THE *IN VIVO* INCORPORATION OF [^{32}P] INORGANIC PHOSPHATE INTO LOMBRICINE AND SERINE ETHANOLAMINE PHOSPHODIESTER OF THE EARTHWORM

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SUMMARY

1. At time intervals varying from one to 16 days after the administration of [^{32}P]inorganic phosphate to earthworms the specific radioactivity of serine ethanolamine phosphodiester (SEP) was greater than that of lombricine in both muscle and gut tissue.

2. In gut the specific radioactivity of both SEP and lombricine was greater than that of the corresponding compounds in muscle at all time intervals after the administration of ^{32}P.

3. In muscle the concentration of lombricine was much greater than that of SEP. In gut the concentrations of these compounds were of the same order and similar to that of SEP in muscle.

4. The incorporation of [^{32}P]inorganic phosphate into the labile P of both ATP and N-phosphoryllombricine was considerably more rapid than the incorporation into the diester P of SEP and lombricine.

5. The rate of incorporation of [^{32}P]inorganic phosphate into SEP was similar to that found for phospholipid and phosphoprotein.

6. It is concluded that in the earthworm SEP is formed from smaller molecules and is the precursor of lombricine.

INTRODUCTION

Serine ethanolamine phosphodiester (SEP, I) has been recently isolated from and identified in the earthworm[1,2], a finding which is consistent with the previous suggestion[2,3] that this compound is the biological precursor of lombricine (II), the N-phosphoryl derivative of which was claimed to be the phosphagen of this species[4,5].

The suggestion that SEP gives rise to lombricine was further strengthened by the finding that earthworm SEP contains the unusual D-isomer of serine[2], which is also present in lombricine[6]. The present communication offers additional support of

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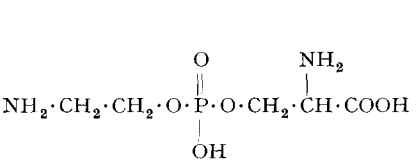
this hypothesis from evidence from experiments on the *in vivo* incorporation of [^{32}P]-orthophosphate into SEP and lombricine in the earthworm. In addition, experiments are reported in which the relatively rapid incorporation of [^{32}P]inorganic phosphate into the labile P of both ATP and N-phosphoryllombricine is contrasted with the very slow incorporation into the diester P of SEP and lombricine.



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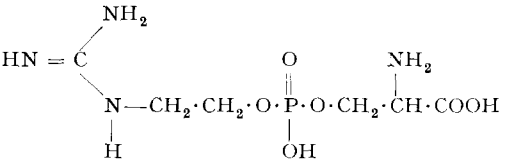

(II)

EXPERIMENTAL

Materials

All reagents were analytical grade and solvents used for chromatography were redistilled before use. Carrier free [^{32}P]inorganic phosphate was obtained from the Atomic Energy Research Establishment, Harwell, England.

Treatment of worms

Earthworms (*Megascolides cameroni*) of average weight 8 g were collected in Australian Capital Territory and were kept at 10° in moist earth taken from their natural habitat. [^{32}P]Inorganic phosphate (10 μC/g) was administered from a microsyringe fitted with fine polythene tubing[7], which was inserted into the gastrointestinal tract for a distance of at least 3 cm. During the incubation procedure the worms were lightly anaesthetised with CO_2. After intubation the worms were returned to the cold room (10°) for periods varying from 24 h to 16 days.

Preparation of tissue extracts

The worms were killed and the gut, together with other viscera, dissected free from the muscular body wall. These two tissues were then homogenized at 0° with two volumes of 1.5 *N* perchloric acid, and the protein precipitate removed by centrifugation. The supernatant was neutralised with 2.5 *N* KOH at 0° and the precipitated $KClO_4$ centrifuged off. Glycogen was removed from the supernatant by treatment with an equal volume of ethanol at 0° and subsequent centrifugation. A portion of the supernatant was taken for the estimation of the specific radioactivity of inorganic phosphate. The remainder was applied to a column (6 × 1 cm) of Zeokarb 225 ion exchange resin (H^+ form) at a rate of 1 ml/min. Phosphoric acid (0.01 *N*) was then passed through the column to remove traces of [^{32}P]inorganic phosphate remaining in the column. This was followed by 0.01 *N* HCl until the effluent was free from inorganic phosphate. The material retained on the resin was eluted with 2.5 *N* ammonium hydroxide (rate 3 ml/min) and the eluate (approx. 50 ml) evaporated to dryness under reduced pressure at 40°. The residue was dissolved in a small volume of distilled water (100 μl and 300 μl in the case of gut and muscle, respectively).

In some experiments the entire worm was frozen in liquid air, ground to a fine

powder in a cooled mortar and extracted with cold perchloric acid as before. The extract was clarified by centrifugation at 0° and immediately adjusted to pH 7.2 by the addition of 2.5 *N* KOH. The precipitated $KClO_4$ was removed by centrifugation at 0° and samples taken from the supernatant for the isolation of SEP and lombricine for the determination of specific radioactivities as described below.

Another sample of the neutralised extract was taken for separation into the "Ba-insoluble" and "Ba-soluble" P fractions as described by LE PAGE[8]. The Ba-insoluble salts were dried under reduced pressure, suspended in a small volume of water and stirred with 2 g of Zeokarb-225 (Na^+ form) until Ba^{++} free as judged by a negative test with Na rhodizonate. The solution was separated from the resin by filtration and the resin washed with a small volume of water. The washings were combined with the filtrate and samples from the resultant solution taken for determination of the specific radioactivities of inorganic phosphate, and the P released as a result of hydrolysis in *N* HCl for 7 min at 100° (P_7)[9]. This latter P fraction has been taken to represent primarily the labile P of ATP.

The "Ba-soluble" fraction was isolated from the supernatant after removal of the Ba-insoluble salts by the addition of four volumes of ethanol. It was dried under reduced pressure, redissolved in water and reprecipitated by the addition of ethanol. The reprecipitated material was dissolved in water and converted to the Na form by passage through a column (1 × 5 cm) of Zeokarb-225 (Na^+ form) ion exchange resin. Samples were removed from the resultant solution for determination of the specific radioactivities of residual inorganic phosphate, and P released (P_9) by hydrolysis for 9 min at 65° in 0.1 *N* HCl (*cf.* ENNOR AND ROSENBERG[9]).

In addition to determination of the amount of P released by such treatment, estimations were made of the amount of lombricine released. The molar ratio P: lombricine varied from 0.97 to 1.01 and thus the P_9 was assumed to have its origin in the phosphoryl group of N-phosphoryllombricine.

The specific radioactivities of the phospholipids and phosphoprotein P were determined after fractionation of the acid-insoluble material by a procedure similar to that of SCHMIDT AND THANNHAUSER[10]. The acid-extracted material was washed repeatedly with *N* $HClO_4$ at 0° until the wash no longer showed radioactivity[11]. The pellet was then extracted with lipid solvents as described by SCHMIDT AND THANNHAUSER[10], and dried under reduced pressure. The fat-free material was hydrolysed in *N* KOH at 37° for 16 h and the inorganic phosphate precipitated according to DELORY[12].

The lipid extracts were combined and dried under reduced pressure at 40°. The lipid was re-extracted with petroleum ether (boiling range: 40°–60°) and the volume of the extract was reduced to 1–2 ml. Acetone (20 ml) was then added, followed by 2 drops of a saturated solution of $MgCl_2$ in ethanol. The precipitated phospholipids were collected by centrifugation, dried and wet ashed.

Chromatography

The entire sample of the concentrated, de-salted gut extract, and 100 μl of the corresponding muscle extract, were used for two-dimensional chromatography (descending) on Whatman No. 3 paper (56 × 56 cm). The solvent pair employed for the separation of lombricine, SEP and the free amino acids was ethanol–formic acid–water (70:10:20, v/v) followed by phenol–water (4:1, w/v). In addition to these,

other standard solvent systems were used for the identification of the amino acids referred to in Figs. 1 and 2.

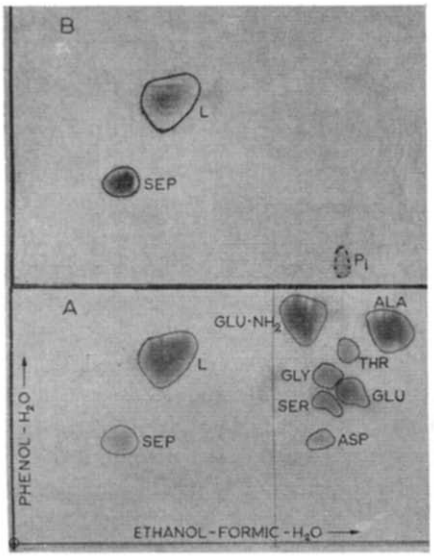

Fig. 1. A. Chromatogram of extract of earthworm muscle prepared 8 days after the administration of [^{32}P]inorganic phosphate ($1.1 \cdot 10^6$ counts/min/g body weight). SEP, serine ethanol amine phosphodiester; L, lombricine; Glu-NH_2, glutamine; Ala, alanine; Thr, threonine; Gly, glycine; Glu, glutamic acid; Ser, serine; Asp, aspartic acid. B. Radioautograph of chromatogram shown in A. P_i, inorganic P. The lines encircling the ninhydrin-stained areas of SEP and lombricine (L) on the chromatogram were superimposed on the radioautograph.

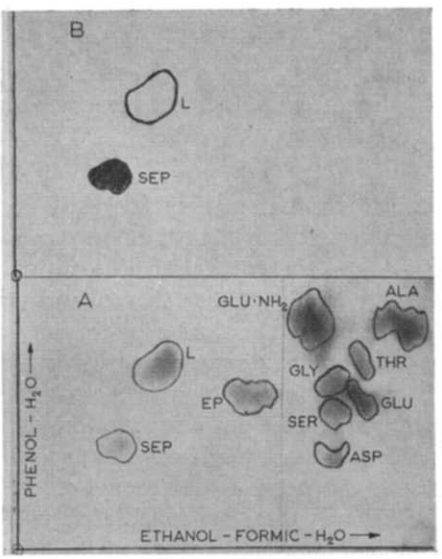

Fig. 2. A. Chromatogram of extract of earthworm gut prepared 8 days after the administration of [^{32}P]inorganic phosphate ($1.1 \cdot 10^6$ counts/min/g body weight). Abbreviations as in Fig. 1. B. Radioautograph of chromatogram shown in A. Abbreviations as in Fig. 1. The lines encircling the ninhydrin-stained areas of SEP and lombricine (L) on the chromatogram were superimposed on the radioautograph.

Chromatograms were developed by spraying with a 0.2 % (w/v) solution of ninhydrin in acetone and heating at 70° for 10 min.

Radioautography

Radioautographs were obtained by exposing Ilford Industrial G film to the chromatograms for 14 days.

Analytical methods

Lombricine was estimated before and after hydrolysis by the procedure described by ROSENBERG, ENNOR AND MORRISON[13] and the specific radioactivity of inorganic phosphate (P_i) according to ENNOR AND ROSENBERG[9].

In order to estimate the amounts and specific radioactivity of P in SEP and lombricine the areas containing these compounds were cut from the developed chromatograms and the compounds eluted with water. The eluates were taken to dryness and the residue ashed with 0.5 ml of a mixture of 2 volumes of 70 % perchloric acid and 3 volumes of concentrated H_2SO_4. The ashed residue was diluted with 2 ml of

water, heated at 100° for 1 h to convert any pyrophosphate into orthophosphate and the specific radioactivity determined as described above.

Radioactivity

Radioactivity was estimated in an M6 liquid counter (20th Century Electronics) and the counting rate was corrected both for background counting rate and decay. Specific radioactivities were expressed as counts/min/μmole.

RESULTS

Typical chromatograms for muscle and gut are presented in Figs. 1A and 2A respectively. It is evident that the muscle extract contains more lombricine than SEP, whereas in the gut extract the two compounds are present in similar amounts. It will also be noted that the amount of SEP (and lombricine) in the gut extract is approximately the same as is the amount of SEP in muscle extract.

The radioautographs corresponding to the chromatograms are shown in Figs. 1B and 2B. In both figures the outlines of the ninhydrin-stained areas on the chromatograms were superimposed on the radioautographs. For muscle tissue (Fig. 1) the darkening of the X-ray film by lombricine was less than that produced by SEP, despite the fact that the amount of lombricine was greater. The specific radioactivity of SEP was therefore much greater than that of lombricine. The intensity of the darkening produced by gut SEP (Fig. 2) was considerably greater than that due to muscle SEP (Fig. 1). Since these two compounds were present in similar amounts, it was concluded that the specific radioactivity of gut SEP was greater than that of muscle SEP.

The labelling of gut lombricine is not shown in the reproduction of the radioautograph, but in the original X-ray film a definite darkening could be seen. A faint spot due to [^{32}P]inorganic phosphate not completely removed in the preparative procedure is visible in the muscle radioautograph. No other radioactive areas were detected.

The above findings are substantiated by the quantitative data (Table I). Whereas the specific radioactivity of the gut inorganic P fell progressively, there was no significant change in the specific radioactivity of the muscle inorganic phosphate after the second day. The specific radioactivities of both SEP and lombricine in muscle

TABLE I

INCORPORATION OF [^{32}P]INORGANIC PHOSPHATE INTO SEP AND LOMBRICINE OF MUSCLE AND GUT OF THE EARTHWORM

Radioactivity administered, $1.1 \cdot 10^6$ counts/min/g body weight.

Time after ^{32}P (days)	*Specific radioactivity (counts/min/μmole)*					
	Muscle			*Gut*		
	P_i	*SEP*	*Lombricine*	P_i	*SEP*	*Lombricine*
1	17,000	270	11	56,700	2,190	40
2	11,700	560	20	29,500	4,310	160
4		840	97		4,990	100
9	12,900	2,140	140	13,400	10,500	470
16	13,000	2,640	250	10,600	7,160	310

rose progressively with time, but the specific radioactivity of SEP was at all times much greater than that of lombricine. The specific radioactivities of both SEP and lombricine in gut were at all times greater than those of the corresponding compounds in muscle, and again the specific radioactivity of SEP was greater than that of lombricine.

Quantitative estimation of the concentration of SEP and lombricine present in muscle and gut indicates (Table II) that in muscle the concentration of lombricine was considerably greater than that of SEP. In gut on the other hand the concentration of SEP and lombricine were of the same order, and similar to the concentration of SEP present in muscle.

TABLE II

CONCENTRATION OF SEP AND LOMBRICINE IN MUSCLE AND GUT OF THE EARTHWORM

μmoles/100 g tissue.

Muscle		*Gut*	
SEP	*Lombricine*	*SEP*	*Lombricine*
15.3	176	10.6	23.8

Some experiments have been carried out in which the relative rates of ^{32}P incorporation into inorganic phosphate, the labile P of ATP (P_7), the labile P of N-phosphoryllombricine (P_9), phosphoprotein and phospholipid P also were determined (Table III).

TABLE III

INCORPORATION OF [^{32}P]INORGANIC PHOSPHATE INTO CERTAIN PHOSPHORUS-CONTAINING COMPOUNDS OF THE EARTHWORM

Radioactivity administered, $1.1 \cdot 10^6$ counts/min/g body weight.

Time after ^{32}P (days)	*Relative specific activity (based on P_i = 100)*					
	P_7 (labile P of ATP)	*P_9 (labile P of N-phosphoryl-lombricine)*	*SEP (diester P)*	*Lombricine (diester P)*	*Phosphoprotein (DELORY[12])*	*Phospholipid P (lipid sol.)*
2	23	—	0.44	0.012	4.5	0.8
2	78	53	0.86	0.073	—	—
2	44.5	36.2	3.98	0.19	—	—
4	173	175	—	0.26	—	—

After two days the relative specific radioactivities of P_7 and P_9 were similar, but less than that of the inorganic P. After four days, although these specific radioactivities were still similar to each other, they were greater than that of inorganic phosphate.

The specific radioactivities of SEP, lombricine, phosphoprotein and phospholipid P were all considerably less than that of the labile P. Whereas the specific radioactivity of SEP was of the same order as that of phosphoprotein and phospholipid P, that of lombricine was much less, in confirmation of the results reported in Table I.

DISCUSSION

The results of the present experiments show that [^{32}P]inorganic phosphate is incorporated into the diester P of both SEP and lombricine. The degree to which such incorporation occurred is consistent with the hypothesis that SEP is a precursor of lombricine in the earthworm and not a degradation product of that substance. This conclusion stems from the fact that the amount of ^{32}P incorporated into lombricine was, at each of the time intervals investigated, less than 0.1 of that which was found in the SEP.

In similar experiments with reptiles AYENGAR AND ROBERTS[14] were unable to detect any incorporation of [^{32}P]inorganic phosphate into muscle SEP, but low levels of radioactivity were reported to be present in kidney SEP 48–72 h after injection. On the basis of these results these workers have concluded that ^{32}P was incorporated very slowly into SEP and that this compound was not synthesised from low molecular weight precursors. Such a finding is in contrast with the present results, where incorporation of [^{32}P]inorganic phosphate occurred in both the muscle and gut of the earthworm. The present findings are also supported by other experiments from this laboratory[15] with [^{14}C]serine, [^{14}C]ethanolamine and [^{3}H]serine, which afford further evidence that in the earthworm SEP is synthesised from low molecular weight precursors.

Some comparison of the metabolic activities of reptilian and earthworm SEP may be obtained from the relative rates of ^{32}P incorporation into SEP and phospholipid P in each species. According to AYENGAR AND ROBERTS[14], reptilian SEP contained no detectable label at the time when phospholipid P attained considerable radioactivity. On the other hand, in the earthworm the labelling of SEP was similar to that of the phospholipid (*cf.* Table III).

There is thus a difference in the relative rates of formation of SEP and phospholipid in the two species. It should, however, be pointed out that SEP in the reptilian muscle contains the L-isomer of serine[16], while in the earthworm the D-enantiomorph[2] is present. It is probable, therefore, that SEP has a different function in each of these species—in fact, one function of earthworm SEP is to give rise to lombricine. This is not shared by reptilian SEP, for lombricine was not detected in any of the reptiles studied (unpublished observations).

The size of the constituent organs of the earthworm has precluded more than a rough breakdown into muscle and gut, which latter comprises gastro-intestinal tract, gonads, pseudo-hearts, etc. Nevertheless large differences in the specific radioactivities of the diester P of SEP and lombricine have been found as between these two tissues and in all cases the higher values were found in the compounds isolated from the gut. It may be concluded therefore that SEP is formed in the gut.

The rate of incorporation of ^{32}P into SEP (and lombricine) is extremely slow compared to the rate of incorporation of this isotope into the labile P of ATP and is indicative of a very slow turnover rate of SEP in the earthworm. The present experiments do not give any precise indication of the speed with which the labile P of ATP or the phosphoryl group of N-phosphoryl lombricine is replaced. It is however of some interest to note that the relative specific radioactivities of the labile P of ATP and N-phosphoryl lombricine are approximately equal—such a result would be expected, for the relationship between these groups is determined by a phosphoryltransferase, some properties of which have recently been described[17,18].

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STUDIES ON THE INCORPORATION OF THYMIDINE INTO DNA BY RAT-LIVER HOMOGENATES *IN VITRO*

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SUMMARY

[^{3}H]thymidine is incorporated into DNA by homogenates of normal and 24 h regenerating liver. Regenerating liver homogenates are somewhat more active than normal liver homogenates. The incorporation of thymidine is not inhibited by ethionine, *p*-fluorophenylalanine, or β-2-thienylalanine when these compounds are added to incubation flasks in solid form. However, chloramphenicol inhibits incorporation by 19 to 91 % at concentrations ranging from 0.38 m*M* to 6.21 m*M*.

The ability of both normal and regenerating liver homogenates to incorporate thymidine into DNA is markedly inhibited by the injection of ethionine 8–14 h before the liver is excised. This suggests that the injected ethionine prevents the synthesis of an enzyme necessary for thymidine incorporation, or inhibits its activity.

INTRODUCTION

It has been demonstrated that the incorporation of [^{3}H]thymidine into regenerating rat liver DNA *in vivo* can be strongly inhibited by the intraperitoneal injection of

Abbreviation: DNA, deoxyribonucleic acid.