The Formation *in vivo* of Lombricine in the Earthworm (*Megascolides cameroni*)

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Lombricine (2-guanidinoethyl 2-amino-2-carboxyethyl hydrogen phosphate, I) was first isolated from earthworms (*Lumbricus terrestris*) by Van Thoi & Robin (1954), who held the view that *N*-phosphoryl-lombricine was the phosphagen of that species. Subsequently it was suggested that the biological precursor of lombricine was serine ethanolamine phosphodiester (2-aminoethyl 2-amino-2-carboxyethyl hydrogen phosphate, II) and that lombricine was probably formed by the transfer of the amidino group of arginine (III) to serine ethanolamine phosphodiester (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959). This suggestion has received support from: (a) the discovery of serine ethanolamine phosphodiester in earthworms (Rosenberg & Ennor, 1959; Ennor, Rosenberg, Rossiter, Beatty & Gaffney, 1960); (b) the finding that the serine moiety of serine ethanolamine phosphodiester (Ennor *et al.* 1960), like that of lombricine (Beatty, Magrath & Ennor, 1959), has the unusual *D*-configuration, and (c) the demonstra-

tion that after the administration of inorganic ^{32}P to earthworms, the specific radioactivity of serine ethanolamine phosphodiester was much greater than that of lombricine (Gaffney, Rossiter, Rosenberg & Ennor, 1960).

This paper describes experiments in which [1:2- ^{14}C]₂ethanolamine, DL-[3- ^{14}C]serine, L-[^3H]serine and D-[^3H]serine were administered to earthworms. It also provides additional evidence that serine ethanolamine phosphodiester is the precursor of lombricine and that the amidino group of the latter is derived from the amidino group of arginine, probably by a transfer of this group to serine ethanolamine phosphodiester with the production of ornithine (IV), according to the transaminase reaction in Scheme 1.

A preliminary account of these experiments has already appeared (Rossiter, Gaffney, Rosenberg & Ennor, 1960).

MATERIALS AND METHODS

Reagents. All reagents were analytical grade, and the ethanol and butanol used in chromatographic solvents were redistilled before use. Serine ethanolamine phosphodiester

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Scheme 1

(SEP) was synthesized by Beatty & Magrath (1959) after the method of Jones & Lipkin (1956). Lombricine was prepared from earthworms as described by Ennor *et al.* (1960).

Radioactive precursors. [1:2- $^{14}\text{C}_2$]Ethanolamine and DL-[3- ^{14}C]serine were obtained from The Radiochemical Centre, Amersham, Bucks, and were used at specific radioactivities of 5 and 2.7 $\mu\text{C}/\mu\text{mole}$ respectively.

L-Serine and D-serine, obtained from commercial sources, were tritiated according to the process of Wilzbach (1957) by The Radiochemical Centre. In order to remove possible decomposition products and exchangeable tritium the tritiated material (100 mg. in each instance) was dissolved in 20 ml. of water and applied to a column (1 cm. $\times$ 10 cm.) of Amberlite CG-120 (mesh 100–200, H^+ form) ion-exchange resin. The column was eluted with a large volume of water, which was collected in 50 ml. fractions. Those fractions containing serine were reappplied to a Zeo-Karb 225 (H^+ form) column (2 cm. $\times$ 10 cm.) and the adsorbed material was eluted with aq. 2.5N- NH_3 solution. The eluate was dried under reduced pressure at a temperature of 40°. Sufficient water and non-radioactive L- or D-serine were added to make a solution with a final concentration of 200 $\mu\text{moles}/\text{ml}$. and a specific radioactivity of 13.5 $\mu\text{C}/\mu\text{mole}$.

L-[amidino- ^{14}C]Arginine was prepared from Na^{14}CN (1 mC) and L-ornithine monohydrochloride as described by Stetten & Bloom (1956). The final product had a specific radioactivity of 0.042 $\mu\text{C}/\mu\text{mole}$.

Animals. Earthworms (*Megascolides cameroni*) of average weight 8 g. were collected in the Australian Capital Territory. They were kept at 10° in cardboard cartons filled with moist earth, and under these conditions were maintained in good condition for many months.

Experimental procedure. The appropriate radioactive precursor (in the amount and with the specific radioactivity listed in the tables) was given to the earthworm from a microsyringe through a fine polythene tube inserted into the anterior end of the gastrointestinal tract for a distance of at least 3 cm. The worm was maintained as described above for periods varying from 24 hr. to 9 days, after which time it was killed and the whole of the gastrointestinal tract and appendages were dissected free from the muscular body wall. These two tissues were treated separately, and are referred to as 'gut' and 'muscle' respectively.

The tissue samples were rapidly weighed and homogenized with 2 vol. of 1.5N-perchloric acid in the micro-cup of a Nalco homogenizer (MSE, Birmingham). After centrifuging, the supernatant fluid was neutralized with 2.5N-KOH and the precipitated KClO_4 was removed by further centrifuging. Ethanol (1 vol.) was added to the supernatant fluid and the mixture was kept at 3° for at least 1 hr. to ensure complete precipitation of glycogen, which was then removed by centrifuging. The supernatant fluid was applied to a column (1 cm. $\times$ 6 cm.) of Zeo-Karb 225 ion-exchange resin (H^+ form), at a rate of 1 ml./min. Water (approx. 20 ml.) was passed through the column until the pH of the effluent had risen to 5, and the washings and effluent were discarded. The material retained by the resin was then eluted with aq. 2.5N- NH_3 soln. at a rate of 3 ml./min. The eluate (approx. 50 ml.) was dried under reduced pressure at 40° and the residue dissolved in a small volume of water (100 μl . for gut and 300 μl . for muscle). These solutions were used in the chromatographic separation of lombricine, SEP and amino acids, and are referred to in the following section as gut or muscle extracts.

In order to evaluate the extent of re-ingestion of excreted radioactive material given, one worm was injected with a quantity of [^{14}C]serine equal to that given to the experimental animals, and was placed in a box filled with moist earth (400 g.). Another worm, which received no injection, was placed in the same box, and the two worms were left in the earth for 8 days. They were then both killed and each worm was treated as described above. The earth from the box was extracted with perchloric acid and the extract was desalted and reduced in volume. The total ^{14}C present in both worms, and in the soil, was assessed. Of the total radioactivity present, the injected worm was found to contain 86.0%, the earth 13.6% and the control worm 0.5%. Complications through re-ingestion of excreted radioactive material were therefore not considered of importance in the present experiments.

Chromatography. Whatman no. 3 filter paper (56 cm. $\times$ 56 cm.) washed with water was used throughout. The papers were handled with plastic forceps to avoid accidental contamination with ninhydrin-reacting substances. For the separation of SEP and lombricine the entire sample of the gut extract or 100 μl . of the muscle extract was applied to the paper between marker spots containing samples of authentic SEP and lombricine. Chromatograms (descending) were run in ethanol-formic acid-water (70:10:20, by vol.) and dried in a current of air at 50°. Lombricine and SEP were located by reference to the marker spots, which were identified by spraying with a 0.2% solution of indane-trione hydrate (ninhydrin) in acetone and heating at 70° for 10 min. In this way it was possible to separate SEP (R_f approx. 0.2) and lombricine (R_f approx. 0.3) from all other ninhydrin-reacting material ($R_f > 0.4$), although in some experiments SEP and lombricine were not completely separated from each other. A complete separation was achieved by applying the eluted samples of SEP or lombricine to a second sheet of filter paper and rechromatographing in phenol-water (4:1, w/v). The SEP (R_f approx. 0.3) and lombricine (R_f approx. 0.55) were identified and eluted as before.

For the separation of free amino acids the entire sample of the gut extract or 100 μl . of the muscle extract was applied to the corner of a sheet of filter paper for two-dimensional chromatography. The first solvent was either ethanol-formic acid-water, as described above, or butanol-water-acetic acid (50:30:20, by vol.). The second solvent was phenol-water (4:1, w/v). In experiments with ^{14}C -labelled precursors the free amino acids, and in some instances SEP and lombricine, were located radioautographically by applying the chromatograms to sheets of Ilford Industrial X-ray film for 2 weeks. Areas on the chromatogram corresponding to darkened areas on the radioautograph were cut out and the material was eluted with water. An alternative procedure for locating the amino acids, which was always used with the ^3H -labelled precursors, was the spraying of the chromatogram with a dilute (0.05%, w/v) solution of ninhydrin in acetone, followed by heating to 70° for 10 min. Ninhydrin-positive areas on the chromatogram corresponding to the required amino acids were cut out and the material was eluted. This method effected a considerable saving in time but the colour of the eluates necessitated the use of an internal standard for the scintillation counting.

The amino acids were identified on the chromatograms by the use of reinforcing markers of authentic compounds, or, in cases of doubt, by rechromatographing the material with

appropriate markers in other standard solvents commonly used for the separation of amino acids.

Counting. A measured portion of the eluted sample of lombricine, SEP or amino acid was pipetted into a 25 ml. glass vial and the water evaporated at a temperature not above 50°. The residue was dissolved in 200 μ l. of 0.5M-*p*-N-(diisobutylresoxyethoxyethyl)-*NN*-dimethyl-*N*-benzylammonium hydroxide (hydroxide of Hyamine 10-X, Rohm and Haas Co., Philadelphia, Pa., U.S.A.) in dry methanol as described by Passmann, Radin & Cooper (1956) and Vaughan, Steinberg & Logan (1957). To this was added 10 ml. of solvent-scintillator containing 4 g. of 2:5-diphenyloxazole and 100 mg. of 1:4-di-(2:5'-phenyloxazolyl)-benzene/l. of toluene. The radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer model 314 (Packard Instrument Co., La Grange, Ill., U.S.A.) with a 10–50 v window and high-voltage tap 4 for ^{14}C -labelled precursors and tap 8 for ^3H -labelled precursors. When the materials were located in the chromatogram with the ninhydrin-detection spray, an internal standard of either [^{14}C]serine or [^3H]serine was used to correct for quenching.

Quantitative determination of serine ethanolamine phosphodiester, lombricine and amino acids. The amount of SEP, lombricine or amino acid in the material eluted from the filter paper was determined by the principles of the method of Connell, Dixon & Hanes (1955), in which NH_3 , the presence of which greatly reduces the reliability of the procedure, is removed by drying the material in the presence of an alkaline buffer. The ninhydrin colour was then developed under the conditions described by Moore & Stein (1954). Any NH_3 present in the water used for the preparation of all reagents was removed by passing the water through a large column of Zeo-Karb 225 ion-exchange resin (H^+ form) immediately before use.

A measured portion of the eluted material was pipetted into a test tube containing 100 μ l. of borate buffer in methanol prepared as described by Connell *et al.* (1955) and the tube left overnight under reduced pressure in a desiccator containing conc. H_2SO_4 . To the tube was then added 0.5 ml. of 30% (v/v) acetic acid to give a final pH of 5, which was found optimum for the development of the ninhydrin colour. The amount of ninhydrin-reacting material in the sample was determined as described by Moore & Stein (1954), authentic samples of SEP, lombricine or serine being used as reference standards.

Hydrolysis of lombricine. In many of the experiments the eluted lombricine was separated into its constituent serine and guanidinoethanol moieties by hydrolysis in a sealed Pyrex tube in 6N-HCl at 110° for 24 hr. After removal of the HCl under reduced pressure in a desiccator containing KOH, the residue was dissolved in 100 μ l. of water and applied to a sheet of filter paper between marker spots containing either (a) serine and lombricine (detected with ninhydrin), or (b) the guanidine derivatives, lombricine, guanidinoethanol and guanidinoethyl phosphate (detected with diacetyl reagent as described by Rosenberg, 1959). The chromatogram was run in phenol-water (4:1, w/v) and dried in the usual way. Under the conditions of the hydrolysis, lombricine was converted into serine (R_F approx. 0.35) and a mixture of guanidinoethanol (R_F approx. 0.8) and guanidinoethyl phosphate (R_F approx. 0.7). Serine and the material on the combined guanidinoethanol and guanidinoethyl phosphate area were eluted and counted as described above.

RESULTS

[1:2- $^{14}\text{C}_2$]ethanolamine. After the administration of [1:2- $^{14}\text{C}_2$]ethanolamine the specific radioactivity of SEP was considerably greater than that of lombricine when these compounds were isolated from an extract of whole worm (Table 1, Expt. 1). A similar relationship was found to exist between the specific radioactivities of these compounds whether isolated from the muscle or from the gut (Table 1, Expt. 2). In addition, the specific radioactivity of each compound was higher in the gut than in the muscle, where the bulk of the radioactivity of lombricine was found to reside in the guanidinoethanol portion of the molecule.

Labelled ethanolamine was detected in the muscle of a worm killed 4 days after injection (Table 2, Expt. 2) and a considerable amount of radioactivity was also found in glutamic acid and glutamine. The

Table 1. *Incorporation of radioactivity from [1:2- $^{14}\text{C}_2$]ethanolamine into serine ethanolamine phosphodiester and lombricine of the earthworm*

The amount administered was 3.7 μ moles of [1:2- $^{14}\text{C}_2$]ethanolamine, containing 7.5×10^6 counts/min./g. body wt. Figures in parentheses represent the percentage of the counts in the relevant portion of the lombricine molecule.

	Specific radioactivity (counts/min./ μ mole)		
	Expt. 1 (3 days) Whole worm	Expt. 2 (4 days)	
		Muscle	Gut
SEP	231 000	67 900	126 000
Lombricine (total)	2 650	1 080	2 580
Lombricine (serine moiety)	—	60 (6%)	—
Lombricine (guanidinoethanol moiety)	—	1 020 (94%)	—

Table 2. *Incorporation of radioactivity from [1:2- $^{14}\text{C}_2$]ethanolamine into the free amino acids of the earthworm*

The amount administered was 3.7 μ moles of [1:2- $^{14}\text{C}_2$]ethanolamine, containing 7.5×10^6 counts/min./g. body wt.

	Specific radioactivity (counts/min./ μ mole)	
	Expt. 1 (3 days) Whole worm	Expt. 2 (4 days) Muscle
Glutamic acid	156 000	38 900
Glutamine	72 000	48 500
Aspartic acid	21 800	7 400
Alanine	18 900	4 100
Glycine	5 900	3 400
Threonine	4 800	6 300
Serine	3 800	3 600
Ethanolamine	—	409 000

Table 3. *Incorporation of radioactivity from DL-[3-¹⁴C]serine into serine ethanolamine phosphodiester and lombricine of the muscle and gut of the earthworm*

The amount administered was 2 μ moles of DL-[3-¹⁴C]serine (10^7 counts/min.)/g. body wt. Figures in parentheses represent the percentage of the counts in the relevant portion of the lombricine molecule.

	Specific radioactivity (counts/min./ μ mole)		
	Expt. 1 (2 days)	Expt. 2 (8 days)	Expt. 3 (9 days)
Muscle			
SEP	31 500	41 700	70 200
Lombricine (total)	900	6 270	15 700
Lombricine (serine moiety)	680	4 450	9 340
	(76 %)	(71 %)	(60 %)
Lombricine (guanidinoethanol moiety)	225	1 820	6 360
	(24 %)	(29 %)	(40 %)
Gut			
SEP	100 000	222 000	422 000
Lombricine	1 730	9 900	27 500
	Specific radioactivity of SEP/specific radioactivity of lombricine		
	Expt. 1 (2 days)	Expt. 2 (8 days)	Expt. 3 (9 days)
Muscle	35	7	5
Gut	58	22	15

Table 4. *Incorporation of radioactivity from DL-[3-¹⁴C]serine into the free amino acids of the muscle of the earthworm*

The amount administered was 2 μ moles of DL-[3-¹⁴C]serine, containing 10^7 counts/min./g. body wt.

	Specific radioactivity (counts/min./ μ mole)	
	Expt. 1 (8 days)	Expt. 2 (9 days)
Glutamic acid	71 100	173 000
Glutamine	62 700	243 000
Aspartic acid	27 800	63 900
Alanine	32 800	57 400
Glycine	10 500	28 700
Threonine	6 700	28 400
Serine	12 800	57 400

other amino acids investigated showed a much lower level of incorporation, which, with serine, is indicative of little tendency of ethanolamine to act as a precursor of this amino acid. A similar conclusion may be drawn from the fact that only a small proportion of the total radioactivity of muscle lombricine could be found in the serine part of the molecule (Table 1, Expt. 2).

DL-[3-¹⁴C]Serine. After the administration of DL-[3-¹⁴C]serine there was a progressive increase in the incorporation of radioactivity into SEP and lombricine in both the muscle and gut (Table 3). The specific radioactivity of SEP at each time interval was considerably greater than that of lombricine for each tissue and the specific radioactivities of both SEP and lombricine in gut were greater than those

of muscle. With time the specific radioactivity of SEP more nearly approached that of lombricine for both tissues, as evidenced by the progressive fall in the SEP/lombricine ratios.

The greater part of the radioactivity of muscle lombricine was in the serine portion of the molecule but, even after only 2 days, 24 % of the radioactivity was in the guanidinoethanol portion, presumably as a result of decarboxylation of serine to form ethanolamine.

The specific radioactivities of the free amino acids of muscle observed after the administration of [¹⁴C]serine bear a relation to each other similar to that found after the administration of [¹⁴C]ethanolamine (Table 2). Thus there was considerable incorporation into glutamic acid and glutamine, but less incorporation into aspartic acid, alanine, glycine and threonine. However, whereas the specific radioactivities of the well-labelled glutamic acid and glutamine were less than that of muscle SEP when [¹⁴C]ethanolamine was the source of the radioactivity (cf. Tables 1 and 2), this was not so when the radioactivity was derived from [¹⁴C]serine (cf. Tables 3 and 4).

The results of a typical experiment with [¹⁴C]serine are illustrated in Figs. 1 and 2. In both figures the lines drawn around the ninhydrin-stained areas of SEP, lombricine and free amino acids on the chromatograms (A) were superimposed on the radioautographs (B). Muscle (Fig. 1) contained considerably more lombricine than SEP but, because of the lower specific radioactivity of lombricine, the darkening of the X-ray

film in the lombricine area was much less. In gut (Fig. 2) the amounts of SEP and lombricine were similar and approximately equal to the amount of SEP in muscle. However, the darkening of X-ray film caused by SEP was much greater for gut (Fig. 2) than for muscle (Fig. 1), because of the greater specific radioactivity of gut SEP. The labelling of lombricine in gut is not evident in the reproduction of the radioautograph shown in Fig. 2, but in the original X-ray film a definite darkening could be seen in this area.

The intensity of the spots corresponding to the free amino acids in both the chromatograms and radioautographs was as anticipated, having regard to the concentrations and specific radioactivities of those substances.

L-[^3H]Serine and *D*-[^3H]serine. 2 and 8 days after the administration of either *L*-[^3H]serine or *D*-[^3H]serine, radioactivity was incorporated into both SEP and lombricine in the muscle and gut (Table 5). As in the previous experiments, the specific radioactivities of both substances were greater in gut than in muscle, and for both tissues the specific radioactivity of SEP was greater than that of

Fig. 1. (A) Chromatogram of extract of earthworm muscle prepared 8 days after the administration of 2 μmoles of DL-[^{14}C]serine containing 10^7 counts/min./g. body wt. SEP, serine ethanolamine phosphodiester; L, lombricine; (Glu-NH₂), glutamine; Ala, alanine; Glu, glutamic acid; Ser, serine; Asp, aspartic acid. (B) Radioautograph of chromatogram shown in (A). The lines encircling the ninhydrin-stained areas on the chromatogram were superimposed on the radioautograph.

Fig. 2. (A) Chromatogram of extract of earthworm gut prepared 8 days after the administration of 2 μmoles of DL-[^{14}C]serine containing 10^7 counts/min./g. body wt. Abbreviations are as given in Fig. 1. (B) Radioautograph of chromatogram shown in (A). The lines encircling the ninhydrin-stained areas on the chromatogram were superimposed on the radioautograph.

Table 5. *Incorporation of radioactivity from either L-[³H]serine or D-[³H]serine into the serine ethanolamine phosphodiester and lombricine of the muscle and gut of the earthworm*

The amount administered was 10 μ moles of L-[³H]serine or D-[³H]serine, containing 4×10^7 counts/min./g. body wt. Figures in parentheses represent the percentage of the counts in the relevant portion of the lombricine molecule.

	Specific radioactivity (counts/min./ μ mole)									
	2 days					8 days				
	L-[³ H]Serine		D-[³ H]Serine			L-[³ H]Serine		D-[³ H]Serine		
Muscle	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6	Expt. 7	Expt. 8	Expt. 9	Expt. 10 Expt. 11
SEP	41 300	35 200	9 190	8 800	24 900	13 400	30 900	52 600	70 000	33 200 23 900
Lombricine (total)	1 670	1 270	1 300	220	550	1 560	1 360	6 500	5 260	2 910 2 600
Lombricine (serine moiety)	575	430	300	140	220	680	460	—	2 750	1 140 —
	(34 %)	(33 %)	(23 %)	(64 %)	(40 %)	(44 %)	(34 %)	—	(52 %)	(39 %)
Lombricine (guanidinoethanol moiety)	1 095	840	1 000	80	330	880	900	—	2 510	1 770 —
	(66 %)	(67 %)	(77 %)	(36 %)	(60 %)	(56 %)	(66 %)	—	(48 %)	(61 %)
Gut	101 000	70 000	181 000	28 600	199 000	80 600	30 300	64 000	191 000	122 000 22 100
SEP	5 470	2 910	9 230	860	8 660	—	—	15 000	7 670	14 700 8 000
Lombricine (total)	920	640	2 310	315	1 660	—	—	—	3 900	2 800 —
Lombricine (serine moiety)	(17 %)	(32 %)	(25 %)	(37 %)	(19 %)	—	—	—	(51 %)	(24 %)
Lombricine (guanidinoethanol moiety)	4 550	2 270	6 920	545	7 000	—	—	—	3 770	11 900 (76 %)
	(83 %)	(78 %)	(75 %)	(63 %)	(81 %)	—	—	—	(49 %)	—

Table 6. *Incorporation of radioactivity from either L-[³H]serine or D-[³H]serine into the free amino acids of the muscle of the earthworm*

The amount administered was 10 μ moles of L-[³H]serine or D-[³H]serine, containing 4×10^7 counts/min./g. body wt. The worms were killed after 2 days.

	Specific radioactivity (counts/min./ μ mole)	
	Expt. 1.	Expt. 2.
	L-[³ H]Serine	D-[³ H]Serine
Glutamic acid	35 900	44 700
Glutamine	53 400	27 000
Aspartic acid	27 900	33 100
Alanine	22 400	15 900
Glycine	42 600	48 600
Serine	262 000	267 000

lombricine. With [³H]serine more of the radioactivity of lombricine was recovered from the guanidinoethanol portion of the molecule than in the experiments with DL-[³⁻¹⁴C]serine. It is probable that the [³H]serine is degraded into a C₂-unit containing C-1 and C-2 of serine, and that it is this C₂-unit that is incorporated into the ethanolamine portion of SEP, and eventually into lombricine. Some evidence for this lies in the observation that 2 days after the administration of [³H]serine the specific radioactivity of the free glycine of muscle was of the same order as that of glutamic acid and glutamine for both isomers (Table 6). This is in sharp contrast with the finding for DL-[³⁻¹⁴C]serine, where the specific radioactivity of the free glycine of muscle was much less than that of either glutamic acid or glutamine (Table 4).

The figures for the specific radioactivities of the other free amino acids of muscle 2 days after the administration of [³H]serine show no consistent differences between the labelling from L-[³H]serine and that from D-[³H]serine (Table 6).

L-[amidino-¹⁴C]Arginine. Experiments were carried out to determine the origin of the amidine-C atom of lombricine. After the injection of L-[amidino-¹⁴C]arginine, considerable radioactivity was recovered from the lombricine of both muscle and gut. Almost all of the radioactivity of the muscle lombricine was in the guanidinoethanol portion of the molecule (Table 7). Negligible radioactivity was found in SEP of both muscle and gut, and in the serine portion of the muscle lombricine; ornithine and glutamic acid also contained negligible radioactivity.

DISCUSSION

On the basis of the structure of lombricine it would be anticipated that administered serine and ethanolamine would both be incorporated, bio-

Table 7. *Incorporation of radioactivity from L-[amidino-¹⁴C]arginine into the serine ethanolamine phosphodiester, lombricine and certain free amino acids of the earthworm*

The amount administered was 20 μ moles of L-[amidino-¹⁴C]arginine, containing 10⁶ counts/min./g. body wt. The worms were killed after 2 days. Figures in parentheses represent the percentage of the counts in the guanidinoethanol portion of the lombricine molecule. 'Negligible' indicates that the net counting rate was less than one-half of the background counting rate.

	Expt. 1. Counts/min./g. of worm		Expt. 2.* Specific radioactivity (counts/min./ μ mole)	
	Muscle	Gut	Muscle	Gut
SEP	Negligible	Negligible	Negligible	Negligible
Lombricine (total)	540	120	329	382
Lombricine (serine moiety)	Negligible	—	Negligible	Negligible
Lombricine (guanidinoethanol moiety)	525 (97%)	—	318 (97%)	348 (91%)
Ornithine	Negligible	—	Negligible	—
Glutamic acid	—	—	Negligible	—
Arginine	12 400	—	38 700	—

* Twice the amount of [¹⁴C]arginine was given.

synthetically, into the molecule. Further, because of the well-known inter-relationship existing between serine and ethanolamine, it would be expected that some of the radioactivity in [3-¹⁴C]-serine would appear in the guanidinoethanol portion of the molecule. The results of the present experiments confirm these expectations.

In all experiments carried out with [¹⁴C]- and [³H]-serine and ethanolamine, the specific radioactivity of SEP was considerably greater than that of lombricine for both gut and muscle tissue. This finding is similar to that obtained with inorganic ³²P and reported elsewhere (Gaffney *et al.* 1960). It is consistent with the view expressed (Ennor & Morrison, 1958; Rosenberg & Ennor, 1959) that SEP is a precursor of lombricine and not a degradation product of that substance. The report (Ennor *et al.* 1960) that the serine moiety of SEP, like that of lombricine, has the unusual D-configuration, is also consistent with this view. In addition, the present experiments make it clear that SEP is built up from small molecules, a finding at variance with the conclusion of Ayengar & Roberts (1957) for the SEP of turtles. However, in contrast with earthworm SEP, the SEP of turtles contains serine with the L-configuration (Roberts & Lowe, 1954).

These experiments confirm the previously reported slow turnover rate of lombricine in the earthworm (Rossiter *et al.* 1960; Gaffney *et al.* 1960). In each instance the amount of radioactivity administered was adjusted to the body weight of the worm. The results clearly show that with all of the precursors studied considerable radioactivity was absorbed from the gut, although some activity (about 14% in the control experiment) was lost with the large quantity of earth excreted by the worm during the course of the experiment. Further,

it was noted that some worms visibly ejected a portion of the administered material through the outer surface of the skin. The variable nature of the amount of radioactive precursors absorbed, or retained when once absorbed, thus did not permit of the plotting of smooth 'precursor-product' curves showing changes in specific radioactivity with time.

Since both L- and D-[³H]serine are incorporated into SEP and lombricine of earthworms (as also is DL-[¹⁴C]serine), it might be anticipated that the D-enantiomorph is incorporated directly and that the L-isomer is changed into the D-form before or at the moment of incorporation into SEP. However, the present experiments provide no data on this point, or on the mechanism of the conversion of L-serine into D-serine.

In the experiments with L-[amidino-¹⁴C]arginine there was negligible radioactivity in SEP and considerable radioactivity in lombricine. Degradation experiments have shown that this radioactivity is restricted to the guanidinoethanol portion of the molecule and are strongly suggestive of the possibility that the amidino group of lombricine is derived from that of arginine (Scheme I).

The site of lombricine formation cannot be determined from these experiments, but with each of the isotopically labelled compounds used ([1:2-¹⁴C₂]-ethanolamine, DL-[3-¹⁴C]serine, L-[³H]serine and D-[³H]serine) the specific radioactivity of SEP was greater in the gut than in the muscle. The indication thus is that SEP is formed in one or more of the components of the gut; a similar conclusion has been reached as a result of experiments with inorganic ³²P (Gaffney *et al.* 1960). The site of the final reaction of SEP to give lombricine has yet to be determined, but the present results suggest that a transaminase reaction is involved.

SUMMARY

1. When [1:2- $^{14}\text{C}_2$]ethanolamine, DL-[3- ^{14}C]-serine, L-[^3H]serine or D-[^3H]serine was administered orally to earthworms, serine ethanolamine phosphodiester and lombricine were found to be radioactively labelled.

2. For each of the precursors the specific radioactivity of serine ethanolamine phosphodiester was greater than that of lombricine in both muscle and gut tissue.

3. The specific radioactivity of serine ethanolamine phosphodiester and lombricine was greater in gut than in muscle.

4. With [1:2- $^{14}\text{C}_2$]ethanolamine most of the radioactivity of muscle lombricine was in the guanidinoethanol portion of the molecule, whereas with DL-[3- ^{14}C]serine most of the radioactivity was in the serine portion.

5. When L-[amidino- ^{14}C]arginine was given, there was negligible radioactivity in serine ethanolamine phosphodiester and considerable radioactivity in lombricine, almost all of which was in the guanidinoethanol portion of the molecule.

6. These findings suggest that serine ethanolamine phosphodiester is the precursor of lombricine in the earthworm and that the amidino group is derived from arginine by the transamidinase-catalysed transfer of the amidino group to serine ethanolamine phosphodiester.

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Structure of the Ribitol Teichoic Acid from the Walls of *Bacillus subtilis*

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Some micro-organisms contain in their walls a considerable amount of a polymer belonging to the group of phosphoric esters known as teichoic acids (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958; Baddiley, 1959; Armstrong *et al.* 1959b). These compounds are polymers of glycerophosphate or ribitol phosphate in which adjacent polyol residues are joined together through phosphodiester linkages. In addition, the glycerol teichoic acids contain alanine in ester linkage with a hydroxyl on the glycerol, whereas ribitol teichoic acids contain both alanine ester linkages and sugars or *N*-acylamino sugars. Hydrolysis of ribitol

teichoic acids with acid, or with alkali followed by phosphatase treatment, suggests that the sugars are attached glycosidically to hydroxyl groups in the ribitol residues.

In the present work the structure of the ribitol teichoic acid from the walls of *Bacillus subtilis* is discussed. A preliminary account of some of these results has been given elsewhere (Armstrong, Baddiley & Buchanan, 1959a).

EXPERIMENTAL

Materials. Prostate phosphatase was kindly supplied by Dr G. Schmidt and β -glucosidase by Dr W. J. Whelan. D-Alanine and D-amino acid oxidase were purchased from

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