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The Purification and Properties of Adenosine Triphosphate-Lombricine Phosphotransferase

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The properties of the enzyme ATP-lombricine phosphotransferase (lombricine kinase), which catalyses the reaction:

have not yet been described in any detail. Pant (1959) studied the above reaction, in the forward direction, with crude homogenates and extracts of acetone-dried powders prepared from whole earthworms (*Lumbricus terrestris* L.). At pH 9.0 these preparations showed a low activity which was slightly increased by the addition of Mg^{2+} ions. In addition to lombricine (D-2-amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate) two other guanidino compounds, taurocyamine (2-guanidinoethanesulphonic acid) and 2-guanidinoethyl phosphate, acted as phosphoryl group acceptors in the reaction. Lombricine-kinase activity was lost on prolonged dialysis, and the addition of concentrated diffusate to the enzyme system did not restore activity. Pant (1959) suggested that this loss of enzymic activity was due to the removal of a labile cofactor by dialysis.

In a preliminary note, Rosenberg, Rossiter, Gaffney & Ennor (1960) described the partial purification of lombricine kinase by ammonium sulphate fractionation of aqueous extracts of the muscle of the earthworm *Megascolides cameroni*. This enzyme preparation catalysed the phosphorylation of synthetic L-lombricine, as well as of D-lombricine and taurocyamine, and was activated by Mn^{2+} , Co^{2+} or Mg^{2+} ions. The isolation in high

yields of crystalline D-lombricine and N-phosphoryl-D-lombricine from earthworms (Ennor, Rosenberg, Rossiter, Beatty & Gaffney, 1960; Ennor & Rosenberg, 1962) has provided an opportunity for a more detailed study of the properties of lombricine kinase. The present paper reports a method for the preparation of highly purified lombricine kinase and some properties of the enzyme from earthworm muscle.

EXPERIMENTAL

Materials and Methods

All reagents were of analytical grade and glass-distilled water was used for the preparation of all solutions.

Magnesium chloride. Solutions of MgCl_2 were made from a stock solution (approx. 0.1M) of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and standardized by titration of H^+ ions displaced from Dowex 50 (H^+ form) by a known volume of the solution.

Ammonium sulphate solution. A solution of $(\text{NH}_4)_2\text{SO}_4$ saturated at 2° was prepared and adjusted to pH 7.4 (measured on a 1:20 dilution by the glass electrode) by the addition of N-NaOH.

Preparation of earthworm muscle. The body wall from large live earthworms (*Megascolides cameroni*), obtained in the Australian Capital Territory and southern New South Wales, was dissected free from viscera and rinsed in cold water. The muscle tissue was frozen in liquid air and stored at -18° until required.

Guanidino compounds. D-Lombricine was isolated from natural sources as described by Ennor *et al.* (1960), dried in air and stored at room temperature. The material was pure as judged by elementary analysis; after chromatography in two dimensions in ethanol-formic acid (98%)-water (7:1:2, by vol.) and in phenol-water (4:1, w/v), it gave a single ninhydrin-positive spot. Solutions of lombricine (0.05M) were adjusted to pH 8.6 by the addition of 0.1N-KOH and stored at -10°.

D-Phospholombricine was prepared enzymically and purified by a modification of the method described by

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Ennor & Rosenberg (1962) for the isolation of phospholombricine from natural sources. The reaction mixture (100 ml.), containing glycine-KOH buffer, pH 9.5 (0.1 M), D-lombricine (0.02 M), ATP (0.03 M) and $MgCl_2$ (4 mM), was incubated at room temperature (24°), and purified lombricine kinase (40 units; see below for definition) was added every hour for 5 hr. After 6 hr., when 80% of the lombricine had been phosphorylated, the reaction mixture was adjusted to pH 7.0 with 5 N-HCl and immediately stirred with 15 g. of acid-washed charcoal (Norit A) for 3 hr. to remove nucleotides. The filtered solution was free of nucleotides as judged by measurement of the extinction at 259 m μ . The charcoal was washed twice with water (20 ml.) and the washings were added to the filtrate (volume 115 ml.). The filtrate which contained phospholombricine (1.5 m-moles) and lombricine (0.25 m-mole) was passed through a column (4 cm. $\times$ 30 cm.) of Dowex 1 (HCO_3^- form; mesh 200-400) at the rate of 3 ml./min. Lombricine appeared in the water washings and was recovered for later use. When the washings were lombricine-free, 300 ml. of 0.15 N- $KHCO_3$ was passed through to remove final traces of lombricine. Phospholombricine was then eluted from the resin with 1.5 N- $KHCO_3$, the effluent being collected in 10 ml. fractions. The fractions containing phospholombricine were pooled, cooled to 1° in an ice bath, and adjusted to pH 3 (glass electrode) by the cautious addition of an aqueous suspension of Dowex 50 (H^+ form; mesh 200-400). The mixture was vigorously aerated throughout this procedure to assist in the removal of CO_2 and, when CO_2 -free, was adjusted to pH 7.5 by the addition of 5 N-NaOH. The suspension was then filtered, the resin was washed twice with water (50 ml.) and the washings were combined with the filtrate. The resultant solution (500 ml.) contained 1.4 m-moles of phospholombricine which was isolated as the magnesium salt as described by Ennor & Rosenberg (1962). The yield, based on the amount of lombricine originally present, was 61%. The final product had a free lombricine content of 0.18%. The magnesium salt (100 mg.) was converted into the sodium salt immediately before use by passage through a column (2 cm. $\times$ 15 cm.) of Dowex 50 (Na^+ form; mesh 200-400). The effluent was collected in 2 ml. fractions and those containing phospholombricine were pooled. The concentration of the phospholombricine solution was assayed and adjusted to the desired concentration, and the solution was stored at -10°.

L-Lombricine, 2-guanidinoethyl phosphate and 2-guanidinoethanol hydrochloride were gifts from Dr D. I. Magrath of this Department.

Taurocyamine, β -guanidinopropionic acid and δ -guanidino-*n*-pentanoic acid were synthesized as described by Morrison, Griffiths & Ennor (1957). L-Arginine monohydrochloride and L-canavanine sulphate were obtained from California Corp. for Biochemical Research. Creatine was from British Drug Houses Ltd.

Nucleotides. ADP, ATP, CDP, UDP, GDP, IDP and deADP were obtained from Sigma Chemical Co. ADP was purified as described by Morrison, O'Sullivan & Ogston (1961) and stored at -10° over $CaSO_4$. Solutions were standardized by measurement of extinctions at 259 m μ (Bock, Ling, Morell & Lipton, 1956) and, after dilution to 10 mM, stored at -10°. ATP, as crystalline disodium salt of 99% purity, was recrystallized twice from aqueous ethanol (Berger, 1956). The dried ATP, which contained less than 0.1% of inorganic phosphate, was stored at -10°

over $CaSO_4$. Solutions were adjusted to pH 8.6 with 1 N-KOH and standardized as for ADP. Stock solutions (25 mM) were stored at -10°.

Buffer systems. Triethanolamine (British Drug Houses Ltd.) was purified by the method of Germann & Knight (1933). *N*-Ethylmorpholine was obtained from Eastman Kodak and was twice distilled under reduced pressure at 38°. It was stored at -10° as a 1 M stock solution.

Inhibitors. *p*-Hydroxymercuribenzoate was obtained from Sigma Chemical Co. Arsenite and dinitrophenol were obtained from British Drug Houses Ltd. Phenyl iodacetate, *o*-iodosobenzoic acid and *N*-ethylmaleimide were gifts.

Determination of lombricine and phospholombricine. Lombricine was determined as described by Rosenberg, Ennor & Morrison (1956) for the determination of arginine. Under the conditions employed a 0.05 mM solution of arginine had an extinction of 0.4 in a cell of 1 cm. light-path.

Phospholombricine was generally determined as lombricine after hydrolysis in 0.1 N-trichloroacetic acid at 100° for 1 min., but, when it was necessary to determine phospholombricine in the presence of relatively large amounts of lombricine (e.g. in studies of the forward reaction), it was measured by determination of the inorganic phosphate released after acid hydrolysis. In some experiments certain components of the reaction mixtures caused interference with the determination of lombricine. Thus the inhibitory effect of cysteine on the colour yield was overcome by the addition of appropriate amounts of *p*-hydroxymercuribenzoate (cf. Ennor & Stocken, 1948), and interference by high glycine concentrations was allowed for by appropriate internal controls. In alkaline solution many metal ions interfered by forming insoluble hydroxides. These were removed by centrifuging, and lombricine was determined in the clear solution.

Determination of orthophosphate. The colorimetric method of King (1932) was used.

Determination of protein. This was done either by the biuret method of Gornall, Bardawill & David (1949) or by the method of Lowry, Rosebrough, Farr & Randall (1951). Crystalline bovine serum albumin was used as a standard.

Measurement of enzymic activity. During the work on the isolation of the enzyme, the velocity of the forward reaction (1) was followed by measuring the rate of phospholombricine formation. The reaction mixture (1.0 ml.) contained glycine-KOH buffer, pH 8.6 (0.1 M), D-lombricine (0.01 M), ATP (5 mM), $MgCl_2$ (5 mM) and enzyme. After equilibration at 30° the reaction was started by the addition of 0.2 ml. of enzyme solution. Enzyme solutions were diluted with cold 1 mM-glycine-KOH buffer, pH 8.6, before use to give a suitable concentration of protein. The reaction was stopped by the addition of 1.0 ml. of 0.2 N-trichloroacetic acid and the tubes were then placed in a boiling-water bath for 1 min. They were then cooled in an ice bath and samples withdrawn for the determination of orthophosphate. Corrections for the small but appreciable hydrolysis of ATP were made as described by Morrison *et al.* (1957).

The velocity of the reverse reaction was followed by measuring the rate of lombricine release from phospholombricine. The reaction mixture (1.0 ml.) contained *N*-ethylmorpholine-HCl buffer, pH 7.0 (0.1 M), phospholombricine (2.5 mM), ADP (2 mM), $MgCl_2$ (5 mM) and

enzyme. After an equilibration period at 30° the reaction was started by the addition of 0.2 ml. of enzyme solution in 1 mM-*N*-ethylmorpholine-HCl buffer, pH 7.0. The reaction was stopped by the addition of 1.0 ml. of 3*N*-NaOH containing EDTA (0.08M), and the lombricine content was determined as described above.

Definitions of activity unit and specific activity. One unit of lombricine-kinase activity is defined as that amount of enzyme which, under the conditions of assay, catalyses the formation of 1 μ mole of phospholombricine from ATP and lombricine in 1 min. Specific activity is expressed in terms of units of lombricine kinase/mg. of protein.

Preparation of ion-exchange celluloses. *NN*-Diethylaminoethylcellulose (DEAE-cellulose) with an anion-exchange capacity of 0.5 m-equiv./g. (Eastman Organic Chemicals) was converted into triethylaminoethylcellulose (TEAE-cellulose) as described by Porath (1957). The dry material was suspended in 0.01 M-*N*-ethylmorpholine-HCl buffer, pH 7.6, and *N*-ethylmorpholine (0.1M) was added as required to maintain the mixture at this pH. After equilibration, the TEAE-cellulose was recovered by centrifuging and resuspended in 0.01 M-*N*-ethylmorpholine-HCl buffer, pH 8.6. Carboxymethylcellulose (Bio-Rad Laboratories) with a cation-exchange capacity of 0.5 m-equiv./g. was equilibrated with 0.01 M-*N*-ethylmorpholine-HCl buffer, pH 7.0, as described above for TEAE-cellulose. All columns were prepared by allowing the substituted cellulose to settle under gravity.

Concentration of dilute protein solutions. Dilute protein solutions such as the eluates from TEAE-columns were concentrated by the pressure-dialysis procedure of Hofsten & Falkbrink (1960).

Purification of lombricine kinase

Unless otherwise stated, all extraction and purification steps were carried out in a cold room at 2° and the buffer used throughout the procedure was *N*-ethylmorpholine-HCl containing EDTA (3 mM). The pH was determined with a glass electrode. All centrifugings were carried out at 0°.

Extraction of the enzyme. Frozen earthworm muscle (100 g.) was dropped into liquid N₂, ground to a fine powder in a cooled mortar and suspended in 300 ml. of 25 mM buffer, pH 7.2. The suspension was slowly stirred for 1 hr. and then treated in a Waring Blendor for 3 min. The mixture was centrifuged at 1500g for 10 min. and the supernatant passed through glass wool to remove floating particles. The residue was resuspended in 200 ml. of buffer, re-extracted in a Waring Blendor for 3 min. and again centrifuged at 1500g for 10 min. The residue was discarded and the supernatant fraction combined with that from the first extraction.

Fraction I. Saturated (NH₄)₂SO₄, pH 7.4, was added to the extract at the rate of 10 ml./min. to give a saturation of 45%. The mixture was stirred throughout the addition and subsequently for 1 hr. It was then centrifuged for 15 min. at 15000g and the residue discarded. Sufficient solid (NH₄)₂SO₄ was added to the supernatant to bring the saturation to 90%. The mixture was rapidly stirred throughout this addition and for a further 30 min., after which it was again centrifuged for 15 min. at 15000g. The supernatant fluid was discarded and the precipitate dissolved in 70 ml. of 0.025M buffer, pH 7.2. This solution was dialysed against four changes of 20 vol. of 0.01M buffer, pH 7.4, for 40 hr.

Fraction II. The clear red solution, which contained about 7 mg. of protein/ml., was reduced to about one-quarter of its volume by pressure dialysis and dialysed against two changes of 20 vol. of 0.01M buffer, pH 8.6, for 8 hr. The small precipitate that formed was removed by centrifuging and the supernatant transferred to the top of a TEAE-cellulose column (3 cm. $\times$ 40 cm.). It was allowed to flow into the upper part of the column, which was then washed with 2 bed volumes of 0.01M buffer, pH 8.6, and the enzyme eluted with 0.5M buffer of the same pH. The red components of the solution remained on the column and the colourless eluate was collected in 10 ml. fractions. Analysis of the latter for both protein content and lombricine-kinase activity revealed that fractions 25-29 contained enzyme of the highest specific activity. These fractions were pooled and the volume was reduced by pressure dialysis to 5 ml., when the protein concentration was about 60 mg./ml. The solution was then dialysed overnight against two changes of 20 vol. of 0.01M buffer, pH 8.6. The small precipitate that formed was removed by centrifuging.

Fraction III. The supernatant solution from the preceding step was applied to another TEAE-cellulose column of smaller size (1 cm. $\times$ 15 cm.) and the procedure described for fraction II was repeated. The eluate was collected in 1 ml. fractions and those fractions (11-17) containing enzyme of the highest specific activity were pooled. The volume of the pooled material was reduced by pressure dialysis until the protein concentration was about 30 mg./ml. The resultant solution was dialysed overnight against two changes of 0.01M buffer, pH 7.0.

Fraction IV. The dialysed solution from the preceding step was stirred for 15 min. with 1 g. (dry wt.) of carboxymethylcellulose (previously equilibrated with 0.01M buffer, pH 7.0, and added in the wet state) and the mixture was centrifuged. The supernatant was discarded and the substituted cellulose washed by suspension in 0.01M buffer, pH 7.0. The suspension was centrifuged, the supernatant discarded and the carboxymethylcellulose resuspended in 5 ml. of 0.5M buffer, pH 7.0, and stirred for 15 min. to extract the enzyme from the cellulose. After centrifuging, the supernatant was dialysed for 12 hr. against 20 vol. of 5 mM buffer, pH 7.5 (containing in this case EDTA at a concentration of 1 mM). The enzyme solution was then subjected to a further 12 hr. dialysis against 20 vol. of 5 mM buffer, pH 7.5, but without EDTA. The dialysed solution was mixed with an equal volume of glycerol and stored at 2°, under which conditions enzymic activity remained unchanged for several months. This solution was used in the experiments reported below.

The summary of the yields and degree of purification of lombricine kinase achieved with a typical preparation (Table 1) shows that the procedure gave about a tenfold purification with an overall recovery of 6%.

RESULTS

Tests for other guanidino phosphotransferases in the lombricine-kinase preparation. Earlier work (Pant, 1959; Rosenberg *et al.* 1960) established that crude preparations of lombricine kinase were capable of catalysing phosphoryl group transfer from ATP to taurocyamine. The possibility that this was due to the presence of two enzymes rather

Table 1. *Summary of yields and specific activities of fractions obtained during the purification of lombricine kinase from 100 g. of earthworm muscle*

Fraction	Details are given in the text.				
	Total protein (mg.)	Total activity (units)	Specific activity (units/mg. of protein)	Purification	Recovery (%)
Extract	4100	13300	3.2	[1.0]	[100]
I: ppt. from 45–90 % saturated (NH ₄) ₂ SO ₄	1830	10600	5.8	1.8	80
II: first TEAE-cellulose eluate	298	4655	15.6	4.9	35
III: second TEAE-cellulose eluate	133	3192	24.0	7.5	24
IV: carboxymethylcellulose eluate	24	798	33.0	10.3	6

Table 2. *Comparison of the initial rates of D-lombricine and taurocyamine by lombricine-kinase preparations at various stages of purification*

Details are given in the text. Activity is expressed as μ moles of phosphoguanidine compound formed/min./ μ g. of protein.

Fraction	Activity		Ratio of activities (2)/(1)
	With lombricine (1)	With taurocyamine (2)	
Extract	3.2	0.72	0.23
I	5.8	1.2	0.21
II	15.6	3.2	0.21
III	24.0	—	—
IV	33.0	8.2	0.25

than one with a broad specificity has been checked by determination of the initial rates of phosphorylation of D-lombricine and taurocyamine at each stage of the purification procedure. The constancy of the ratio of specific activities (Table 2) suggests that the phosphorylation of lombricine and taurocyamine was the function of a single enzyme.

Assays for other enzymic activities. Fraction IV of the enzyme preparation (20 μ g. of protein in 1 ml. of reaction mixture) was tested under appropriate conditions for the presence of Mg²⁺ ion-activated ATP-phosphohydrolase, ATP-AMP-phosphotransferase and phosphoamide-hydrolase activities. The Mg²⁺ ion-activated ATP-phosphohydrolase was determined in routine assays of the forward reaction by omitting lombricine from the reaction mixture and determining inorganic phosphate after incubation for 30 min. Since no inorganic phosphate was released, the preparation was judged to be free from this enzyme. ATP-AMP-phosphotransferase activity was also absent when tested for by the routine assay procedure (except that ADP was substituted for ATP) adopted for the forward reaction, i.e. no formation of phospholombricine could be detected. Phosphoamide-hydrolase activity was tested for by determination of any lombricine released from phospholombricine under the standard assay conditions (except that ADP was omitted) for the reverse reaction: no such activity was detected.

Electrophoretic studies. Fraction IV migrated as a single component when examined in an Antweiler micro-electrophoresis apparatus under the following conditions: 90v and 1.5 mA; time 20 min.; temperature 1°; protein concentration 5 or 10 mg./ml. The buffers used (ionic strength 0.1) were potassium phosphate (pH 6.0 and 9.8), veronal-hydrochloric acid (pH 8.6).

Properties of lombricine kinase. Several attempts were made to employ ethanol fractionation, as described by Kuby, Noda & Lardy (1954), as a purification procedure. All such attempts failed. Thus the addition of ethanol to the crude extract at -5°, although causing no loss of activity, led to no worthwhile purification. When ethanol fractionation was attempted on crude extracts made 0.1M with respect to Mg²⁺ ion, the enzyme was completely inactivated. Preparations of lombricine kinase (fraction IV) may be dialysed against 0.02M-N-ethylmorpholine-hydrochloric acid buffer, pH 7.5, for 24 hr. at 2° with only slight loss of activity. Freeze-drying of preparations in non-volatile buffers caused almost 40 % loss of activity, and freezing and thawing at pH 8.6 in N-ethylmorpholine-hydrochloric acid buffer produced complete inactivation.

Effect of enzyme concentration on reaction velocity. The initial velocity was directly proportional to the enzyme concentration for both the forward and reverse reactions in the ranges 0–20 and 0–2.5 μ g. of protein/ml. respectively. The maximum rate of the reverse reaction was approximately five times that of the forward reaction when both reactions were measured under standard conditions.

Effect of pH on reaction velocity. The effects of pH and buffer species on the initial velocity in the forward and reverse reactions are shown in Figs. 1 and 2 respectively. The pH optimum for the forward reaction was about 8.6 and at this pH the reaction rate was slightly higher in glycine-potassium hydroxide than in triethanolamine-hydrochloric acid buffer. In the reverse reaction (Fig. 2) the pH optimum was about 7.2, the maximum rates being similar in imidazole-hydrochloric acid and N-ethylmorpholine-hydrochloric acid buffers.

Effect of lombricine and phospholombricine concentration on the reaction velocity. The effects of the concentration of both D- and L-lombricine on the initial velocity of the forward reaction are shown in Fig. 3. Although the apparent K_m values (9.1 mM and 11.7 mM for D- and L-lombricine respectively) were of similar magnitudes, the maximum velocity was greater with L-lombricine than with D-lombricine: 49 and 31 μ moles of lombricine were phosphorylated/mg. of protein/min. respectively.

Fig. 1. pH-activity curves for the forward reaction catalysed by lombricine kinase in the presence of 0.1M-glycine-KOH (▲) and 0.1M-triethanolamine-HCl buffer (○). Activities were determined as described in the Materials and Methods section. The enzyme sample used was 10 μ g. of protein (specific activity, 40); the reaction time was 1 min. at 30°.

Fig. 2. pH-activity curves for the reverse reaction catalysed by lombricine kinase in the presence of 0.1M-imidazole-HCl (■) and 0.1M-N-ethylmorpholine-HCl buffer (▲). Enzyme activities were determined as described in the Materials and Methods section. The enzyme sample used was 2 μ g. of protein (specific activity, 40); the reaction time was 1 min. at 30°.

The effect of the concentration of phospholombricine on the initial velocity of the reverse reaction is shown in Fig. 4. The apparent K_m for phospholombricine was 7.4 mM, and the maximum velocity corresponded to 200 μ moles of lombricine formed/mg. of protein/min.

Effect of metal ions on the reaction velocity. Lombricine kinase was activated by Mn^{2+} , Mg^{2+} , Co^{2+} or Ca^{2+} ions in both the forward and reverse reactions (Table 3). No activation was detected with Ni^{2+} , Cu^{2+} , Fe^{3+} , Fe^{2+} , Al^{3+} , Sn^{2+} , Ba^{2+} , Cd^{2+} , Zn^{2+} and Be^{2+} ions when these were tested in both the forward and reverse reactions.

Fig. 3. Effects of D- and L-lombricine concentration on the initial velocity of the forward reaction catalysed by lombricine kinase. The experimental conditions were as described in the Materials and Methods section except that the concentration of lombricine was varied. The enzyme sample used was 10 μ g. of protein (specific activity, 40); the reaction time was 1 min. at 30°. Velocity is expressed in arbitrary units. ●, D-Lombricine; ▲, L-lombricine.

Fig. 4. Effect of phospholombricine concentration on the initial velocity of the reverse reaction catalysed by lombricine kinase. The experimental conditions were as described in the Materials and Methods section except that the concentration of phospholombricine was varied. The enzyme sample used was 0.5 μ g. of protein (specific activity, 40); the reaction time was 1 min. at 30°. Velocity is expressed in arbitrary units.

Substrate specificity of lombricine kinase. The specificity of lombricine kinase for other guanidino compounds was investigated in the forward reaction. In addition to L-lombricine and taurocyamine,

2-guanidinoethyl phosphate was also phosphorylated. Creatine, arginine, guanidinoacetate, guanidinoethanol, δ -guanidinobutyrate, β -guanidinopropionate, γ -guanidino-*n*-pentanoate and canavanine were not phosphorylated.

Table 3. *Activation of lombricine kinase by bivalent metal ions*

Each metal ion was used at a concentration of 5 mM. Activities were determined as described in the Materials and Methods section, and expressed as percentages of the activity with Mn^{2+} ions. The enzyme sample used was 5 μ g. of protein (specific activity, 40); the reaction time was 5 min. at 30° and 3 min. at 23° for the forward and reverse reactions respectively.

Metal ion	Relative activity (%)	
	Forward reaction	Reverse reaction
Mn^{2+}	[100]	[100]
Mg^{2+}	95	90
Co^{2+}	21	18
Ca^{2+}	27	8

Table 4. *Specificity of lombricine kinase for nucleotides*

Activities are expressed as percentages of that with ADP and were determined as described in the Materials and Methods section with each nucleotide at a concentration of 1 mM. The enzyme sample used was 20 μ g. of protein (specific activity, 40); the reaction time was 6 min. at 30°.

Nucleotide	Relative activity (%)
ADP	[100]
deADP	16
CDP	2.9
GDP	2.4
IDP	3.5
UDP	1.8

Table 5. *Effect of thiol reagents and metabolic inhibitors on the activity of lombricine kinase*

Enzyme activity was determined in the reverse direction under the conditions described in the Materials and Methods section. The inhibitor was preincubated for 5 min. with the enzyme in the presence of the reaction components other than ADP, and the reaction was started by the addition of the latter. The reaction time was 5 min. at 30°.

Inhibitor	Concn. of inhibitor (μ M)	Inhibition (%)
<i>p</i> -Hydroxymercuribenzoate	0.05	20
	0.1	100
<i>p</i> -Hydroxymercuribenzoate + cysteine* (2 μ M)	0.05	100
Phenyl iodoacetate	1000	100
<i>o</i> -Iodosobenzoate	300	100
<i>N</i> -Ethylmaleimide	300	100
Arsenite	1000	0
2,4-Dinitrophenol	1000	0

* Added together with *p*-hydroxymercuribenzoate.

The specificity of lombricine kinase for nucleotides was also investigated. No significant reaction occurred with CDP, GDP, IDP or UDP; the very small activity observed with these could be due to contaminating traces of ADP. However, deADP was phosphorylated, but at a much slower rate than ADP (Table 4).

Effect of inhibitors on lombricine-kinase activity. Enzyme activity was inhibited by a variety of inhibitors known to react with thiol groups. No inhibition was observed with arsenite or dinitrophenol (Table 5).

DISCUSSION

The purification procedure described in the present work, although giving low yields of lombricine kinase, is readily reproducible and gives a product that is homogeneous as judged by electrophoretic studies. Lombricine kinase, although unstable if stored in 0.25 M-*N*-ethylmorpholine-hydrochloric acid buffer, pH 7.5, at 2°, has been stored without any detectable loss of activity for 4 months in a mixture (1:1, v/v) of this buffer and glycerol. The enzyme is also stable to dialysis at 2° over periods up to 24 hr. This latter finding is in contrast with that of Pant (1959), who reported a progressive decline in activity during prolonged dialysis and interpreted this as possibly being due to the loss of some labile cofactor. However, Pant's extracts were prepared from either whole fresh earthworms (*Lumbricus terrestris* L.) or acetone-dried powders of similar material and, since no purification was attempted, it is likely that such extracts contained proteolytic enzymes which would destroy the lombricine kinase.

In general, the properties of the enzyme are very similar to those of other guanidino phosphotransferases (Ennor & Morrison, 1958). Thus, when tested in both the forward and reverse reactions (eqn. 1), the enzyme shows optimum activities at pH 8.6 and 7.2 respectively. Under conditions of optimum pH and with the enzyme saturated with the guanidino substrates (cf. Figs. 3 and 4) the rate of the reverse reaction was approximately five times that of the forward reaction, and this, too, is similar to the findings with other ATP-guanidino phosphotransferases. Another point of similarity lies in the fact that the enzyme has an absolute requirement for a bivalent metal ion. Thus, under conditions where all bivalent metal ions would be expected to form a complex with EDTA, there was no detectable enzyme activity. Like the other guanidino phosphotransferases, the present enzyme

is completely inhibited by low concentrations of such thiol reagents as *p*-hydroxymercuribenzoate and *N*-ethylmaleimide. Since the inhibition by the former reagent was not reversed by a large excess of cysteine, some doubt arises as to whether the inhibitory effects were, in fact, due to interaction with the thiol groups of the enzyme. The failure of arsenite to inhibit lombricine-kinase activity is reminiscent of the lack of effect of arsenite on arginine kinase (Morrison *et al.* 1957) and of lewisite on creatine kinase (Rosenberg & Ennor, 1955).

ADP and deADP are the only nucleotides capable of acting at a significant rate as phosphoryl group acceptors when tested with *N*-phospholombricine as a donor. Several guanidino compounds have been tested for their abilities to serve as phosphoryl group acceptors, and in confirmation of the results reported by Pant (1959) and Rosenberg *et al.* (1960) it was found that taurocyamine and 2-guanidinoethyl phosphate could both function as acceptors. We have shown that a single enzyme is responsible for the phosphorylation of both lombricine and taurocyamine, and, since the preparation is apparently homogeneous, it is probably responsible for the phosphorylation of 2-guanidinoethyl phosphate. So far as is known, the latter compound is not found in Nature, but taurocyamine is the natural substrate for taurocyamine kinase which is present in *Arenicola marina* (Thoai, 1957) and *A. assimilis* (Ennor & Morrison, 1958). Thus lombricine kinase has a broader specificity for guanidino substrates than have the other guanidino kinases. However, though taurocyamine (2-guanidinoethanesulphonic acid) is active as a substrate, the carboxylic acid analogue (β -guanidinopropionic acid) is completely inactive.

SUMMARY

1. A method is described for the preparation, in an electrophoretically homogeneous state, of ATP-lombricine phosphotransferase from the muscular body wall of the earthworm (*Megascolides cameroni*).

2. The enzyme displayed optimum activity in the forward and reverse reactions at pH 8.6 and 7.2, respectively, and was activated in both directions by Mg^{2+} , Mn^{2+} , Ca^{2+} or Co^{2+} ions.

3. The enzyme catalyses the phosphorylation of L-lombricine at a faster rate than that of D-

lombricine, the natural substrate. Taurocyamine and 2-guanidinoethyl phosphate can also function as phosphoryl group acceptors.

4. Both ADP and deADP can act as phosphoryl group acceptors.

5. The enzyme is similar in its general characteristics to other previously described ATP-guanidino phosphotransferases.

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