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A New Phosphagen, *N'*-Phosphorylguanidinoethylphospho-*O*-(α-*N*,*N*-dimethyl)serine (Phosphothalassemine)†

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ABSTRACT: A new guanidine compound, L-thalassemine, the corresponding phosphagen, phospho-L-thalassemine, and the corresponding phosphagen kinase, ATP:L-thalassemine phosphotransferase, have been found in the body wall muscle of an echiuroid worm, *Thalassema neptuni*. L-Thalassemine has been isolated from *Thalassema* muscle as the free-base monohydrate: $C_8H_{20}N_4O_6P \cdot H_2O$, mp 184°, $[\alpha]_D^{26}$ −11.2°. Its structure was elucidated as the guanidinoethylphospho-*O*-(α-*N*,*N*-dimethyl)serine or α-*N*,*N*-dimethyllombricine and confirmed by synthesis from L-lombricine. Lombricine (guanidinoethylphospho-*O*-serine), which is likely to be the precursor of thalassemine, was isolated from *Thalassema* viscera and found to be of the same L series as thalassemine from *Thalassema* muscle and as lombricine isolated from another echiuroid, *Urechis caupo*, in contrast with lombricine present in various oligochetous and polychetous annelids, which was shown to be the D isomer. The biological origin of the methyl groups of thalassemine from L-[*methyl*-^{14}C]methionine in *T. neptuni* was established from *in vivo* experiments. Phosphothalassemine was identified in *Thalassema* muscle. The phosphagen kinase responsible for the phosphorylation of thalassemine was characterized in the same tissue and some properties of the enzyme were investigated.

The mono- and disubstituted guanidines which can be phosphorylated into muscular phosphagens are amino acid (arginine) or amino acid degradation products, guanidinoacetic acid and creatine originating from glycine, and amidinohypotaurine (hypotaurocyamine) and amidinotaurine (taurocyamine) from cysteine. Another compound issued from serine, guanidinoethanol, is phosphorylated into guanidinoethyl phosphate which is either esterified with serine giving rise to guanidinoethylphospho-*O*-serine (lombricine) (I) or methylated by methionine into guanidinoethyl methyl phosphate (opheline) (II) (see review by Thoai and Robin, 1969).

Actually opheline is found only in a marine polychete, *Ophelia neglecta* (Thoai *et al.*, 1963), while lombricine, isolated for the first time from *Lumbricus terrestris* (Thoai and Robin, 1954), is widely distributed in all oligochetes (Robin and Roche, 1965), in polychetes (Thoai *et al.*, 1963), and in two echiuroids, *Urechis caupo* (Robin, 1964) and *Bonellia viridis* (Thoai *et al.*, 1967).

In the muscle of the last worm, lombricine is present only in traces. The main muscular product is a β-aspartyllombricine, called bonellidine (III) (Thoai *et al.*, 1967). Even though the N'-phosphorylated derivative has not been found in *Bonellia*, it may be presumed that bonellidine, the only guanidine com-

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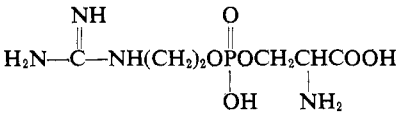
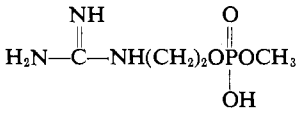


I



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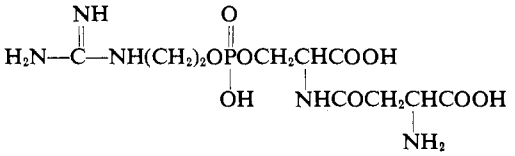


III

pound present in the muscle, would participate in the formation of the phosphagen of this echiuroid.

From the characterization of the three phosphoric diesters, two of these from echiuroids, it may be asked if other analogous products could be found, specially in this group of worms.

The work was started with another echiuroid, *Thalassema neptuni*, living in cold marine coasts, contrary to the close species, *B. viridis*, which lives in less cold mediterranean water. From the muscle of *Thalassema*, a new guanidine derivative was isolated, as a free compound and as a labile phosphorylated one (phosphagen). The new products, called thalassemine and phosphothalassemine, were respectively identified as a lombricine derivative, dimethylated at the α-amino group of the serine moiety, and as *N'*-phosphorylguanidinoethylphospho-*O*-(α-*N*,*N*-dimethyl)serine.

The precursor of the new compound, lombricine, was isolated from *Thalassema* viscera and its optical properties exhibit interesting similarities or differences with lombricine from oligochetes, polychetes, and echiuroids.

Administration of L-[*methyl*-^{14}C]methionine to living *T. neptuni* yielded radioactive thalassemine, which was localized in muscle.

Thalassemine was phosphorylated from ATP into the corresponding phosphagen by the muscle extract, the specificity of which was characterized.

Experimental Procedures

Materials. L- and D-guanidinoethylphospho-*O*-serine (L- and D-lombricine) were the natural products isolated from *U. caupo* and *L. terrestris*, respectively, according to Robin (1964). Guanidinoethyl phosphate and guanidinoethylmethyl phosphate (opheline) were prepared as described by Thoai *et al.* (1963). Taurocyamine was obtained through amidination of taurine with *S*-methylisothiourea (Schütte, 1943). Hypotaurocyamine was prepared according to Desvages and Thoai (1968). ATP (A grade) was supplied by Calbiochem. All other chemicals were of the best analytical grade and were obtained from B.D.H., Calbiochem, and Merck Co. L-[*methyl*-^{14}C]Methionine (specific activity 50.5 Ci/mole) was obtained from the C.E.A. (Saclay, France). Cellulose plates for thin-layer chromatography were purchased from Carl Schleicher & Schüll, D-3354, Dassel, Germany. Pure lombricine kinase was prepared from *L. terrestris* muscle (Thoai *et al.*, 1970). The marine worms, *T. neptuni* and *Ophelia bicornis*, were collected on the Atlantic coast of Brittany, and kindly supplied by Professor L. Amoureux, Department of Zoology, Catholic University, Angers, France, and by the Marine Laboratory, Concarneau, France.

Analytical Procedures. Tissue extracts for thin-layer chromatography were prepared by extracting the tissues with three parts (v/w) of 2% acetic acid. Thin-layer chromatography was operated on cellulose plates. The solvents and the location reagents (α-naphthol–hypobromite reagent for monosubstituted guanidines, α-naphthol–diacetyl reagent for *N,N*-disubstituted guanidines, ninhydrin reagent for primary amines, molybdic acid–SH_2 reagent for phosphoric compounds) were as previously described for paper chromatography (Robin, 1964).

High-voltage electrophoresis was conducted on Whatmann No. 1MM paper, in a Shandon Gross type apparatus. The migration was effected for 60 min at 5000 V in the following buffers: 0.5% pyridine, 5% acetic acid (pH 3.5), 10% pyridine, 0.4% acetic acid (pH 6.5), or 0.05 M sodium borate (pH 9.2). The location reagents were those used for thin-layer chromatography.

Optical rotations were determined at the temperature indicated on a Perkin-Elmer 141 polarimeter, in water.

Guanidine compounds were estimated by the diacetyl–α-naphthol method (Rosenberg *et al.*, 1956). Phosphoric compounds were estimated as described by Thoai *et al.* (1970). The labile phosphate and thalassemine from phosphothalassemine were estimated through determination of the products liberated by acid hydrolysis (0.1 N HCl, 1 min, 100°). The impure barium salt of the phosphagen was extracted with cold water and barium was eliminated as the insoluble sulfate; phosphate and thalassemine were separately titrated in the extract before and after acid hydrolysis.

The ^{14}C samples were counted in a Model 3003 Packard Tri-Carb scintillation spectrometer, using the Bray (1960) liquid scintillator.

Isolation of Crystallized L-*Thalassemine from Thalassema Muscle.* Frozen *Thalassema* muscle (84 g) was homogenized in a Sorvall homogenizer with three volumes (v/w) of 2% acetic acid. The homogenate was heated for 5 min in boiling water, cooled, and centrifuged. The supernatant was reserved and the residue was reextracted two times with one volume of 1% acetic acid according to the same process. The supernatants were combined, diluted with two volumes of deionized water, and applied to a column of Dowex 50-X2 100–200 mesh H^+ form (1.8 × 38 cm) at a flow rate of 80 ml/hr. The column was washed with water and eluted with 1 N ammonia. Fractions of 5 ml were collected and analyzed by thin-layer chromatography. Those containing thalassemine were pooled and concentrated under vacuum. The residue was dissolved in 7 ml of 0.04 M pyridine–0.4 M formic acid buffer (pH 2.6); the mixture was adjusted to pH 2.6 with concentrated HCl, centrifuged, and applied to a column of Dowex 50-X4 200–400 mesh pyridine form (3.2 × 65 cm) equilibrated with the above pyridine–formate pH 2.6 buffer. The column was developed with the same buffer, 10-ml fractions being collected. The breakthrough of the developer occurred at 1 l. The effluent was analyzed by thin-layer chromatography and the thalassemine-containing fractions were concentrated under vacuum. The residue was dissolved in a small volume of 0.05 M acetic acid and applied to a column of phosphocellulose H^+ form (1.25 × 42 cm) equilibrated with 0.05 M acetic acid. The column was developed with 0.05 M acetic acid and the effluent was collected in 2-ml fractions at a flow rate of 20 ml/hr. Fractions 36–71, containing thalassemine freed from ninhydrin-reacting impurities, were concentrated under

vacuum, and the product was recrystallized from water by addition of methanol: yield 50 mg, mp 184°, $[\alpha]_D^{26}$ −11.2° (*c* 0.980, H_2O). *Anal.* Calcd for $C_8H_{20}N_4O_6P \cdot H_2O$: C, 30.38; H, 6.69; N, 17.71; P, 9.79; H_2O, 5.69. Found: C, 30.90; H, 6.70; N, 18.70; P, 10.20; H_2O, 5.30.

Isolation of Crude Phosphothalassemine from Thalassema Muscle. The phosphagen was isolated from 10 g of *Thalassema* muscle as the water-soluble ethanol-insoluble barium salt according to Robin (1964) and purified as follows. The crude barium salt was extracted by, successively, 1 and 0.5 ml of ice-cold water and the insoluble fraction was discarded by centrifugation in the cold. The combined supernatants were added with 0.2 ml of 20% Br_2Ba, the pH being maintained slightly alkaline with NaOH (pink to phenolphthalein). After 30 min at 0°, the mixture was centrifuged, the precipitate was discarded, and the supernatant was added with 1.5 ml of absolute ethanol at −10°. After standing 15 min at −10°, the precipitate was collected by centrifugation, washed with ethanol and ether, dried under vacuum, and kept in the cold. The yield of phosphagen in the purified material was low (about 1%), but the mixture contained no free guanidine base nor free phosphate and was suitable for the identification of phosphothalassemine.

Isolation of L-*Lombricine from Thalassema Viscera. Thalassema* viscera (150 g), collected at 0°, were homogenized with two volumes (v/w) of 2% acetic acid, heated for 5 min in boiling water, cooled, and centrifuged at 34,800*g*. The residue was reextracted twice with one volume of 1% acetic acid as above. The combined supernatants were diluted with two volumes of deionized water and adsorbed on a column of Dowex 50-X8 20–50 mesh H^+ form (1.96 × 50 ml). The column was washed with deionized water and eluted with 1 M ammonia, the elution of lombricine being controlled by thin-layer chromatography. The lombricine-containing fractions were evaporated under vacuum, the residue was taken up in 9 ml of a 0.04 M pyridine–0.4 M formic acid mixture (pH 2.6), adjusted to pH 2.6 with concentrated HCl, and the insoluble fraction was centrifuged. The supernatant was placed on a column of Dowex 50-X4 200–400 mesh pyridinium form (3.2 × 55 cm) previously equilibrated with the above pyridine–formic acid mixture (pH 2.6). The column was developed with 900 ml of the same mixture (pH 2.6) and thereafter with 900 ml of a 0.1 M pyridine–0.3 M formic acid mixture (pH 3.1). The elution of lombricine occurred at the end of the second developer. The Sakaguchi-positive fractions, contaminated with a number of ninhydrin-reacting compounds, were concentrated under vacuum; the residue was dissolved in a small volume of 0.05 M acetic acid, placed on a column of phosphocellulose (1.25 × 42 cm) previously equilibrated with 0.05 M acetic acid. Lombricine, eluted from the column with the same acetic acid solution and still containing traces of ninhydrin-positive impurities, was concentrated under vacuum. The residue was taken up in a minimal volume of water and sparingly added with ethanol. Lombricine crystallized from the ethanol–water mixture before the contaminating compounds, which were completely removed after three recrystallizations: yield 5 mg (mp 233°), $[\alpha]_D^{25}$ −11.3° (*c* 0.380, H_2O).

Isolation of D-*Lombricine from Ophelia bicornis Muscle. Ophelia bicornis* muscle (25 g) was extracted and the extract was fractionated as described above for the isolation of lombricine from *Thalassema* viscera: yield 11 mg (mp 229–230°), $[\alpha]_D^{25.4}$ +14.4° (*c* 0.917, H_2O).

Synthesis of L-*Thalassemine* (*α-N,N-Dimethylombricine*). The compound was synthesized from L-lombricine through catalytic reductive condensation with formaldehyde (Bowman and Stroud, 1950). L-Lombricine (67.5 mg; 0.25 mmole) and formaldehyde (15 mg; 0.5 mmole) were dissolved in 5 ml of water and 135 mg of 5% palladium on charcoal was added. The mixture was hydrogenated for 5 hr at room temperature, centrifuged at 34,800*g*, and the residue was washed repetitively with 0.05 M acetic acid. The supernatants, containing *N,N*-dimethyllombricine besides small amounts of *N*-monomethyllombricine as shown by thin-layer chromatography, were concentrated under vacuum and fractionated on a column of phosphocellulose (1.1 × 27 cm) previously equilibrated with 0.05 M acetic acid and developed with the same solvent. Fractions of 2 ml were collected at a flow rate of 20 ml/hr. *N,N*-Dimethyllombricine (fractions 16–22) was collected free from *N*-monomethyllombricine (fractions 26–31), concentrated under vacuum, and recrystallized from water and methanol: yield 35 mg (44%), mp 184°, $[\alpha]_D^{26}$ −11.3° (*c* 0.919, H_2O). *Anal.* Calcd for $C_8H_{20}N_4O_6P \cdot H_2O$: C, 30.38; H, 6.69; N, 17.71; P, 9.79. Found: C, 30.30; H, 7.00; N, 17.60; P, 9.50.

Enzymatic Assays. The ATP:guanidine phosphotransferase activity of *T. neptuni* muscle on various guanidine compounds was assayed on a crude extract prepared as follows. Muscle (1 g) was homogenized with 3 ml of cold 10^{-3} M EDTA, and the mixture was stirred 15 min at 0° and centrifuged; the supernatant was diluted $^1/_{75}$th in 0.05 M glycine–NaOH buffer (pH 8.5) just before the determination of the enzymatic activity. The activity of lombricine kinase from *L. terrestris* muscle was assayed on the purified enzyme. The standard conditions for the determination of the enzymatic activity were as described by Thoai *et al.* (1970), except that the final concentration of the guanidine substrate in the reaction mixture was 0.05 M. For a final volume of 1 ml, the addition of enzyme was 0.1 ml of crude extract diluted $^1/_{75}$th for *Thalassema* muscle assays and 0.1 ml of enzyme containing 0.5 μg of protein for the *Lumbricus* enzyme assays.

In Vivo Experiments with L-[*methyl*-^{14}C]*Methionine.* Thirteen *T. neptuni* (total weight 17 g) received into the celomic cavity a total of 40 μCi of L-[*methyl*-^{14}C]methionine dissolved in 0.48 ml of seawater. After standing 24 hr in seawater at +6°, the animals were sacrificed and the body wall muscle and viscera were collected. Both tissues were separately extracted and the extracts were fractionated as described above for the isolation of thalassemine from *Thalassema* muscle. The volumes of the ion exchangers utilized were respectively 5 ml for the column of Dowex 50-X2 H^+ 100–200 mesh (0.8 × 10 cm), 22 ml for that of Dowex 50-X4 200–400 mesh pyridine form pH 2.6 (1.6 × 11 cm), and 3 ml for that of phosphocellulose equilibrated with 0.05 M acetic acid (0.8 × 6 cm), the elution volumes being reduced proportionally. The 0.3-ml fractions collected from the phosphocellulose column were analyzed by chromatography to detect the peak of thalassemine and thalassemine was estimated in the positive fractions. Accordingly, the radioactivity of the effluent was measured by liquid scintillation.

Results and Discussion

Distribution of Guanidine Compounds in Thalassema Tissues. The results obtained from thin-layer chromatographic analysis of tissue extracts are summarized in Table I. *Thalassema* muscle was shown to contain a new biological monosubstituted guanidine compound which was called thalassemine. That compound was the only guanidine derivative present in muscle extracts, and was found only in that tissue. No thalassemine was detected in viscera, which contained, on the other

hand, substantial amounts of lombricine and some arginine. The presence of thalassemine as the unique guanidine component in *Thalassema* muscle suggested that it might play the role of phosphate acceptor in that tissue. As will be shown below, that hypothesis was confirmed by isolating and identifying the compound and establishing its contribution to the formation of the muscular phosphagen in *T. neptuni*.

The presence of lombricine and arginine in viscera and eggs is not exceptional in worms (Thoai and Robin, 1969). In the same way, the presence of creatine in spermatozoa is a common phenomenon among these animals. In most worms, as in many invertebrates, mature testes and spermatozoa are loaded with creatine, which acts as phosphoryl acceptor for ciliated spermatozoa (Greenwald, 1946; Roche *et al.*, 1957). But what is noticeable in Table I, besides the strict distribution of thalassemine in muscle, is the presence of three different guanidine compounds, thalassemine, lombricine, and creatine, in muscle, eggs, and spermatozoa, respectively, presuming the presence of three different phosphagens in these tissues. Usually, the muscle phosphagen in worms appears to be the same either as that of eggs or spermatozoa, the only exception being *O. neglecta* which seems to contain different phosphagens in body wall muscle, eggs, and spermatozoa (Thoai and Robin, 1969). *T. neptuni* provides therefore a new example of the diversity of the distribution of guanidine compounds in worms.

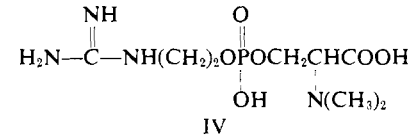
Properties and Structure of Thalassemine. Crystallized thalassemine isolated from *T. neptuni* muscle was obtained as the free-base monohydrate: sharp needles, mp 184°. The natural product is the L isomer, $[\alpha]_D^{26}$ −11.2° (water), freely soluble in water and insoluble in absolute methanol and usual organic solvents. The general formula $C_8H_{20}N_4O_6P \cdot H_2O$ was deduced from the elementary analysis (Found: C, 30.90; H, 6.70; N, 18.70; P, 10.20; H_2O, 5.30). The compound reacted with the α-naphthol–hypobromite reagent (Sakaguchi, 1925) specific for monosubstituted guanidines and with the molybdic acid–SH_2 reagent (Hanes and Isherwood, 1949) characteristic for phosphoric compounds; it did not react with the ninhydrin reagent, nor with 2,4-dinitrofluorobenzene (Dubin, 1960), which excluded the presence of a primary or secondary amino group.

Acid hydrolysis of thalassemine (1 N HCl, 100°) yielded a monosubstituted guanidino compound which was chromatographically identified with β-guanidinoethyl phosphate $C_3H_9N_3O_4P$, suggesting for the unknown moiety of thalassemine the composition $C_5H_{13}NO_3$. From three possible structures (*N*-methylthreonine, *N*-methylhomoserine, and *N,N*-dimethylserine) only the last one was compatible with the absence of primary and secondary amino group in thalassemine, as shown by the lack of reaction with ninhydrin and with 2,4-dinitrofluorobenzene. In that case, thalassemine would be the guanidinoethylphospho-*O*-(α-*N,N*-dimethyl)serine, that is the α-*N,N*-dimethyl derivative of lombricine.

The identity of thalassemine with α-*N,N*-dimethyllombricine was established by comparison to the synthetic product prepared from L-lombricine (see Experimental Procedures). The two compounds had the same melting point (184°) and the mixture melting point (184°) was not lowered. The specific rotation of the natural and of the synthetic products, −11.2 and −11.3°, respectively, were similar, and both compounds had the same chromatographic and electrophoretic behavior in all solvent mixtures and buffers assayed. The infrared (ir) spectra of L-thalassemine and synthetic α-*N,N*-dimethyllombricine were superimposable. We can therefore attribute to thalassemine the structure IV closely related to that

TABLE I: Distribution of Guanidino Compounds in *Thalassema neptuni* Tissues.

Tissue	Guanidino Compound			
	Thalassemine	Lombricine	Arginine	Creatine
Muscle	++++	0	0	0
Viscera	0	++	Traces	0
Eggs	0	+	0	0
Spermatozoa	0	0	0	++



IV

of lombricine (guanidinoethylphospho-*O*-serine) (I) and of bonellidine (guanidinoethylphospho-*O*-(β-aspartyl)serine) (III). All three compounds are *O*-serine esters of guanidinoethyl phosphate, the only difference lying on the substitution at the α-NH_2 of the serine moiety which is unsubstituted in lombricine, monosubstituted by a β-aspartyl residue in bonellidine, disubstituted by two methyl groups in thalassemine.

N-Mono- or dimethyl derivatives of L-serine do not seem to be widely distributed in living organisms. The presence of *N*-monomethyl-L-serine as one of the dominant free amino acids in gilfblaar, *Dichapetalum cymosum*, was reported by Eloff and Grobbelaar (1969), but, to our knowledge, the natural occurrence of *N,N*-dimethylserine free or combined has never been pointed out previously.

As was reported above the specific optical rotation of thalassemine is −11.2° which means that the serine moiety of its precursor in *Thalassema*, lombricine, belongs also to the L series, even though lombricine from oligochetes exhibits an optical rotation of +14.4° (Beatty and Magrath, 1960). Therefore it was interesting to know if the D-serine is specific for oligochetes and if the serine moiety of lombricine from other animal groups is similar to that of oligochetes or to that of aminoethylphospho-*O*-serine detected in the turtle by Roberts and Lowe (1954).

The monosubstituted guanidine isolated from *Thalassema* viscera was identified with lombricine *via* its chromatographic and electrophoretic behavior, and its mp 234° (lombricine from *U. caupo*, mp 236°, mmp 234°). The compound was found to be the L isomer, $[\alpha]_D$ −11.2°, in water.

The results obtained from the determination of the optical rotation of a number of lombricines isolated from different materials and reported in Table II indicate that the products from *Thalassema* and from *Urechis* are of the same L series while these from oligochetes (*Lumbricus*, *Octolasium*, and *Allolobophora*) and from polychetes (*Ophelia*) are the D isomers.

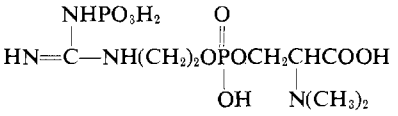
Characterization of Phosphothalassemine in T. neptuni Muscle. The identity of the phosphagen from *Thalassema* muscle with phosphothalassemine was established qualitatively by thin-layer chromatography of the impure barium salt of the phosphagen, comparatively to standards. When an aqueous solution of the phosphagen was chromatographed, no monosubstituted guanidine was detectable on the chromatogram, unless this had been previously heated 1 hr at 85° to

TABLE II: Optical Rotation of Guanidinoethylphospho-*O*-serine Compounds of Various Animal Origin.

Compound	Animal Origin: Phylum	Classes	Genus and Species	Tissue	$[\alpha]_D$ (Water) (deg)	Reference
L-Thalassemine	Echiuroidea		*Thalassema neptuni*	Body wall muscle	−11	This work
L-Lombricine	Echiuroidea		*Thalassema neptuni*	Viscera	−11.3[a]	This work
	Echiuroidea		*Urechis caupo*	Whole animal	−13.9	This work
D-Lombricine	Annelida	Oligocheta	*Allolobophora caliginosa*	Body wall muscle	+14.5	Beatty and Magrath (1960)
	Annelida	Oligocheta	*Lumbricus terrestris*	Body wall muscle	+14	This work
	Annelida	Oligocheta	*Octolasium cyaneum*	Body wall muscle	+14.5	Beatty and Magrath (1960)
	Annelida	Polycheta	*Ophelia bicornis*	Body wall muscle	+14.4	This work

[a] This value is only a rough estimate owing to the small quantity of material available.

hydrolyze the phosphagen and liberate the guanidine moiety. After mild hydrolysis (0.1 N HCl, 100°, 1 min) the phosphagen yielded thalassemine; no other guanidino compound (*e.g.*, lombricine) was detected in the hydrolyzed extract. Quantitatively, the ratio micromoles of labile P per micromoles of labile thalassemine estimated in the phosphagen extract was found to be 1.04 (calcd 1.00). Therefore and by analogy with other phosphagens, the structure V can be assigned to the phosphagen of *T. neptuni* muscle (phosphothalassemine).



The characterization of a new phosphagen, phosphothalassemine, in *T. neptuni* muscle brings to six the number of the phosphagens specific for worms (Annelids, Echiuroids, Sipunculids), besides the unspecific phosphoarginine and phosphocreatine (review by Thoai and Robin, 1969).

Characterization of ATP:Thalassemine Phosphotransferase in Thalassema Muscle. The ATP: guanidine phosphotransferase activity of *Thalassema* muscle was assayed toward a number of guanidine acceptors: L-thalassemine, D- and L-lombricine, opheline, guanidinoethyl phosphate, taurocyamine, hypotaurocyamine, which are interspecific substrates for a number of phosphagen kinases (Thoai, 1968). According to the similarity of structure between thalassemine and lombricine, the specificity of lombricine kinase from *L. terrestris* was comparatively studied toward the same substrates. The results are summarized in Table III.

For both enzymes, the highest activity is obtained with L-lombricine as substrate, although that compound is the natural substrate for none of these enzymes. On the other hand, substantial differences are noticed between the specificity of the two enzymes: thalassemine kinase from *Thalassema* is more active on L-thalassemine than on D-lombricine and inversely lombricine kinase from *Lumbricus* is more active on D-lombricine than on L-thalassemine; the former is only active on guanidinoethyl phosphate derivatives, while the latter exhibits a high activity toward taurocyamine. The efficiency of the guanidine substrates as phosphate acceptors is, by decreasing order, for thalassemine kinase: L-lombricine > L-thalassemine > guanidinoethyl phosphate > D-lombricine > opheline > taurocyamine = hypotaurocyamine, and for lombricine kinase: L-lombricine > D-lombricine > L-thalassemine >

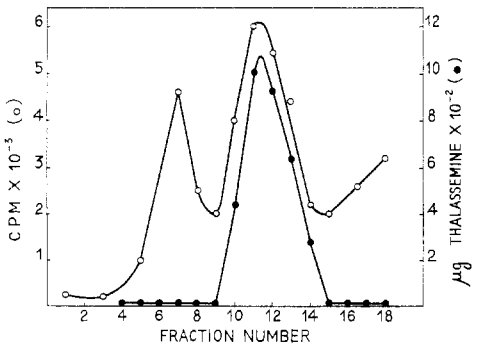


FIGURE 1: Phosphocellulose column chromatography of radioactive thalassemine isolated from *T. neptuni* body wall muscle 24 hr after administration of L-[*methyl*-^{14}C]methionine. Thalassemine was first purified on Dowex 50 columns and then chromatographed on phosphocellulose. Details of the procedure are given under Experimental Procedures. ^{14}C countings and thalassemine determinations were performed on aliquots (0.01 ml) of each column fraction and calculated for the whole fraction (0.3 ml).

TABLE III: Comparative Activity of Thalassemine Kinase from *Thalassema neptuni* and of Lombricine Kinase from *Lumbricus terrestris* toward a Number of Guanidino Substrates.

Thalassemine Kinase: Substrate	Act. (%)	Lombricine Kinase: Substrate	Act. (%)
L-Thalassemine	100	D-Lombricine	100
L-Lombricine	135	L-Lombricine	176
Guanidinoethyl phosphate	45	L-Thalassemine	88
D-Lombricine	40	Taurocyamine	41
Opheline	10	Guanidinoethyl phosphate	15
Taurocyamine	0	Opheline	0
Hypotaurocyamine	0	Hypotaurocyamine	0

taurocyamine > guanidinoethyl phosphate > opheline = hypotaurocyamine.

From these results, it is shown that the enzyme responsible for the phosphorylation of thalassemine in *T. neptuni* muscle is a new phosphagen kinase, which was called ATP: thalassemine phosphotransferase or thalassemine kinase.

Biosynthesis of Thalassemine from L-[*methyl*-^{14}C]*Methionine.* The thalassemine isolated from the body wall muscle of the animals administered *in vivo* with L-[*methyl*-^{14}C]methionine was found to be labeled. The peak of thalassemine and the principal peak of radioactivity detected in the effluent of the phosphocellulose column were exactly superimposable (Figure 1). Furthermore, on all chromatograms and electrophoregrams all of the radioactivity was located on the spot of thalassemine detected with the Sakaguchi reagent.

As precedently noticed with the untreated animals (Table I), no thalassemine was present in the viscera from the animals injected with L-[*methyl*-^{14}C]methionine.

We can therefore conclude that L-methionine is the transmethylating agent responsible for the formation of the methyl groups of thalassemine. The absence of labeled thalassemine in viscera suggests that the methylating process takes place in the muscle tissue, as was precedently demonstrated for the O-methylation of guanidinoethyl phosphate into opheline in *O. neglecta* tissues (Thoai *et al.*, 1964).

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