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The Isolation of Lombricine and its Possible Biological Precursor

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In connexion with experiments on the chemical and biological synthesis of 2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate (lombricine) it was desirable to have, as a reference compound, adequate amounts of the naturally occurring material. The isolation of lombricine from the earthworm (*Lumbricus terrestris*) has been described by Van Thoai & Robin (1954), but the low yield reported stimulated a search for an improved method. The simplified procedure described in this paper results in much greater yields of analytically pure material. In addition, experiments are described on the detection and characterization of 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate which, it is suggested, is the immediate precursor of lombricine.

MATERIALS AND METHODS

All reagents used were analytical-grade and solvents used for chromatography were purified by fractional distillation. The reference compounds, 2-guanidinoethyl phosphate, 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate (serine ethanolamine phosphodiester, SEP) and lombricine, were synthesized by D. I. Magrath & I. M. Beatty (unpublished work), who kindly provided samples for chromatographic purposes. Those containing serine were synthesized from the L-isomer. All other compounds were commercial

preparations. The reference standards used were chromatographically homogeneous in all solvent systems employed.

The earthworms used were collected in the Canberra area and were of mixed species but were predominantly *Allolobophora caliginosa* and *Octolasion cyaneum*.

The method described by Rosenberg, Ennor & Morrison (1956) for the estimation of arginine was used to determine the concentration of guanidino compounds present in earthworm extracts. These compounds will be referred to as 'guanidine-reacting material'.

Chromatography. All exploratory chromatograms were run in ascending fashion on washed no. 3 Whatman paper, 28 cm. × 28 cm., unless specifically mentioned. Washing was carried out by immersing the ends of the papers in a tank of water and allowing the water to run off for several hours after the front had reached the bottom edges. Two-dimensional chromatograms involving reinforcement with reference compounds were run on pairs of papers cut from one sheet, and care was taken to develop each in the same sequence of solvent systems and in the same direction relative to the original sheet. This was found to produce identical patterns in any one pair of solvent systems.

When chromatography was employed for preparative purposes, solutions of the unknown compounds were applied to the origin as 3–5 mm. wide bands on sheets 60 cm. × 60 cm. The requisite band was located with the aid of markers, cut out and eluted by the method of Reith (1957). This enabled elution from a band approx. 200 cm.² in area in a volume of about 1 ml.

The solvent systems employed, together with the approximate development time, at 23° for ascending chromatograms on paper 28 cm. × 28 cm. were:

I. Water-saturated phenol	13–14 hr.
II. Butan-1-ol–acetic acid–water (5:3:2, by vol.)	8–9 hr.
III. Acetone–acetic acid–water (2:2:1, by vol.)	3–4 hr.
IV. Diisopropyl ether–acetic acid–ethanol–water (4:4:1:2, by vol.)	8–9 hr.
V. Methyl Cellosolve (2-methoxyethanol)–water (9:1, by vol.)	8–9 hr.
VI. Methanol–formic acid–water (8:1:1, by vol.)	4 hr.
VII. Ethanol–formic acid–water (7:1:2, by vol.)	8–9 hr.
VIII. Ethanol– α -ammonium acetate buffer, pH 3.8 (7:3, v/v)	7–8 hr.
IX. Methyl Cellosolve–butan-2-one–acetic acid–water (15:40:6:24, by vol.)	8–9 hr.
X. <i>tert.</i> -Butanol–butan-2-one–water–diethylamine (10:5:10:1, by vol.)	18 hr.
XI. Water-saturated phenol–ethanol–aq. ammonia (sp.gr. 0.88)–water (150:40:10:1, by vol.)	12 hr.

Guanidine derivatives and inorganic phosphate were detected on chromatograms as described by Rosenberg (1959*a* and 1959*b* respectively).

Amino acids and related compounds were detected by dipping the paper in a 0.2% solution of ninhydrin in acetone, followed by heating in the usual manner.

RESULTS

Isolation of lombricine

Live earthworms were frozen in liquid air and stored at –60°. The frozen material (1 kg.) was disintegrated in 200 g. batches in a Waring Blendor with 300 ml. of 0.75*N*-HClO₄. The suspensions were filtered by the aid of suction and the filter pads from each batch re-extracted in the Blendor with 200 ml. of extractant. The combined filtrates amounted to 3 l. Tests carried out on a third extraction revealed the presence of only small amounts of guanidine-reacting material and the residues were discarded. The combined extracts were brought to, and maintained at, 75° for 15 min. to hydrolyse any *N*-phosphorylated guanidine which may have been present. The extract, which contained considerable amounts of inorganic matter and glycogen in colloidal solution, was then cooled to 20° and treated with an equal volume of ethanol. Immediate precipitation occurred and clarification was achieved by passage through a Sharples centrifuge. The residue which contained no guanidine-reacting material was dis-

carded and the clear, straw-coloured supernatant was passed at a rate of 14 ml./min. through a column (70 cm. × 4 cm.) of Zeo-Karb 225 (H⁺ form) which retained all the guanidine-reacting material. The effluent was discarded and the column washed (20 ml./min.) with 1 l. of 0.01 *N*-HCl in 50% ethanol. The washings, which were yellow in colour but which contained no trace of guanidine-reacting material, were discarded.

The material retained on the column was eluted with aq. 2.5*N*-NH₃ (flow-rate 11 ml./min.). The entire guanidine-reacting material was collected in 650 ml. and the major proportion of the NH₃ removed by distillation at 25° under reduced pressure. The solution (pH 10) was then diluted with water to 2 l. and passed through (5 ml./min.) a column (50 cm. × 312 cm.) of Dowex-2 (OH[–] form). The effluent, which contained arginine, but no lombricine, as determined by paper chromatography in solvent system VII, was discarded. The column was then washed with water (1 l.) and the lombricine eluted with *N*-HCl (5 ml./min.) in a total volume of 700 ml. An equal volume of ethanol was added and this solution again passed at 10 ml./min. through a column (40 cm. × 4 cm.) of Zeo-Karb 225 (H⁺ form). The entire amount of guanidine-reacting material was retained and the effluent was discarded. The column was washed with 0.01 *N*-HCl in 50% (v/v) ethanol and eluted with aq. *N*-NH₃ in a total volume of 500 ml. The eluate was distilled under reduced pressure at 25° to remove NH₃ and its volume reduced to 250 ml. The resultant solution was adjusted to pH 6.5 by the addition of 5*N*-HCl and a small amount of suspended material removed by centrifuging. The solution was freeze-dried, the bulk of the solid redissolved in water in a final volume of 25 ml. and the small amount of insoluble material again removed by centrifuging. Ethanol (1 vol.) was added and the resultant cloudy suspension clarified by filtration. Additional ethanol (3 vol.) was then added and crystallization commenced immediately. The mixture was cooled to 3° and stored at this temperature for 3 hr. before filtration. The filtrate was retained and examined for the presence of SEP (see below). The crystals were redissolved in about 15 ml. of water and recrystallized by the cautious addition of ethanol (3 vol.). The crystals (white needles) were recovered by filtration, washed with ethanol and acetone and dried *in vacuo* over P₂O₅. Yield 650 mg., m.p. 224° (decomp.). [Found (for lombricine): C, 26.5; H, 5.71; N, 20.64; P, 11.6. C₆H₁₅O₅N₄P requires C, 26.6; H, 5.5; N, 20.8; P, 11.5%.]

The product was checked chromatographically in solvent systems I and VII. It was free from other guanidines but contained barely detectable traces of ninhydrin-positive material which ran ahead of lombricine.

Detection of the possible lombricine precursor

The proposed structure of lombricine (Van Thoi & Robin, 1954) suggests that the corresponding amine, SEP, may be the biological precursor of lombricine, which could be formed from the former compound by a transamidation reaction involving arginine. Such a suggestion has already been made (Ennor & Morrison, 1958). Because of the similar nature of the acidic and basic groups of the two compounds, SEP was expected to behave in a manner similar to that of lombricine in the isolation procedure and to be present in the mother liquor from which the initial yield of lombricine was obtained. This liquor was therefore examined for the presence of SEP.

The mother liquor was treated with acetone (4 vol.) and allowed to stand overnight at 3°. The cloudy suspension was centrifuged and as the supernatant contained comparatively little ninhydrin-positive material it was discarded. The gummy precipitate was dissolved in 50 ml. of water, and the solution brought to boiling point, treated with charcoal and filtered. The filtrate was freeze-dried, the residue dissolved in 2 ml. of water and a small amount of flocculent precipitate removed by centrifuging. A sample of this solution was analysed by two-dimensional chromatography in solvent systems V and I. On treatment with ninhydrin, at least 16 spots were detected and one (R_F 0 in solvent V and 0.34 in solvent I) behaved similarly to a synthetic sample of SEP. This spot was also reinforced when SEP was added before development.

Samples of the solution were then chromatographed in a number of solvents, and it was found that in solvent system VII the major proportion of the ninhydrin-positive material ran with R_F 0.5. Since in this system authentic SEP had R_F 0.2, it was used to achieve a partial purification of the material contained in the mother liquor by preparative chromatography (see Methods).

A sample of the partially purified material was then checked chromatographically (solvent system I). Only three ninhydrin-positive spots were

detected. The fastest-running component (R_F 0.55), which also gave a positive 1-naphthol-butane-2:3-dione reaction, was identified as lombricine. The intermediate component (R_F 0.34) corresponded in position to SEP, but the third component (R_F 0.22) was not identified. In view of this separation the remainder of the material was purified by chromatography in solvent system I and obtained in a final volume of 0.4 ml. This solution was then examined chromatographically in a number of solvent systems, and in all showed the presence of only one ninhydrin-reacting component with an R_F identical with that of an authentic sample of SEP (Table 1).

A sample of the solution was subjected to two-dimensional chromatography in solvent systems I and V. Similar chromatograms were developed with samples of authentic SEP and a mixture of the latter and the isolated material. In each experiment only one ninhydrin-reacting spot was obtained and its position was precisely similar in all chromatograms. The intensity of the colour reaction with the mixture was considerably greater than with either sample alone.

Hydrolysis. Samples of the eluate (0.04 ml.) were heated in sealed tubes at 105° for 30 min., 2 hr. and 4 hr. with 0.06 ml. of 10N-HCl. The hydrolysates were evaporated to dryness on watch-glasses heated on a steam bath. The residues were exposed to NH_3 to neutralize traces of residual HCl and dissolved in water (0.05 ml.). Samples of these hydrolysates were spotted on papers, together with a sample of authentic SEP which had been similarly treated and markers of serine, 2-aminoethyl serine phosphate, 2-aminoethyl phosphate and orthophosphate. The chromatogram was developed in solvent system II and then treated with ninhydrin. The ninhydrin-reacting areas were outlined in pencil and the chromatogram was treated with the appropriate reagents to render visible the inorganic phosphate and phosphorus-containing compounds. The chromatogram (Fig. 1) indicates that, in addition to inorganic phosphate, three ninhydrin-reacting compounds were released by acid hydrolysis. Two of these compounds corre-

Table 1. R_F values of 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate and related compounds

Solvent system (see Methods)	R_F						
	Serine	Serine phosphate	Ethanolamine	2-Aminoethyl phosphate	Lombricine	SEP	Isolated compound
I	0.43	0.12	0.53-0.69	0.46	0.55	0.34	0.34
II	0.44	0.23	0.53	0.29	0.23	0.15	0.15
III	0.62	0.44	0.84	0.55	0.31	0.22	0.22
IV	0.49	0.31	0.62	0.41	0.26	0.23	0.22
V	0.17	0.04	0.44	0.06	0	0	0
VI	0.57	0.42	0.68	0.40	0.18	0.13	0.12
VII	0.64	0.42	0.75	0.46	0.28	0.20	0.19
VIII	0.46	0.22	0.70	0.44	0.23	0.19	0.20

sponded in R_f to the serine and ethanolamine markers, but the fastest-running compound was unidentified. A similar pattern was revealed when a chromatogram was developed in solvent system X (Fig. 2).

Although the identity of the isolated material seems to be established, it was nevertheless thought desirable to gain more information about the fastest-running ninhydrin-reacting compound. Consequently samples of the serine, serine ethanolamine phosphodiester and 2-aminoethyl phosphate markers were heated with 6N-HCl under conditions similar to those used for the hydrolysis of SEP. These solutions were evaporated to dryness and taken up in a small volume of water as before. Samples of these solutions were applied to papers, together with the appropriate markers and hydrolysates of SEP and the isolated material. The chromatograms were developed in solvent systems II, VIII, X, XI and treated with ninhydrin and phosphate reagents. The results with the first

Fig. 1. Products of acid hydrolyses of authentic 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate and isolated material. Outlined areas, ninhydrin-reacting material; shaded areas, phosphorus-containing material. Under the conditions employed SEP was not hydrolysed and thus the phosphorus moiety was not demonstrable. 1, Serine phosphate; 2, 2-aminoethyl phosphate; 3, serine; 4, ethanolamine; 5, SEP; 6, 8 and 10, 30 min., 2 hr. and 4 hr. hydrolysates of authentic SEP respectively; 7, 9 and 11, 30 min., 2 hr. and 4 hr. hydrolysates of isolated material; 12, inorganic phosphate. Solvent system II.

Fig. 2. Products of acid hydrolyses of authentic 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate and isolated material. Outlined and shaded areas as in Fig. 1. 1, Serine phosphate; 2, 2-aminoethyl phosphate; 3, serine; 4, ethanolamine; 5 and 6, authentic SEP and isolated material respectively; 7 and 8, as for 5 and 6 but after 4 hr. acid hydrolysis; 9, inorganic phosphate. Solvent system X.

Fig. 3. Products of acid hydrolyses of 2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate and related compounds. Outlined and shaded areas as in Fig. 1. 1, Serine phosphate; 2, serine phosphate hydrolysed; 3, serine; 4, mixture of serine and ethanolamine, acid-treated (see text); 5, ethanolamine; 6, 2-aminoethyl phosphate hydrolysed; 7, 2-aminoethyl phosphate; 8, SEP; 9 and 10, hydrolysates of authentic SEP and isolated material respectively; 11, inorganic phosphate. Solvent system VIII.

three solvent systems were similar; that for solvent system VIII is reproduced in Fig. 3. Serine and ethanolamine were unchanged after acid treatment and serine phosphate yielded the two expected products, serine and inorganic phosphate. However, with 2-aminoethyl phosphate the products were inorganic phosphate, ethanolamine and another ninhydrin-reacting compound which ran ahead of ethanolamine and corresponded in position to the fastest-moving component arising from the hydrolysis of SEP and the isolated material.

When developed in solvent system XI the chromatogram (Fig. 4) confirmed the above results, but in this system the fast-running component, produced as a result of the hydrolysis of 2-aminoethyl phosphate, SEP and the isolated material, was not detected.

On theoretical grounds it seems likely that chloroethylamine could be formed according to the following reactions:

Fig. 4. Acid hydrolysates of 2-aminoethyl 2-amino-2-carboxyethylhydrogen phosphate and 2-aminoethyl phosphate. Outlined and shaded areas as in Fig. 1. 1, inorganic phosphate; 2, serine phosphate; 3, 2-aminoethyl phosphate; 4, serine; 5, ethanolamine; 6, 2-aminoethyl phosphate hydrolysed; 7 and 8, authentic SEP and isolated material respectively; 9 and 10, as for 7 and 8 respectively, but after 4 hr. acid hydrolysis. Solvent system XI.

A sample of chloroethylamine was prepared and its chromatographic behaviour was identical with that of the fast-running component when examined in three solvent systems.

Guanylation. A sample (0.04 ml.) of a solution containing about 1 mg. of SEP/ml. was mixed with 0.02 ml. of a solution of *O*-methylisourea (2 mg./ml.). Sufficient 2*N*-NaOH was added to render the mixture just alkaline to thymolphthalein and the mixture was allowed to react for 24 hr. at room temperature. A sample (10 μ l.) of the neutralized reaction mixture was chromatographed in solvent systems I and IX and then sprayed with the 1-naphthol-butanedione reagent or treated with ninhydrin. Apart from *O*-methylisourea two 1-naphthol-butanedione-positive spots were detected. One of these was present in just detectable amounts and was not identified, but the other, which constituted the bulk of the guanidine-reacting material, was identified as lombricine by comparison with the natural material. Only two ninhydrin-positive spots were detected: one corresponded to SEP and the other to lombricine.

DISCUSSION

The present identification of SEP in extracts of earthworm tissue depends on several lines of evidence: the similarity in behaviour of the material with SEP when subjected to two-dimensional chromatography and when unidimensionally chromatographed in a number of solvent systems, the chromatographic similarity of the products of acid hydrolysis with those of the synthetic material, and finally the fact that guanylation of the isolated compound yielded a product similar in behaviour to natural lombricine. Beatty & Magrath (1959) have established that lombricine may be synthesized in high yield by guanylation of synthetic SEP with *O*-methylisourea. Theoretically guanylation of SEP could yield two products in the absence of protective groups on the α -NH₂ but, as Beatty & Magrath (1959) found, one product is formed almost exclusively. It would thus seem that the α -NH₂ group is unreactive under the conditions employed.

The appearance of serine and ethanolamine as a result of hydrolysis of SEP was to be expected and confirms the findings of Roberts & Lowe (1954). These latter findings were, however, not confirmed in that, in the present experiments, (a) it did not prove possible to detect the phosphate esters of ethanolamine or serine and (b) a third ninhydrin-reacting spot with an *R_F* greater than that of ethanolamine was detected. From the preliminary results obtained it seems probable that this spot is β -chloroethylamine.

The present experiments give no indication whether SEP isolated from earthworms contains