3. *The Synthesis of* D- *and* L-2-*Amino*-2-*carboxyethyl* 2-N′-*Phosphonoguanidinoethyl Hydrogen Phosphate* (*Phospholombricine*).[1]

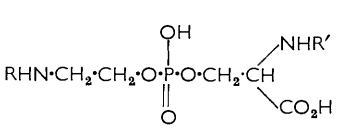
By Iva M. Beatty, D. I. Magrath, and A. H. Ennor.

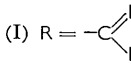
The D- and the L-form of 2-amino-2-carboxyethyl 2-*N*′-phosphonoguanidinoethyl hydrogen phosphate have been synthesised by the reaction of D- or L-2-amino-2-carboxyethyl 2-aminoethyl hydrogen phosphate with *O*-methyl-*N*-phosphonourea in alkaline solution, and have been compared with the natural product isolated from earthworms. Two by-products of the synthesis, 2-aminoethyl 2-carboxy-2-*N*′-phosphonoguanidinoethyl hydrogen phosphate and 2-carboxy-2-*N*′-phosphonoguanidinoethyl 2-*N*′-phosphonoguanidinoethyl hydrogen phosphate, have been identified and characterised. The structure of the *N*-phosphonoguanidino-group of phosphagens is discussed in the light of these results.

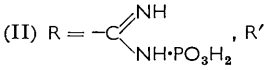
The isolation from earthworms (*Lumbricus terestris*) of the phosphorus-containing guanidine derivative, lombricine, was first reported by Thoai and Robin,[2] who ascribed to it the structure, 2-amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate (I). This structure was confirmed by synthesis [3,4] and the serine portion was shown to have the D-configuration.*,[3-5]

Thoai *et al.* also isolated [2,6] from earthworms an impure *N*-phosphonoguanidino-compound which, on mild acid hydrolysis, yielded equimolecular proportions of phosphoric acid and lombricine. They allocated to this compound the structure, 2-amino-2-carboxyethyl 2-*N*′-phosphonoguanidinoethyl hydrogen phosphate (phospholombricine * (II), and suggested that it acted as a phosphagen in these creatures. Phospholombricine was subsequently isolated from earthworms (*Octolasium cyaneum* and *Allolobophora caliginosa*) as a pure magnesium salt by Ennor and Rosenberg,[7] its properties being consistent with the structure allocated by the earlier workers.

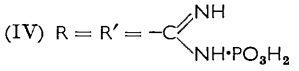
In the present Paper, the synthesis of D- and L-2-amino-2-carboxyethyl 2-*N*′-phosphonoguanidinoethyl hydrogen phosphate is described and a comparison is made between these and the natural product.



(I) R =  , R′ = H.

(II) R =  , R′ = H.

(III) R = R′ = H.

(IV) R = R′ = 

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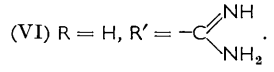
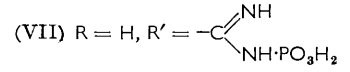
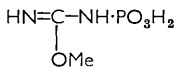
(VI) R = H, R′ =  .

(VII) R = H, R′ = 

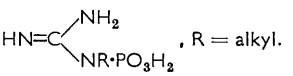
Lombricine has been conveniently synthesised [4] by the reaction of *O*-methylurea with 2-amino-2-carboxyethyl 2-aminoethyl hydrogen phosphate (serine ethanolamine phosphodiester) (III) in alkaline solution. An analogous method for the synthesis of phospholombricine, by use of *O*-methyl-*N*-phosphonourea (VIII),[8] was therefore indicated.

* The terms lombricine and phospholombricine refer herein to either enantiomorph.

[1] Preliminary communication: Beatty, Ennor, and Magrath, *Nature*, 1960, **188**, 1026.
[2] Thoai and Robin, *Biochim. Biophys. Acta*, 1954, **14**, 76.
[3] Beatty and Magrath, *Nature*, 1959, **183**, 591.
[4] Beatty and Magrath, *J. Amer. Chem. Soc.*, 1960, **82**, 4983.
[5] Beatty, Magrath, and Ennor, *Nature*, 1959, **183**, 591; Beatty, Ennor, Rosenberg, and Magrath, *J. Biol. Chem.*, 1961, **236**, 1028.
[6] Thoai, Roche, Robin, and Thiem, *Compt. rend. Soc. Biol.*, 1953, **147**, 1670.
[7] Ennor and Rosenberg, *Biochem. J.*, 1962, **83**, 14.
[8] Cramer and Vollmar, *Chem. Ber.*, 1958, **91**, 919.



(VIII)



(IX)

Treatment of either the D- or the L-form of the phosphate (III) with *O*-methyl-*N*-phosphonourea in aqueous alkali gave a mixture which was separated by ion-exchange chromatography. The major product gave positive colour reactions with the molybdate [9] and ninhydrin spray reagents, indicating the presence of phosphorus and free amino-groups, respectively, but it did not react with the Sakaguchi [10] or α-naphthol–biacetyl [11] reagent, indicating the absence of free guanidino-groups (cf. ref. 1). When it was heated in 0·1N-hydrochloric acid at 100° for 2 minutes, complete conversion into phosphoric acid and a guanidino-base occurred. The latter was identified as lombricine by paper chromatography and electrophoresis, and by its behaviour on degradation by nitrous acid [4] and in 6N-acid [4] at 110°. Potentiometric titration of the product (ammonium salt) gave pK_a values of *ca.* 2, 4·6, and *ca.* 8·8 (cf. lombricine,[3,4] *ca.* 2 and 8·9), consistent with the presence of carboxyl, secondary phosphonohydroxyl-, and α-amino-groups, respectively. Thus it may be concluded that the main product of the reaction is 2-amino-2-carboxyethyl 2-*N*′-phosphonoguanidinoethyl hydrogen phosphate (II).

A product with chemical, chromatographic, and electrophoretic properties, pK_a values, and infrared spectrum (ammonium salt) identical with those of the product described above was obtained (14% yield) by directly phosphorylating D-lombricine with phosphoryl chloride in alkaline solution, a method which has been widely used in the synthesis of phosphagens [12–14] (cf. also ref. 15). The significance of this identity in relation to the structure of synthetic phosphagens prepared in this way is discussed below.

L-Phospholombricine was characterised as its ammonium salt, and the D-isomer as its ammonium, lithium, magnesium, and sodium salts. All save the ammonium salts were difficult to purify for analysis, but satisfactory preparations were finally obtained by precipitation from aqueous, carbon dioxide-free solution at pH 6—6·5 with an organic solvent, usually ethanol. Elementary analysis indicated a high carbon content in all samples and this is attributed to the retention by the material of varying amounts of the organic solvent. The presence of ethanol in the lithium salt of the D-isomer, precipitated from aqueous solution with ethanol, was established by using an analytical procedure [16] involving alcohol dehydrogenase.* Such difficulties in obtaining preparations which give satisfactory analytical results are not uncommon in dealing with phosphagens (cf. refs. 12 and 17).

D-Phospholombricine isolated from earthworms [7] had the same chemical, chromatographic and electrophoretic properties as those of the synthetic products described above and, in addition, its lithium salt had an infrared absorption spectrum identical with that of the lithium salt of synthetic material (D-isomer), thus confirming the structure put forward for phospholombricine by Thoai and his co-workers.

The nature of two by-products of the synthesis of phospholombricine from serine ethanolamine phosphodiester was investigated. One of these did not react on chromatograms with either α-naphthol–biacetyl or ninhydrin but did so with the molybdate reagent. Paper electrophoresis showed that it carried a net negative charge in buffers B—E (see

* Detection of ethanol by the Zeisel determination was unsatisfactory as lombricine itself gave a spuriously positive result.

[9] Hanes and Isherwood, *Nature*, 1949, **164**, 1107.
[10] Archer and Crocker, *Biochim. Biophys. Acta*, 1952, **9**, 704.
[11] Griffiths, Morrison, and Ennor, *Biochem. J.*, 1957, **65**, 612.
[12] Marcus and Morrison, *Biochem. J.*, 1964, **92**, 429.
[13] Thoai and Thiem, *Bull. Soc. Chim. biol.*, 1957, **39**, 355.
[14] Thiem, Thoai, and Roche, *Bull. Soc. Chim. biol.*, 1962, **44**, 285.
[15] Ennor and Morrison, *Physiol. Rev.*, 1958, **38**, 631.
[16] Bonnichsen and Theorell, *Scand. J. Clin. Lab. Invest.*, 1951, **3**, 58.

Table 2). After purification of the compound by ion-exchange chromatography, its magnesium and lithium salts were prepared under the conditions used for the preparation of similar salts of phospholombricine. Analysis of these was consistent with the structure 2-carboxy-2-*N'*-phosphonoguanidinoethyl 2-*N'*-phosphonoguanidinoethyl hydrogen phosphate (IV).

In 0·1N-hydrochloric acid at 100° this material liberated phosphoric acid and a guanidino-base in 2 minutes. The latter base was isolated from the hydrolysate by ion-exchange chromatography. It was identified as 2-carboxy-2-guanidinoethyl 2-guanidinoethyl hydrogen phosphate (V) by analysis, electrophoretic, chromatographic, and " spray " characteristics, and by mixed m. p. with authentic material.* In addition, the two samples behaved in the same way on treatment with 6N-hydrochloric acid at 110° and had the same infrared absorption spectrum.

Like phospholombricine, the second by-product investigated did not react with α-naphthol–biacetyl but did so with the molybdate and the ninhydrin reagent. Its behaviour on paper chromatography and electrophoresis was also very similar to that of phospholombricine, although a clear-cut separation of the two was obtained on electrophoresis in alkaline buffers (see Tables 1 and 2). Hydrolysis of the crude material in 0·1N-acid converted it into a mixture of phosphoric acid and a guanidino-base which exhibited paper chromatographic and electrophoretic behaviour similar to that of lombricine; however, separation of the two was possible in several systems (see Tables 1 and 2). A small amount

TABLE 1.

Compound	R_F values in solvents: A	B	C	D	E	F	G	H
Lombricine (I)	0·31	0·16	0·23	0·28	—	0·32	0·29	0·18
Isolombricine (VI)	0·36	0·19	0·22	0·34	—	0·32	0·29	0·18
2-Carboxy-2-guanidinoethyl 2-guanidinoethyl hydrogen phosphate (V)	0·42	0·22	0·27	0·16	—	0·23	0·32	0·20
Phospholombricine (II)	—	—	—	0·10	0·63	0·18	0·07	0·05
Phosphoisolombricine (VII)	—	—	—	—	0·64	0·17	0·07	0·06
2-Carboxy-2-*N'*-phosphonoguanidinoethyl 2-*N'*-phosphonoguanidinoethyl hydrogen phosphate (IV)	—	—	—	—	—	0·10	0·04	0·05

TABLE 2.

Compound	Migration distance (MD; cm.) * in buffers: A	B	C	D	E
Lombricine (I)	−2·0	−0·9	−1·2	+3·2	+3·5
Isolombricine (VI)	−3·5	−0·9	−1·2	+1·1	+2·1
2-Carboxy-2-guanidinoethyl 2-guanidinoethyl hydrogen phosphate (V)	−3·4	−0·8	−1·2	−2·0	−2·2
Phospholombricine (II)	—	+6·6	+9·2	+9·7	+9·8
Phosphoisolombricine (VII)	—	+6·5	+9·2	+8·6	+8·8
2-Carboxy-2-*N'*-phosphonoguanidinoethyl 2-*N'*-phosphonoguanidinoethyl hydrogen phosphate (IV)	—	+10·1	+13·2	+10·8	+10·3

* + = Migration towards anode. − = Migration towards cathode.

of the base was isolated from the acid hydrolysate by ion-exchange chromatography, and its elementary analysis and behaviour in hot 6N-acid † were consistent with its being 2-aminoethyl 2-carboxy-2-guanidinoethyl hydrogen phosphate (isolombricine) (VI). It may be assumed, therefore, that the by-product itself was 2-aminoethyl 2-carboxy-2-*N'* phosphonoguanidinoethyl hydrogen phosphate (phosphoisolombricine) (VII).

The relative proportions (*ca.* 20 : 1) in which phospholombricine and the isomer (VII)

* The synthesis of 2-carboxy-2-guanidinoethyl 2-guanidinoethyl hydrogen phosphate and 2-carboxy-2-guanidinoethyl dihydrogen phosphate, and the behaviour of these and related compounds towards acid and alkali, will be reported later.

† See footnote on p. 13.

were isolated from the reaction mixture show that *O*-methyl-*N*-phosphonourea reacts preferentially, as does *O*-methylurea,[4] with the terminal amino-group of serine ethanolamine phosphodiester. It is interesting that Cramer *et al.* in their recent synthesis [17,18] of phosphoarginine by reaction of ornithine with *O*-methyl-*N*-phosphonourea, obtained, as a by-product, αδ-di-(*N*′-phosphonoguanidino)valeric acid, analogous in structure to the di-*N*-phosphonoguanidino-compound (IV) obtained in the present synthesis. These authors were unable, however, to detect in their reaction mixtures, the presence of the corresponding isomeric mono-*N*-phosphonoguanidino-compound, *i.e.*, the compound analogous to phosphoisolombricine (VII). Thus here, too, it is the terminal and not the α-amino-group which is the more reactive towards this reagent.

Although the structure (as in II) of the phosphonoguanidino-group of phospholombricine accords with that accepted for other phosphagens, both natural and synthetic, until the application of *N*-phosphonourea [1,15,17,18] and thiourea derivatives [19] to syntheses in this field, no conclusive evidence existed [1]—in the case of phosphagens derived from monoalkylguanidines—to exclude the alternative structure (IX) in which the phosphono-residue is attached to the already substituted nitrogen atom. Further, existing evidence for the identity of natural phosphagens derived from monoalkylguanidines with the respective synthetic products is inconclusive, resting on such data as similarity in behaviour on treatment with 0·1N-acid, comparison of melting points (of the salts; not mixed [13]), or R_F values (in one or two solvent systems [14]), and the fact that some synthetic preparations act as substrates for the respective transphosphorylating enzyme.[14,20]

The results reported here (cf. also ref. 1), together with those recently reported by Cramer and his collaborators,[17] now confirm the accepted formulation for the phosphonoguanidino-group, both for the products formed by the direct phosphorylation of monoalkylguanidines and for natural phospholombricine. The synthetic *N*-phosphono-derivatives of glycocyamine [17,19] and arginine [17,18] prepared by Cramer and his associates, whilst of analogous structure to the above, have not yet been compared with the corresponding natural materials.

Experimental

The techniques for paper chromatography, the detection of phosphorus-containing-,[9] guanidino-,[10,11] and amino-compounds on paper chromatograms and electrophoretograms and in fractions from ion-exchange columns, and for the determination of m. p.s (corrected), and pK_a values were as previously described.[4] Infrared spectra were determined on KBr discs using a Perkin-Elmer model 21 double-beam infrared spectrophotometer equipped with sodium chloride optics. For the detection of *N*-phosphonoguanidino-compounds on paper, the dried papers were kept in an atmosphere of hydrochloric acid vapour for 0·5—1 hr., then hung in a current of air for several hr. before being sprayed with the α-naphthol–biacetyl reagent; [11] this procedure is designated herein as α-naphthol–biacetyl (H^+). The ninhydrin reagent [4] used for the detection of amino-compounds on buffered papers contained 5% of acetic acid. In the assessment of paper chromatographic and electrophoretic homogeneity of samples, several systems and all appropriate spray reagents were used.

Use was made of the following solvent systems: A, acetone–acetic acid–water (2 : 2 : 1); B, ethyl methyl ketone–Methylcellosolve (2-methoxyethanol)–acetic acid–water (40 : 15 : 6 : 24); C, butan-1-ol–acetic acid–water (50 : 20 : 30); D, ethyl methyl ketone–Methylcellosolve–3N-ammonia (2 : 7 : 3); E, methanol–aqueous ammonia (*d* 0·91)–water (60 : 10 : 30); F, propan-1-ol–aqueous ammonia (*d* 0·91)–water (60 : 30 : 10); G, ethanol–M-ammonium acetate buffer, pH 7·2 (70 : 30); H, propan-1-ol–M-ammonium acetate buffer, pH 7·2 (70 : 30); J, propan-1-ol–2M-ammonium acetate buffer, pH 4·7 (70 : 30).

Paper electrophoresis was carried out with Whatman No. 3 MM paper and either an LKB electrophoresis apparatus (type 3276; LKB, Stockholm, Sweden) or an apparatus constructed

[17] Cramer, Scheiffele, and Vollmar, *Chem. Ber.*, 1962, **95**, 1670.
[18] Cramer, Vollmar, and Scheiffele, *Angew. Chem.*, 1960, **72**, 211.
[19] Cramer and Vollmar, *Chem. Ber.*, 1959, **92**, 392.
[20] Morrison, Ennor, and Griffiths, *Biochem. J.*, 1958, **68**, 447; Thoai, Robin, and Pradel, *Biochim. Biophys. Acta*, 1963, **73**, 437.

as described by Gross [21] (plate length 20 in. and pressure 1·5 lb./sq. in.). All migration distances (MD) quoted refer to 0·5-hr. runs on the latter apparatus at a potential gradient of 59 v/cm.; no correction has been made for electro-osmotic flow (towards cathode) which was 0·8—1·0 cm. for buffers A and B, 1·2—1·5 cm. for buffer C, and 1·9—2·2 cm. for buffers D and E. Buffers used were: A, 0·025M-phthalate–hydrochloric acid, pH 2·3; B, 0·05M-disodium hydrogen phosphate (852 ml.)–0·02M-citric acid (1548 ml.), pH 3·9; C, 0·033M-phosphate, pH 7·3; D, 0·05M-borate, pH 9·5; E, 0·05M-borate, pH 10·6. R_F values and MD's are given in Tables 1 and 2, respectively.

Dowex-50W and Dowex-1 (X4, 200—400 mesh; The Dow Chemical Co., Midland, Michigan) and Amberlite CG-120 (type 1, 100—200 mesh; Rohm and Haas Co., Philadelphia, Penn.) ion-exchange resins were used. Solvents were normally evaporated at $<40°/\sim 20$ mm. in a rotary evaporator (W. Buchi, Flaivil, Switzerland).

With all *N*-phosphonoguanidino-compounds infrared band overlap was marked, leading to broad absorption in the 2600—3600, 1580—1700, and 1000—1200 $cm.^{-1}$ regions.

Before elementary analysis, unless otherwise stated, compounds were dried for several days at 20—25°/0·05 mm. over calcium chloride and potassium hydroxide, then for several days over phosphorus pentoxide; all *N*-phosphono-compounds were weighed under anhydrous conditions. Nitrogen was determined by the Kjeldahl method, and total and labile phosphorus (P_T and P_L, respectively, the latter being defined as phosphorus split off by 0·1N-hydrochloric acid at 100° in 3 min.) by King's method,[22] in the former case after digestion of the sample according to the instructions of Ennor and Rosenberg.[7] Metals were determined by atomic absorption spectrophotometry. Lombricine was estimated in 0·1N-acid hydrolysates of phospholombricine by the method of Rosenberg *et al.*; [23] lombricine so liberated is equivalent to the amount of phospholombricine originally present and has been designated herein as L_{eq}. Empirical formulæ given for the magnesium, lithium, and sodium salts of phospholombricine are based on N = 4, any excess of carbon being attributed to the presence of ethanol, and those for the ammonium salts on P = 2 and potentiometric-titration data.

Ethanol was determined in samples of phospholombricine by the method of Bonnichsen and Theorell,[16] except that yeast alcohol dehydrogenase (Nutritional Biochemicals Corporation, Cleveland, Ohio), made up in phosphate buffer (0·01M; pH 7·5), was substituted for liver alcohol dehydrogenase, and the digestion carried out in pyrophosphate buffer (0·01M; pH 8·8). Standard ethanol concentrations were analysed at the same time and acetone-precipitated lithium phospholombricine was used as a control.

O-Methyl-*N*-phosphonourea [8] (VIII) was recrystallised by addition of ethanol to a cold aqueous solution and had m. p. 159—160° (decomp.; lit.,[8] 155—156°).

D- *and* L-2-*Amino*-2-*carboxyethyl* 2-*Aminoethyl Hydrogen Phosphate* (III).—The preparation differed from that described [4] in the following details; the yield and m. p. quoted were obtained for a preparation of L-isomer and are typical. The molecular proportions of monophenyl phosphorodichloridate, *N*-benzyloxycarbonylaminoethanol, *N*-benzyloxycarbonylserine benzyl ester, and pyridine used were 0·03, 0·035, 0·025, and 0·15, respectively. After being dried at 30—40°/0·05 mm., the crude product (79·7 g., from 32·9 g. of the serine ester) was hydrogenated as before, to give a colourless gum (32·4 g.) which was applied in water (18 ml.) to Dowex-50 (NH_4^+) resin (6·55 × 66 cm.). The column was washed with water until 128 × 25 ml. fractions had been collected, and then with N-aqueous ammonia until ninhydrin-positive material ceased to be eluted.

Evaporation of fractions 52—77 yielded a gum which was dissolved in warm water (5 ml.) and the solution diluted with methanol (60 ml.). After refrigeration overnight, the supernatant liquor was decanted and the plastic residue triturated under methanol until it solidified. The ester (III) was collected by centrifugation and dried in a vacuum over potassium hydroxide. It had m. p. 141—142° (13·3 g., 58% based on the serine ester). Chromatography in solvents (R_F 0·20; lit.,[24] 0·21) A and F (R_F 0·39) disclosed traces of two unidentified ninhydrin-positive substances. These were removed by using the potassium perchlorate gradient-elution technique described by Ennor *et al.*,[25] with Amberlite CG-120 resin (H^+ form) and 1 l. of water in the

[21] Gross, *J. Chromatog.*, 1961, **5**, 194.
[22] King, *Biochem. J.*, 1932, **26**, 292.
[23] Rosenberg, Ennor, and Morrison, *Biochem. J.*, 1956, **63**, 153.
[24] Jones and Lipkin, *J. Amer. Chem. Soc.*, 1956, **78**, 2408.
[25] Ennor, Rosenberg, Rossiter, Beatty, and Gaffney, *Biochem. J.*, 1960, **75**, 179.

mixing vessel and a reservoir containing, in succession, 2 l. of 0·1, 0·25, 0·5, 0·75, 1·0, 1·25, and 1·5N-perchloric acid.

Fractions 31—48 from the Dowex-50(NH_4^+) column were combined and on evaporation gave a semi-crystalline mass (9·18 g.). Chromatography (solvents A and F) showed this to be bis-(2-amino-2-carboxyethyl) hydrogen phosphate [24] (R_F 0·12 and 0·30, respectively; lit.,[24] 0·12 in solvent A), in admixture with some phosphoserine and traces of phosphoethanolamine and serine. It was not further purified.

Fractions 186—202 on evaporation yielded a pale yellow syrup which on drying for one week over potassium hydroxide crystallised. This (6·13 g.) was dissolved in water (8 ml.), and the solution adjusted to pH 5—6 with dilute hydrochloric acid, concentrated to incipient crystallisation, and diluted with cold ethanol (20 ml.). After refrigeration, the crystals were collected, washed with ice-cold water–ethanol (2 : 23 v/v) and dried. This *bis-(2-aminoethyl) hydrogen phosphate monohydrochloride* (5·7 g.) had m. p. 249—250° (Found: C, 22·0; H, 6·4; Cl, 16·15; N, 12·8; P, 14·3. $C_4H_{14}ClN_2O_4P$ requires C, 21·8; H, 6·4; Cl, 16·1; N, 12·7; P, 14·0%), $\nu_{max.}$ 759w, 801s, 883w, 934s, 994s, 1017s, 1083s, 1148m, 1206s, 1282w, 1319w, 1374w, 1416w, 1459w, 1546m, 1644m, 2105w, 2455w, 2520m, 2662m, 2710m, 2895s, 2970s, and 3390sh cm.$^{-1}$. It was homogeneous on chromatograms (solvents A and F, R_F 0·31 and 0·54, respectively). Jones and Lipkin [24] isolated this compound in the form of two different perchlorates, containing severally 1 and 0·33 mol. of acid per mol. of ester. The former had R_F 0·59 in system A, whereas the latter gave two ninhydrin-positive spots of R_F 0·43 and 0·60.

2-Amino-2-carboxyethyl 2-N′-Phosphonoguanidinoethyl Hydrogen Phosphate (Phospholombricine) (II).—L-*Isomer.* An aqueous solution of the L-ester (III) (0·399 g., 0·00175 mol.), and *O*-methyl-*N*-phosphonourea (0·170 g.) at pH ~12 was kept at room temperature for 72 hr. Additional *O*-methyl-*N*-phosphonourea (0·17 g. and 0·08 g. after 12 and 54 hr., respectively; total of 0·0027 mol.) was added and the pH was re-adjusted to ~12 with 5N-sodium hydroxide after each addition. The solution was then cooled in ice and, with good stirring, adjusted to pH 7·6—7·8 with Dowex-50(H^+) resin. The latter was filtered off and washed with water until the washings were ninhydrin-negative. The filtrate and washings were combined and applied to Dowex-50(H^+) resin (4·2 × 31·7 cm.) at 2°. The column was washed with water at the same temperature and 5-ml. fractions were collected. When the effluent became acidic, subsequent fractions were collected in tubes containing sufficient concentrated aqueous ammonia to render the fractions alkaline. These fractions were combined and freeze-dried, and the fluffy residue was dried over potassium hydroxide at 0·05 mm. It weighed 0·53 mg. [79% based on ester (III) and calculated as pure diammonium salt] and was chromatographically and electrophoretically homogeneous (see Tables 1 and 2). For analysis, the ammonium salt was obtained as very hygroscopic crystals by addition of methanol to a cold aqueous solution (Found: C, 19·55; H, 6·0; N, 20·10; P, 15·5. Calc. for $C_6H_{14\cdot2}O_9N_4P_2,1{\cdot}8NH_4,0{\cdot}5CH_3{\cdot}OH$: C, 19·7; H, 5·9; N, 20·5; P, 15·6%), $\nu_{max.}$ 777w, 890sh, 969m, 1056s, 1084s, 1220m, 1309w, 1349w, 1413m, 1457m, 1628s, 3020s, 3180s, and 3370s cm.$^{-1}$. Potentiometric titration gave pK_a values at, ~2, 4·6, and ~8·8 and confirmed that the material was a mixture of mono- and di-ammonium salts (1 : 4). The compound did not react with the α-naphthol–biacetyl or Sakaguchi [10] reagent. Hydrolysis in 0·1N-hydrochloric acid for 1 min. at 100° liberated phosphoric acid (R_F 0·41 and 0·23 in solvents C and J, respectively; MD's 7·8 and 14·6 cm. in buffers A and D, respectively) and a guanidino-base having the same R_F values as lombricine and behaving in the same way [4] on treatment with nitrous acid and in 6N-sulphuric acid at 110°.

D-*Isomer.*—(*a*) *From* D-*lombricine and phosphoryl chloride.* A solution of D-lombricine (4·05 g., 0·015 mol.) in 1·76N-sodium hydroxide (50 ml.) in a three-necked flask (250 ml.) at 0° was stirred during the consecutive addition of 10N-sodium hydroxide (10·8 ml.), water (15·4 ml.), and, after 5 min., phosphoryl chloride (1·75 ml.). These additions were repeated at 10-min. intervals until a total of 8·75 ml. (0·095 mol.) of phosphoryl chloride had been added. The mixture was then cooled to −5° for 30 min., the precipitated salt was removed and washed with ice–water (2 × 10 ml.), and the filtrate and washings were combined. Estimation of lombricine in samples of this solution before and after heating at pH 1 for 3 min., indicated that *ca.* 21% phosphorylation of the guanidino-group of lombricine had occurred. The pH of the solution was adjusted to 7·6 by concentrated hydrochloric acid, with stirring and cooling in ice. Ethanol (160 ml.) was added and the precipitate removed by centrifugation. Addition of more ethanol (250 ml.) to the supernatant liquid precipitated an oil which after 1 hr. at 5° was collected by centrifugation and dissolved in water (25 ml.). The solution was applied to a

column (4·6 cm. diam.) of Dowex-50(H^+) resin (650 ml. wet resin) which had been equilibrated at 2°. The column was then washed with water (10 ml. fractions), and the ammonium salt (0·81 g., 14% calc. as pure diammonium salt) was isolated from the effluent as described above for L-phospholombricine. It gave one spot on chromatography and electrophoresis, with R_F values and MD's identical with those of the products described above and below. Precipitated (as before) from water with methanol, it was obtained as hygroscopic crystals (Found: C, 20·2; H, 6·15; N, 19·7; P, 14·8. Calc. for $C_6H_{14\cdot23}N_4O_9P_2,1\cdot77NH_4,CH_3\cdot OH$: C, 20·4; H, 6·2; N, 19·6; P, 15·0%) whose infrared spectrum was identical with that of the ammonium salt of the L-isomer described above. Potentiometric titration gave pK_a values at ~2, 4·6, and 8·8 and confirmed the sample to be a mixture of the mono- and di-ammonium salts (1 : 3·25).

Again the product did not react with the α-nahthol–biacetyl andd Sakaguchi reagents. Hydrolysis in 0·1N-hydrochloric acid at 100° for 3 min. resulted in its complete conversion into phosphoric acid and lombricine, both identified by paper chromatography.

(*b*) *From the ester* (III) *and* O-*methyl*-N-*phosphonourea*. An aqueous solution (25 ml.; pH *ca.* 11·5) of D-ester (III) (2·28 g., 0·01 mol.) and *O*-methyl-*N*-phosphonourea (3·08 g., 0·02 mol.) was kept at room temperature for 33 hr. After being treated with Dowex-50(H^+) resin as described for L-phospholombricine, the solution was applied to Dowex-1(HCO_3^-) resin (6·62 × 84 cm.) which was then washed with an increasing concentration of potassium hydrogen carbonate [7] (2·5 l. of water in the mixing vessel, 12% potassium hydrogen carbonate solution in the reservoir), and the effluent was collected in 50-ml. fractions.

Fractions 106—116 were combined, and, with stirring and a stream of nitrogen passing through the solution at 2—5°, Dowex-50(H^+) resin was slowly added until the pH had dropped to 4—4·5. The pH of the solution was then adjusted to 7·6—7·8 with 10% aqueous potassium hydroxide, and the resin was filtered off and washed with water. The filtrate and washings were combined (1 l.) and a 20% solution of magnesium acetate in 50% aqueous ethanol (50 ml.) added to the ice-cold solution, followed by ice-cold ethanol (2·5 l.). After 3 hr. at 5° the precipitate of crude magnesium phospholombricine was collected by centrifugation, washed successively with 90% ethanol, absolute ethanol, and acetone, and then dried over potassium hydroxide *in vacuo*. It [2·3 g., 43% based on the ester (III) and calculated on P content] [Found: C, 16·2; H, 5·0; Mg, 11·5; N, 10·5; P_L 6·0; P_T 11·6; L_{eq} 52%] was homogeneous on paper chromatography and electrophoresis, with R_F values and MD's identical with those of the preparations described above.

A solution of the crude magnesium salt (53 mg.) in carbon dioxide-free distilled water (0·5 ml.) was cooled in ice, and N-hydrochloric acid slowly added with good stirring until the pH had dropped to *ca.* 6. A stream of nitrogen was passed through the solution for a few minutes, the pH re-adjusted to *ca.* 6, and the operation was then repeated twice more, before a final adjustment of the pH to 6—6·5. Carbon dioxide-free ethanol (4 ml.) was added to the solution at 0° and the solid which separated was triturated, collected by centrifugation, and washed three times with ethanol by suspension and re-centrifuging. After being dried the *magnesium salt* (36·1 mg.) (Found: C, 22·1; H, 4·5; Mg, 7·2; N, 14·0; P_L 8·3; P_T 15·8; L_{eq} 65·4. $C_6H_{14}MgN_4O_9P_2,0\cdot7C_2H_5\cdot OH$ requires C, 22·0; H, 4·5; Mg, 6·0; N, 13·85; P_L 7·65; P_T 15·3; L_{eq} 66·8%) was homogeneous on chromatography and electrophoresis and had $\nu_{max.}$ 728w, 785m, 820sh, 895w, 954w, 1008m, 1047s, 1069s, 1110s, 1233m, 1355w, 1424w, 1462w, 1522w, 1639s, 2965sh, 3190sh, and 3330s cm.$^{-1}$.

Lithium salt. The crude magnesium salt (0·2 g.) was dissolved in water (5 ml.) and N-hydrochloric acid was added as before until the pH had dropped to *ca.* 7·6. The solution was then applied Dowex-50(Li^+) resin (0·9 × 6·1 cm.), and the column washed with water until the elution of ninhydrin-positive material had ceased. The effluent (17 ml.) was concentrated to ~5 ml., the pH was adjusted to 7·2, and the solution evaporated to a syrup. Carbon dioxide-free water (1·3 ml.) was added and, after the pH of the solution had been adjusted to 6—6·5 as described for the magnesium salt, the lithium salt (0·137 g.) was precipitated with ethanol (13 ml.) (Found: C, 20·7; H, 4·6; Li, 3·7; N, 14·4; P_L 8·1; P_T 16·3; L_{eq} 71·8. Calc. $C_6H_{14}Li_2N_4O_9P_2,0\cdot4C_2H_5\cdot OH,0\cdot6H_2O$: C, 20·7; H, 4·5; Li, 3·6; N, 14·4; P_L 7·95; P_T 15·9; L_{eq} 69·4%). It was homogeneous as judged by the usual criteria and had $\nu_{max.}$ 727w, 785m, 894w, 989m, 1050sh, 1069s, 1104s, 1234m, 1316w, 1355w, 1423w, 1462w, 1527w, 1645s, 2965sh, 3190sh, and 3340s cm.$^{-1}$.

Acetone in place of ethanol as precipitant gave a solid (though not so readily as with ethanol) which, after being dried at 0·05 mm. for 6 days over paraffin–potassium hydroxide, then 3 days

over phosphorus pentoxide, had the following analysis: C, 19·64; H, 4·3; N, 14·11. (Calc. for $C_6H_{14}Li_2N_4O_9P_2$,0·2CH_3·CO·CH_3,1·5H_2O: C, 19·6; H, 4·55; N, 14·1%).

In the alcohol dehydrogenase method [16] for the determination of ethanol, the acetone-precipitated product (1·03 mg. in the digestion mixture) did not reduce nicotinamide–adenine dinucleotide, whereas the alcohol-precipitated product (1·244 mg. in the digestion mixture) gave a reduction corresponding to the presence of *ca.* 0·22 mole of ethanol per mole of lithium salt.

A lithium salt (alcohol-precipitated) was prepared from a sample of the barium salt of phospholombricine obtained from earthworms,[7] kindly supplied by Dr. H. Rosenberg (Found: C, 20·0; H, 4·6; Li, 3·8; N, 14·2; P_L 7·9; P_T 15·9; L_{eq} 71·8. Calc. for $C_6H_{14}Li_2N_4O_9P_2$,0·3C_2H_5·OH,H_2O: C, 20·1; H, 4·5; Li, 3·5; N, 14·25; P_L 7·9; P_T 15·75; L_{eq} 68·7%). Its infrared absorption, chromatographic and electrophoretic behaviour, and reaction towards the various spray reagents were identical with those of the lithium salt of synthetic D-phospholombricine described above.

Sodium salt. The crude magnesium salt (55 mg.) was dissolved in water (1·4 ml.) and N-acetic acid was added at 0° with stirring until pH ~7·8 was reached. The solution was then passed through Dowex-50(Na^+) resin (0·64 × 5·8 cm.). The sodium salt (0·376 g.), isolated as described above (Found: C, 19·0; H, 3·8; N, 13·2; Na, 10·4; P_L 7·4; P_T 14·8; L_{eq} 63·0. Calc. for $C_6H_{14}N_4Na_2O_9P_2$,0·3C_2H_5·OH,0·75H_2O: C, 19·0; H, 4·2; N, 13·2; P_L 7·3; P_T 14·6; L_{eq} 63·8%), was homogeneous on chromatography and electrophoresis and had $\nu_{max.}$ 785w, 839sh, 896w, 951sh, 977m, 1049sh, 1066m, 1099s, 1236m, 1314w, 1354w, 1420w, 1463w, 1527w, 1644s, and 3360s cm.$^{-1}$.

2-*Aminoethyl* D-2-*Carboxy*-2-*guanidinoethyl Hydrogen Phosphate* (*Isolombricine*) (VI).—Fractions 100—105 from the Dowex-1(HCO_3^-) column used in the preparation of D-phospholombricine were subjected to a procedure similar to that described for the fractions containing phospholombricine. A solid mixture of magnesium salts of phospholombricine and a ninhydrin-, molybdate-, and α-naphthol–biacetyl (H^+)-reacting substance, together with smaller amounts of other substances, was obtained. The second main component was shown to be 2-aminoethyl 2-carboxy-2-*N*′-phosphonoguanidinoethyl hydrogen phosphate (phosphoisolombricine) (VII) (see Tables 1 and 2) by hydrolysis in 0·1N-acid in 1 min. at 100° to phosphoric acid and isolombricine. The crude mixture (0·34 g., representing *ca.* 70% of the yield from two preparations of D-phospholombricine of the scale described above) was dissolved in 0·1N-hydrochloric acid (3 ml.) and the pH adjusted to *ca.* 1 with 4N-acid. The solution was heated on the steam-bath for 3 min., cooled, neutralised with 5N-sodium hydroxide, and centrifuged and the supernatant liquid was concentrated to *ca.* 5 ml. and added to Dowex-50(NH_4^+) resin (3·5 × 31 cm.). The column was washed with water, and the effluent collected in 10 ml. fractions. Fractions 25—30 were combined and evaporated to a glass (35 mg.) which was re-chromatographed over a column of Dowex-50(NH_4^+) of the same size. Evaporation of fractions 28—29 gave crystals (17·6 mg.) which were dissolved in a little hot water, to which warm ethanol (3 vol.) was next added. The crystals of 2-*aminoethyl* D-2-*carboxy*-2-*guanidinoethyl hydrogen phosphate* which separated on cooling were filtered off, washed with 80% ethanol, and dried at 0·05 mm. over potassium hydroxide. It (13·6 mg.) (Found: C, 26·3; H, 5·9; N, 20·2; P, 11·2. $C_6H_{15}N_4O_6P$,0·3H_2O requires C, 26·15; H, 5·7; N, 20·3; P, 11·2%) had $\nu_{max.}$ 690m, 750w, 784m, 845w, 907w, 936m, 1024s, 1040s, 1082s, 1178m, 1230s, 1274sh, 1321m, 1355m, 1392m, 1424w, 1460m, 1466m, 1525m, 1618s, 1664s, 1697m, 1716m, 2935s, 3110s, 3260s, and 3375s cm.$^{-1}$ and was homogeneous on chromatography and electrophoresis (for R_F values and MD's, see Tables 1 and 2); its behaviour in 6N-hydrochloric acid at 110° was consistent with the proposed structure (see footnote, p. 13).

D-2-*Carboxy*-2-N′-*phosphonoguanidinoethyl* 2-N′-*Phosphonoguanidinoethyl Hydrogen Phosphate* (IV).—Fractions 93—99 from the Dowex-1(HCO_3^-) column used in the preparation of D-phospholombricine, on being subjected to a procedure similar to that used in the isolation of the latter, yielded a crude magnesium salt (0·82 g.) of compound (IV) (Found: P, 13·4; Mg, 15·7%). This salt (0·3 g.) was suspended in water (15 ml.) and cooled in ice, and N-hydrochloric acid was added with stirring until the pH had dropped to ~7·5. The solution was applied to Dowex-1(Cl^-) (1·72 × 27 cm.) which was then treated with a linear sodium chloride gradient * (1 l. of 0·4M-sodium chloride in reservoir, 1 l. of water in mixer) (cf. ref. 12). The effluent was

* Phosphoisolombricine, phospholombricine, and compound (IV) were efficiently separated by such a column (with 0·3M-sodium chloride as eluant) and were eluted in that order, at sodium chloride concentrations of ~0·09, 0·18, and 0·24M, respectively.

collected in 25-ml. fractions. Fractions (51—54) containing compound (IV) were combined and after being neutralised were reduced in volume to *ca.* 10 ml. Magnesium acetate solution (5 ml.) was then added, followed by ice-cold ethanol (35 ml.). The precipitate was collected by centrifugation, washed once by re-suspension in ethanol–water (4 : 1), then re-dissolved in water (4 ml.), and cold ethanol (16 ml.) was added. The precipitate was collected, washed once by re-suspension in ethanol–water (5 : 1) and once in absolute ethanol, and dried over potassium hydroxide/calcium chloride at 0·05 mm. This material (105·65 mg.) was homogeneous on chromatography and electrophoresis; R_F values and MD's are recorded in Tables 1 and 2.

It (39 mg.) was dissolved in carbonate-free distilled water (1 ml.), and the magnesium salt re-precipitated as described for magnesium phospholombricine and dried at 0·05 mm. for 1 week over calcium chloride–potassium hydroxide and for 4 days over phosphorus pentoxide (35 mg.) (Found: C, 19·8; H, 3·9; Mg, 9·1; N, 13·8; P_L 11·1; P_T 16·8. Calc. for $C_7H_{15}Mg_2N_6O_{12}P_3,1{\cdot}5C_2H_5{\cdot}OH,H_2O$: C, 19·9; H, 4·35; Mg, 8·0; N, 13·9; P_L 10·3; P_T 15·4%); it had $\nu_{max.}$ 726w, 796w, 954w, 1004m, 1062sh, 1089sh, 1122s, 1330w, 1372w, 1417w, 1461w, 1637s, 3220s, and 3330s cm.$^{-1}$.

Lithium salt. This was prepared from the purified magnesium salt (60 mg.) as described for the preparation of lithium phospholombricine. The salt was finally precipitated from concentrated aqueous solution (~0·2 ml., pH 6—6·5) by the addition of cold carbon dioxide-free ethanol (4 ml.). It was washed twice with ethanol–water (10 : 1), once with absolute ethanol, then dried in the same way as the magnesium salt. The lithium salt (48·3 mg.) was chromatographically and electrophoretically homogeneous (Found: C, 19·1; H, 4·0; Li, 5·0; N, 14·2; P_L 11·0; P_T 16·2. Calc. for $C_7H_{15}Li_4N_6O_{12}P_3,1{\cdot}2C_2H_5{\cdot}OH,2H_2O$: C, 19·3; H, 4·5; N, 14·3; Li, 4·7; P_L 10·5; P_T 15·8%) and $\nu_{max.}$ 726w, 798w, 991m, 1059sh, 1124s, 1233m, 1330w, 1372w, 1417w, 1461w, 1637s, 2480sh, 3210sh, and 3338m cm.$^{-1}$.

D-2-*Carboxy*-2-*guanidinoethyl* 2-*Guanidinoethyl Hydrogen Phosphate* (V).—An aqueous solution of the impure magnesium salt of compound (IV) (100 mg.) at pH 1 was heated on the steam-bath for 5 min. After being cooled, the solution was neutralised with 5N-sodium hydroxide and applied to Dowex-50(NH_4^+) resin (2·2 × 13·3 cm.; 100 ml. of wet resin). The column was washed with water, and 10-ml. fractions were collected. Fractions 8—11 were combined and evaporated, leaving crystalline D-2-*carboxy*-2-*guanidinoethyl* 2-*guanidinoethyl hydrogen phosphate* (V) (28 mg.). This was chromatographically and electrophoretically homogeneous (see Tables 1 and 2). Recrystallised from aqueous methanol and dried at 0·05 mm. over phosphorus pentoxide, it had m. p. 223—224° (decomp.), raised to 225° on admixture with an authentic sample [D-isomer, m. p. 230° (decomp.)] (Found: C, 27·2; H, 6·0; N, 27·0; P, 10·0. $C_7H_{17}N_6O_6P$ requires C, 26·9; H, 5·5; N, 26·9; P, 9·9%). Its infrared absorption and behaviour on paper chromatography, electrophoresis, and in 6N-acid at 110° were identical with those of the authentic compound (see footnote, p. 13).

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