Isolation of Lombricine and its Enzymic Phosphorylation

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Van Thoai & Robin (1954) isolated a new mono-substituted guanidine diester from *Lumbricus terrestris* L., named lombricine, which played the role of phosphagen in the worms during muscular contraction. According to them the compound was guanidinoethyl seryl phosphate (I).

This paper deals with a modified method for the isolation of lombricine in improved yield and a study of some of its properties and its transphosphorylation under various conditions.

EXPERIMENTAL

Materials

Worms were collected locally when weather conditions favoured, otherwise they were obtained from L. Haig and Co., Beam Brook, Newdigate, Surrey, and identified as *Lumbricus terrestris* L.

Ion-exchange resins. C₅₀D was a gift from Phillips and Pain-Vermorel, 31, Rue de la Vanne, Montrouge, Seine, France. It is a cross-linked divinylbenzene-polystyrene resin and a fairly strongly acidic cation-exchanger with SO₃H⁻ as the functional group. As the resin is supplied in the sodium form it was converted into its hydrogen form by soaking it in about twice its volume of HCl (10%, v/v) for 2–3 hr. with occasional stirring. The material was then transferred to a column 45 cm. in length and 2.5 cm. in diameter. Excess of acid was drained off and water passed up the tube to remove all air bubbles and the unduly fine particles of resin. When the washings leaving the column became clear, it was washed free from HCl by a downward flow of water to pH 5. In the text this column is referred to as the 'cation column C₅₀D'.

A₃₀₀D was also given by Phillips and Pain-Vermorel. It is a divinylbenzene-polystyrene resin with amino and hydroxyamino groups as the functional groups and is a moderately basic anion-exchanger. The resin being supplied in the chloride form was used after soaking it in water (2 bed-volumes) for 2–3 hr. and back-washing in a column of 45 cm. in length and 2.5 cm. in diameter as described above. This column is referred to in the text as the 'A₃₀₀D anion column'.

Methods

Chromatography. Paper partition chromatography of Consden, Gordon & Martin (1944) was used. Both the one-dimensional ascending method and the two-dimensional technique of Datta, Dent & Harris (1950) were employed. Small volumes (20–30 μl.) of aqueous solutions of pure amino acids and worm extracts at various stages of isolation of lombricine were dried on Whatman no. 1 filter-paper sheets of suitable size (30 cm. × 20 cm.) in a stream of warm air (40–50°) from a hair-drier. Additions were made, 2–3 μl. at a time, drying between each addition and restricting the spot size to about 0.5 cm. in diameter.

One-dimensional chromatography. The chromatograms were developed at room temperature (22°) for about 6–8 hr. and the solvent was allowed to ascend a distance of about 23–25 cm. The operation was conducted in an air-tight glass aquarium tank fitted with a lid. The atmosphere of the tank was kept saturated with the vapours of the solvent.

Two-dimensional ascending chromatograms. Sheets (20 cm. × 20 cm.) were developed first with 80% (w/v) aq. phenol for 7–8 hr. in an atmosphere of NH₃ provided by the presence of a beaker of aq. NH₃ (sp.gr. 0.88). The second development was with 90% (v/v) aq. methyl Cellosolve (2-methoxyethanol; Bender, 1951) for 4–5 hr. as irrigating solvents.

Detection of spots and their identification. After the complete evaporation of the solvents from the chromatograms, the paper sheets were uniformly sprayed with a 0.5% (w/v) solution of ninhydrin in butanol. Spots, usually shades of purple, appeared after heating the papers for 5 min. at 110°. The amino acid spots were identified by comparing them with the two-dimensional array of spots obtained by preparing a chromatogram of known pure amino acids both singly and as mixtures under the same conditions. This chromatogram, referred to as a 'map of spots' by Dent (1948) for the particular pair of solvents used, was kindly supplied by Dr D. S. P. Patterson.

The spots on the one-dimensional chromatograms were identified by running spots of known pure substances under identical conditions on the same sheet of paper as markers along with a mixture of the known and the unknown amino acids.

For the detection of guanidine derivatives the chromatograms were first sprayed with a mixture of solutions containing 0.2 ml. of NaOH (40%, w/v), 0.2 ml. of ethanolic solution of α-naphthol (0.1%, w/v) and 0.2 ml. of urea solution (40%, w/v) in 10 ml. of water. The mixture was freshly prepared at the time of spraying. After the papers had been dried at room temperature they were sprayed with a solution of NaOBr obtained by diluting (1:5, v/v) a stock solution of 0.9% (v/v) bromine in 10% (w/v) NaOH.

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Appearance of pink spots indicated the presence of guanidine derivatives.

Buffers. Phthalate and borate buffers were prepared from 0.2M-potassium hydrogen phthalate and 0.2M-boric acid in 0.2M-KCl respectively by adjusting the pH with 0.2N-NaOH (Hawk, Oser & Summerson, 1954). Glycine (pH 9) was prepared by titrating a molar solution of glycine with N-NaOH. 2-Amino-2-hydroxymethylpropane-1:3-diol (tris, pH 7) was made from 0.25M-solution by addition of 0.1N-HCl (Gomori, 1955).

Isolation of lombricine from worms. Live worms (1 kg.) were freed from mud by washing them in cold water and were then homogenized with 1 l. of ice-cold (distilled) water in a Waring Blendor for 5 min. The homogenate was adjusted to pH 4 by adding conc. H_2SO_4 dropwise and boiled vigorously for 5 min. After cooling and centrifuging, the clear supernatant was removed and stored. The residue was washed with boiling water (200 ml.) and centrifuged and the supernatant was added to that first collected. This washing procedure was repeated once. To the combined supernatants was then added excess of basic lead acetate solution (20%, w/v; 450 ml.) till there was no further precipitation. The mixture was well stirred and left overnight at room temperature. The clear supernatant was sucked off and the precipitate (lead proteinate) centrifuged down and washed three times with dilute basic lead acetate solution (5%, w/v; 100 ml. portions). The precipitate was discarded and the washings were collected and added to the clear supernatant. A current of H_2S was then passed through the worm extract for 5-6 hr. The mixture was centrifuged and the supernatant retained; the precipitated lead sulphide was washed thoroughly (4-5 times) with saturated aq. H_2S to extract the guanidine bases adsorbed. The combined lead-free extracts were concentrated to about 200 ml. under reduced pressure and filtered. The extract (pH 8.5) was brought to pH 4 by the dropwise addition of conc. H_2SO_4 and was passed through the cation column $C_{50}D$ at the rate of 8-10 drops (approx. 0.8-1.0 ml./min). Acidic amino acids passed in the effluent, whereas the basic and the

neutral ones were adsorbed on the column. After the column had been washed with water until the pH of the effluent rose to 5 it was eluted with aq. 0.15N- NH_3 till the eluate became negative to the Sakaguchi test. The eluate contained the base lombricine, some neutral acids like alanine, serine and leucine, etc., and traces of the stronger bases arginine and lysine.

Ammonia was then removed by concentrating the eluate *in vacuo* to 100 ml. This concentrate (pH 8.3) was passed through the anion column $A_{300}D$ which allowed lombricine along with traces of arginine to pass into the effluent, whereas all the other remaining amino acids, e.g. lysine, serine, alanine, tyrosine and phenylalanine, were adsorbed on the column. The column was washed with water until the effluent became negative to the Sakaguchi test and the combined effluents were evaporated to dryness *in vacuo*. The residue, consisting mainly of lombricine, was dissolved, in the smallest possible quantity (50-60 ml.) of hot aqueous ethanol (50%, v/v) and filtered. Absolute ethanol was then added in small quantities (approx. 150 ml.) till a turbidity was produced and the solution was left at 0° overnight. The white crystalline base (0.85 g.) was filtered off, washed with ethanol 2-3 times and finally with ether and dried. The solid material was recrystallized several times from aqueous ethanol and finally from water. Yield 0.60 g.; m.p. 228° [cf. yield 0.06 g.; m.p. 223-224° (Van Thoi & Robin, 1954)].

Isolation of lombricine from lombricine flavianate. As a modification to the method described above, lombricine was isolated by converting the base present in the concentrated eluate from the $C_{50}D$ column into lombricine flavianate by treating it with flavianic acid (2:4-dinitro-1-naphthol-7-sulphonic acid; Stein, Moore & Bergmann, 1944) and by passing a 2% (w/v) solution of the crystallized flavianate through the anion column $A_{300}D$ to remove the flavianic acid. The Sakaguchi-positive effluent was collected, concentrated *in vacuo* and purified by repeated crystallization from water. Yield 0.317 g./kg. of worms; m.p. 225-226°.

The material obtained by both the methods described ran as a single spot on the paper chromatogram in all the solvents tested (2-9, Table 1). The spot gave positive reactions with ninhydrin and Sakaguchi reagents. Spraying with the molybdate reagent of Hanes & Isherwood (1949) showed the presence of phosphate.

The melting point was unchanged on mixing with an authentic specimen of lombricine (kindly supplied by Mme Y. Robin of Collège de France, Paris) (Found: C, 26.4; H, 5.6; N, 20.67; P, 11.7. $C_6H_{15}O_8N_4P$ requires C, 26.6; H, 5.5; N, 20.85; P, 11.4%).

The flavianate of lombricine, prepared in the usual manner, crystallized from water in small shining orange needles and melted above 250° with decomposition (Found: N, 14.3; P, 5.4. $C_{16}H_{19}O_{13}N_{18}SP$ requires N, 14.8; P, 5.5%).

Attempts to prepare the picrate and the benzoyl derivative of lombricine proved unsuccessful.

Enzymic experiments

Acetone-dried powder of worms. This was prepared by the method of Green, Needham & Dewan (1937). Worms (50 g.) were weighed in an ice-cold beaker and thoroughly ground up in an ice-cold mortar with 100 ml. of water at 0° and allowed to stand for 10 min. Ice-cold acetone (300 ml.) was then stirred into the worm extract for 2-3 min. and the

Table 1. R_f of lombricine developed with different solvent systems

Solvent	R_f
1	No movement
2	0.06
3	0.15
4	0.11
5	0.05
6	0.05
7	0.17
8	0.48
9	0.06

The proportions of the components of the solvent systems are by volumes. 1, butan-1-ol-water-acetic acid (73:17:10); 2, pyridine-isoamyl alcohol-water-acetic acid (80:40:40:10); 3, pyridine-isoamyl alcohol-water (80:40:70); 4, pyridine-isoamyl alcohol-water-20% (v/v) aq. NH_3 , sp.gr. 0.88 (80:40:40:10); 5, phenol saturated with water; 6, propan-1-ol-water-20% (v/v) aq. NH_3 , sp.gr. 0.88 (73:7:20); 7, methanol-water-acetic acid (80:20:10); 8, phenol (80%, w/v)- NH_3 (see text); 9, aqueous methyl Cellosolve (90%).

suspension filtered through a Büchner funnel, with gradually increasing suction. When the powder became fairly dry it was tipped back into the mortar, re-extracted with ice-cold acetone (125 ml.), thoroughly mixed and filtered off in the same filter paper used previously. The brick-red powder obtained was washed with a little cold acetone (20 ml.) and sucked almost dry, care being taken to avoid undue exposure of the powder to the atmosphere. It was then dried *in vacuo* over liquid paraffin and stored at 0°.

Preparation of the enzyme from the acetone-dried powder. A sample of the powder (5 g.) was mixed with 15 ml. of water in a mortar to obtain a homogeneous paste. This was then dialysed at 0° for 15 hr. against water in a cellophan sac. The contents of the sac were centrifuged to remove the insoluble matter and the clear supernatant was used for enzymic experiments.

Preparation of the worm homogenate. Clean live worms (300 g.) were cooled to -5° in a beaker for about 30 min. and homogenized in a Waring Blendor with water (150 ml.) at 0°. The very frothy homogenate was squeezed through a cheese cloth, the pasty residue being discarded. The strained, frothy, viscous liquid (about 120 ml.) obtained was dialysed at 0° for 15 hr. in a number of cellophan sacs against 15 l. of water in a glass tank. The large amount of insoluble material was centrifuged off and discarded. After the clear, pink supernatants (150 ml.) had been mixed, they were stored in 10 ml. portions at -20°.

Transphosphorylation of lombricine. Six tubes with different incubation systems were set up (Table 2) by adding, in order, 0.1 ml. of 0.01 M-MgSO₄, 0.75 ml. of 0.1 M-lombricine, 0.75 ml. of 0.1 M-arginine, 0.15 ml. of 0.1 M-adenosine triphosphate (ATP), 0.30 ml. of buffer solution and 1 ml. of the enzyme solution. The final volume of all the tubes was made up to 3 ml. with water and the tubes were incubated for 15 min. at 37°, after which they were cooled in an NaCl-ice mixture. When cool, 1 ml. of trichloroacetic acid (32%, w/v) was added to each tube and the contents centrifuged at 0°. The supernatant was decanted from the precipitated protein and immediately neutralized with NaOH (40%, w/v), phenolphthalein being used as indicator. Then 2 ml. of aq. 10% (w/v) CaCl₂ at 0°, saturated with Ca(OH)₂, was added and the tubes were allowed to stand at 0° for 15 min. The precipitate of inorganic phosphates was spun off and discarded, the supernatant filtered and the volume was made up to 10 ml. with water. To 5 ml. of this, 1 ml. of trichloroacetic acid (10%, w/v) was added and the phosphorylated compound was

hydrolysed for 1 min. in a boiling-water bath. After cooling, 2.5 ml. of ammonium molybdate [2.5% (w/v) in 5N-H₂SO₄] was added to each tube, followed by Fiske-Subbarow reducing agent (1 ml.). The volume was made up to 10 ml. and the blue colour developed after 15 min. was compared against a standard solution containing 0.1 mg. of P/ml. in a Gallenkamp colorimeter no. 3615 with 608 mμ (red) filter.

RESULTS

Enzymic phosphorylation of lombricine was attempted with acetone-dried powder of the worms from pH 4.6 to 10 (phthalate buffers, 4.6-6; tris, 7; borate buffers, 8-10 and glycine buffer 9), dialysing the enzyme against water for different lengths of time (15 and 48 hr.) and employing different makes of ATP (L. Light and Co., and Sigma Chemical Co. Ltd.).

Only in glycine-HCl buffer (pH 9.0) was there an appreciable transphosphorylation. The amount of phosphagen P formed under these conditions was roughly one-half that obtained with a worm homogenate under the same conditions (Table 2). Increasing the dialysis time from 15 to 48 hr. decreased the amount of phosphagen P formed by the acetone-dried powder preparation by 60%, while the phosphagen P formed by the worm homogenate fell by 40%. Omission of Mg²⁺ ions from the reaction mixture caused a decreased formation of phosphagen P. This effect was greatest after prolonged dialysis (48 hr.). The reduction was 34% with the acetone-dried powder and 38% with the homogenate, whereas after 15 hr. dialysis the reduction was only 20 and 10% respectively.

By using the worm homogenate in glycine-HCl buffer (pH 9), guanidinoethanol phosphate and taurocyamine were successfully phosphorylated, the phosphagen P formed in 5 ml. each of the incubated mixtures being 19.60 and 18.40 μg. respectively. However, when arginine, creatine, guanidine, guanidinoethanol and glycoeyamine were similarly treated they did not accept the phosphate groups at the expense of ATP.

Table 2. *Transphosphorylation of lombricine with acetone-dried powder and worm homogenate with M-glycine buffer at pH 9*

The complete incubation system comprised: 0.10 ml. of 0.01 M-MgSO₄, 0.75 ml. of 0.1 M-lombricine, 0.15 ml. of 0.1 M-ATP, 0.3 ml. of buffer and 1 ml. of enzyme solution. The values are presented after subtracting the apparent phosphagen P formed in the absence of lombricine. No appreciable phosphagen P was formed when enzyme or ATP was omitted or when arginine was substituted for lombricine.

Enzyme source	Time of dialysis (hr.)	μg. of phosphagen P formed in incubation systems	
		With Mg ²⁺	Without Mg ²⁺
Acetone-dried powder	15	10.20	8.20
Acetone-dried powder	48	4.20	2.80
Worm homogenate	15	28.50	22.40
Worm homogenate	48	15.92	9.80

DISCUSSION

The progressive loss of activity of the enzyme preparations during dialysis suggested that some cofactor was being removed from the enzyme by the treatment. This was tested by adding a concentrate of the diffusate to the enzyme preparations, but no reactivation of the enzyme preparations was obtained. This suggests that, if loss of activity of the enzymes is due to a loss of a cofactor, then this cofactor must be labile under the conditions of dialysis and subsequent concentration of the aqueous diffusate.

The bases tested as acceptors in the transphosphorylation reaction may be divided into two classes: those containing acidic groups such as phosphate or sulphate and those without acidic groups. It may be of significance that only the acid-containing bases (lombricine, guanidinoethanol phosphate and taurocyamine) were phosphorylated.

SUMMARY

1. Lombricine has been isolated in good yield from *Lumbricus terrestris* L. with ion-exchange resins. Lombricine flavianate has been prepared.

2. Transphosphorylation from adenosine triphosphate to lombricine has been achieved with dialysed enzyme preparations from worm homogenates or acetone-dried powders. The addition of Mg^{2+} ions was necessary for maximum enzyme activity.

3. Of the bases other than lombricine tested (guanidinoethanol phosphate, taurocyamine, arginine, creatine, guanidine, guanidinoethanol and

glycoccyamine) as phosphate acceptors, only guanidinoethanol phosphate and taurocyamine were active.

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Pepsin-Catalysed Transpeptidation of the Amino-Transfer Type

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Some *N*-acyldipeptides containing tyrosine or phenylalanine or both are known to be hydrolysed by pepsin (Fruton & Bergmann, 1939; Harington & Pitt-Rivers, 1944; Dekker, Taylor & Fruton, 1949; Casey & Laidler, 1950; Baker, 1951). Direct cleavage of the peptide bond, producing an acylamino acid and a free amino acid, was the only reaction assumed to take place. Analysis of the products formed by peptic digestion of substrates of the above type, containing a *C*-terminal tyrosine, showed that both transpeptidation and hydrolysis

occur. It could furthermore be demonstrated that the transpeptidation involves an amino transfer, where the amino moiety of the tyrosine peptides used as substrates is transferred to an acceptor with a free carboxyl group (Hanes, Hird & Isherwood, 1950).

EXPERIMENTAL

Materials

Twice-crystallized pepsin from Mann Research Laboratories Inc., New York, was used throughout.