicates that the excess counts found in the racemate were not due to the presence of D-[¹⁴C]-serine.

Stability of lombricine in worm homogenates

Some attention has been given to the question of whether the D-serine found arises from the breakdown of lombricine or D-serine ethanolamine phos-

Table 3. *Specific radioactivities and physical characteristics of serine isolated from earthworm extracts to which L-[¹⁴C]-serine had been added*

	DL-Serine (crop I)	L-Serine (crop II)
Yield (mg.)	105	140
M.p. (decomp.)	244°	222°–223°
[α] _D ²⁵ in water	0.0 (c, 4.0)	–7.2 (c, 5.97)
Specific radioactivity (counts/min./mg.)	8,790	16,970

phodiester or both, particularly as Robin (1954) has reported the ability of the insoluble particles of worm homogenates to degrade lombricine to guanidinoethyl phosphate and serine. A homogenate was prepared by homogenizing one live worm (*Megascolides cameroni*) (7 g.) in 5 ml. of water. This crude homogenate was made to 15 ml. by the addition of water and was tested for its ability to degrade lombricine and D-serine ethanolamine phosphodiester. The reaction mixtures tested contained 1.0 ml. of homogenate and 1.0 ml. of either phosphate buffer (0.1 M, pH 7.0) or pyrophosphate buffer (0.1 M, pH 8.3) and 5 mg. of test substance in a total volume of 5.0 ml. The tubes with appropriate controls were incubated for 4 hr., when 2.0 ml. of 3.5N-HClO₄ was added. The resultant mixtures were centrifuged and the supernatant fluid was brought to pH 7.0 by the addition of 2N-KOH at 0°. The precipitates of KClO₄ were removed by filtration and the filtrates evaporated to dryness *in vacuo*. The residues were dissolved in 1.0 ml. of cold water and filtered to remove insoluble KClO₄. Portions of the resultant clear solutions were spotted on to paper together with markers of authentic samples of lombricine, 2-guanidinoethyl phosphate, 2-guanidinoethanol and serine, and the chromatograms were developed in solvents II and III and sprayed with ninhydrin and α -naphthol-diacetyl reagents respectively. In addition, two-dimensional chromatograms were developed in solvent systems I and II and sprayed with ninhydrin. The unidimensional chromatograms showed the presence of trace amounts of 2-guanidinoethyl phosphate in all the incubated mixtures together with traces of an unidentified α -naphthol-diacetyl-positive compound with R_f 0.9, which was greater than that of 2-guanidinoethanol, the next fastest-running marker compound. Lombricine was present in all reaction mixtures and the intensity of the lombricine spots showed no lessening with either increasing incubation time or with pH. The two-dimensional chromatograms were confirmatory in the sense that there was no

evidence of any disappearance of lombricine, or D-serine ethanolamine phosphodiester, or of any appearance of the expected degradation products.

DISCUSSION

The unique nature of the finding (Rosenberg & Ennor, 1960) of a D-amino acid in the free amino acid pool of animal tissue makes it desirable that the evidence for its presence should be accompanied by unequivocal proof that the 'unnatural' isomer could not have arisen as a result of racemization possibly induced by the isolation procedure.

The subject of the present investigation was the earthworm, which has already been shown to contain lombricine and D-serine ethanolamine phosphodiester, in both of which substances the serine component is present as the D-isomer (Beatty *et al.* 1959; Ennor *et al.* 1960; Beatty *et al.* 1961). In addition therefore to the possibility that the D-serine may have arisen as a result of racemization, there is a possibility that it may have arisen as a result of a bio-degradation of lombricine or D-serine ethanolamine phosphodiester or both. Both of these possibilities have been investigated in the present work.

The effect of alkali in producing racemization of serine appears to have met with general acceptance (cf. Neuberger, 1948; Desneulle, 1953). This is based on the report of Daft & Coghill (1931), who claimed without proof that L-serine in protein hydrolysates was racemized on treatment with barium hydroxide. Crawhall & Elliott (1951), using optical rotation as a criterion, failed to detect any change in the optical purity of serine in N-sodium hydroxide for 22 hr. at room temperature. The present results confirm and amplify those of Crawhall & Elliott (1951) in that they demonstrate the optical stability of serine to alkali by using an enzymic method which affords a greater degree of sensitivity than the measurement of optical rotation. Barium hydroxide was shown to be without effect on the optical purity of L-serine. The exposure of 238 μ moles of L-serine to aqueous 2.5N-ammonia solution at room temperature for 48 hr. did lead to a detectable uptake of oxygen when tested with D-amino acid oxidase. If it be assumed that this was due to D-serine the amount present could not have been more than 1 μ mole and thus would represent less than 0.5 % conversion of L-serine into the D-enantiomorph. Racemization as a result of exposure of L-serine to alkaline solvents can therefore clearly be excluded as an explanation for the large amount (25 %) of the D-isomer found in association with the L-enantiomorph.

When ammoniacal solvents were replaced by acidic ones no detectable degree of racemization occurred and, under these conditions, the occur-

Table 4. *Effect of repeated recrystallization on the specific radioactivity of D-serine*

D-Serine was isolated from a mixture of 40 mg. of DL-[¹⁴C]serine (cf. Table 3) and 70 mg. of authentic D-serine.

Compound		Specific radioactivity (counts/min./mg.)
Original DL-serine		8790
D-Serine	first crystallization	288
	second crystallization	238
	third crystallization	75*
	fourth crystallization	37*

* In these recrystallizations about 1 mg. of unlabelled L-serine was added as carrier.

rence of D-serine was again demonstrated. The further possibility that racemization may be 'catalysed' by some compound(s) in the earthworm extract has also been investigated. In these experiments L-[¹⁴C]-serine was added to perchloric acid suspensions of earthworm tissue and use was made of the difference in solubilities of D- and L-serine and the racemic mixture (cf. Minthorne & Koeppe, 1960). Theoretically the latter would be expected to possess precisely one-half of the specific radioactivity of the L-serine present in the extract and experimentally this has been found nearly to be the case. In actual fact the DL-serine contained 8790 counts/min./mg. as compared with the theoretical expectation of 8490 counts/min./mg. That this difference was probably due to experimental error and not to racemization was indicated by the progressive fall in radioactivity of the isolated D-serine after successive recrystallizations. The nature of this fall and the relatively small amount of radioactivity which remained after the fourth recrystallization strongly suggest that a zero level of radioactivity would have been reached had further recrystallizations been possible. These, however, were precluded because of the small amount of material which remained at this stage. In view of these results it is concluded that the D-serine isolated could not have originated in a racemization induced by acid, alkaline or resin treatment of the earthworm extracts.

There remains the final possibility that the D-serine may have arisen as a result of a post-mortem enzymic breakdown of lombricine or D-serine ethanolamine phosphodiester or both. This possibility cannot be dismissed from consideration with the same degree of certainty as can those already discussed, for, although the present experiments lend no support, the fact remains that complete breakdown of lombricine was achieved in Robin's (1954) experiments at pH 8.0 in 3 hr. Robin (1954) assumed that the breakdown observed was enzymic in nature, but no evidence was adduced to support this assumption and in this connexion it is noteworthy that lombricine (and serine ethanolamine phosphodiester) is extremely labile at room temperature in alkaline solution in the presence of traces of Cu²⁺ ion (Beatty & Magrath, 1960).

The presence of D-amino acids has been recognized in some micro-organisms and in the products of mould and bacterial metabolism, but in none of these has a free D-amino acid been found. So far as we are aware the presence of a free D-amino acid has been described only once before in Nature, when D-alanine was detected in insect (*Oncopeltus fasciatus*) haemolymph by Auclair & Patton (1950).

The present results establish beyond reasonable doubt that D-serine is a component part of the free

amino acid pool in the earthworm, but whether this arises from a catabolic reaction involving lombricine (and D-serine ethanolamine phosphodiester), a synthesis of this isomer *de novo*, a racemization of L-serine or some other process, cannot at present be determined. Experiments designed to determine its origin and its mode of incorporation into D-serine ethanolamine phosphodiester are in progress.

SUMMARY

1. D-Serine has been isolated as the racemate from perchloric acid extracts of earthworms.
2. It has been shown that the D-serine isolated is unlikely to have arisen as a result of racemization due to the isolation procedures or of any enzymic breakdown of lombricine or of D-serine ethanolamine phosphodiester.
3. D-Serine is presumed to be a part of the free amino acid pool in the earthworm.
4. Experimental evidence has been adduced to show that, contrary to accepted beliefs, the optical homogeneity of serine is unaffected by exposure to alkaline solvents.

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