The above aqueous filtrate, from which the genin VI was separated, was combined with the water washes and extracted three times with 10-ml. portions of chloroform. The latter, which contained a small quantity of the genin VI not originally separating, was discarded. The aqueous solution was evaporated *in vacuo* at 20° to remove the remainder of the methanol and the resulting aqueous solution heated at 65° for 30 minutes. To this was added 375 mg. of barium carbonate and the resulting suspension was stirred for 1 hour, filtered through a layer of barium carbonate, the filtrate evaporated to dryness at 25° and the resulting sirupy residue dissolved in 5 ml. of methanol. After treating with a small amount of Norit A, the methanolic solution was filtered, evaporated to dryness and the residue redissolved in a small volume of ethyl acetate. After the addition of a little *n*-pentane, the solution was seeded with a small quantity of digitoxose (I). After standing overnight, the crude, crystalline material was separated and recrystallized from ethyl acetate–*n*-pentane, giving 6.5 mg. (30%) of pure I, m.p. 100–103°, and giving no depression when admixed with an authentic specimen.

Determination of the Specific Rotations of the Chloride IIIa and the Bromide IIIb.—For the preparation of IIIa and IIIb for this study, 357 mg. (0.6 mmole) of the tri-*p*-nitrobenzoate II was weighed out in each case and treated according to the original directions.[6] After one crystallization from ether–dichloromethane (1:1), both halides were separated from the solvent by decantation and dried in high vacuum at room temperature. Dry nitrogen was admitted and the flasks were immediately stoppered. The flasks were carefully weighed, the halides dissolved in a minimum quantity of dry dichloromethane and diluted to 10 ml. accurately. Rotational measurements were made without delay. The chloride IIIa gave an $[\alpha]^{25}D$ + 185.9 ± 0.3° (*c* 2.10, $CHCl_3$) and the bromide IIIb an $[\alpha]^{25}D$ + 139.1 ± 0.7° (*c* 1.75, $CHCl_3$). The readings were not altered after 1 hour. It was possible to recover both halides in part.

Methyl 2,6-Dideoxy-3,4-di-O-*p*-nitrobenzoyl-β-D-*ribo*-hexoside (IV).—Crystalline bromide IIIb prepared as in the preceding experiment from 1190 mg. (2.0 mmoles) of the tri-*p*-nitrobenzoate II was dissolved in a minimum amount of dry dichloromethane and added rapidly to 100 ml. of absolute methanol. After 15 minutes, the methanol was evaporated *in vacuo* at 30°. The sirupy residue was dissolved in 5 ml. of methanol and the methanol evaporated again. This procedure was repeated three times more and the sirup which still contained traces of hydrogen bromide was dried at room temperature under a reduced pressure of 10^{-3} mm. The sirup thus dried was dissolved in methanol, treated with Norit A and filtered. The filtrate was evaporated *in vacuo* to a small volume and allowed to crystallize for 20 hours. The material, which amounted to 342 mg. (37% based on II), melted at 124–134°. Four recrystallizations from absolute ethanol gave pure IV, m.p. 132.5–135.5°, $[\alpha]^{25}D$ + 128.0 ± 0.3° (*c* 0.30 methanol). *Anal.* Calcd. for $C_{21}H_{20}O_{19}N_2$: C, 54.77; H, 4.38; N, 6.08. Found: C, 54.56; H, 4.58; N, 6.20.

By an identical procedure, 1190 mg. of II was converted to the chloride IIIa which, in turn, was subjected to methanolysis. Recrystallization of the resulting material likewise gave pure IV, m.p. 132.5–136°, undepressed when admixed with a specimen from the preceding preparation.

Deacylation of IV.—A solution of 74.8 mg. of methyl 2,6-dideoxy-3,4-di-*O*-*p*-nitrobenzoyl-β-D-*ribo*-hexoside (IV) in 25 ml. of absolute methanol was prepared and 20 ml. of this solution was transferred to a 1-dm. "reaction type" polarimeter tube. An initial reading was made, giving an $[\alpha]^{25}D$ + 128°. To this was added 1 ml. of 0.01 *N* methanolic sodium methoxide and, after 25 minutes, no further change in the optical rotation could be observed. After standing overnight, the reading was redetermined; this gave an $[\alpha]^{25}D$ −5.12°, based on the molecular weight of a methyl 2,6-dideoxy-D-*ribo*-hexopyranoside (V). The contents of the tube were evaporated *in vacuo* to a small volume, and diluted with ether and water. After separating, the ether phase was dried over magnesium sulfate and evaporated, leaving a crystalline residue which, when recrystallized from a little ethanol, gave pure methyl *p*-nitrobenzoate. The water layer was neutralized with dilute sulfuric acid and treated with 2 g. of Amberlite MB-1 ion-exchange resin. After filtering and evaporating to dryness, a clear sirup was obtained, which could not be brought to a crystalline state. The sirup did not reduce ammoniacal silver oxide.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, JOHN CURTIN SCHOOL OF MEDICAL RESEARCH, THE AUSTRALIAN NATIONAL UNIVERSITY, CANBERRA, A.C.T., AUSTRALIA]

The Synthesis of D-, L- and DL-2-amino-2-carboxyethyl 2-Guanidinoethyl Hydrogen Phosphate (Lombricine) and the Identity of the Natural Compound with the D Isomer

BY IVA M. BEATTY[1] AND DAVID I. MAGRATH

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The D, L and DL forms of 2-amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate have been synthesized and the D isomer has been shown to be identical with the natural product, lombricine. The route involved the selective guanidination of D-, L- and DL-2-amino-2-carboxyethyl 2-aminoethyl hydrogen phosphate, respectively. The characterization of the products and their metal-catalyzed degradation in alkaline solution are discussed.

Thoai and Robin[2] in 1954 reported the isolation from earthworms (*Lumbricus terrestris*) of a new phosphorus-containing guanidine derivative, lombricine, to which, from evidence based on elementary analysis, a study of the functional groups present and paper chromatographic identification of the products of acid hydrolysis, they allocated the structure, 2-amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate (I). The amount of material isolated was small and the configuration of the 2-amino-2-carboxyethyl, *i.e.* the serine, moiety was not investigated.

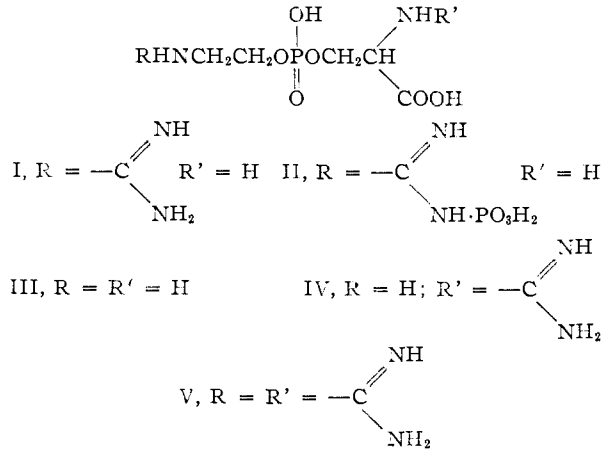
The corresponding phosphagen, 2-amino-2-carboxyethyl 2-N-phosphorylguanidinoethyl hydrogen phosphate (phosphoryllombricine, II) was also shown[2,3] to be present in the muscle. On mild acid hydrolysis it yielded phosphoric acid and a guanidine derivative, shown by paper chromatography to be identical with lombricine.

Synthesis of lombricine was, from our point of view, desirable for two reasons: first, to provide final confirmation of the structure allocated by Thoai and Robin[2] and to extend further the identification to the configuration of the serine moiety; secondly, to provide substrate material for a study of the enzymes associated with its biochemical function in muscle. The latter requirement has

(1) Australian National University Scholar.

(2) N. v. Thoai and Y. Robin, *Biochim. Biophys. Acta* **14**, 76 (1954).

(3) N. v. Thoai, J. Roche, Y. Robin and N. v. Thiem, *Compt. rend. soc. biol.*, **147**, 1670 (1953).



since been largely met in respect to the naturally-occurring isomer as a result of the isolation of lombricine from earthworms by Rosenberg and Ennor,[4] in much higher yield than achieved by the French workers. Further, the ready availability of the natural compound[5] enabled a thorough comparison to be made between it and the compounds whose synthesis is described below.

Of the many available synthetic approaches to a molecule of the lombricine type, a route involving the synthesis and subsequent guanidination of 2-amino-2-carboxyethyl 2-aminoethyl hydrogen phosphate (serine ethanolamine phosphodiester, SEP,[4] III) seemed the most straightforward. The synthesis of the DL and L forms of the latter compound had been reported[6] and the success of the method appeared, therefore, to depend solely on the feasibility of selectively guanidinating the terminal amino group, a precedent for which already existed in the established procedure for the synthesis of, *e.g.*, arginine from ornithine,[7] where the α-amino group is protected from guanidination by chelation with copper.

In addition, the key intermediate in the above reaction scheme, SEP, was of considerable interest in its own right. It had been found to occur naturally in turtle tissue,[8] and *a priori* it seemed likely that it might function as the immediate biological precursor of lombricine in the earthworm.[9] The subsequent isolation of SEP from earthworms[4,10] is consistent with this hypothesis and, in addition, the compound has now been found to occur widely throughout the lower animals.[11–13] Direct comparison[10] of the material isolated from earthworms with the synthetic compounds has shown it to be the D isomer; in all other cases so far investigated, the naturally occurring compound has proved to be the L isomer.[8,12,13]

The practicability of the approach outlined above has now been demonstrated by its successful application to the synthesis of DL-, L- and D-lombricine, described below. A preliminary report of the synthesis of the DL and L compounds has already been published.[14] DL-, L- and D-SEP were synthesized according to Jones and Lipkin's procedure[6] which involves the staggered reaction of the corresponding N-benzyloxycarbonylserine benzyl ester and N-benzyloxycarbonylethanolamine with monophenylphosphorodichloridate. Ion-exchange chromatography was, however, introduced in the final stages of purification, Dowex-50 resin, in either the acid or ammonium form, being used. In the latter case, application of a concentrated aqueous solution of the impure material obtained from a single cellulose fractionation to the column of resin, followed by simple elution with water, furnished the diester in pure form. This technique proved very useful throughout the course of the work, both in the preparation of SEP and lombricine isomers, and of certain reference compounds, and has since been successfully applied[10,12,13] in the isolation of some of these compounds from natural sources and of D-serine from an acid hydrolysate of natural lombricine.[12,15]

Attempted guanidination of the copper complex of DL-SEP with O-methylurea in alkaline solution gave a complex mixture of products, and it was subsequently found that SEP and natural lombricine, though stable for at least 90 hr. at room temperature in aqueous solution at *p*H 10, were rapidly degraded under these conditions when present in the form of their chelates with copper[16] (see below).

Guanidination of unprotected SEP could conceivably lead to the formation of a mixture of the two isomeric monoguanidinated products (I and IV) and the diguanidino compound (V). However, there exist in the literature several examples of the preferential guanidination of particular amino groups in polyamino compounds, such as diamino acids,[17] peptides[18] and proteins,[19] and although no clear explanation of this preferential guanidination seems to have been advanced, it has been suggested[20,21] that the relative basicities of the relevant amino groups may play a role.[22] Since the terminal amino group of SEP is probably the more basic,[23] it was considered possible that a preferen-

(4) H. Rosenberg and A. H. Ennor, *Biochem. J.*, **73**, 521 (1959).

(5) The natural lombricine used throughout this work was kindly made available by Professor A. H. Ennor and Dr. H. Rosenberg.

(6) E. E. Jones and D. Lipkin, THIS JOURNAL, **78**, 2408 (1956).

(7) F. Turba and K. Schuster, *Z. physiol. Chem.*, **283**, 27 (1948); A. C. Kurtz, *J. Biol. Chem.*, **180**, 1253 (1949).

(8) E. Roberts and I. P. Lowe, *ibid.*, **211**, 1 (1954).

(9) A. H. Ennor and J. F. Morrison, *Physiol. Revs.*, **38**, 631 (1958).

(10) A. H. Ennor, H. Rosenberg, I. M. Beatty and T. Gaffney, *Biochem. J.*, **75**, 179 (1960).

(11) P. Ayengar and E. Roberts, *Federation Proc.*, **16**, 147 (1957).

(12) I. M. Beatty, A. H. Ennor, D. I. Magrath and H. Rosenberg, in preparation.

(13) A. H. Ennor, A. M. Morgan and H. Rosenberg, unpublished data.

(14) I. M. Beatty and D. I. Magrath, *Nature*, **183**, 591 (1959).

(15) I. M. Beatty, D. I. Magrath and A. H. Ennor, *ibid.*, **183**, 591 (1959).

(16) Preliminary experiments indicated that cobalt was also active in promoting the degradation.

(17) E. Schulze and E. Winterstein, *Ber.*, **32**, 3191 (1899); *Z. physiol. Chem.*, **34**, 128 (1902); E. Winterstein and A. Küng, *ibid.*, **59**, 138 (1909); K. Odo, *J. Chem. Soc. Japan, Pure Chem. Sect.*, **74**, 1 (1953); *cf.* C. Lempert, *Chem. Revs.*, **59**, 667 (1959).

(18) J. P. Greenstein, *J. Biol. Chem.*, **109**, 541 (1935); *J. Org. Chem.*, **2**, 480 (1937).

(19) L. K. Ramachandran, *Biochim. Biophys. Acta*, **32**, 557 (1959), and references therein.

(20) E. Schütte, *Z. physiol. Chem.*, **279**, 52 (1943).

(21) J. Roche, M. Mourgue and R. Baret, *Bull. soc. chim. biol.*, **36**, 85 (1954).

(22) Factors such as accessibility must also be considered when dealing with large complicated molecules such as proteins.[19]

(23) The inductive effect of the carboxyl group reduces the basicity of α-situated amino groups.

TABLE I

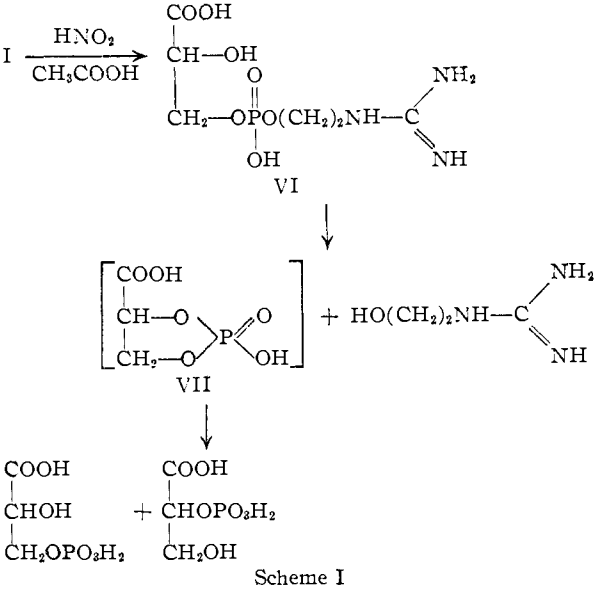
Compound	R_f values in solvent A	B	C	D	E	F	G	H	J
Ethanolamine	0.75	0.58	0.57	0.66	..	..	0.73	0.63	..
Serine	.53	.37	.41	.35	..	..	.54	.34	..
Phosphoethanolamine	.47	.30	.33	.35	..	..	.30	.08	..
Phosphoserine	.35	.23	.26	.10	..	..	.26	.04	..
SEP (DL, D or L)	.19	.12	.22	.28	0.34	0.65	.37	.12	0.21
N-Amidinoserine	.67	.47	.49	.56	.45	.51	.43	.27	.49
2-Guanidinoethanol (hydrochloride)	.77	.66	.63	.78	.69 (with streak to .42)	.42 – .66 (streak)	.50 .62	.34 .44	.74
2-Guanidinoethyl phosphate	.57	.36	.40	.57	.11	.49	.21	.06	.20
Synthetic lombricine (DL, D or L)	.30	.15	.26	.51	.29	.58	.32	.11	.26
Natural lombricine	.30	.15	.27	.50	.30	.60	.32	.11	.26

tial guanidination of this amino group might, therefore, be effected. In fact, D, L and DL forms of I were obtained in yields of 62, 72 and 54%, respectively, by reaction of the corresponding form of SEP with two equivalents[24] of O-methylurea in aqueous solution at *p*H 11, followed by purification of the product over Dowex-50 (NH_4^+) resin.

All three synthetic products gave analytical figures, and color tests showing the presence of phosphorus (molybdate spray reagent[26]) and of free amino (ninhydrin) and monosubstituted guanidino (Sakaguchi[27] and α-naphthol-diacetyl[28] spray reagents) groups, in agreement with their formulation as I. The melting points of the D and L compounds, and of natural lombricine, agreed within experimental error, but that of the DL compound was significantly lower. Small differences between the infrared absorption spectrum of the DL compound (solid state) and corresponding spectra of the D and L compounds and of natural lombricine, which were identical, can be attributed to differences in the structure of the crystal lattice.[29] Comparison of pK_a values obtained for all four compounds (*ca.* 2 and 8.9 in each case)[30] with those found for the SEP isomers (*ca.* 2, 8.8 and 10 in each case), showed that it was the more strongly basic amino group, *i.e.* the terminal one,[23] of SEP that had been guanidinated, thus supporting structure I for the compounds.

The three synthetic compounds, and natural lombricine, had identical R_f values on paper chromatograms run in a variety of solvent systems (see Table I). Similarly, acid hydrolysates (6 *N* H_2SO_4, 110°, 8 hr.) of all four gave identical patterns of ninhydrin-, α-naphthol/diacetyl- and molybdate-reacting spots which corresponded with markers of authentic serine, 2-guanidinoethanol and 2-guanidinoethyl phosphate, thus confirming similar observations made by Thoai and Robin[2] in the case of natural lombricine and extending them to the three synthetic products.

The position of the guanidino group in the molecule of the synthetic compounds and of natural lombricine was also ascertained in another way. Treatment of I with nitrous acid in dilute acetic acid, *i.e.* under conditions not affecting the guanidino group,[31] should lead to replacement of the α-amino group by a hydroxyl group,[32] giving 2-guanidinoethyl 2-hydroxy-2-carboxyethyl hydrogen phosphate (VI). In contrast to the parent compound, hydrolysis of VI would be expected to occur readily, to give a mixture of 2-guanidinoethanol and 2- and 3-phosphoglyceric acids, *via* the intermediate formation of glyceric acid 2,3-cyclic phosphate[33] (VII; see Scheme I). On the other hand, a mixture of N-amidinoserine and 2-hydroxyethyl phosphate would be expected from the isomer, IV.



Scheme I

(24) Two equivalents of reagent were used to increase the rate of reaction and offset the slow decomposition of O-methylurea in alkali.[25]

(25) W. L. Hughes, H. A. Saroff and A. L. Carney, THIS JOURNAL, **71**, 2476 (1949); C. H. Chervenka and P. E. Wilcox, *J. Biol. Chem.*, **222**, 635 (1956); W. A. Klee and F. M. Richards, *ibid.*, **229**, 489 (1957).

(26) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(27) R. Archer and C. Crocker, *Biochim. Biophys. Acta*, **9**, 704 (1952).

(28) D. E. Griffiths, J. F. Morrison and A. H. Ennor, *Biochem. J.*, **65**, 612 (1957).

(29) A. Weissberger, "Techniques of Organic Chemistry," Vol. IX, "Chemical Applications of Spectroscopy," Interscience Publishing Co., New York, N. Y., 1956, p. 324.

(30) The pK_a of the guanidino group was too high to be determined.

(31) A. Hynd and M. G. MacFarlane, *Biochem. J.*, **20**, 1264 (1926).

(32) P. A. Levene and A. Schormüller, *J. Biol. Chem.*, **106**, 595 (1934); A. Neuberger in "Advances in Protein Chemistry," Vol. IV, M. L. Anson and J. T. Edsall, eds., Academic Press, Inc., New York, N. Y., 1948, p. 297.

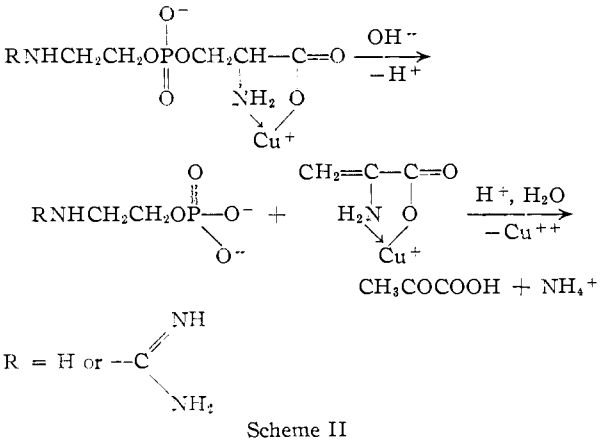
(33) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952), and references therein; D. M. Brown and A. R. Todd, *ibid.*, 2040 (1953); D. Lipkin, P. T. Talbert and M. Cohn, THIS JOURNAL, **76**, 2871 (1954); C. Dekker and J. Lecocq, *Experientia*, **15**, 27 (1959).

Reaction of either the synthetic D isomer or natural lombricine with nitrous acid in 20% acetic acid, though incomplete, lead, as ascertained by paper chromatographic examination of the reaction mixture, to the almost exclusive formation of a ninhydrin-negative, α-naphthol/diacetyl-positive, phosphorus-containing substance, shown to be VI by its ready hydrolysis (30 min., *N* HCl, 100°) to 2-guanidinoethanol and an α-naphthol/diacetyl-negative, phosphorus-containing spot behaving as 3-phosphoglyceric acid in a number of solvent systems; no phosphoric acid was formed. Under the acid conditions employed for the hydrolysis, phosphoglyceric acid is known to exist primarily in the form of the 3 isomer.[34] No clear evidence for the presence of the 2 isomer was obtained from the chromatograms, though the solvent systems used included some claimed[35-37] to effect a good separation of the two isomers.

Finally, comparison of the specific rotations of the two optically-active synthetic isomers with that of natural lombricine showed the latter to be identical with the D isomer. This conclusion was confirmed[15] by the isolation of D-serine from an acid hydrolysate of the natural compound, full details of which will be reported in a later communication.[12] 2-Guanidinoethyl phosphate was also isolated in a crystalline state from the hydrolysate and characterized by elementary analysis and infrared spectroscopy, thus confirming the earlier identification of this degradation product of lombricine by paper chromatography.[2,14]

As previously mentioned, the copper complexes of both SEP and lombricine were unstable in alkaline solution at room temperature. From chromatographic studies it appeared that the former gave phosphoethanolamine, probably *via* an unstable intermediate, as a major degradation product and the latter, 2-guanidinoethyl phosphate directly. Small amounts of other ninhydrin- or α-naphthol/diacetyl-reacting products, which possibly included phosphoserine, were also formed but in neither case was phosphoric acid or serine observed on the chromatograms, though the copper complex of the latter appeared not to be appreciably degraded under the conditions used. In addition, there was no evidence for the presence of any ethanolamine in the SEP hydrolysate or of any 2-guanidinoethanol in the lombricine hydrolysate, suggesting that phosphoserine, if it is formed, does not come directly from the parent molecule by simple hydrolytic fission.

Although the data at present are insufficient to permit of any definite conclusions regarding the mechanism of degradation in either case, the formation of the major degradation products, phosphoethanolamine and 2-guanidinoethyl phosphate, respectively, without concomitant production of serine, is perhaps best explained in terms of an elimination reaction of the type undergone by esters of β-hydroxy carbonyl derivatives.[38] Such a mechanism has been advanced[39] to account for the great lability of phosphoryl groups attached through ester linkage to the serine residues of proteins[40] and to explain the facile hydrolysis of certain fully substituted derivatives of phosphoserine[41] under mildly alkaline conditions. The degradation of SEP or lombricine by this mechanism may be represented as shown in Scheme II.



Scheme II

In the above scheme, the strongly electronegative metal ion is envisaged as functioning in the same way as the substituents attached to the α-amino and carboxyl groups of serine in the examples quoted above, *i.e.* to reduce, by induction, electron density at the α-carbon atom and thus facilitate removal of the attached proton,[42] with subsequent elimination of the phosphoryl residue (*cf.* the postulated role of the metal in the reactions of amino acids, in particular, serine and phosphoserine,[43] catalysed by pyridoxal and metal ions[44]). However, even though such a mechanism seems to fit closely the facts relating to the degradation of lombricine and might reasonably be expected to apply equally in respect to SEP degradation, the precise significance of the "intermediate" in the latter case remains to be explained. The R_f of this material (0.07 in solvent A, 0.04 in solvent B) relative to those of SEP and its conceivable degradation products (see Table I) suggests that its molecular weight is of the same order as that of SEP, but whether it is to be regarded as a true intermediate in the hydrolysis or as the product of a recombination of initially formed smaller fragments cannot be decided until further evidence as to its structure is available.

Whatever the mechanism, the implications of this metal-catalyzed degradation with regard to the stability of other naturally occurring phosphoric

(34) C. E. Ballou and H. O. L. Fischer, THIS JOURNAL, **76**, 3188 (1954); C. E. Ballou in "Biochem. Preparations," Vol. V, D. Shemin, Ed., John Wiley and Sons, Inc., New York, N. Y., 1957, p. 66.

(35) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

(36) D. C. Mortimer, *Can. J. Chem.*, **30**, 653 (1952).

(37) R. W. Cowgill, *Biochim. Biophys. Acta*, **16**, 614 (1955).

(38) D. M. Brown, M. Fried and Sir Alexander R. Todd, *J. Chem. Soc.*, 2206 (1955), and references therein.

(39) L. Anderson and J. J. Kelley, THIS JOURNAL, **81**, 2275 (1959).

(40) R. E. Ferrel, H. S. Olcott and H. Fraenkel-Conrat, *ibid.*, **70**, 2101 (1948); D. K. Mecham and H. S. Olcott, *ibid.*, **71**, 3670 (1949).

(41) G. Riley, J. H. Turnbull and W. Wilson, *Chem. & Ind. (London)*, 1181 (1953); *J. Chem. Soc.*, 1373 (1957).

(42) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 420.

(43) J. B. Longenecker and E. E. Snell, *J. Biol. Chem.*, **225**, 409 (1957).

(44) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76**, 648 (1954); E. E. Snell in "Vitamins and Hormones," Vol. XVI, R. S. Harris, G. F. Marrian and K. V. Thimann, Eds., Academic Press, Inc., New York, N. Y., 1958, p. 77.

esters, *e.g.*, phosphoserine and phosphatidylserine, would seem worthy of investigation.

Experimental

Paper chromatography was routinely carried out at room temperature, using the ascending technique and Whatman No. 1 paper. Authentic marker substances were always included on the chromatograms. Guanidino compounds were located on the paper with either the Sakaguchi[27] or α-naphthol/diacetyl[28] spray reagents, amino compounds by spraying with 0.25% ninhydrin in water-saturated 1-butanol and then heating the paper for 5–10 min. at *ca.* 90° and phosphates by the Hanes and Isherwood reagent.[26]

Use was made of the following solvent systems: A, acetone–acetic acid–water (2:2:1)[6]; B, methyl ethyl ketone–methyl cellosolve–acetic acid–water (40:15:6:24)[45]; C, 1-butanol–acetic acid–water (50:20:30); D, phenol saturated with water (4:1, w./v.); E, methyl ethyl ketone–methyl cellosolve–3 *N* ammonia (2:7:3)[46]; F, methanol–aqueous ammonia (sp. gr. 0.91)–water (60:10:30)[35]; G, 1-propanol–aqueous ammonia (sp. gr. 0.91)–water (60:30:10); H, 1-propanol–aqueous ammonia (sp. gr. 0.91)–water (73:20:7); J, ethanol–ammonium acetate buffer, *p*H 7.2 (70:30). Typical R_f values for key substances in this investigation, using freshly made-up solvent systems, are given in Table I.

Dowex-50 × 4, 200-400 mesh and Dowex-3 × 4, 20–50 mesh (The Dow Chemical Co., Midland, Mich.) and Amberlite IRC-50 (Rohm and Haas Co., Philadelphia, Penna.) ion-exchange resins were used. Elution of substances from columns of cellulose powder (prepared as described by Jones and Lipkin[6]) or ion-exchange resins was followed by spotting each fraction on Whatman paper and spraying with the appropriate reagent, and then examining selected fractions by paper chromatography.

Melting points are corrected unless otherwise stated and were determined in a metal block type of apparatus enabling three determinations to be made simultaneously. Many of the compounds studied melted with decomposition, the m.p. varying appreciably with rate of heating and state of subdivision. Melting points for such compounds were determined using unsealed tubes, dropped into the apparatus at a temperature 20–30° below the expected m.p. and then heated at a rate of *ca.* 2° per min.; in such cases the m.p. of a reference compound was always determined at the same time.

Samples were prepared for analysis by drying at 0.1–0.5 mm. over phosphorus pentoxide; lombricine, 2-guanidinoethyl phosphate and N-amidinoserine were heated at 110° and SEP at 80°. Although SEP was weighed out under anhydrous conditions, difficulty was sometimes experienced in obtaining satisfactory hydrogen figures; this is attributed to the absorption of moisture during the subsequent addition to the sample of vanadium pentoxide, which was necessary in order to ensure complete combustion. Nitrogen was determined by the Kjeldahl method and phosphorus either colorimetrically[46] or gravimetrically.[47]

pK_a values were determined potentiometrically. Infrared spectra were obtained on potassium bromide discs using a Perkin–Elmer Model 21 double beam infrared spectrophotometer equipped with sodium chloride optics.

DL-, D- and L-Serine were commercial samples (British Drug Houses Ltd., Poole, England; California Corporation for Biochemical Research, Los Angeles, Calif., CfP grade; and Schwarz Labs., Inc., Mt. Vernon, N.Y., O.S. grade, respectively) and were recrystallized where necessary. $[\alpha]^{23.5}D$ for the D isomer, + 7.4 (c = 2.02 g., H_2O) and $[\alpha]^{23}D$ for the L isomer, − 6.94 (c = 4.04 g., H_2O); both had m.p. 221° dec. DL-Phosphoserine and phosphoethanolamine were products of California Corporation for Biochemical Research, C.P. grade. 2- and 3-Phosphoglyceric acids were obtained as their barium salts from Sigma Chemical Co., St. Louis, Missouri; for use as reference compounds on paper chromatograms they were converted to their ammonium salts by shaking with an aqueous suspension of Dowex-50 (NH_4^+) resin. Natural lombricine was isolated from earthworms of mixed species, predominantly *Allolobophora caliginosa* and *Octolasium cyaneum*.[4]

2-Guanidinoethanol hydrochloride (m.p. 103–104°; *cf.* 107°[48]) and N-amidino-DL-serine (gradually charred > 210°; *cf.* m.p. 200° dec.[49] and 205° dec.[48]) were prepared by the reaction of O-methylurea hydrochloride[50] with ethanolamine in dry ethanol and DL-serine in aqueous solution at *ca.* *p*H 9, respectively. In the latter case, the reaction mixture was neutralized with Amberlite IRC-50 (H^+) resin and the product isolated by fractionation of the concentrated filtrate on Dowex-50 (NH_4^+) resin, with water as eluent. Both compounds gave correct analytical figures for C, H and N.

2-Guanidinoethyl Phosphate.—Phosphoethanolamine (2 g.) was dissolved in sodium hydroxide (*N*; 16 cc.), O-methylurea hydrochloride (1.6 g., 1 eq.) added, and the *p*H of the solution adjusted to between 10 and 11 by the addition of more alkali (6 cc.). The flask was stoppered and the reaction mixture set aside at room temperature for 3–4 days, the *p*H being checked night and morning and re-adjusted to approx. the original value where necessary. The reaction mixture was then applied to a column (3.5 × 10.5 cm.) of Dowex-50 (H^+) resin and the column washed with water. The portion of the eluate containing the required product (730–1880 cc.) was evaporated to dryness under reduced pressure, giving a white, crystalline residue (2.192 g., 84.4%). Recrystallization from aqueous methanol gave sheaves of fine needles, 1.938 g., m.p. 201–202° dec., with shrinking above 195°. It was recrystallized once more for analysis, m.p. 202–204° dec.

Anal. Calcd. for $C_3H_{10}N_3O_4P$: C, 19.68; H, 5.50; N, 22.95; P, 16.92. Found: C, 19.53; H, 5.60; N, 22.64; P, 16.65.

It crystallized from aqueous ethanol as stout, colorless rods, m.p. 207–208° dec. and gave infrared absorption peaks (sh = shoulder) at 3375, 3140, 2640, 2445 (sh), 2320, 1687, 1664, 1630, 1597, 1460, 1452, 1394, 1357, 1296, 1253, 1185, 1118, 1097, 1054, 1035, 1018, 937, 915, 766, 725 and 700 cm.$^{-1}$.

2-Guanidinoethyl phosphate has previously been prepared[2] in unstated yield by the action of phosphoryl chloride on 2-guanidinoethanol but its characterization rested solely on paper chromatographic data.

DL-, D- and L- 2-Amino-2-carboxyethyl 2-Aminoethyl Hydrogen Phosphate (SEP).—The racemic and optically active forms of SEP were synthesized according to the method of Jones and Lipkin,[6] slightly modified in the case of the D and L compounds.[51] R_f values of SEP in a number of solvent systems are given in Table I.

A. DL-SEP.—This form was obtained as colorless prisms from aqueous ethanol, m.p. 193–194° dec. Jones and Lipkin quote m.p. 180–181° uncor., dec.

Anal. Calcd. for $C_5H_{13}N_2O_6P$: C, 26.33; H, 5.75; N, 12.28; P, 13.58. Found: C, 26.53; H, 5.80; N, 12.24; P, 13.59.

pK_a values, *ca.* 2, 8.8 and 10.0; infrared absorption peaks (sh = shoulder) at 3400, 2940, 2640, 2340 (sh), 2090, 1640, 1580 (sh), 1530, 1460, 1415, 1368, 1344, 1313, 1304, 1238, 1212, 1185 (sh), 1166, 1137, 1075, 1037, 1011, 993, 932, 914, 885, 831, 789 and 752 cm.$^{-1}$, in excellent agreement with the values reported by Jones and Lipkin.

B. L-SEP.—From 8.24 g. of N-benzyloxycarbonyl-L-serine benzyl ester[52] there was obtained 1.9 g. (33.3%) of crude product from the cellulose column fractionation. This was dissolved in water and applied to a column (3.2 × 7 cm.) of Dowex-50 (H^+) resin. Elution with water, which removed a little phosphoserine, and then with *N* aqueous ammonia, furnished the mixed diester contaminated with a little phosphoethanolamine. Crystallization at this stage proved unsatisfactory and the material was re-chromatographed on a column of Dowex-50 (NH_4^+) resin of the same size, with water as eluant. Evaporation of the relevant fractions gave a material which crystallized from aqueous methanol as colorless, hygroscopic needles (microcrystalline), 1.34 g. (23.4%), m.p. 142–143° dec. (*cf.* 139–141° uncor. dec.[6]).

(45) G. Porcellati, *J. Neurochem.*, **2**, 128 (1958).

(46) I. Berenblum and E. Chain, *Biochem. J.*, **32**, 295 (1938).

(47) A. Steyermark, "Quantitative Organic Microanalysis," The Blakiston Co., New York, N. Y., 1951, p. 192.

(48) M. Mourgue, *Bull. soc. chim. France*, 181 (1948).

(49) J. Kapfhammer and H. Müller, *Z. physiol. Chem.*, **225**, 1 (1934).

(50) F. Kurzer and A. Lawson, *Org. Syntheses*, **34**, 67 (1954).

(51) The authors are indebted to Dr. D. E. Griffiths for the preparation of N-benzyloxycarbonylethanolamine, used in the synthesis of these compounds.

(52) E. Baer and J. Maurukas, *J. Biol. Chem.*, **212**, 25 (1955).

Anal. Calcd. for $C_5H_{13}N_2O_6P$: C, 26.33; H, 5.75; N, 12.28; P, 13.58. Found: C, 26.27; H, 6.36; N, 12.20; P, 13.57.

$[\alpha]^{23.5}D$ −14.8 (c = 0.811, H_2O); Jones and Lipkin quote $[\alpha]^{23.5}D$ −15.0 (c = 2.2, H_2O). pK_a values, *ca.* 2, 8.8 and 10.0; infrared absorption peaks at 3400, 2940, 2340 (sh), 2080, 1684, 1640, 1532, 1462, 1415, 1372, 1343, 1305, 1225, 1073, 1038, 1023 (sh), 994 (sh), 912 (sh), 896, 833 and 779 cm.$^{-1}$, in good agreement with the data of Jones and Lipkin.

C. D-SEP.—From 8.24 g. of N-benzyloxycarbonyl-D-serine benzyl ester[52] there was obtained 2.36 g. of the crude mixed phosphodiester from the cellulose column. Chromatography on a column (3.2 × 10 cm.) of Dowex-50 (H^+) resin as described above, but this time washing with water (*ca.* 500 cc.) until all ninhydrin-reacting impurities had been removed before stripping with N aqueous ammonia yielded a product which was obtained from aqueous methanol as a hygroscopic, microcrystalline powder, 1.0 g. (20%), m.p. 143–144° dec.

Anal. Calcd. for $C_5H_{13}N_2O_6P \cdot 1/2\ CH_3OH$: C, 27.05; H, 6.19; N, 11.48; P, 12.68. Found: C, 26.85; H, 6.25; N, 11.43; P, 12.86.

$[\alpha]^{23.5}D$ + 18.2 (c = 0.659, H_2O); pK_a values, *ca.* 2, 8.8 and 10.0; infrared spectrum was identical with that of the L isomer; for full curve see ref. 10.

2-Amino-2-carboxyethyl 2-Guanidinoethyl Hydrogen Phosphate (Lombricine).—In preliminary experiments, it was found that reaction of the copper complex of DL-SEP with O-methylurea at pH 8.5 or 10 and room temperature gave a mixture of products, including substances behaving on chromatograms as phosphoethanolamine and 2-guanidinoethyl phosphate. In the absence of copper, a guanidino compound having the same R_f values as natural lombricine in several solvent systems was readily formed at pH 9, 11 or 12, the rate of reaction and the yield being increased when two, rather than one, equivalents of reagent were used.

A. DL-Lombricine.—DL-SEP (0.228 g., 0.001 mole) and O-methylurea hydrochloride (0.221 g., 0.002 mole) were dissolved in water (2.5 cc.) and the pH of the solution adjusted to *ca.* 11 with 2 N sodium hydroxide. The solution was kept at room temperature for 8 hr., the pH being readjusted to 11 from time to time with alkali and then neutralized with hydrochloric acid. Chromatography (solvent B) of the reaction mixture showed that conversion of SEP to a product corresponding on the chromatograms to lombricine was complete. There was also present a small amount of another guanidino compound, R_f 0.21.

Following an unsuccessful attempt to purify the product by chromatography on sheets of Whatman 3 MM paper, the material eluted (0.260 g.) from the relevant portion of the papers was dissolved in a minimum of water and applied to a column (3.5 × 24 cm.) of Dowex-50 (NH_4^+) resin. The column was washed with water and fractions containing the required product were combined and evaporated to dryness under reduced pressure. A crystalline residue (0.146 g., 54%) was obtained which gave a single α-naphthol/diacetyl- and ninhydrin-positive spot on chromatograms run in solvent B. The compound crystallized readily from aqueous methanol as colorless needles, 0.101 g., m.p. 217–218° dec.

Anal. Calcd. for $C_6H_{15}N_4O_6P$: C, 26.67; H, 5.60; N, 20.73; P, 11.47. Found: C, 26.46; H, 5.51; N, 20.90; P, 11.56.

pK_a values, *ca.* 2 and 8.9; infrared absorption peaks[53] at 3414 (sh), 3370, 3212, 3078, 2980, 2900, 2835, 2681, 2634, 2340 (sh), 2050, 1687, 1642, 1595 (sh), 1545, 1492, 1472, 1414, 1377, 1350, 1317, 1228, 1175, 1146, 1100, 1087, 1050, 1037, 997, 933, 890, 847 and 760 cm.$^{-1}$.

B. L-Lombricine.—L-SEP (0.254 g.) was reacted with O-methylurea hydrochloride (2 eq.) as described for the DL compound. The neutralized reaction mixture was applied directly to a column (3.5 × 24 cm.) of Dowex-50 (NH_4^+) resin and the column washed with water, 5 cc. fractions being collected. Chromatographic examination of the fractions in solvent B indicated the presence in trace amounts of 2-guanidinoethyl phosphate (fraction 18), SEP and an unidentified ninhydrin-positive, phosphorus-containing substance (fractions 24-25) and two α-naphthol/diacetyl-positive compounds (R_f values 0.27 and 0.21, fractions 49-54 and 72-100, respectively). Fractions 28-48, which contained a single α-naphthol/diacetyl- and ninhydrin-positive compound behaving identically with natural lombricine on the chromatograms, were combined and evaporated to dryness under reduced pressure. After drying in a desiccator, the residue weighed 0.216 g. (72%) and crystallized readily from aqueous methanol as colorless needles, 0.170 g., m.p. 228–229° dec.

Anal. Calcd. for $C_6H_{15}N_4O_6P$: C, 26.67; H, 5.60; N, 20.73; P, 11.47. Found: C, 26.64; H, 5.56; N, 20.74; P, 11.57.

$[\alpha]^{23.5}D$ −13.0 (c = 0.81, H_2O); pK_a values, *ca.* 2 and 8.9; infrared absorption peaks[53] at 3400 (sh), 3364, 3208, 3073, 2957, 2900, 2678, 2330 (sh), 2045, 1692, 1642, 1595, 1532, 1492, 1472, 1418, 1378, 1352, 1322, 1230, 1180, 1146, 1102, 1087, 1048, 1035, 1003, 934, 893, 849 and 762 cm.$^{-1}$.

C. D-Lombricine.—The D isomer was prepared in the same manner as described for the L isomer. From 0.228 g. of D-SEP there was obtained, from the Dowex-50 (NH_4^+) column (3.5 × 30.5 cm.), 0.170 g. (62%) of a chromatographically homogeneous product. It was recrystallized from aqueous methanol, giving colorless needles, m.p. 225–226° dec.

Anal. Calcd. for $C_6H_{15}N_4O_6P$: C, 26.67; H, 5.60; N, 20.73; P, 11.47. Found: C, 26.62; H, 5.47; N, 20.68; P, 11.35.

$[\alpha]^{23.5}D$ + 16.1 (c = 0.805, H_2O); pK_a values, *ca.* 2 and 8.8; infrared spectrum was identical with those of the L isomer and natural lombricine.

Characterization of Natural Lombricine.—Natural lombricine[4] was recrystallized from aqueous methanol, giving colorless needles, m.p. 227–228° dec.; subsequent recrystallization from aqueous ethanol raised this to 231–232° dec. (*cf.* 223–224°[2] and 224° dec.[4]). It had R_f values in a number of solvent systems identical with those of the synthetic compound (DL, L or D isomer; see Table I).

$[\alpha]^{23.5}D$ + 14.5 (c = 0.93, H_2O); pK_a values *ca.* 2 and 8.9; infrared spectrum was identical with those of the synthetic D and L isomers.

Acid Hydrolysis of Synthetic DL-, and D- and L-Lombricine and of Natural Lombricine.—The compounds (10 mg. of each) were dissolved in 0.2 cc. volumes of 6 N sulphuric acid and the solutions heated in sealed tubes at 110° for 9 hr. Aqueous barium hydroxide (0.5%) was then added to the cold hydrolysate until no further precipitate was formed, the mixture centrifuged and the supernatant submitted to paper chromatography in solvents B and C. An identical pattern of α-naphthol/diacetyl-, ninhydrin- and molybdate-reacting spots, corresponding with markers of authentic serine, 2-guanidinoethyl phosphate and 2-guanidinoethanol, was observed for all four compounds. In addition, there was also present in all four hydrolysates a trace of another ninhydrin-reacting material of an R_f (0.46 in both systems) similar to that of alanine.[54]

Action of Nitrous Acid in Aqueous Acetic Acid on D-2-Amino-2-carboxyethyl 2-Guanidinoethyl Hydrogen Phosphate and on Natural Lombricine.—Solutions of D-2-amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate (10 mg.) and natural lombricine (10 mg.) in 0.6 cc. volumes of 20% acetic acid were halved and to each half, contained in 5 cc. test tubes and cooled in ice, was added sodium nitrite (3 mg.). The solutions were kept in the ice-bath for 30 min. and then at room temperature for a further 30 min. One of each pair was immediately spotted onto chromatograms which were developed in systems B, C, E, F, G and K. Solvent was removed from the remaining pair under reduced pressure and the gummy residues were kept under vacuum with the pump running for 30 min. at room temperature. N-Hydrochloric acid (0.3 cc.) was then added to each and the tubes stoppered and heated in a boiling water-bath for 30 min.[57] After cooling, both solutions were carefully

(53) For better resolution, the spectrum above 2400 cm.$^{-1}$ was determined with a lithium fluoride prism (Perkin–Elmer Model 12-C instrument).

(54) Ninhydrin-reacting substances of similar R_f values to alanine in a number of systems were also formed on heating serine or phosphoserine in 6 N sulfuric acid at 110°. Though alanine is known to be formed from serine under alkaline conditions,[55] its formation under acid conditions does not appear to have been reported.[56]

(55) F. S. Daft and R. D. Coghill, *J. Biol. Chem.*, **90**, 341 (1931); T. Wieland and L. Wirth, *Ber.*, **82**, 468 (1949).

(56) See, however, M. Damodaran and B. V. Ramachandran, *Biochem. J.*, **35**, 122 (1941).

neutralized with concentrated ammonia solution and submitted to paper chromatography along with the unhydrolyzed reaction mixtures (above) and appropriate reference compounds (2-guanidinoethanol, 2-guanidinoethyl phosphate, N-amidino-DL-serine and 2- and 3-phosphoglyceric acids).

Both reaction mixtures, before hydrolysis, gave identical patterns of ninhydrin-, α-naphthol/diacetyl- and molybdate-reacting spots in all solvent systems. In addition to unchanged starting material, one major and four minor α-naphthol/diacetyl-positive, ninhydrin-negative substances were observed. Of the four minor constituents, present in trace amounts only,[58] one had R_f values in all systems identical with those of 2-guanidinoethyl phosphate; its concentration was not appreciably altered by the hydrolysis. Another had R_f values identical with those of 2-guanidinoethanol. The other two (R_f values, 0.45 and 0.54, respectively; solvent C) were not identified; one appeared to have been completely, and the other partially, destroyed by the hydrolysis.

The major product (R_f values 0.37, 0.35, 0.33, 0.59, 0.32 and 0.33), which contained phosphorus, was almost completely destroyed by the hydrolysis. Its destruction was accompanied by the appearance on the chromatograms of a very strongly α-naphthol/diacetyl-reacting spot, of the same R_f as 2-guanidinoethanol in systems C, B and J[60] and of a ninhydrin- and α-naphthol/diacetyl-negative, phosphorus-containing substance having R_f values in all 6 systems (0.40, 0.35, 0.13, 0.52, 0.23 and 0.11, respectively) identical with or very similar to those of 3-phosphoglyceric acid (0.42, 0.36, 0.13, 0.52, 0.24 and 0.11, respectively). Further, this latter spot was in every instance reinforced by the addition of authentic 3-phosphoglyceric acid to the hydrolysate before chromatography.

There is no substance observed on the chromatograms which could be definitely identified as 2-phosphoglyceric acid. This may have been due to an adverse effect of the ammonium chloride present in the hydrolysate on the chromatographic separation of 2- and 3-phosphoglyceric acids. However, it was not possible to clearly separate mixtures of the two isomers in any of these solvent systems. Chromatograms of the hydrolysates using the system of Cowgill[37] did carry a small spot of a phosphorus-containing substance whose R_f relative to that of the main phosphorus-containing constituent of the hydrolysate, *i.e.*, 3-phosphoglyceric acid, suggested that it might be the 2 isomer. However, the R_f values of both were considerably displaced from those of authentic markers of these two compounds in this system.

Alkaline Degradation of the Copper Complex of: A. SEP.—An aqueous solution of SEP (5 mg.) was warmed on a steam-bath with excess basic copper carbonate for 20 min. Unreacted copper carbonate was removed by centrifugation and the deep blue supernatant adjusted to *p*H 10 with 0.1 *N* sodium hydroxide, the final volume of the solution being approx. 1.5 cc. A similar solution of SEP, but without the addition of copper carbonate, was prepared at the same time. The solutions were then set aside at room temperature, the *p*H being maintained at *ca.* 10 by periodic adjustment with 0.1 *N* sodium hydroxide. Samples of both solutions were taken after 3, 19, 26, 50, 71 and 94 hr. standing and after acidification and removal of copper with hydrogen sulfide, where appropriate, were submitted to paper chromatography in solvent A.

While no degradation of SEP in the absence of copper was observed, little remained after 50 hr. in the solution which contained copper. At 19 hr. there was present on the chromatogram, in addition to much unchanged SEP, a considerable amount of a slow-running ninhydrin- and molybdate-reacting substance of R_f 0.07 (0.04 in solvent B). The concentration of this material had greatly increased after 26 hr. but thereafter slowly fell, although it was still present in appreciable amount after 94 hr. There was also present on chromatograms run after 19 hr., a trace of ninhydrin-positive, phosphorus-containing material with the same R_f as phosphoethanolamine. Unlike the slow-running product, its concentration gradually increased throughout the course of the experiment. After 50 hr. there also appeared on the chromatograms a trace of another ninhydrin-positive, phosphorus-containing substance with an R_f (0.33) slightly lower than that of phosphoserine; it gradually increased in amount with time but its final concentration was very much less than that of the substance behaving as phosphoethanolamine. No phosphoric acid or material of an R_f similar to serine or ethanolamine was observed on any of the chromatograms.

B. **Lombricine.**—Aqueous alkaline solutions of lombricine (5 mg.) and its copper complex (from 5 mg. lombricine) were prepared and examined by paper chromatography (systems A, B and C), after standing for 6, 18, 42 and 96 hr., as described above for SEP.

A trace of 2-guanidinoethyl phosphate was present in the copper-free solution of lombricine after 42 hr. but did not increase in concentration on longer standing. In the copper-containing solution, after 18 hr. standing, very little lombricine remained and none after 42 hr. After 6 hr., there was present an α-naphthol/diacetyl-positive, phosphorus-containing substance of the same R_f as 2-guanidinoethyl phosphate in all three systems. Its concentration increased until all the lombricine had been destroyed and it constituted the major α-naphthol/diacetyl-reacting material formed. In addition, a very small spot (R_f 0.33) of another guanidino compound, which appeared to be faintly positive to the molybdate spray, was observed just below 2-guanidinoethyl phosphate on chromatograms of the 6 hr.-old reaction mixture in solvent C; it could still be seen after 18 hr. on chromatograms developed in solvents A (R_f, 0.47) and C. On the latter chromatograms there also appeared a faint ninhydrin-positive spot of R_f 0.37 in system A and 0.35 in system C. No phosphoric acid, serine or 2-guanidinoethanol was observed on any of the chromatograms.

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(57) Neither natural lombricine nor 2-guanidinoethyl phosphate was affected under these conditions.

(58) As it is known[59] that the reaction of nitrous acid with serine is not entirely restricted to the replacement of the α-amino group by a hydroxy group, the formation of small amounts of by-products in the present case is not surprising.

(59) F. Bettzieche, *Z. physiol. Chem.*, **150**, 177 (1925).

(60) Systems E, F and G caused either streaking or double-spotting of the 2-guanidinoethanol hydrochloride marker.