THE SYNTHESIS OF LOMBRICINE

AND RELATED COMPOUNDS

A THESIS

submitted for the Degree

of

DOCTOR OF PHILOSOPHY

in the

Australian National University

by

IVA MAY BEATTY

March, 1962
This thesis embodies work carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, from March, 1958, to March, 1961, during the tenure of a Research Scholarship for which I am indebted to the Council of the University.
Statement

The regulations of The Australian National University require that a statement be made describing which parts of the work in this thesis have been carried out by myself. This may best be done by quoting from a letter from my Supervisor, Dr. D. I. Magrath, to the Registrar of the University:

".......The identification of lombricine from earthworms and of serine ethanolamine phosphodiester (SEP) isolated from a variety of natural sources (Chapter II) was the result of collaboration with Professor A.H. Ennor, Dr. H. Rosenberg and Miss M. Morgan, who isolated the materials and carried out the enzymic experiment referred to in Section II. Apart from additional collaboration with me in the synthesis of 2-guanidinoethyl phosphate and N-amidino-serine (Chapter II), in the investigation of the reaction of nitrous acid with lombricine (Chapter II) and in preliminary experiments relating to the reactivity of O-methyl-N-phosphorylisourea (Chapter III), the remainder of the work described in the thesis was carried out exclusively by the candidate".

Candidate's signature:

Iva M. Beatty.
Acknowledgements

I wish to express my thanks to Dr. D.I. Magrath for his constant interest and helpful supervision during the course of this work. It is a pleasure to acknowledge my debt of gratitude to Professor A.H. Ennor and Dr. H. Rosenberg, who investigated the biochemical aspects relating to the work, for their cooperation, continued interest and stimulating discussion, and to Dr. R.L. Blakley, whose help and encouragement are much appreciated. I thank other members of the Departments of Biochemistry, Medical Chemistry and Physiology, and of the Biological Inorganic Chemistry Unit, for many profitable discussions.

Grateful acknowledgement is also made to Dr. D. Perrin, Mr. F.V. Robinson and Mr. H. Satrapa, to Dr. E. Spinner and Mr. D.T. Light, to Dr. J.E. Fildes and staff, for pK\textsubscript{a} determinations, infrared spectra and micro-analyses, respectively, and to Mr. B. Thorpe for most of the phosphorus analyses.

Finally, I am indebted to Mrs. P. Lanigan for her care and skill in the typing of this thesis and to Mr. V. Paral and staff for the preparation of photographic prints.
ABBREVIATIONS

The following abbreviations have been used in this thesis:

ATP  adenosine-5' triphosphate
ADP  adenosine-5' diphosphate
AMP  adenosine-5' monophosphate
CTP  cytidine-5' triphosphate
NAD  nicotinamide-adenine dinucleotide*
DNA  deoxyribonucleic acid
RNA  ribonucleic acid
P1   orthophosphate
PP   pyrophosphate
DCC  dicyclohexylcarbodi-imide
SEP  2-amino-2-carboxyethyl 2-aminoethyl hydrogen phosphate

# TABLE OF CONTENTS

**CHAPTER I. INTRODUCTION**

**Section I. Lombricine and Related Naturally-Occurring Compounds**

- Phosphorylated guanidines which act as phosphagens 2
- Phospholombricine as a phosphagen 6
- Other functions of guanidino compounds 8
- Biosynthesis of guanidino compounds 9
- SEP as the probable precursor of lombricine 12
- Compounds with a phosphodiester linkage 13

**Section II. The Structure of Lombricine. Methods for the Synthesis of Guanidino Compounds and Phosphodiesters**

- The structure of lombricine 17
- Synthesis of guanidino compounds 19
- Synthesis of phosphodiesters 34
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Section I.</td>
<td>The Synthesis of DL-, L- and D-SEP and the Identity of the Naturally-Occurring Isomers</td>
<td>59</td>
</tr>
<tr>
<td>Section II.</td>
<td>Synthesis of DL-, L- and D-Lombricine from DL-, L- and D-SEP and the Identity of the Natural Compound with the D Isomer</td>
<td>60</td>
</tr>
<tr>
<td>Section III.</td>
<td>The Biochemical Significance of D-Lombricine and D-SEP of Earthworms and of L-SEP in Reptilian and Avian Species</td>
<td>76</td>
</tr>
<tr>
<td>Experimental</td>
<td></td>
<td>92</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter III.</th>
<th>THE SYNTHESIS OF D-2-AMINO-2-CARBOXYETHYL 2-(N'-PHOSPHORYLGUANIDINO)-ETHYL HYDROGEN PHOSPHATE AND THE CORRESPONDING L ISOMER</th>
<th>101</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis of Phosphorylguanidines</td>
<td></td>
<td>146</td>
</tr>
<tr>
<td></td>
<td></td>
<td>148</td>
</tr>
</tbody>
</table>
The Synthesis of 2-Amino-2-carboxyethyl
2-(N'-phosphorylguanidino)ethyl
hydrogen phosphate

Appendix

Experimental

REFERENCES
LIST OF FIGURES, TABLES, FORMULAE AND SCHEMES

Page followed.

Figure II.1 (2 pages) 117
Figure II.2 (2 pages) 130
Figure III.1 191

Table II.1 72
Table II.2 102
Table III.1 184

Chapter I
III - XI 2
Scheme I.1 9
Scheme I.2 10
XVII - XXII 13
XXIII - XXIV 14
XXV - XXVII 17
Scheme I.3 and Scheme I.4 19
XXVIII - XXXI 20
XXXII - XXXIV 26
XXXV - XXXVI 27
Scheme I.5 29
XXXVII - XLI 29
Scheme I.6 36
Scheme I.7 36
Scheme I.8 37
Scheme I.9 38
<table>
<thead>
<tr>
<th>Scheme</th>
<th>Page followed</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVII - LX</td>
<td>39</td>
</tr>
<tr>
<td>Scheme I.10</td>
<td>41</td>
</tr>
<tr>
<td>Scheme I.11</td>
<td>43</td>
</tr>
<tr>
<td>Scheme I.12</td>
<td>44</td>
</tr>
<tr>
<td>Scheme I.13</td>
<td>45</td>
</tr>
<tr>
<td>Scheme I.14</td>
<td>46</td>
</tr>
<tr>
<td>Scheme I.15</td>
<td>47</td>
</tr>
<tr>
<td>Scheme I.16 and Scheme I.17</td>
<td>49</td>
</tr>
<tr>
<td>Scheme I.18</td>
<td>51</td>
</tr>
<tr>
<td>Scheme I.19</td>
<td>54</td>
</tr>
<tr>
<td>Scheme I.20</td>
<td>54</td>
</tr>
<tr>
<td>Scheme I.21</td>
<td>55</td>
</tr>
<tr>
<td>Scheme I.22</td>
<td>55</td>
</tr>
<tr>
<td>Chapter II</td>
<td></td>
</tr>
<tr>
<td>Scheme II.1</td>
<td>60</td>
</tr>
<tr>
<td>XIV - XVI</td>
<td>78</td>
</tr>
<tr>
<td>Scheme II.2</td>
<td>82</td>
</tr>
<tr>
<td>XXII - XXIV</td>
<td>82</td>
</tr>
<tr>
<td>XXV - XXXI</td>
<td>87</td>
</tr>
<tr>
<td>XXXII - XXXIX</td>
<td>88</td>
</tr>
<tr>
<td>Scheme II.3</td>
<td>89</td>
</tr>
<tr>
<td>Scheme II.4</td>
<td>90</td>
</tr>
<tr>
<td>XLII</td>
<td>95</td>
</tr>
</tbody>
</table>
Chapter III

II - IV 149

V 156

Scheme III.1 159

Scheme III.2 160

Scheme III.3 160

XV - XIX 163

Scheme III.4 and Scheme III.5 164

Scheme III.6 167

Scheme III.7 170

XXVIII - XXXII 175

XXXIII - XXXV 179

XXXVI - XXXIX 180

XL - XLV 181
CHAPTER I

INTRODUCTION

SECTION I. LOMBRICINE AND RELATED NATURALLY-OCcurring COMPOUNDS

The isolation from earthworms (Lumbricus terrestris) of a new phosphorus-containing guanidino compound, lombricine, was reported in 1954 by Thoai and Robin, who, on the basis of evidence to be discussed later (Section II), allocated the structure, 2-amino-2-carboxyethyl 2-guanidino-ethyl hydrogen phosphate (I).

\[
\begin{align*}
\text{I. Lombricine} \\
\begin{array}{c}
\text{OH} \\
\text{P-NH} \\
\text{HO}
\end{array}
\begin{array}{c}
\text{C-NH-CH_2-CH_2-O-P-O-CH_2-CH-COOH} \\
\text{NH}
\end{array}
\begin{array}{c}
\text{OH} \\
\text{NH}_2
\end{array}
\end{align*}
\]

II. Phospholombricine

The presence in earthworm muscle of a phosphorylated derivative of lombricine was also demonstrated (Thoai, Roche, Robin and Thiem, 1953a) and it was assigned the structure, II (Thoai and Robin, 1954; Robin, 1954).

\[
\begin{align*}
\text{II. Phospholombricine} \\
\begin{array}{c}
\text{OH} \\
\text{P-NH} \\
\text{HO}
\end{array}
\begin{array}{c}
\text{C-NH-CH_2-CH_2-O-P-O-CH_2-CH-COOH} \\
\text{NH}
\end{array}
\begin{array}{c}
\text{OH} \\
\text{NH}_2
\end{array}
\end{align*}
\]
When the work to be described in this thesis was commenced, little was known of the biochemistry of lombricine. A reasonable assessment of its biological function and consequent importance could, however, be obtained from a consideration of its relationship to other naturally-occurring compounds.

According to the structure allocated by Thoai and Robin, lombricine (I) possesses a guanidino group, a "mixed" phosphodiester linkage and an α-amino acid (serine) residue.

In this section the occurrence, the metabolic role and biosynthesis of compounds related to lombricine by virtue of these structural features, are discussed.

**Phosphorylated guanidines which act as phosphagens**

In addition to phospholombricine, four phosphorylated guanidines are known to occur in animal tissues which also contain the parent guanidine. These are derived from the monoalkylguanidines, arginine (III), guanidinoacetic acid (glycocyamine, IV) and taurocyamine (V), and the dialkylguanidine, creatine (VI). The structural formula for phosphocreatine and the formulae generally accepted for the phosphorylated derivatives of the monoalkylguanidines, are as shown (VII-X). It is pertinent to point out here that, with the exception of synthetic phospholombricine (II) (see Chapter III) and phosphorylated derivatives of arginine and guanidino-
III. $R = H$; Arginine

VII. $R = H_2PO_3$; Phosphoarginine

IV. $R = H$; Guanidinoacetic acid

VIII. $R = H_2PO_3$; Phosphoguanidinoacetic acid

V. $R = H$; Taurocyamine

IX. $R = H_2PO_3$; Phosphotaurocyamine

VI. $R = H$; Creatine

X. $R = H_2PO_3$; Phosphocreatine

XI. Hirudonine
acetic acid recently synthesised by Cramer and co-workers (Cramer, Vollmar and Scheiffele, 1960; Cramer and Vollmar, 1959), the accepted position for the phosphoryl residue in all previously described preparations of phosphorylated derivatives of monoalkylguanidines, both natural and synthetic, although perhaps correct, remains unproven. The general question of the precise location of the phosphoryl group in these products will be discussed in Chapter III.

The phosphorylated guanidines are commonly called "phosphagens". Although the term as originally used referred to phosphocreatine and reflected the fact that phosphocreatine is acid-labile and yields orthophosphoric acid, the expression is now used to denote compounds which serve as stores of phosphate-bond energy for the synthesis of ATP and, in this sense, the term has been applied to compounds other than phosphorylated guanidines, such as polyphosphates, which in micro-organisms appear to serve as energy reservoirs. Ennor and Morrison (1958) have suggested that "the term 'phosphagen' should be regarded as a generic name embracing all (and restricted to) those naturally-occurring phosphorylated guanidino compounds which function as stores of phosphate-bond energy from which phosphoryl groups may be transferred to ADP to form ATP as a result of enzymic catalysis".

Since it is believed that the energy for muscular
contraction is derived from ATP (Weber and Portzehl, 1954; Szent-Györgyi, 1953, 1958; Morales, Botts, Blum and Hill, 1955; Morales, 1956), the role of phosphagens in the maintenance of an ATP-level in muscle assumes some importance. Although there is now considerable evidence to indicate that phosphagens are intimately associated with the process of muscular contraction, the precise biochemical mechanism of the physiological process has yet to be elucidated.

The isolation of a phosphorylated guanidine is presumptive evidence that the compound acts as a phosphagen in the organism. More convincing evidence may be provided by the demonstration that there is present in the organism an enzyme capable of catalysing the reaction,

\[
\text{Guanidine base} + \text{ATP} \rightleftharpoons \text{phosphorylated guanidine} + \text{ADP}.
\]

Phosphotransferases, the enzymes responsible for the phosphate group transfer reactions (International Union of Biochemistry, 1961), have been described for phospho-creatine, phosphoarginine, phosphoguanidinoacetic acid and phosphotaurocyamine (see Ennor and Morrison, 1958; Morrison, Ennor and Griffiths, 1957). ATP:creatine phosphotransferase and ATP:L-arginine phosphotransferase have been obtained in a crystalline state by Kuby, Noda and Lardy (1954) and Elodi and Szorenyi (1956). Some of
the properties of partially purified preparations of ATP:guanidinoacetate phosphotransferase from extracts of *Nereis diversicolor* and ATP:taurocyamine phosphotransferase from *Arenicola marina* have been described by Thoai (1957).

Although other naturally-occurring guanidino compounds besides those already mentioned are known, there is no evidence that these can be phosphorylated enzymically to yield a phosphagen. However, it has been claimed (Robin, Thoai and Pradel, 1957; Thoai, 1960) that hirudonine, which has the structure, 4-(3-guanidinopropyl)-aminobutylguanidine (XI) (Robin and Thoai, 1961), is the only guanidino compound present in the muscles of the leech, *Hirudo medicinalis*, and on these grounds it has been postulated (Robin, Thoai and Pradel, 1957; Thoai, 1960) that the phosphorylated derivative of hirudonine is the phosphagen in this organism. Proof of the presence of such a phosphorylated derivative or of an enzyme capable of catalysing the transfer reaction described above is, however, lacking. A phosphorylated guanidine present in the protozoan, *Tetrahymena geleii*, should also be mentioned. Seaman (1952) showed that this compound was different from phosphoarginine and the annelid phosphagens, which were later identified as phospho-guanidinoacetic acid and phosphotaurocyamine, and Thoai and Roche (1957) have expressed the opinion that the
compound is indeed distinct from any of the known phosphagens.

**Phospholombri cine as a phosphagen**

The occurrence of lombricine and its phosphorylated derivative (phospholombricine) in the muscle of the earthworm suggested (Thoai, Roche, Robin and Thiem, 1953; Thoai and Robin, 1954) that the latter functions as a phosphagen in this annelid. While the work to be described in this thesis was in progress the presence of ATP:lombricine phosphotransferase in extracts of the muscles of earthworms was demonstrated (Pant, 1959; Rosenberg, Rossiter, Gaffney and Ennor, 1960), thus proving the role of phospholombricine as the phosphagen in earthworms.

Phospholombricine is distinguished from most other phosphagens by its apparently very restricted distribution in nature. Phosphocreatine has long been recognised as the only phosphagen in vertebrates and occurs in all vertebrates which have been examined. For some time phosphoarginine was thought to be the characteristic phosphagen of invertebrates, where it has been found in species of many classes and phyla, but more recently phosphocreatine has also been found in invertebrates (see Ennor and Morrison, 1958; Thoai, 1960). Studies on the distribution of phosphocreatine and phosphoarginine within invertebrate phyla led to the discovery of
phosphoguanidinoacetic acid, phosphoaurocysmine (Thoai, Roche, Robin and Thiem, 1953b) and phospholombricine (Thoai, Roche, Robin and Thiem, 1953a) in one of these phyla. These three phosphagens, as well as phospho-creatine and phosphoarginine, are found in various species within the phylum, Annelida (Greenwald, 1946; Hobson and Rees, 1955; Robin, Pradel, Thoai and Roche, 1959).

Phospholombricine has been detected only in the class, Oligochaetes. However, a guanidino compound with chromatographic behaviour similar to that of lombricine has been detected (Robin, Thoai, Pradel and Roche, 1956) in the eggs of Audouinia tentaculata (a sedentary polychaete). This compound was not the only guanidino compound present in the eggs and it is not known whether it is concerned with the formation of a phosphagen.

Although phospholombricine does not, therefore, appear to be widely distributed in nature, it is nevertheless a member of an important class of naturally-occurring compounds which has attracted considerable attention. The role of phospholombricine as a phosphagen made further studies of the structure and metabolism of lombricine and phospholombricine desirable. In order to make available materials for this purpose, the synthesis of lombricine and phospholombricine was undertaken and
will be described in later chapters of this thesis.

**Other functions of guanidino compounds**

The possibility that phosphorylated guanidino compounds may have biological functions other than the transfer of phosphoryl groups to ADP, by which means they act as reservoirs of phosphate bond energy for muscular contraction, cannot be excluded (cf. Ennor and Morrison, 1958). Indeed, several other pathways for the synthesis and degradation of one phosphagen, phospho-creatine, have been suggested, though these have not been substantiated (see Ennor and Morrison, 1958; Morrison and Ennor, 1960; Morrison and Doherty, 1961; Van Pilsum and Hiller, 1959).

The possibility that guanidino compounds have functions other than formation of phosphagens is suggested by the fact that they are more widely distributed in animal tissue than are the corresponding phosphorylated derivatives. This is certainly so in the case of arginine, which is a normal constituent of both vertebrate and invertebrate tissues, whereas phosphoarginine occurs only in some tissues of certain invertebrates. In addition to its function as a phosphagen precursor, arginine is involved in protein biosynthesis, nitrogen excretion by the urea cycle, and the biosynthesis of other guanidino compounds (see below).

Guanidinoacetic acid and taurocyamine, the guanidine
bases of the phosphagens of certain invertebrates, are also found in vertebrate tissues where the corresponding phosphagens do not occur (Dubnoff, 1941; Thoai, Roche and Olomucki, 1954; Thoai, Olomucki, Robin, Pradel and Roche, 1956). A well-established role of guanidinoacetic acid, additional to its part in phosphoguanidinoacetic acid formation in invertebrates, is that of precursor for creatine in vertebrates (Cantoni and Vignos, 1954). The precise function of taurocyamine in mammalian tissues is unknown.

No role unrelated to phosphagen function has yet been demonstrated for lombricine or phospholombricine, but the above examples of guanidino compounds which are involved in processes other than direct phosphagen synthesis suggest that this is a possibility which must also receive consideration in the case of lombricine.

**Biosynthesis of guanidino compounds**

Knowledge of the pathways by which guanidino compounds are synthesised is not complete but a consideration of the known biosynthetic routes shows that in all these routes the guanidino compounds are formed either directly or indirectly from arginine.

In vertebrate tissue arginine is formed by the series of reactions shown in Scheme I.1 (Brown and Cohen, 1959). The carbamyl group of carbamyl phosphate (XII), which is synthesised from ammonia, bicarbonate
\[
\begin{align*}
\text{NH}_3 + \text{bicarbonate} + 2\text{ATP} & \xrightarrow{\text{carbamyl phosphate synthetase}} \text{carbamyl phosphate} + 2\text{ADP} + \text{P}_i \\
\text{carbamyl phosphate} + \text{ornithine} & \xrightarrow{\text{transcarbamylase}} \text{citrulline} + \text{P}_i \\
\text{citrulline} + \text{aspartate} + \text{ATP} & \xrightarrow{\text{condensing enzyme}} \text{argininosuccinate} + \text{AMP} + \text{PP} \\
\text{argininosuccinate} & \xrightarrow{\text{cleavage enzyme}} \text{arginine} + \text{fumarate} \\
\end{align*}
\]

SCHEME I.1. The Biosynthesis of Arginine.
and ATP (Marshall, Metzenberg and Cohen, 1958), is transferred to ornithine (XIII) to form citrulline (XIV) (Burnett and Cohen, 1957). The latter compound condenses with aspartic acid in the presence of ATP to form an intermediate compound, argininosuccinic acid (XV), which subsequently undergoes conversion to arginine and fumaric acid (Petrack and Ratner, 1958). The reactions outlined in Scheme 1.1 represent the de novo synthesis of the amidino group. Although it has also been claimed (Szorenyi, Elodi and Deutsch, 1954) that arginine is synthesised from citrulline and ammonia in the presence of an extract of lobster muscle, it is not clear how the energy is supplied for this reaction (cf. Petrack, Sullivan and Ratner, 1957).

Attempts have been made (Thoai, 1960) to demonstrate the enzymic formation of other guanidino compounds, viz., creatine and taurocyamine, by analogous routes to that for arginine, but no evidence has yet been provided that such pathways exist.

Guanidino compounds are known to arise from arginine directly or indirectly by transfer of the amidino group of arginine to an acceptor amino compound, e.g., Scheme 1.2, or by degradation or transformation of the carbon chain of arginine. Arginine has a carbon chain unrelated to those of the other known phosphagen bases, including lombricine, and the enzymic synthesis of these guanidino
SCHEME 1, 2. A Transamidination Reaction.
compounds would not be expected to occur by the latter route. Therefore, such routes need not be considered here.

Transamidination reactions from arginine to the acceptors, glycine, ornithine, canaline and hydroxylamine, have been demonstrated (see Ennor and Morrison, 1958). The reactions are reversible (Fuld, 1956) and are inhibited by ornithine (Sorm, Sebesta and Tursky, 1952).

Creatine is formed by methylation of guanidinoacetic acid (Cantoni, 1951; Cantoni and Vignos, 1954), which is synthesised enzymically in vertebrate tissue by transamidination (Borsook and Dubnoff, 1941) and thus arises indirectly from arginine. The pathway for creatine synthesis in invertebrates, however, has not been completely defined. Extracts of echinoids and ophiuroids are able to convert guanidinoacetic acid to creatine in the presence of methyl group donors (Thoai and Robin, 1951a, 1951b), but the biosynthesis of guanidinoacetic acid has not yet been demonstrated in these organisms (Robin, 1954; Thoai, 1960). Failure to demonstrate transamidination involving arginine and glycine, and also taurine, in invertebrate tissue, has been attributed (Ennor and Morrison, 1958) to the degradation of the amidine group donor, arginine, to
ornithine and urea in the crude extracts. Abbott and Awapara (1960) claim to have shown that in the invertebrate, *Arenicola cristata*, taurocyamine is formed by transamidination of taurine by arginine, but their results do not stand critical analysis (cf. Gaffney, 1962).

**Serine ethanolamine phosphodiester as a probable precursor of lombricine**

Ennor and Morrison (1958) suggested that lombricine is synthesized either by a pathway involving transamidination from arginine to serine ethanolamine phosphodiester (SEP, XVI), or by a route similar to that by which arginine itself is formed, which would also involve SEP.

```
0
||
NH_2-CH_2-CH_2-O-P-O-CH_2-CH-COOH
  |
OH NH_2
```

XVI. SEP

At the time Ennor and Morrison made this suggestion SEP had not been shown to occur in earthworms, though it had been isolated from the muscles of the turtle, *Pseudemys elegans*, (Roberts and Lowe, 1954) and from snake and alligator muscle (Ayengar and Roberts, 1957). Its structure was deduced from degradation studies (Roberts and Lowe, 1954) and was later confirmed by chemical synthesis (Jones and Lipkin, 1956). Roberts and Lowe (1954) further assigned an L-configuration to the serine moiety of SEP isolated from turtles. These studies
13.

on the structure and configuration of SEP will be discussed fully in Chapter II.

**Compounds with a phosphodiester linkage**

Lombricine is unique among other naturally-occurring guanidino compounds in its possession of a phosphodiester linkage. SEP and lombricine share this structural detail with several other important groups of naturally-occurring compounds such as phospholipids, polymers of glycerophosphate and of ribitol phosphate, derivatives of these polymers (teichoic acids), the nucleic acids (polynucleotides), polyribose phosphates, and lastly, vitamin $B_{12}$ and its analogues.

Of these compounds, certain types of phospholipids, namely the cephalins (phosphatidylethanolamine, XVII) and phosphatidylserine (XVIII), closely resemble lombricine: the similarity to SEP is even more evident. These and related phospholipids such as lecithins (phosphatidylcholine, XIX), phosphatidylmyoinositol (XX) and the more complex inositol-containing phospholipids, plasmalogen (XXIa or XXIb; postulated structures, see Malkin, 1961), sphingomyelin (XXII) and related compounds, and polyglycerol phospholipids (see Klenk and Debuch, 1959), are all believed to be derivatives of L-$\alpha$-glycerophosphoric acid (Baer and Maurukas, 1955). In phosphatidylserine the amino acid residue has the L-configuration (Folch, 1948).
CH₂-O-COR
|     |     |     
| CH-O-COR
|     |     |     
| CH₂-O-PO-O-CH₂-CH₂-NH₂
| OH |     |     

XVII.

CH₂-O-COR
|     |     |     
| CH-O-COR
|     |     |     
| CH₂-O-PO-O-CH₂-CH₂-N-(CH₃)₃
| OH | OH |     

XIX.

CH₂-O-CH=CH-R'
|     |     |     
| CH-O-COR
|     |     |     
| CH₂-O-PO-O-R''
| OH |     |     

XXIa.

CH(OH)CH=CH-(CH₂)₁₂-CH₃
|     |     |     
| CH-NH-COR
|     |     |     
| CH₂-O-PO-O-CH₂-CH₂-N-(CH₃)₃
| OH | OH |     

XXII.

CH₂-O-COR
|     |     |     
| CH-O-COR
|     |     |     
| CH₂-O-PO-O-CH₂-CH₂-NH₂
| OH |     | COOH 

XVIII.

CH₂-O-COR
|     |     |     
| CH-O-COR
|     |     |     
| CH₂-O-PO-O-CH₂-CH₂-N-(CH₃)₃
| OH | OH |     

XX.

CH₂-O-CH=CH-R'
|     |     |     
| CH-O-COR
|     |     |     
| CH₂-O-PO-O-R''
| OH |     |     

XXIb.

CH₂-O-COR
|     |     |     
| CH-O-COR
|     |     |     
| CH₂-O-PO-O-R''
| OH |     |     

XXII.

R-CO-O = fatty acid residue
R' = C₁₆H₃₃
R'' = (CH₂)₂NH,
(CH₂)₂N(OH)(CH₃)₃ or
CH₂CH(COOH)NH₂
XXIII \( R = O-D-\text{alanyl}-\beta-D-\text{glucosyl} \) residue or
\( O-D-\text{alanyl} \text{N-acytelyglucosaminyl} \) residue

XXIVa \( R = \text{OH} \)
Base = cytosine, uracil, adenine or guanine

XXIVb \( R = \text{H} \)
Base = cytosine, thymine, adenine or guanine
Some micro-organisms have been found to contain in their cell walls and membranes considerable amounts of glycerol or ribitol phosphate polymers in which adjacent polyol residues are linked by a phosphodiester bridge. Those polymers (e.g., XXIII) which contain D-alanyl residues are termed teichoic acids (Armstrong, Baddiley, Buchanan, Kelemen and Neuhaus, 1959) in order to distinguish them from simple glycerophosphate polymers isolated from bacterial walls and membranes (Mitchell and Moyle, 1951; McQuillen, 1955), as well as from certain other ribitol phosphate derivatives (Rebers and Heidelberger, 1959). Partial structures have been assigned to three types of teichoic acids (Armstrong, Baddiley and Buchanan, 1960; Armstrong et al., 1959; Baddiley, 1959).

The nucleic acids, the acidic units of nucleoprotein, are polynucleotides in which the constituent nucleotide residues are joined in phosphodiester linkage as in XXIV. The two types of nucleic acids, the ribonucleic acids (RNA, XXIVa) and the deoxyribonucleic acids (DNA, XXIVb), are believed to be concerned with the biosynthesis of cytoplasmic protein and the transmission of hereditary characters respectively (Davidson, 1960). A polymer of ribose phosphate, lacking the base constituents of the nucleic acids but having in
their place a second similar polyribose phosphate chain, is also known (Overend and Stacey, 1955).

The phosphodiester linkage of the complex molecules of vitamin $B_{12}$ and its analogues (see Smith, 1960) joins the "nucleoside" residue and $\text{l-amino-2-propanol}$, the latter being in amide linkage with a propionic acid residue of the porphyrin-like (corrin) nucleus.

This summary of the naturally-occurring phosphodiesters indicates how common the phosphodiester linkage is in compounds which are of biological importance and which serve in diverse roles in living organisms. The possibility that lombricine might have functions additional to that of precursor of phospholombricine has already been suggested. Such other functions might conceivably be related to its phosphodiester structure.

The close similarity of structure between L-SEP of reptilian origin and the phospholipids led Roberts and Lowe (1954) to suggest that L-SEP might serve as a donor of serine, ethanolamine, phosphoserine or phospho-ethanolamine for the biosynthesis of cephalins or phosphatidylserine. The species which are known to contain L-SEP are vertebrates in which phosphocreatine is the phosphagen and, therefore, SEP is unlikely to function in these species as the precursor of lombricine unless this guanidine, if present, possessed some other role than that of a phosphagen precursor. However, it
is known that lombricine is not present in these species (Ennor and Rosenberg, personal communication) and that SEP of reptilian tissues is not, therefore, concerned with lombricine biosynthesis.

Although the reactions by which phosphodiesters are known to be formed enzymically are of interest in that they suggest possible pathways for the biosynthesis of this bond both in lombricine and SEP, it is outside the scope and purpose of this thesis to discuss the biosynthesis of phosphodiesters.

SECTION II. THE STRUCTURE OF LOMBRICINE.

METHODS FOR THE SYNTHESIS OF GUANIDINO COMPOUNDS AND PHOSPHODIESTERS

In the previous section the biochemical significance of lombricine and phospholombricine has been discussed and attention has been directed to the fact that synthesis of these compounds was desirable to provide material so that their biochemistry could be further explored. Because the established procedure for the synthesis of phosphorylated guanidines involves the phosphorylation of the parent guanidine (see Chapter III), synthesis of lombricine would provide material for use as an intermediate in the synthesis of phospholombricine.

At the commencement of the work to be described in this thesis, the complete structural identification
of lombricine had not been made. The structure, I, had been allocated by Thoai and Robin (1954) on the basis of elementary analysis, a study of the functional groups present in the molecule and examination of the products of acid hydrolysis. Elementary analysis of the compound provided an empirical formula, \( \text{C}_6\text{H}_{15\text{.}}\text{O}_6\text{N}_4\text{P} \). It gave positive Sakaguchi and ninhydrin reactions, indicating the presence of monosubstituted guanidino and free amino groups, respectively. Prolonged acid hydrolysis (8 hr. in 6 N \( \text{H}_2\text{SO}_4 \) at 110°) did not result in any increase in the number of free amino groups, but gave rise to a number of hydrolysis products. These were identified as orthophosphoric acid, 2-guanidinoethyl phosphate (XXV), 2-guanidinoethanol (XXVI) and serine (XXVII) on the basis of a comparison of their \( R_f \) values in six solvent systems with those of appropriate reference samples. It should be noted, however, that the characterisation of one of these reference compounds, viz., 2-guanidinoethyl phosphate, was quite inadequate (see Experimental, Chapter II).

From the above evidence it was concluded (Thoai and Robin, 1954) that lombricine was 2-amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate (I); the configuration of the serine moiety was not, however, investigated. No further work was published by the French group, presumably because of a scarcity of material,
the yield of lombricine being only 60 mg./kg. earthworms. Subsequently, Rey (1956) and Nilsson (1957) isolated small amounts of lombricine from earthworm extracts and identified the compound by its paper chromatographic behaviour and by the degradative procedure described by Thoai and Robin (1954).

Synthesis of lombricine was desirable, therefore, to provide final confirmation of the structure allocated by Thoai and Robin (1954) and to extend the identification to the configuration of the serine moiety.

A consideration of the possible approaches to the synthesis of lombricine must take into account two distinct issues: firstly, the incorporation into the molecule of a guanidino group, and secondly, the synthesis of the mixed phosphodiester linkage. Any approach must, in addition, be applicable to the synthesis of a compound with an optically-active α-amino acid residue. The manner in which these separate issues have been met in the present work, which has resulted in a successful synthesis of lombricine, will be described in the following Chapter. However, before describing the synthesis and the subsequent characterisation of lombricine, it is appropriate to survey the methods which have been developed for the synthesis of guanidino compounds and phosphodiesters in general, with special
emphasis on those of biological interest.

**The synthesis of guanidino compounds**

The two methods most commonly employed in the synthesis of guanidino derivatives are based on the Erlenmeyer synthesis (Scheme 1.3) from an amine salt and cyanamide (Erlenmeyer, 1868) and the Rathke synthesis (Scheme 1.4) from an amine and an alkylisothiouronium or alkylisouronium salt (Rathke, 1881, 1884). Guanidination of amino compounds by other reagents including guanidine and guanidinium salts is less frequently used but should also be considered in this survey. In all these methods the overall process of guanidination involves the substitution of an amino group by an amidino group, \(- \text{C}(=\text{NH})\text{NH}_2\).

A chemical synthesis of lombricine could be achieved by guanidination of the appropriate amino group of SEP, i.e., the ethylamino group. As SEP also contains an amino group in the \(\alpha\) position to a carboxylic acid group, it is pertinent to make a few general remarks about the behaviour of such \(\alpha\)-amino groups towards guanidinating reagents before proceeding to a survey of the methods of guanidination.

When the amino group of an \(\alpha\)-amino acid or its ester is guanidinated the reaction leads, depending on the kind of reagent, the conditions of the reaction and
SCHEME 1.3. Erlenmeyer Synthesis

\[
\begin{align*}
\text{R}^1 \text{NH}_2 \cdot \text{X} \quad \text{C-NH}_2 \\
\text{R-NH}_2 \cdot \text{X} + \text{C-NH}_2 & \rightarrow \text{R-N-C-NH}_2 \\
\end{align*}
\]

SCHEME 1.4. Rathke Synthesis

\[
\begin{align*}
\text{R}^1 \text{NH} + \text{R}'' \text{S-C-NH}_2 & \rightarrow \text{R-N-C-NH}_2 + \text{R}'' \text{SH} \\
\text{R-NH} + \text{R}'' \text{S-C-NH}_2 & \rightarrow \text{R-N-C-NH}_2 + \text{R}'' \text{SH} \\
\text{R}^1 \text{NH} + \text{R}'' \text{O-C-NH}_2 & \rightarrow \text{R-N-C-NH}_2 + \text{R}'' \text{OH} \\
\end{align*}
\]

\[\text{R} = \text{alkyl group}\]
\[\text{R}^1 = \text{H, or alkyl group}\]
\[\text{R}'' = \text{alkyl group}\]
\[\text{X} = \text{Br}^-, \text{Cl}^-, \text{I}^- \text{ or HSO}_4^-\]
the stability relationship of the expected reaction products, either to an \( \alpha \)-guanidino acid or to the corresponding anhydride derived by an intramolecular cyclisation reaction. The anhydride of guanidinoacetic acid, 2-amino-4-imidazolidinone (XXVIII), is commonly called glycocyamidine and thus the whole group of cyclised derivatives of \( \alpha \)-guanidino acids are designated glycocyamidines. In general, the transformation of glycocyamidines to \( \alpha \)-guanidino acids is difficult to achieve because the bond between \( N^1 \) and \( C^2 \) is often prone to attack by the same reagents which hydrolyse the bond between \( C^4 \) and \( N^3 \), but certain of these compounds, including creatinine (1-methylglycocyamidine) and glycocyamidine, may be hydrolysed to \( \alpha \)-guanidino acids by careful treatment with bases (see Lempert, 1959).

Because both amino groups of SEP might, therefore, be expected to be susceptible to guanidination, particular reference will be made in the following discussion to known examples of selective and preferential guanidination of other amino groups in polyamino compounds containing \( \alpha \)-amino acid residues.

(a) \textit{Erlenmeyer synthesis and related methods}. Several procedures for the guanidination of an amine by the use of cyanamide or its derivatives are recorded in the literature. Refluxing an alcoholic solution of an amine
XXVIII.

XXIX.

XXX.

XXXI.
salt with cyanamide for several hours is a common procedure but frequently gives poor yields of guanidino compounds (e.g., Braun, 1933; Lawson, 1956; King and Tonkin, 1946). Guanidinium salts are also obtained by the reaction of cyanamide with an amine salt in aqueous solution at room temperature, the yields being improved in some cases by the presence of free amine in the above reaction mixture (Odo, 1950; Odo and Ichikawa, 1955). Poor yields of guanidino compounds obtained by these procedures have been attributed principally to the dimerisation of cyanamide to dicyandiamide, \( \text{NH}_2\text{C(\text{NH}_2)NHCN} \) (Bannard, Casselman, Cockburn and Brown, 1958; Sugino, Shirai and Aoyagi, 1942; Taylor and Baker, 1937a), although fusion of the latter compound with amine salts has itself been used as a preparative procedure (Werner and Bell, 1922; Traube and Gorniak, 1929; Mold, Ladino and Schantz, 1953; Sugino, Shirai and Aoyagi, 1942; Odo and Sugino, 1942). Fusion of an amine salt with excess cyanamide has been found to be effective for the preparation of certain guanidino derivatives (Sugino et al., 1942; Odo and Sugino, 1942).

The synthesis of guanidino compounds from amines which may undergo degradation on heating, such as \( \alpha \)-amino acids and peptides, has been accomplished by prolonged standing of their concentrated aqueous solutions with cyanamide in the presence of ammonia, e.g., \( \alpha-\text{N-benzoylornithine} \) and \( \delta-\text{N-benzoylornithine} \) yield arginine and
isoarginine derivatives, respectively (Sorensen, 1910). On the other hand, under these conditions the products directly obtained from \(N\)-substituted \(\alpha\)-amino acids are \(N^1\)-substituted glycocyanamidines and not the corresponding \(\alpha\)-guanidino acids; presumably, substitution confers greater stability on the ring compound (Lempert, 1959). The fact that \(N\)-ethylglycine (XXIX) yields 1-ethylglycocyanamide (XXX; Duvillier, 1886; see, however, Armstrong, 1956), whereas \(N\)-methylglycine (sarcosine, XXXI) furnishes creatine (Bloch and Schoenheimer, 1939; Bloch, Schoenheimer and Rittenberg, 1941) and not 1-methylglycocyanamide, is due to the insolubility of creatine hydrate in the reaction mixture (Armstrong, 1956).

Although only mono- and asym. di-substituted guanidines can be prepared from cyanamide by reaction with primary and secondary amines, respectively, \(N,N'\)-substituted guanidines should be obtainable using substituted cyanamides. Mono-substituted cyanamides have been used occasionally as guanidinating reagents to prepare sym substiuted guanidines, but disubstituted cyanamides frequently fail to react in an analogous fashion (Lecher and Demmler, 1927; King and Tonkin, 1946; Bhatnagar, Chopra, Narang and Ray, 1937; Ackermann, 1935; Ackermann and Müller, 1935).

Cyanogen bromide and iodide (BrCN, ICN) have been used for the preparation of \(N,N'\)-dialkylguanidines from
monoalkylamines (Neivelt, Mayo, Tiers, Smith and Wheland, 1951, and references therein; Angyal and Warburton, 1951; Mold, Ladino and Schantz, 1953). Guanidination is accomplished in two steps through an intermediate substituted cyanamide. However, only salts of monoalkylamines were isolated after reaction with cyanogen chloride (Neivelt et al., 1951, and references therein).

Finally, carbodi-imides (RN=C=NR) undergo an addition reaction with ammonia or amines to yield sym, di- or tri-substituted guanidines, respectively (Taylor and Baker, 1937b; Khorana, 1953).

(b) Rathke synthesis. Phillips and Clarke (1923) reported that the sulphate of S-methylisothiourea, which Arndt (1921) had prepared by methylation of thiourea (see Shildneck and Windus, 1943), gave excellent yields of methylguanidine and N,N-dimethylguanidine from methyl- and dimethyl-amine, respectively, in concentrated aqueous solution at 30°. The method was similar to that of earlier workers, Rathke (1881, 1884), Wheeler and Merriam (1903), Wheeler and Jamieson (1908) and Schenck (1912). Schotte, Priewe and Roescheisen (1928) described the method as a Rathke synthesis.

The general procedure of Phillips and Clarke (1923) has been applied to the synthesis of a variety of guanidino compounds by such workers as Braun (1933), King
and Tonkin (1946), Bannard, Casselman, Cockburn and Brown (1958), but the yields by this method are not always satisfactory. Wheeler and Merriam (1903) were probably the first workers to synthesise an \( \alpha \)-guanidino acid from an \( \alpha \)-amino acid by a Rathke synthesis, and King (1930), using their procedure, claims to have synthesised guanidinoacetic acid in 90% yield from glycine, S-methylisothiourea hydrochloride and an equivalent amount of alkali. Slight excess of sodium hydroxide was employed in Steib's synthesis of guanidino derivatives from alkylisothiouronium salts (Steib, 1926) and the reactants are now commonly dissolved in sufficient aqueous alkali (2 N NaOH) to constitute an excess (Brand and Brand, 1942; Izumiya, 1951; Ratner, Petrack and Rochovansky, 1953; Halpern, 1952, 1953). However, the best yields of guanidino acids by this general method appear to be obtained when the reaction of S-methylisothiouronium salts and an amino acid is performed in ammoniacal solution (Fischl, 1934). Thus Schütte (1943) records an 83% yield of creatine from sarcosine and this procedure has been generally adopted (e.g., Mourgue, 1948; Mourgue and Baret, 1955, 1956).

Kapfhhammer and Müller (1934) guanidinated amino acids and peptides with O-methylisourea in methanolic solution at room temperature; this method was also used
by Greenstein (1935) and Schütte (1943). The latter author reported a low yield (20.6%) of creatine from sarcosine and \( \text{O-} \)methylisourea after a reaction time of two weeks and the yield was not improved by raising the temperature of the reaction; cf., the high yield of creatine above. On the other hand, \( \text{O-} \)methylisourea was more reactive than its \( \text{S-} \)analogue or cyanamide in guanidinating the amino groups of proteins (Hughes, Saroff and Carney (1949). In aqueous protein solutions the maximum rate of reaction was found to be at pH 10.5-11; below pH 10 the rate decreased rapidly. As \( \text{O-} \)methylisourea has a \( pK_a \) value of 9.7, and amino groups of proteins generally have \( pK_a \) values of 9-10, Hughes, Saroff and Carney (1949) concluded that the reaction involved the un-ionised form of one or probably both of the reactants. Although guanidination of proteins is effected more rapidly at high pH values, such conditions are detrimental to the reagent and to many proteins. In alkaline solution \( \text{O-} \)methylisourea slowly hydrolyses to methanol and unidentified crystalline deposits (Hughes et al., 1949; Chervenka and Wilcox, 1956; Klee and Richards, 1957).

In an extension of the method, \( \text{N-} \)substituted derivatives of \( \text{S-} \)alkylisothiourea have been used (Schenck, 1912; King and Tonkin, 1946; Angyal and Warburton, 1951; Mold, Ladino and Schantz, 1953) to prepare a variety of
multisubstituted guanidines, including $N_N',N''$-substituted
derivatives which cannot be synthesised by the
Erlenmeyer method.

(c) Reaction of amino compounds with other guanidinating
reagents.

(i) Triacetylanhydroarginine (XXXII), the product
obtained by boiling arginine with acetic anhydride
(Bergmann and Koster, 1926), readily forms guanidine
derivatives under anhydrous conditions by transfer of its
diacetylated amidino group from the $\alpha$-piperidone ring to
the nitrogen of methylamine (Bergmann and Zervas, 1928),
ethyl glycinate or sarcosine ethyl ester (Bergmann and
Zervas, 1927; 1928).

(ii) 1-Amidino-3,5-dimethylpyrazole (XXXIII),
prepared from aminoguanidine and 2,4-pentanedione (Scott
and Reilly, 1952) was introduced by Scott, O'Donovan and
Reilly (1953) as a guanidinating reagent for amino
compounds. As in the case of triacetylanhydroarginine,
the mechanism of the reaction of XXXIII with primary or
secondary amines involves cleavage of the N-C bond external
to the heterocyclic ring. The yield of phenylguanidine,
prepared from the reagent (XXXIII) and excess aniline in
a refluxed aqueous solution, was 96%. A comparison of
this method with that using S-methylisothiourea or
cyanamide indicated that this new reagent could prove
XXXII

\[ 
\begin{align*}
\text{NHCOCH}_3 \\
\text{C} = \text{NCOCH}_3 \\
\text{NHCOCH}_3
\end{align*}
\]

\[ \xrightarrow{\text{CH}_3\text{NH}_2} \]

\[ 
\begin{align*}
\text{NHCOCH}_3 \\
\text{C} = \text{NCOCH}_3 \\
\text{NHCOCH}_3
\end{align*}
\]

XXXIII

\[ 
\begin{align*}
\text{CH}_3
\end{align*}
\]

\[ \xrightarrow{\text{RNH}_2} \]

XXXIV

\[ 
\begin{align*}
\text{H}_2\text{N} \\
\text{C} = \text{SO}_2
\end{align*}
\]

\[ \xrightarrow{\text{NH}_2\text{CH}_2\text{COOH}} \]

\[ 
\begin{align*}
\text{H}_2\text{N} \\
\text{C} = \text{NH} - \text{CH}_2 - \text{COOH}
\end{align*}
\]
extremely useful in preparing guanidino compounds (Bannard, Casselman, Cockburn and Brown, 1958). It has now been tested for its suitability as a reagent for guanidinating proteins. Two advantages have been pointed out by Habeeb (1960): firstly, it is more stable than O-methylisourea, and secondly, it permits guanidination of protein under milder alkaline conditions (pH 9.5) than with O-methylisourea.

(iii) Aminoiminomethanesulphinic acid (formamidine-sulphinic acid) (XXXIV). Walter (1955) studied the reaction of this compound with amino acids and isolated guanidinoacetic acid in 36% yield after reaction of the reagent with glycine in ammoniacal solution.

(iv) Nitroguanidines. \( N \)-alkyl- and \( N,N' \)-dialkyl-guanidines have been prepared from nitroguanidine (XXXV) and \( N \)-alkyl-\( N' \)-nitroguanidine (XXXVI), respectively, by reaction with amines (McKay, 1952; Davis and Elderfield, 1933).

(v) Guanidine and guanidinium salts. When glycine is heated (100-160°) with guanidine carbonate, guanidinoacetic acid is formed and ammonia evolved (McKay, Braun and Hatton, 1954). From the substituted \( \alpha \)-amino carboxylic acids, \( \alpha \)-phenylglycine and sarcosine, cyclised derivatives are obtained; presumably the elevated temperature favours the formation of the substituted glycocyamidines, 5-phenyl-
\[
\begin{align*}
\text{NH}_2 - C = \text{NNO}_2 + \text{RNH}_2 &\rightarrow \left[ \begin{array}{c}
\text{NH}_2 \\
\text{NHR}
\end{array} \right] \\
\text{XXXV.} \\
\text{H}_2\text{O} &\rightarrow \left[ \begin{array}{c}
\text{NH} \\
\text{NH}_2 - C - \text{NHR}
\end{array} \right] \\
\text{NH}_2 - C = \text{NNO}_2 + \text{RNH}_2 &\rightarrow \left[ \begin{array}{c}
\text{NH}_2 \\
\text{NHR}
\end{array} \right] \\
\text{XXXVI.} \\
\text{H}_2\text{O} &\rightarrow \left[ \begin{array}{c}
\text{NH} \\
\text{R'}\text{NH} - C - \text{NHR}
\end{array} \right]
\end{align*}
\]
glycocyanidine and creatinine, rather than the corresponding α-guanidino acids (see Lempert, 1959). During these reactions, thermolysis of guanidine may produce cyanamid which would then function as the actual guanidinating reagent.

On the other hand, guanidine reacts extremely readily at very low temperatures with methyl or ethyl esters of glycine or sarcosine to give glycocyamidine and creatinine, respectively (Traube and Ascher, 1913; Abderhalden and Sickel, 1928a; 1928b; McKay, Braun and Hatton, 1954). Lempert (1959) has pointed out that it is unlikely that guanidine decomposes to cyanamid under the conditions of the reactions (−15° and 0°) or that the formation of cyanamid, if it did occur, could account for the high yield (65%) of creatinine obtained, since the ethyl ester of sarcosine gives only 29% yield of creatinine by reaction with cyanamid (Abderhalden and Sickel, 1928b). Therefore, guanidine itself, or possibly an acyl derivative of guanidine, would appear more likely to function as the actual guanidinating reagent. Although the precise mechanism of the reaction is not clear, the reaction does involve the loss of one guanidinium nitrogen atom, as indicated by the formation of creatinine from sarcosine, and the acylation of another guanidinium nitrogen atom, a reaction which is known to occur readily.
between guanidine and esters of carboxylic acids (Traube, 1910; Traube and Ascher, 1913), or with methyl or ethyl esters of α-guanidino acids, e.g., creatine ethyl ester which, when liberated from its hydrochloride, cyclises intramolecularly with the formation of creatinine (see Mold, Gore, Lynch and Schantz, 1955). Possible mechanisms of the reaction are shown in Scheme I.5.

An interesting reaction which is analogous to the acylation reactions mentioned above is the formation of a glycocyamidine derivative (XXXVIII) and ornithine methyl ester (XXXIX) from the methyl ester of arginine when the latter compound is liberated from its hydrochloride (XXXVII) by titration with alkali (Zervas and Bergmann, 1928). It suggests the possible instability of the corresponding carboxylic ester of lombricine (XL) and its conversion to the carboxylic ester of SEP (XLI) by an analogous intermolecular reaction and subsequent disproportionation.

Finally, guanidine reacts with α-chloro acids, e.g., α-chloroacetic, in the presence of two molecular proportions of alkali at moderate temperatures (35–40°) to give the corresponding α-guanidino acid (Garst and Nossel, 1956).

(d) Guanidination of polyamino compounds; selective and preferential guanidination of particular amino groups. Guanidination of a diamino compound could give rise to
SCHEME I.5. Possible Mechanisms for the Formation of Creatinine from Guanidine and Sarcosine Ethyl Ester
XXXVII.

\[
\begin{align*}
2 \text{NH}_2\text{-C-NH-(CH}_2\text{)}_3\text{-CH-COOCH}_3 & \xrightarrow{\text{OH}^-} \\
\text{NH}_2\text{.C1}^- & \quad \text{NH}_2
\end{align*}
\]

XXXVIII.

\[
\begin{align*}
\text{NH}_2\text{-C-NH-(CH}_2\text{)}_3\text{-CH-CO} & + \text{NH}_2\text{-(CH}_2\text{)}_3\text{-CH-COOCH}_3 \\
\text{NH} & \quad \text{NH} & \quad \text{NH}
\end{align*}
\]

XXXIX.

\[
\begin{align*}
\text{OH} & \quad \text{NH}_2\text{-C-NH-CH}_2\text{-CH}_2\text{-O-P-O-CH}_2\text{-CH-COOCH}_3 \\
\text{NH} & \quad \text{O} & \quad \text{NH}_2
\end{align*}
\]

XL.

\[
\begin{align*}
\text{OH} & \quad \text{NH}_2\text{-C-NH-CH}_2\text{-CH}_2\text{-O-P-O-CH}_2\text{-CH-CO} & + \\
\text{NH} & \quad \text{O} & \quad \text{NH} & \quad \text{NH}
\end{align*}
\]

\[
\begin{align*}
\text{OH} & \quad \text{NH}_2\text{-CH}_2\text{-CH}_2\text{-O-P-OCH}_2\text{-CH-COOCH}_3 \\
\text{O} & \quad \text{NH}_2
\end{align*}
\]

XLI.
two isomeric monoguanidinated products and a diguanidino compound. It is precisely for this reason that the reaction of ornithine (α,δ-diaminovaleric acid) with cyanamide gives low yields of arginine (Schulze and Winterstein, 1899, 1902) and similarly, a mixture of mono- and di-guanidino derivatives is obtained from lysine and α,β-diaminopropionic acid (Winterstein and Künig, 1909).

Later workers developed methods for selectively protecting amino groups. Sörensen (1910) used α-N-benzoylornithine as an intermediate in an Erlenmeyer synthesis of arginine and similarly prepared isoarginine from δ-N-benzoylornithine. Selective guanidination using α-N-tosyl-lysine and cyanamide, and ε-N-benzoyl-lysine and cyanamide or N-ethylisothiourea, was achieved by Steib (1926). Homoarginine, the product obtained by Steib after protecting groups had been removed from the guanidinated intermediates, was also synthesised from α-N-benzoyl-lysine and O-methylisourea by Greenstein (1937) and Stevens and Bush (1950). The difficulty inherent in this general method is the preparation of the protected amino compounds to be guanidinated, since these are obtained by selective removal of one protecting group from a di-amino compound which has both amino groups protected (see references above).
The simplest and most successful method for protecting the α-amino group of ornithine and similar compounds is that of Kurtz (1937, 1949) and Turba and Schuster (1946, 1948). The former obtained arginine in 77% yield from the reaction of the copper complex of ornithine, in which the α-amino function is masked, with O-methylisourea in dilute alkali at room temperature. Guanidination of the terminal amino group of lysine (Kurtz, 1949; Stevens and Bush, 1950; Mourgue and Baret, 1955) and α,β-diaminopropionic acid (Kurtz, 1949; see, however, Greenstein and Winitz, 1961) has been similarly effected. The method utilising copper for the protection of the α-amino group of a carboxylic acid is less effective when an S-alkylisothiourea is used as guanidinating reagent because of the formation of copper sulphides (Kurtz, 1949).

It is possible, however, to achieve preferential guanidination of the terminal amino group of an α,ω-diamino carboxylic acid. Although the diguanidino derivative was obtained by Odo and Ichikawa (1955) as the main product of the fusion of lysine hydrochloride with cyanamide at 140-170°, at room temperature, lysine, or preferably a mixture of lysine and lysine hydrochloride, reacted with aqueous cyanamide to give predominantly homoarginine (41% with lysine, 51% with the mixture),
the amount of the diguanidino derivative formed being correspondingly reduced (9% and 13% yield, respectively). The yield of homoarginine, nevertheless, is not particularly attractive.

There are indications from the work of Odo (1953) that the $\delta$-amino group of ornithine reacts preferentially, if not exclusively, with $\delta$-methylisothiouronium chloride in aqueous solution, since arginine was prepared in 68% yield from ornithine itself. Using a similar method Izumiya (1951) and Odo and Ichikawa (1955) have prepared homoarginine from lysine in 78% and 70% yields, respectively.

Preferential guanidination of $\varepsilon$-amino groups of lysine residues in lysylglutamic acid was observed by Greenstein (1935), who reacted the peptide with excess $\theta$-methylisourea in absolute methanol. The material isolated was predominantly the $\varepsilon$-guanidino derivative but was thought to contain traces of a compound with an $\alpha$-guanidino residue (Greenstein, 1937). A large number of proteins have been guanidinated using $\theta$-methylisourea (see Ramachandran, 1959; Habeeb, 1960) and it appears that the $\varepsilon$-amino groups of the lysine residues of proteins react and that the $\alpha$-amino group of a terminal amino acid residue is not guanidinated (Hughes, Saroff and Carney, 1949; Chervenka and Wilcox, 1956; Klee and Richards, 1957; Shields, Hill and Smith, 1959; see, however, Evans and
Structural characteristics of the protein molecule could contribute, however, to this apparent specificity (Roche, Mourgue and Baret, 1954). No investigation of the guanidination of an α-amino acid such as lysine or ornithine by O-methylisourea seems to have been undertaken, but it might be expected that this reagent would react with such compounds in the same way as S-methylisothiourea.

Summary. Cyanamide and S-alkylisothiourea have been extensively used as guanidinating reagents in syntheses from amino compounds, while O-methylisourea has been used primarily for the guanidination of proteins and for selective guanidination of ω-amino groups in polyamino compounds in which α-amino carboxylic acid residues are protected by chelation with copper. Recent literature suggests that more basic amino groups are more reactive towards guanidinating reagents, particularly S-methylisothiourea, than are α-amino groups of amino acids. Of the other reagents that have been employed, 1-amidino-3,5-dimethylpyrazole, the most recently tested guanidinating reagent, seems the most useful and may subsequently become more widely used.

The method of selective guanidination using O-methylisourea suggested that it might be practical to synthesise lombricine from the copper complex of SEP.
The synthesis of phosphodiesters

Synthesis of a phosphoric ester entails, in principle, the transfer of a phosphoryl group to a hydroxyl group with elimination of a molecule of water. The methods of phosphorylation in use before 1950 have been well summarised by Kosolapoff (1950). Many of these were unsatisfactory, involving rather drastic reaction conditions which led to low yields. Since then, however, methods for the synthesis of phosphoric acid derivatives have developed extraordinarily rapidly, principally as a consequence of a systematic attack on nucleotide chemistry undertaken by Sir Alexander Todd and his colleagues at Cambridge, and by former members of that school in other laboratories.

A large number of compounds have been introduced as phosphorylating agents, the use of which in preparing phosphoric acid derivatives from alcohols has been fully discussed in the recent reviews of Todd (1959a) and Cramer (1960). The following discussion will deal only with those methods of phosphorylation which lead to the formation of phosphodiester bond. These include methods used for the synthesis of phosphodiesters in the phospholipid and nucleotide fields. The synthesis of polynucleotides has been treated specifically in reviews by Todd (1959b, 1961), Khorana (1960) and Cramer (1961).
and will not be discussed here.

A prerequisite to the formation of a phosphodiester linkage is the protection of reactive groups other than the hydroxylic function of the esterifying component, since phosphorylating agents in general lack specificity. The protecting groups introduced prior to phosphorylation must be capable of removal under conditions which do not cause cleavage of the phosphodiester bond or undesired reaction at any other site within the molecule. The use of protecting groups will be illustrated in the methods described.

Phosphodiesters may be grouped into two classes, namely, the symmetrical phosphodiesters (XLII), which have two identical esterifying groups, and the unsymmetrical (or mixed) phosphodiesters (XLIII), which are formed from two different hydroxylic components.

\[
\begin{align*}
\text{XLII.} & \\
(RO)_2P-OH & \quad (RO)-P-(OR') \\
\text{XLIII.} & \\
\end{align*}
\]

Naturally-occurring phosphodiesters (see Section I) are predominantly of the latter class and it is to this class that SEP and lombricine belong. The synthesis of mixed phosphodiesters is more difficult than the synthesis of symmetrical phosphodiesters, and in the following
discussion special consideration is given to the problems associated with the formation of the former, and to suitable methods for their synthesis. For the purposes of this discussion the methods of synthesis have been classified according to the reaction by which the phosphodiester bond is formed and they may be divided into two main types as shown below:

**Type 1.** Syntheses which involve the reaction of an activated form of the phosphate ester of one of the hydroxylic components with the hydroxyl group of the second hydroxylic compound (Reaction 2, Scheme I.6).

**Type 2.** Syntheses which utilise an exchange reaction between the silver salt of a phosphate ester of one of the hydroxylic components and the halide derived from the second esterifying component (Reaction 2, Scheme I.7).

Methods of both types may be further subdivided according to the phosphorylation procedure (Reaction 1, Schemes I.6 and I.7) used to prepare the activated form or the silver salt of the phosphate ester.

**Method Type 1 (a).** Use of a polyfunctional phosphorylating agent: a stepwise reaction for the formation of the phosphodiester bond.

The most successful approach of this type utilises a protected bifunctional phosphorylating reagent, e.g.,
SCHEME 1.6. Synthesis of Phosphodiesters: Type 1

Type 1 (a): $A = \text{Cl}, B = \text{OC}_{6}H_{5}$, etc., Cl.

Type 1 (b): $A = \text{Cl}, B = \text{OCH}_{2}C_{6}H_{5}$.

Type 1 (c): $A = -\text{O-PO-}(-\text{OC}_{6}H_{5})_{2}$, etc., $B = \text{OCH}_{2}C_{6}H_{5}$.

Type 1 (d): $A = -\text{O-PO-0-C(}^{+}\text{NHC}_{6}H_{11})\text{NHC}_{6}H_{11}$, etc., $B = O^{-}$. 
ROH

\[ \text{Reaction 1} \]

\[
\begin{align*}
\text{RO-P-OAg} & + R'X & \rightarrow & \text{RO-P-OR'} \\
\text{B} & & & \text{B}
\end{align*}
\]

\[ X = \text{halogen} \]

\[ B = \text{OC}_6\text{H}_5, \text{etc.} \]

SCHEME 1.7. Synthesis of Phosphodiesters: Type 2
monophenyl phosphorodichloridate (XLIV), and was developed by Baer and his colleagues (see Baer, 1956) for the synthesis of mixed phosphodiesters in the phospholipid field. Stepwise reaction of two different alcohols with monophenyl phosphorodichloridate (see Scheme I.8), followed by the removal of the phenyl group by hydrogenation, furnishes the mixed phosphodiester (L). A serious disadvantage of this method is that the bifunctional reagent (XLIV) reacts with the first alcohol to form the neutral triester (XLVI) in addition to the diester phosphorochloridate (XLV). Therefore, the desired neutral triester (XLVIII), formed by the reaction of XLV with the second alcohol, is contaminated with the triester (XLVI), hydrogenation of which would give rise to a symmetrical phosphodiester (XLVII). In addition to this by-product, another symmetrical phosphodiester (LI) corresponding to the neutral triester (XLIX) derived from unreacted reagent (XLIV) and the second alcohol, is formed.

Although the use of a bifunctional phosphorylating agent does not appear particularly attractive for the synthesis of a mixed phosphodiester because of this unavoidable formation of symmetrical phosphodiesters, the success of the method depends on the fact that stepwise replacement of the halogen atoms becomes progressively more difficult, particularly in the presence of quinoline
**Scheme 1.8. Synthesis of Phosphodiesters:**

Method Type 1(a) using Monophenyl Phosphorodichloridate
(Baer and Kates, 1948), so that the formation of the required unsymmetrical phosphodiester is favoured.

Baer and Kates (1950) effected a synthesis of phosphatidylcholine in 46% yield by the successive phosphorylation of the two hydroxylic compounds, D-1,2-diglyceride (LII) and choline chloride (LV), with monophenyl phosphorodichloridate (XLIV), as shown in Scheme I. In the condensation of XLIV and LII in the presence of pyridine (equimolecular proportions) an appreciable amount of diglyceride phenyl phosphate (LIV) was formed in addition to the desired activated form of the monoglyceride ester (LIII). Addition of choline chloride (LV) to the reaction mixture in the presence of a large excess of pyridine resulted in the formation of the phenyl ester of phosphatidylcholine chloride (LVI), which, after tedious separation from the diglyceride phenyl ester (LIV), was converted to phosphatidylcholine by hydrogenation over a platinum catalyst. The same method, but using N-benzylloxycarbonylethanolamine and the benzyl ester of N-benzylloxycarbonyl-L-serine in place of choline chloride, and both platinum and palladium catalysts to remove the protecting groups, yielded phosphatidylethanolamine in 37-51% yield (Baer, Maurukas and Russel, 1952) and phosphatidylserine in 23% yield (Baer and Maurukas, 1955), respectively.
SCHEME I.9. Synthesis of Phosphatidylcholine:
Method Type 1(a) using Monophenyl Phosphorodichloridate
The type of synthesis outlined in Scheme 1.8 is not generally applicable to the synthesis of phosphodiesters since the reagent will not always effect the formation of the phosphodiester bond between particular hydroxylic compounds, and when it does, it is not always practicable to remove the phenyl group originating from monophenyl phosphorodichloridate by hydrogenolysis without causing some undesired alteration to the phosphodiester molecule.

In syntheses involving the use of this method in the nucleotide field both these drawbacks are evident. Platinum-catalysed hydrogenation causes concomitant reduction of the purine and pyrimidine rings of nucleoside residues, while the alternative procedure for the removal of phenyl groups, namely, alkaline hydrolysis, may result in some degradation of nucleoside ester bonds. The latter procedure has been employed in the syntheses of the symmetrical nucleoside phosphodiesters, uridylyl-(5'-→ 5')-uridine (LVII) and thymidylyl-(5'-→ 5')-thymidine (LVIII) from the appropriately protected nucleosides by phosphorylation with XLIV and mono-p-nitrophenyl phosphorodichloridate, respectively (Gulland and Smith, 1948; see, however, Brown, Haynes and Todd, 1950, for the identification of LVII; Razzell and Khorana, 1959). It is also possible to prepare in low yield an unsymmetrical dinucleoside phosphate with a C₅-C₅ linkage (LIX) or a nucleoside-5' alkyl
OH
\[ \text{CH}_2 \text{O} - \text{PO} - \text{OCH}_2 \]

LVII

\( \text{Uracil} \)

OH
\[ \text{CH}_2 \text{O} - \text{PO} - \text{OCH}_2 \]

LVIII

\( \text{Thymine} \)

OH
\[ \text{CH}_2 \text{O} - \text{PO} - \text{OCH}_2 \]

R, R' = \text{Base}

LIX

R'' = \text{alkyl group}

LX
phosphate (LX) by this method (see Gilham and Khorana, 1958). On the other hand, a similar synthesis of the analogous dinucleoside phosphates with a $C_5-C_3$ bond as found in nucleic acids (see XXIV) has been reported to be unsatisfactory (Gilham and Khorana, 1958). This failure is presumably due to the inertness of the $C_3$ hydroxyl group of the nucleoside to phosphorylating agents of this type (see Hayes, Michelson and Todd, 1955).

In the phospholipid field, hydrogenation following the formation of the phenyl ester of an unsaturated phospholipid (e.g., LVI, $O-COR = \text{unsaturated fatty acid residue}$) is not practical for synthesising a phospholipid with unsaturated fatty acid residues. Baer, Buchnea and Newcombe (1956) synthesised an unsaturated phosphatidylcholine by successive phosphorylation of a suitably protected derivative of glycerol (D-acetone glycerol) and ethylene chlorohydrin with monophenyl phosphorodichloridate, removal of all protecting groups by hydrogenation and mild acid hydrolysis, followed by the incorporation of the unsaturated fatty acid residues into the purified barium salt of glycercylphosphoryl ethylene chlorohydrin, and finally, treatment with trimethylamine under pressure.

Other procedures developed in recent years for the synthesis of unsaturated phospholipids avoid the use of protected phosphorylating agents and, for the protection
of the esterifying components, use only those groups which can be removed without resorting to hydrogenation. Thus, most syntheses of these compounds have been carried out using the polyfunctional phosphorylating agent, phosphoryl chloride. (See, however, syntheses described under Type 1 (b), Type 1 (c) and Type 2). The major drawback to the use of this reagent lies in its polyfunctional nature; it reacts with an alcohol to yield a mixture of products from which the monoester, the sym,diester and a small amount of triester are obtained after the addition of water to the reaction mixture (Kosolapoff, 1950). Stepwise reaction with two different alcohols yields, therefore, a complex mixture of products.

In early attempts using phosphoryl chloride to synthesise saturated phospholipids the mixture of products was inefficiently separated, but purification techniques introduced by Rose (1947), Hunter, Roberts and Kester (1948) and Bevan and Malkin (1951) gave products which were considered to be of satisfactory purity. More recently, Hirt and Berchtold (1957) synthesised phosphatidyl-ethanolamine (see Scheme I.10) by condensation of a saturated D-1,2-diglyceride (LXII) and 2-phthalimidoethyl phosphorodichloridate (LXIII), the latter compound having been isolated from the reaction of 2-phthalimidoethyl alcohol (LXI) and excess phosphoryl chloride. (It would
**Scheme 1.10. Synthesis of Phosphatidylethanolamine:**

**Method Type 1(a) using Phosphoryl Chloride**
appear, however, from the analysis for chloride, that the dichloridate (LXIII) may have been impure). In the condensation reaction, which gives rise to the diester phosphorochloridate (LXIV), the formation of a neutral triester with two glyceride moieties and a phthalimidoethyl moiety is unavoidable and this product (LXV) has to be removed in the purification procedures. Nevertheless, this method (also used for a synthesis of phosphatidylcholine by Hirt and Berchtold, 1958) was important, since the only protecting group, the phthalyl group, can be removed by treatment with hydrazine. The method has since been applied (see Malkin, 1961) to the synthesis of unsaturated phospholipids.

The disadvantages in the use of phosphoryl chloride as a phosphorylating agent have now been partly overcome by the introduction of silicic acid column chromatography, originally developed for the separation of natural phospholipids (see Malkin, 1961). Baer and Buchnea (1959) have prepared a phosphatidylethanolamine in 40% yield by stepwise reaction of an unsaturated 1,2-diglyceride and 2-phthalimidoethyl alcohol with phosphoryl chloride in quinoline, followed by removal of the protecting group and fractionation of the products by silicic acid column chromatography.

Method Type 1 (b). Use of a procedure which converts an
alcohol to a phosphorochloridate via the phosphite intermediate without the concomitant production (as in Method Type 1 (a) ) of the corresponding _sym_ .phosphodiester; subsequent reaction of the phosphorochloridate with a second hydroxylic compound (see Scheme I.11).

The alkyl benzyl phosphorochloridate (LXVIII) is prepared according to the method of Todd (Corby, Kenner and Todd, 1952; Kenner, Todd and Weymouth, 1952) from the corresponding phosphite (LXVII) obtained by the reaction of a suitably protected alcohol with the mixed anhydride, O-benzylphosphorous O,O-diphenylphosphoric anhydride (LXVI), in 2:6 lutidine. This phosphorochloridate (LXVIII), which is analogous to Type 1 (a) intermediates, is then used to phosphorylate directly a second hydroxylic component to give the triester (LXIX) which is subsequently debenzylated by treatment with anhydrous sodium iodide, hydrogenation or alkaline hydrolysis, to yield the required mixed diester.

This method thus avoids the formation of symmetrical phosphodiesters, the disadvantage of the syntheses which employ phosphoryl chloride or a phosphorodichloridate to prepare the required phosphorochloridate. The occurrence of side reactions (discussed below) concomitant with the formation of the phosphodiester from the benzyl phosphorochloridate is, however, a feature of this method of synthesis.
**SCHEME I,11. Synthesis of Phosphodiesters:**

Method Type 1(b) using a Phosphite Intermediate
The first reported synthesis of a C\textsubscript{3}–C\textsubscript{5}, phospho-
diester bond between nucleoside residues was achieved
(Michelson and Todd, 1955) by this method (see Scheme I.12).
Treatment of a protected deoxyribonucleoside, 5'-O-acetyl-
thymidine (LXX) with the mixed anhydride (LXVI) yielded the
protected nucleoside benzyl phosphite (LXXI) which was
converted by treatment with N-chlorosuccinimide to the
corresponding phosphorochloridate, 5'-O-acetylthymidine-
3' benzyl phosphorochloridate (LXXII). The latter
compound was reacted with 3'-O-acetylthymidine (LXXIII)
to form the protected dinucleoside benzyl phosphate
(LXXIV) from which acetyl and benzyl groups were removed
by mild acid and alkaline hydrolysis, furnishing the
unsymmetrical dinucleoside phosphate, thymidylyl-(3'→5')-
thymidine (LXXV). The method was adapted for the synthesis
of dithymidine nucleotide by replacement of the protected
nucleoside (LXX) with the protected nucleotide,
thymidine-5' dibenzyl phosphate.

The yields of these products and of similarly
prepared dinucleoside phosphates with C\textsubscript{2}–C\textsubscript{5}, or C\textsubscript{5}–C\textsubscript{5},
internucleotidic bonds (Michelson, Szabo and Todd, 1956;
Hall, Todd and Webb, 1957) are uniformly low (8–20%),
despite precautionary measures to exclude adventitious
moisture. Hydrolysis of the alkyl benzyl phosphoro-
chloridate (LXVIII from Scheme I.11) would lead to the
Scheme 1.12. Synthesis of thymidylyl-(3'→5')-thymidine:

Method Type 1(b) using a phosphite intermediate
formation of the symmetrical pyrophosphate (LXXVI) as shown in Scheme I.13. The main by-product of the reaction outlined in Scheme I.12 was indeed a symmetrical dinucleoside pyrophosphate (dithymidine-3')$_2$P$_1$P$_2$-pyrophosphate, which would be formed from the compound analogous to LXXVI by the hydrolytic procedures employed for the removal of the acetyl and benzyl protecting groups. Moreover, Hall, Todd and Webb (1957) have indicated a pathway by which pyrophosphates may be formed even in the absence of water. The alkyl benzyl phosphorochloridate (LXVIII) is unstable in the presence of the tertiary base employed in the reaction, decomposing by loss of a benzyl group to the monoester phosphorochloridate (LXXVIII, Scheme I.13). Condensation of the latter with the diester phosphorochloridate (LXVIII) followed by hydrolysis, would then give rise to the symmetrical pyrophosphate (LXXVII) via its chloro benzyl ester derivative (LXXIX). A further possibility for pyrophosphate formation is suggested (Hall, Todd and Webb, 1957) by the observation that a phosphoro-chloridate will react with a phosphotriester even in the absence of moisture, with the production of a pyrophosphate (Hall and Jacobson, 1948). Thus, the triester (LXIX from Scheme I.11) will give the fully esterified pyrophosphate (LXXX) and benzyl chloride, as shown in Scheme I.13, since benzyl groups are known to be attacked
Scheme 1.13. Possible side reactions in Method Type 1 (b)
more readily than alkyl groups in the presence of chloride ions (Lecocq and Todd, 1954). The occurrence of this side reaction in an attempted synthesis of phosphatidylcholine from choline chloride and diglyceride benzyl phosphorochloridate (Brown and Hammond, 1960) may explain the failure to obtain the desired product in a satisfactorily pure state, particularly as there was some evidence that benzyl chloride was formed during the reaction. Moreover, phosphatidylethanolamine was successfully synthesised via the same phosphorochloridate by reaction with N-benzyloxy carbonylethanolamine or phthalimido ethanol.

This synthesis of phosphatidylethanolamine (Scheme I.14) mentioned above is important, as it constitutes the first application of a method originally developed for nucleotidic phosphodiesters to the synthesis of a phospholipid. In this synthesis Brown and Hammond (1960) introduced a modification of the procedure commonly employed which enabled them to obtain a 30% yield of phosphatidylethanolamine from a saturated D-1,2-diglyceride (LXXXI). The benzyl phosphite of the latter compound (LXXXII) was prepared in the way previously described and then treated simultaneously with N-chlorosuccinimide and N-benzyloxy carbonylethanolamine (LXXXIII). This procedure, rather than stepwise addition of the reactants, was
SCHEME I.14. Synthesis of Phosphatidylethanolamine:
Method Type 1(b) using a Phosphite Intermediate
employed in order to reduce the extent of the side reactions due to the expected instability of the benzyl phosphorochloridate corresponding to the phosphite intermediate (LXXXII). The benzyl and N-benzyloxycarbonyl groups of the phosphatidylethanolamine derivative (LXXXIV) were removed by hydrogenation. Alternatively, phthalimidoethanol (LXXXV) was used in place of LXXXIII, and the subsequent removal of the benzyl group and the phthaloyl residue from the resulting protected triester (LXXXVI) effected by treatment with sodium iodide in hot acetone and by reaction with hydrazine, respectively. The latter procedure is adaptable to the synthesis of an unsaturated phosphatidylethanolamine.

**Method Type 1 (c).** This method involves the conversion of a phosphorochloridate derived from one hydroxylic compound and prepared as described in Type 1 (b), to a mixed anhydride (LXXXVII), by reaction with a salt of a strong acid: subsequent reaction of this mixed anhydride with a second hydroxylic compound (Scheme I.15).

The phosphorylating function of the mixed anhydride (LXXXVII) is derived from the weaker acidic component since the main driving force of the reaction in the presence of a base is the expulsion of the more stable anion, i.e., that of the stronger acid (Todd, 1959a, 1959b). Thus, mixed anhydrides of an alkyl benzyl diester of
SCHEME I.15. Synthesis of Phosphodiesters:
Method Type 1(c) using a mixed anhydride intermediate
phosphoric acid with a stronger acid, e.g., diphenyl phosphoric acid, trifluoroacetic acid or toluene-$p$-sulphonic acid, effect phosphorylation of an alcohol to yield a triester (LXXXVIII) which, on debenzylation, gives an unsymmetrical phosphodiester.

Mixed anhydrides have been very successfully employed in the synthesis of $C_5^1$-$C_5^1$ internucleotidic bonds but have been ineffective in attempts made so far to form $(C_3^1$-$C_5^1)$-linked nucleoside derivatives (Hall, Todd and Webb, 1957; Michelson and Todd, 1955). Thus, when benzyl 2$'$:3$'$-$O$-isopropylideneadenosine-$5'$ phosphoro-chloridate, prepared via the corresponding phosphite, was reacted in the presence of 2:6 lutidine with diphenyl hydrogen phosphate and 2$'$:3$'$-$O$-isopropylideneuridine, a product was obtained which gave adenylyl-$(5'\rightarrow 5')$-uridine in excellent yields (70-87%; see Hall, Todd and Webb, 1957) after the protecting groups were removed by mild acid and alkaline hydrolysis. On the other hand, treatment of 5$'$-$O$-acetylthymidine-$3'$ benzyl phosphoro-chloridate (LXXII, see Scheme I.12) with diphenyl phosphoric acid and 3$'$-$O$-acetylthymidine (LXXIII) was shown (Michelson and Todd, 1955) to be ineffective for the synthesis of thymidylyl-$(C_3^1\rightarrow C_5^1)$-thymidine (LXXV). It will be remembered that the latter compound was formed by the direct phosphorylation of the protected derivative of thymidine nucleoside (LXXIII) with the appropriate
phosphoro chloridate (LXXII) according to the method outlined under Type 1 (b), Scheme I.12, but that yields of dinucleoside phosphates were low (8-20%). This is in contrast to the high yields of ($C_5^'-C_5^'$)-linked dinucleoside phosphates obtained via the mixed anhydride intermediate as described above. In certain syntheses, therefore, a mixed anhydride may react much more smoothly than does the corresponding phosphoro chloridate.

Method Type 1 (d). Phosphorylation of one hydroxylic component to form a monoester; activation in situ of the latter by dicyclohexylcarbodi-imide (DCC, LXXXIX) or another appropriate reagent and its subsequent condensation under anhydrous conditions with a second hydroxylic component (Scheme I.16).

Before this method for unsym. phosphodiester synthesis was introduced it had already been shown that carbodi-imides readily reacted with phosphomonoesters to form diester pyrophosphates (Khorana and Todd, 1953; Khorana, 1954) and that ribonucleoside-$2'$ (or $3'$) phosphates, which possess an adjacent cis-hydroxy group, reacted with DCC in aqueous pyridine at room temperature to form ribonucleoside-$2',3'$ cyclic phosphates (Dekker and Khorana, 1954; Tener and Khorana, 1955; Khorana, Tener, Wright and Moffatt, 1957). The synthesis of these cyclic phosphodiesters in the ribonucleotide series had previously been accomplished by treatment of the phosphomonoester with
SCHEME I.16. Synthesis of a Phosphodiester:
Method Type 1(d) using DCC

\[
\begin{align*}
\text{ROH} + (\text{HO})_2\text{PO-OCH}_2\text{CH}_2\text{CN} & \xrightarrow{\text{DCC}} \text{ROPO(OH)}_2 + \text{C}_6\text{H}_{11}\text{N}=\text{C}=\text{NC}_6\text{H}_{11} + \text{R'}\text{OH} \\
\text{RO-PO-OH} + \text{C}_6\text{H}_{11}\text{NH- CO-NHC}_6\text{H}_{11} & \xrightarrow{\text{DCC}} \text{ROPO-OR'} + \text{C}_6\text{H}_{11}\text{NH- CO-NHC}_6\text{H}_{11}
\end{align*}
\]

SCHEME I.17. Preparation of a Phosphomonoester
using DCC and \(\beta\)-cyanoethyl phosphate
trifluoroacetic acid, and proceeds via the intermediate formation of a mixed anhydride of the phosphomonoester (Brown, Magrath and Todd, 1952). Preliminary reports of the extension of these methods to the synthesis of unsymmetrical phosphodiesters were made by Khorana and his colleagues, who used p-toluenesulphonyl chloride (Khorana, Tener, Moffatt and Pol, 1956) or DCC (Khorana, Razzell, Gilham, Tener and Pol, 1957) to activate the phosphomonoesters. A detailed account of the procedures used was subsequently published in 1958 (Gilham and Khorana, 1958).

The preparation of the phosphomonester may be accomplished using a variety of phosphorylating agents, the selection of an appropriate reagent depending on the nature of the alcohol and its protecting groups. Phosphorylating reagents commonly employed are diphenyl and dibenzyl phosphorochloridates, as well as the corresponding substituted dibenzyl and diphenyl derivatives, and tetra-benzyl and tetra-p-nitrophenyl pyrophosphates. These reagents yield a triester in which two of the ester groupings are protecting groups and may be removed by appropriate procedures to yield the desired monoester. The method of phosphorylation using a mixture of β-cyanoethyl phosphate and DCC (see Scheme I.17), recently devised by Tener (1961) for the phosphorylation of the relatively
unreactive $C_3$ hydroxyl function of a deoxyribonucleoside, is of particular interest as it involves in the preparation of the monoester the application of the method of synthesis of phosphodiester bonds at present under discussion. Thus, from the diester (XCl) formed by the activation of $\beta$-cyanoethyl phosphate (XC) with DCC and subsequent reaction with the hydroxylic compound, the monoester corresponding to the latter is obtained by hydrolysis, since this procedure effects the removal of the $\beta$-cyanoethyl group.

The manner in which DCC is postulated to participate in the formation of phosphodiesters from phosphomonoesters has been discussed by Khorana (Smith, Moffatt and Khorana, 1958; Gilham and Khorana, 1958) and Todd (1959a, 1959b, 1961; see also Schofield and Todd, 1961). The first stage of the reaction is represented (see Scheme I,18) by the formation of the adduct (XCII) between the monoester and DCC. This intermediate reacts with a second monoester anion to give the symmetrical pyrophosphate (XCIII). In anhydrous medium and provided a strong base is not present, it is postulated that this is transformed into an active intermediate of the type XCIV, which then phosphorylates the alcohol, $R'O\text{H}$, directly or indirectly, to yield the unsymmetrical phosphodiester. This assumption is necessary since the symmetrical diester pyrophosphate (XCIII) is not
SCHEME I.18. The Mechanism of Activation by DCC.
itself capable of phosphorylating an alcohol. According to Todd's theory, phosphorylation of the alcohol by XCIV proceeds by way of breakdown of the latter to the hypothetical alkyl monomeric metaphosphate, ROPO₂, which then assumes the role of the true phosphorylating reagent (Todd, 1959a, 1959b, 1961; Schofield and Todd, 1961).

In aqueous medium, or in the presence of a strong base, diester synthesis is inhibited and the symmetrical pyrophosphate (XCIII) is the end product of the reaction (Gilham and Khorana, 1958).

There is, however, a second mechanism for the formation of phosphodiesters from a monoester, DCC and an alcohol in anhydrous medium which is not inhibited by the presence of a strong base and thus does not involve a pyrophosphate intermediate. The reaction is dependent on the presence of a large excess of the alcohol (e.g., as solvent) and gives quantitative yields based on the monoester. Presumably in this case, the phosphomonoester adduct (XCII) reacts exclusively with the alcohol to form the phosphodiester directly (Gilham and Khorana, 1958).

The method of phosphodiester synthesis using DCC to activate a monoester has so far proved the most satisfactory way of synthesising internucleotidic bonds (Khorana, 1960). In comparison to the low yields of \( (\text{C}_{3'}-\text{C}_{5'}) \)-linked nucleoside derivatives obtained by other Type 1 methods,
high yields (about 70%) of dideoxyribonucleoside phosphates and the corresponding dinucleotides have been realised by this method (Gilham and Khorana, 1958). The "tailoring" of the method to effect the first synthesis of a 3',5'-diribonucleoside phosphate, uridylyl-(3'→5')-uridine, in high yields (Smith and Khorana, 1959) is a fine achievement since the tailored method provided a neat solution to the difficulty of protecting the vicinal 2'-hydroxyl group of ribonucleosides. Further, the method has proved adaptable to the synthesis of small oligonucleotides. The application of this method to the synthesis of the latter compounds has recently been reviewed by Khorana (1960) and will not be discussed here.

Other reagents that have been used for the activation of a phosphomonoester for subsequent reaction with alcohols include ketene imides (Cramer, 1960) and trichloroacetonitrile (Cramer and Weimann, 1960), and the mechanisms of phosphodiester formation presumably involve intermediates similar to those of the carbodi-imide method (Todd, 1961).

Method Type 2 (a). Phosphorylation of an alcohol by a phosphorodichloridate (a bifunctional reagent) in quinoline to yield a diester of phosphorochloridic acid (a diester phosphorochloridate) from which the silver salt of the corresponding diester of phosphoric acid is obtained: subsequent reaction of the latter with the halide
corresponding to the second hydroxylic component in an anhydrous inert solvent (Scheme I.19).

The method utilising an exchange reaction between a silver salt and a halide in the synthesis of a phosphodiester was introduced in work on phospholipid synthesis by Malkin and his colleagues (Bevan, Malkin and Tiplady, 1957; Baylis, Bevan and Malkin, 1958). These workers prepared silver salt intermediates (XCVII) by the use of monophenylphosphorodichloridate, which (as noted earlier) leads unavoidably to the formation of neutral triester (XCVI) in addition to the phosphorochloridic acid diester (XCV). The latter compound, however, hydrolyses readily in aqueous solution and the phosphodiester thus formed can then be separated from the neutral triester and converted to the silver salt (XCVII).

Malkin and co-workers synthesised phosphatidylethanolamine and phosphatidylserine by the following routes (see Scheme I.20). Reaction of monophenyl phosphorodichloridate with \textit{N}-benzyloxy carbonyl ethanolamine (XCIXa) and with the benzyl ester of \textit{N}-benzyloxycarbonylserine (XCIXb) in the presence of quinoline gave satisfactory yields (44\% and 67\%, respectively) of the corresponding silver salts (Ca and Cb, respectively). Diacylglycerol-\(\alpha\)-iodohydrin (CI) was allowed to react in boiling benzene or xylene solution with Ca and with Cb,
SCHEME I.19. Synthesis of Phosphodiesters:

Method Type 2(a) using Monophenyl Phosphorodichloridate
ROH

C₆H₅OPocl₂

RO-PO-Cl + (RO)₂-PO-OC₆H₅

(1) H₂O

(2) Ag⁺

RO-PO-0Ag + CH₂-O-COR → CH₂-O-COR

CH-O-COR

CH₂I

CH-O-COR

CH₂-O-PO-OR

OC₆H₅

CH₂-O-COR

CH-O-COR

CH₂-O-PO-OR

OC₆H₅

a: R = -CH₂CH₂NHCOC₆H₅

b: R = -CH₂CH(COOC₆H₅)NHCOC₆H₅

SCHEME I.20. Synthesis of Phosphatidylethanolamine and Phosphatidylserine: Method Type 2(a)
and the protecting groups were removed from the resulting triesters by hydrogenation to give phosphatidylethanolamine (CIIa) and phosphatidylserine (CIIb), respectively.

**Method Type 2 (b).** Phosphorylation of a halide derivative corresponding to one hydroxyl component with dry silver dibenzyl phosphate, debenzylolation of the resulting triester to a diester, and conversion of the latter to its silver salt; its subsequent reaction with the halide corresponding to the second hydroxylic component (Scheme I.21).

This route of synthesis precludes the formation of a symmetrical phosphodiester derived from either hydroxylic component of the required mixed phosphodiester. Indeed, the mixed phosphodiester is formed specifically.

Hoefnagel, Stegerhoek and Verkade (1960) have synthesised phosphatidylethanolamine by this elegant method (see Scheme I.22). The silver salt of benzyl diacylglycerol-\(\alpha\)-phosphate (CV) was prepared in 75-85\% yield from diacylglycerol-\(\alpha\)-iodohydrin (CII) via the corresponding dibenzyl ester (CIV) (Gielkens, Hoefnagel, Stegerhoek and Verkade, 1958). By the reaction of equimolar quantities of the silver salt (CV) thus prepared and 2-((dibenzylamino)-ethyl bromide (CVI) and subsequent hydrogenation of the product (CVII), phosphatidylethanolamine was obtained in 80-90\% yield.

Application of this method to the synthesis of
RI + AgO-PO-(OCH₂C₆H₅)₂ →

RO-PO-(OCH₂C₆H₅)₂ + AgI

(1) NaI
(2) IR 120(H⁺)

RO-PO-OH
OCH₂C₆H₅

Ag⁺

RO-PO-OAg + R'I → RO-PO-OR'
OCH₂C₆H₅

RO-PO-OR'
OCH₂C₆H₅

OH

SCHEME I.21. Synthesis of Phosphodiesters:
Method Type 2(b) using silver dibenzyl phosphate
**Scheme 1.22. Synthesis of Phosphatidylethanolamine:**

Method Type 2(b) using silver dibenzyl phosphate
unsymmetrical nucleoside phosphodiesters is restricted by the inaccessibility of the appropriate halogeno intermediates. While Elmore and Todd (1952) succeeded in preparing adenylyl-(5'→5')-uridine by reacting the silver salt of 2':3'-O-isopropylideneadenosine-5' benzyl phosphate with 2':3'-O-isopropylidene-5'-deoxy-5'-iodo-uridine and removing the protecting groups, there do not appear to be any other reports of dinucleoside phosphate synthesis by this method. Michelson and Todd (1955) have reported, however, that attempts to prepare a dinucleoside phosphate containing a (C₃'-C₅') inter-nucleotidic linkage by treating the silver salt of deoxycytidine-3' benzyl phosphate with a suitably protected 5'-deoxy-5'-iodothymidine were unsuccessful.

General remarks. In the above discussion, the various procedures for the synthesis of phosphodiesters have been grouped into two main classes, namely, those in which an activated form of a phosphoryl derivative of one hydroxylic component is reacted with a suitably protected form of the second hydroxylic component (Type 1), and those in which the silver salt of a phosphoric acid ester derived from one hydroxylic component is reacted with a halide corresponding to the other hydroxylic compound (Type 2). Variation in the type of active intermediate involved in the formation of the relevant phosphodiester, or in the
synthetic route used for preparing this intermediate, has been made on the basis of the subdivision of these two main classes.

Classification could also be made according to other criteria. Thus, a classification which distinguishes whether the active intermediate is derived from a phosphomonoester or a phosphodiester has been made by Todd (1959a), and an underlying difference in basic mechanism of phosphorylation postulated. The present approach stresses the essential similarity in principle of all the Type 1 methods on the one hand, and of the Type 2 methods on the other, each particular method of which simply represents an attempt to overcome technical difficulties standing between a full realisation of the principles involved, either by improving the efficiency of the synthetic route leading to the phosphorylating intermediate, or by the introduction of a more active or selective intermediate. Perhaps the most important consideration in the development of suitable procedures is the need for specificity in the synthesis of mixed phosphodiesters in order to avoid concomitant production of symmetrical phosphodiesters.

It should be emphasised that the synthetic methods were developed in order to prepare mixed phosphodiesters belonging to the two important classes of naturally-
occurring compounds, phospholipids and polynucleotides. The potentialities of the various procedures in respect to a synthesis of lombricine or SEP are discussed in the next chapter. However, it may be anticipated that, because these unsymmetrical phosphodiesters bear a close resemblance to phosphatidylserine and phosphatidyl-ethanolamine, their synthesis could very likely be achieved by methods analogous to those suitable for the synthesis of phospholipids.
CHAPTER II


Of the many synthetic approaches to a molecule of the lombricine type, a route involving the synthesis and subsequent guanidination of SEP seemed the most straightforward. The latter compound had already been synthesised in its DL and L forms (Jones and Lipkin, 1956) and the success of the method depended solely on the feasibility of selective or preferential guanidination of the terminal amino group of SEP.

In addition, the synthesis of SEP, the key intermediate in the above reaction scheme, would provide reference material for the possible detection of SEP in earthworms and other animals, and substrate material for enzyme studies designed to determine whether this compound functioned as the biological precursor of lombricine.

In this chapter, the synthesis of the D, L and DL forms of 2-amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate (lombricine) via the guanidination of the corresponding isomers of SEP is described, together with the characterisation of all six preparations. The synthetic compounds are compared with those isolated from natural sources and the biological significance of the
natural compounds in relation to their optical configuration is discussed.

Lombricine and SEP were isolated by Professor Ennor and his colleagues and made available for chemical studies. The development of a procedure (Rosenberg and Ennor, 1959; Ennor, Rosenberg, Rossiter, Beatty and Gaffney, 1960) by which lombricine could be isolated from earthworms in much higher yields than obtained previously (Thoai and Robin, 1954; Robin, 1954; Pant, 1959) not only enabled thorough comparison between it and the synthetic compounds, but also made feasible the isolation of serine and 2-guanidinoethyl hydrogen phosphate from its acid hydrolysate. A complete structural identification of lombricine was therefore made by synthesis and degradation.


The route of synthesis used by Jones and Lipkin (1956) for the synthesis of DL- and L-SEP was accomplished (Scheme II.1) by the stepwise reaction of the corresponding \( \text{N}-\text{benzyloxy} \text{carbonylserine benzyl ester (II)} \) and \( \text{N}-\text{benzyloxy-carbonylthanolamine (V)} \) with monophenyl phosphoro-dichloridate. Hydrogenation of the resulting mixture of neutral phosphate esters, followed by the separation of the components of the reaction mixture by chromatography
on cellulose columns, led to the isolation of DL- and L-SEP (XI) in 36% and 21% yields, based on the serine intermediate. This method is similar to that used by Baer and co-workers for the synthesis of phosphatidylserine and phosphatidylethanolamine (see Chapter I, Section II, Synthesis of Phosphodiesters, Method Type 1 (a)).

Possible alternative approaches to the synthesis of SEP

Of the methods of synthesising mixed phosphodiesters which had appeared in the literature before the present work commenced, none appeared to offer definite advantages over that used by Jones and Lipkin (1956). Syntheses utilising phosphoryl chloride were less attractive because of the polyfunctional nature of this reagent. The yields of mixed nucleoside phosphodiesters obtained by the method using a phosphite intermediate (Type 1 (b)) were uniformly low (8-20%) and, on this basis, it was unlikely that the method could be applied to give SEP in any better yield than had been obtained by Jones and Lipkin (1956). Results published (Brown and Hammond, 1960) while the present work was in progress show that the method does not give a better yield of phosphatidylserine and phosphatidylethanolamine (Scheme I.14) than that obtainable using monophenyl phosphorodichloridate.
Malkin and his colleagues had introduced the silver salt-halide exchange reaction (Method Type 2) for the synthesis of phosphatidylserine and had prepared the silver salt of a protected phosphoserine derivative (XCIXb, Scheme I.20) for use in this synthesis (Bevan, Malkin and Tiplady, 1957). Such a compound would be a suitable intermediate in the synthesis of SEP. Alternatively, a silver salt of a protected derivative of phosphoethanolamine, prepared by a similar method or from 2-phthalimidoethyl phosphorodichloridate synthesised by the method of Hirt and Berchtold (1957), could have been used. However, the selection of a suitably protected halogeno derivative of ethanolamine or serine appeared to present difficulties and the fact that the method for preparing the silver salts utilised the bifunctional reagent, monophenyl phosphorodichloridate (Bevan, Malkin and Tiplady (1957), or phosphoryl chloride (Hirt and Berchtold, 1957), and gave low yields, indicated that this route of synthesis, if practical, would not be much more efficient than the route used by Jones and Lipkin, 1956). Recent work confirms the reservations regarding this method of synthesis for SEP. Baylis, Bevan and Malkin (1958) prepared a silver salt of protected phosphoethanolamine (XCIXa, Scheme I.20) and Theodoropoulos, Gazopoulos and Souchleris (1960) described a different
route of synthesis to a fully protected derivative of phosphoserine from which it was possible to obtain the sodium salt of the p-nitrobenzyl ester of a phosphoserine derivative. However, the yields of these compounds were not very satisfactory. Although a suitable silver salt for use in the synthesis of SEP could possibly be obtained in high yield from 2-dibenzylaminoethyl bromide (Gump and Nikawitz, 1950; Hoefnagel, Stegerhoek and Verkade, 1960) using the method (see Type 2 (b)) described by Gielkens, Hoefnagel, Stegerhoek and Verkade, 1958), recent findings (Hoefnagel, Stegerhoek and Verkade, 1960) have shown that the selection of a suitable halide intermediate for the exchange reaction with a silver salt does present difficulty. In the synthesis of phosphatidylethanolamine (cf. Scheme I.22) the halide, 2-(benzyloxycarbonylamino)-ethyl bromide (Katchalski and Ishai, 1950), which has a benzyloxycarbonyl protecting group on the amino group attached to the β-carbon atom, could not be used successfully.

A mixed anhydride derived from the benzyl phosphoro-chloridate of N-benzyloxycarbonylethanolamine or N-benzyloxycarbonylserine benzyl ester (prepared via the corresponding phosphite) and diphenyl hydrogen phosphate, might react with N-benzyloxycarbonylserine benzyl ester or N-benzyloxycarbonylserine benzyl ester or N-benzyloxycarbonylethanolamine, respectively, as in Type 1 (c), but such syntheses do not always give better yields than those
of Type 1 (a) or 1 (b). In preliminary experiments it was found (Magrath, 1958) that a complex mixture of products was obtained by this method and that only a small amount of SEP was formed.

Finally, the method employing dicyclohexylcarbodi-imide, which has so far proved the most satisfactory in the nucleotide field for the synthesis of mixed phosphodiesters (Type 1 (d) ) but which was not available when the work described in this thesis was commenced, may provide the best approach to the synthesis of SEP. Thus, e.g., the N-benzyloxy carbonyl derivative of phospho-ethanolamine, in the presence of a suitable base, DCC and N-benzyloxy carbonylserine benzyl ester, might react under anhydrous conditions to give the desired protected derivative of SEP from which the protecting groups may be readily removed by catalytic hydrogenation.

The procedure of Jones and Lipkin (1956), with some modification in the isolation and purification procedures, was therefore adopted for the synthesis of DL-, L- and D-SEP described below.

The synthesis of DL-, L- and D-SEP

The protected serine and ethanolamine intermediates, I, II and V, were prepared by known methods, with the exception of the benzyl esters of N-benzyloxy carbonyl-DL-, D- and L-serine (II), which were obtained by a
modification of the method of Baer and Maurukas (1955) in higher yields (80-86%) than previously reported. Some time after these esters were synthesised a modification of the method was described by Baer, Buchnea and Stancer (1959) which gives comparable yields and also reduces the reaction time considerably.

From these intermediates, DL-, L- and D-SEP were obtained in yields of 20-28%, which are comparable to those reported by Jones and Lipkin (1956). The low yields result in part from the formation of symmetrical phosphodiesters during the stepwise reaction of equimolar proportions of \(N\)-benzylxoxycarbonylserine benzyl ester, \(N\)-benzylxoxycarbonylethanolamine and monophenyl phosphoro-dichloridate. The formation of the bis-serine derivative, IV, (in Step 1, Scheme II.1), leaves an excess of monophenyl phosphorodichloridate from which the bis-ethanolamine derivative (VI) may be formed (see Step 2). Appreciable amounts of a compound with an \(R_f\) comparable to that of bis-2-amino-2-carboxyethyl hydrogen phosphate (XIII), which was described by Jones and Lipkin (1956), were detected by chromatography of the reaction mixture after hydrogenolysis. The presence of bis-2-aminoethyl hydrogen phosphate (XII) among the components of the reaction mixture was not definitely demonstrated, but the compound has been reported by Jones and Lipkin (1956).
to be unstable.

The instability of phosphoric acid triesters under acidic conditions may also lower the yield, particularly as the hydrogenation of the mixture of triesters to remove benzylxycarbonyl, benzyl and phenyl groups is carried out in the presence of perchloric acid. Compounds such as phosphoethanolamine, phosphoserine, serine and ethanolamine, which are likely degradation products of the triesters, were, in fact, present in the reaction mixture after hydrogenation. To minimise this degradation the hydrogenation of the mixture of triesters should be carried out as rapidly as possible by removing the protecting groups simultaneously rather than in the sequential manner of Scheme II.1. In the synthesis of phospholipids these protecting groups are usually removed in one operation by the use of a mixture of palladium and platinic oxide catalysts (e.g., Baer and Maurukas, 1955; Baer, 1957) or by using platinic oxide catalyst alone (e.g., Riley, Turnbull and Wilson, 1957), but in some cases the blocking groups have been removed sequentially so that the course of the reactions could be followed more conveniently. This procedure may also be expedient to avoid the loss of both catalysts if a poison for one catalyst is present. Simultaneous removal of the groupings in the present syntheses was not
attempted because of the presence of a platinum catalyst poison. This was indicated by the fact that in the case of derivatives of L- or D-serine, but curiously not of DL-serine, the platinum catalyst, which was added to the mixture of triesters after palladium-catalysed hydrogenation, did not initiate hydrogenation, but after its removal, addition of a second portion of catalyst caused complete hydrogenation to occur. Jones and Lipkin (1956) recorded similar interference in the preparation of L-SEP and found that treatment of the reaction mixture before hydrogenation with acid-washed charcoal (Norit A, the amount not specified) was effective for the removal of catalyst poison. It was not clear, however, whether both stages of the hydrogenation were affected in the preparation described by Jones and Lipkin (1956) or if only the platinum-catalysed hydrogenation was inhibited, as in the present syntheses. Because the hydrogenation during the preparation of DL-SEP proceeded smoothly, there seemed no reason why there should be any interference in the hydrogenation of similar reaction mixtures containing D- and L-serine derivatives, provided that similar precautions were taken to remove quinoline, which is known to be a catalyst poison, from the reaction mixtures. Moreover, it might be expected that the poison would have been adsorbed on the charcoal (Norit A) used
as a carrier for the palladium catalyst. Presumably, however, the carrier charcoal is not thus active, or the amount is insufficient to remove all traces of the poison.

Following the hydrogenation, the separation of DL-SEP from other products was accomplished by chromatography on a cellulose column and a crystalline product was obtained. However, the L and D compounds were not obtained in a chromatographically pure condition after elution from a similar cellulose column. Failure to obtain L- and D-SEP in a pure state by methods identical to those employed for the racemic compound may be explained by slight differences between the solubility characteristics of the optically-active compounds and those of the racemate. Rather than resort to the use of a second fractionation on cellulose, which Jones and Lipkin (1956) undertook for the further purification of L-SEP, ion-exchange chromatography was introduced, 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 elution with water, furnished the diester in pure form. Subsequently, chromatography on Dowex-50 resin was applied to the fractionation of the
hydrogenated material directly, thus avoiding the use of cellulose entirely. The technique using Dowex-50 (NH₄⁺) resin and elution with water proved very useful throughout the course of this work, both in the preparation of SEP and lombricine isomers and of certain reference compounds. It has since been successfully applied in the isolation of some of these compounds from natural sources (see Ennor, Rosenberg, Rossiter, Beatty and Gaffney, 1960; Beatty, Ennor, Rosenberg and Magrath, 1961; Rosenberg and Ennor, 1961a) and of D-serine from an acid hydrolysate of natural lombricine (see below).

All three synthetic products were fully characterised by means of elementary analysis, melting points, optical rotation, infrared absorption spectrum and pKₐ values. The data confirm and extend those quoted by Jones and Lipkin (1956) for the L and DL compounds.

DL-SEP crystallised in colourless prisms from aqueous ethanol, but attempts to crystallise the optically-active isomers in a similar manner were unsuccessful; both were obtained as colourless, hygroscopic needles (microcrystalline) from aqueous methanol.

Chromatography of each of the three products in a number of solvent systems (see Experimental, Table II.2) demonstrated the presence of only one compound, which
contained phosphorus and gave a positive reaction with ninhydrin. The three synthetic compounds had identical \( R_f \) values in all the solvent systems employed.

Elementary analysis revealed that D-SEP, but not DL- or L-SEP, crystallised with solvent of crystallisation (one half mole of methanol). Subsequently it was found that other samples of D- and L-SEP formed solvated crystals, and that there was a variation in the nature and extent of solvation, presumably due to slight variations in the conditions of recrystallisation. These observations are consistent with those of Jones and Lipkin (1956), who found that DL-SEP recrystallised from aqueous ethanol contained no solvate molecules, while L-SEP, which could not be crystallised from aqueous ethanol, contained one-half mole of formamide when crystallised from the latter solvent.

The compounds melted with decomposition. The melting points of L- and D-SEP agreed within experimental error and were comparable to the value previously reported (Jones and Lipkin, 1956) for L-SEP; that of the DL compound was significantly higher than that of the L or D compound and somewhat higher than the value obtained by Jones and Lipkin.

In aqueous solution L-SEP was laevorotatory and the specific rotation was in good agreement with that quoted
by Jones and Lipkin (1956). While D-SEP gave a positive but slightly higher specific rotation, the nature of the compounds makes it difficult to obtain accurate and consistent specific rotations.

The infrared spectrum of the solid racemic compound dispersed in potassium bromide differed appreciably from the corresponding spectra of the D and L compounds, which were identical in spite of the presence of solvate molecules in the D-isomer. This difference (see Experimental, Fig. II.1) can be attributed to differences between the structure of the crystal lattice of the racemic compound and that of the optically-active isomers (Weissberger, 1956). The spectra of DL- and L-SEP showed good agreement with the spectral data (Nujol mull) given for these compounds by Jones and Lipkin (1956). It seems that the presence of solvate molecules in D-SEP (methanol) and in the L-SEP of Jones and Lipkin (1956) (formamide) has not influenced the infrared absorption spectrum of SEP in any detectable manner.

The $pK_a$ values obtained by potentiometric titration were ca. 2, 8.8 and 10.0. The $pK_a$ value of ca. 2 was assigned to the carboxylic acid group and those of 8.8 and 10.0 were tentatively assigned to the $\alpha$-amino and terminal amino groups of SEP, respectively; cf. $pK_a$ values for serine, ethanolamine, phosphoserine and
phosphoethanolamine given in Table II,1. Determination of the \( pK_a \) value of the amino group of lombricine (8,9; see Section II) strongly indicates that this assignment of the \( pK_a \) values found for SEP is correct. It is, of course, to be expected that SEP would possess a fourth dissociation constant due to the ionisation of the phosphorus hydroxy group, the expected \( pK_a \) value of which would be less than 2 (Kumler and Eiler, 1943; Jordan, 1960; Bunton, Mhala, Oldham and Vernon, 1960). This \( pK_a \) was too low to be determined.

Chromatographic examination of an acid hydrolysate (6 N HCl, 105°, 4 hr.) of DL-SEP showed ninhydrin and molybdate-reacting spots which corresponded with markers of authentic serine, ethanolamine, phosphoserine, phosphoethanolamine and orthophosphoric acid, thus confirming similar observations made by Roberts and Lowe (1954). It is apparent, therefore, that cleavage of the phosphodiester linkage occurs on either side of the phosphorus atom leading on the one hand to phosphoethanolamine and serine, and on the other to phosphoserine and ethanolamine, and that the monoesters are subsequently hydrolysed with liberation of orthophosphoric acid. However, the presence in the hydrolysate of traces of unidentified ninhydrin-positive materials indicated that some other reaction, or reactions, occurred. Indeed, Rosenberg and
### TABLE II.1

pK\textsubscript{a} Values of SEP and Related Compounds

<table>
<thead>
<tr>
<th></th>
<th>Carboxylic acid</th>
<th>Secondary phosphoric acid</th>
<th>α-Amino</th>
<th>Amino</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine*</td>
<td>2.21</td>
<td></td>
<td>9.15</td>
<td></td>
</tr>
<tr>
<td>Ethanolamine*</td>
<td></td>
<td></td>
<td></td>
<td>9.4</td>
</tr>
<tr>
<td>Phosphoserine**</td>
<td>2.2</td>
<td>5.82</td>
<td>9.9</td>
<td></td>
</tr>
<tr>
<td>Phosphoethanolamine**</td>
<td></td>
<td>5.82</td>
<td></td>
<td>10.6</td>
</tr>
<tr>
<td>SEP</td>
<td>ca.2</td>
<td></td>
<td>8.8</td>
<td>10.0</td>
</tr>
</tbody>
</table>

*Ansell (1959)

**Determined by Dr. D. Perrin
Ennor (1959) have degraded a sample of L-SEP by treatment with hydrochloric acid to serine, ethanolamine and a third ninhydrin-reacting substance which from preliminary results appeared to be \( \beta \)-chloroethylamine. It was postulated that the latter compound could be formed via a \( \beta \)-elimination reaction from phosphoethanolamine and this hypothesis was supported by the degradation of phosphoethanolamine, under conditions similar to those used for SEP, to a substance whose chromatographic behaviour was identical to that of authentic \( \beta \)-chloroethylamine.

In the present work, the principal interest in the reactivity of the SEP molecule centred around the two amino groups. That the \( \alpha \)-amino group, in conjunction with the carboxyl group, formed a complex with cupric ion in alkaline solution was apparent from the rapid formation of the deep blue colour characteristic of a copper complex of an \( \alpha \)-amino acid. However, at room temperature in aqueous solution at pH 10, under which conditions SEP is stable, the copper complex of SEP was found to be rapidly degraded to phosphoethanolamine. A possible mechanism for this type of degradation, which also occurs in the case of lombricine, will be discussed later.
The characterisation of the naturally-occurring isomers of SEP

It has already been stated that SEP was discovered in extracts of turtle muscle and that it was assigned an L-configuration. However, it seems a little doubtful whether the published data (Roberts and Lowe, 1954) justify the conclusion regarding the configuration. It was demonstrated by paper chromatographic techniques that D-amino acid:O₂ oxidoreductase (deaminating), the enzyme commonly called D-amino acid oxidase, did not destroy the serine in the mixture of products from the action of snake venom (from Crotalus adamanteus) on a sample of SEP which had been isolated from an alcoholic extract of turtle tissue by paper chromatography in two solvent systems. However, no controls are reported which would eliminate the possibility that the D-amino acid oxidase was inhibited by some other component in the mixture. Subsequent comparison with synthetic L-SEP prepared by Jones and Lipkin (1956) is restricted to paper chromatographic behaviour and degradation by snake venom (Jones and Lipkin, 1956; Ayengar and Roberts, 1960).

The presence of small amounts of SEP in earthworms was demonstrated by Rosenberg and Ennor (1959) from evidence based on the chromatographic behaviour of the compound compared with that of synthetic SEP, of its
hydrolysis products and of its amidino derivative (prepared as described in Section II, below). Larger amounts of the compound were later isolated from earthworms (Ennor, Rosenberg, Rossiter, Beatty and Gaffney, 1960), from chicken (Morgan and Beatty, 1960), and from the muscles of the river turtle (Chelodina longicollis) and the crocodile (Beatty, Ennor, Rosenberg and Magrath, 1961).

The isolation of reasonable amounts of SEP from these sources enabled a complete structural identification to be made by comparison with the fully characterised synthetic D- and L-SEP described above. On treatment with acid (6 N HCl, 105°C, 4 hr.) each compound behaved exactly as did synthetic SEP, giving rise to serine, ethanolamine, phosphoserine, phosphoethanolamine and orthophosphoric acid as the major products of degradation. The melting points (decomposition) of SEP from all four sources were comparable to that of D- or L-SEP, and the infrared spectra of the solid samples of SEP obtained from earthworms, turtle and crocodile were identical with that of synthetic D- or L-SEP. As in the case of synthetic D- and L-SEP, the compounds crystallised from aqueous methanol in the form of solvated crystals; elementary analyses were in good agreement with those demanded by theory, assuming particular contributions by
solve molecules for each compound: earthworm SEP, one-half mole of methanol; crocodile SEP, one mole of methanol; turtle SEP, one-half mole of methanol and one-half mole of water.

One important difference distinguished the SEP derived from earthworms from that isolated from reptilian and avian sources. The aqueous solution of the former was dextrorotatory, while solutions of SEP from the latter sources were laevorotatory. Since L-SEP is the laevorotatory isomer, the earlier conclusion of Roberts and Lowe (1954) that L-serine is present in SEP isolated from river turtles was substantiated and, in addition, L-SEP was identified as a constituent of the tissues of the crocodile and chicken. The results also prove conclusively that SEP isolated from earthworms is the D-isomer. The significance of these results will be discussed in Section III below.


The procedure introduced by Kurtz (1937) and applied (Kurtz, 1949; Turba and Schuster (1946; 1948) to the synthesis of a monoguanidinated derivative of a diamino compound containing an α-amino carboxylic acid grouping, utilised the effective bonding in alkaline
solution between this grouping and cupric ion and left the second amino group free to react (see Chapter I, Section II). Application of this procedure to the guanidination of the terminal amino group of SEP proved impossible because of the instability in alkaline solution of SEP in the form of its copper complex (Section I). Thus, the attempted guanidination of the copper complex of SEP with 0-methylisourea at pH 10 gave a complex mixture of products including phosphoethanolamine and 2-guanidinoethyl hydrogen phosphate, but none of the required product. The presence of 2-guanidinoethyl hydrogen phosphate in the reaction mixture could be accounted for by guanidination of phosphoethanolamine, a degradation product of SEP, or by degradation of the required product, lombricine. Subsequently it was discovered that natural lombricine is degraded even more rapidly than SEP in the presence of cupric ions and that 2-guanidinoethyl hydrogen phosphate is formed (see later discussion of this degradation). It was evident, therefore, that degradation of SEP and of any guanidinated product which formed under the experimental conditions, was responsible for the failure of this synthetic approach.

Other methods described in Chapter I, Section II, for the selective guanidination of particular amino groups in polyamino compounds have not been as successful.
as the copper-masking method and were not considered. Instead, attention was given to the possibility of preferentially guanidinating the terminal amino group of SEP.

The guanidination of DL-, L- and D-SEP

Guanidination of unprotected SEP could conceivably lead to the formation of two isomeric monoguanidinated products, XIV and XV, as well as the diguanidino compound, XVI. However, there exist in the literature several examples of the preferential guanidination of particular amino groups in polyamino compounds by use of O-methylisourea or the S-analogue (discussed in Chapter I, Section II). It has been suggested (Schutte, 1943; Roche, Mourgue and Baret, 1954) that the relative basicities of the relevant amino groups determine which is preferentially guanidinated, although in proteins or other large molecules factors such as the accessibility of amino groups may also influence the course of guanidination. In all examples recorded in the literature, with one exception, which is of doubtful authenticity (Mourgue, 1948), the more basic amino group is preferentially guanidinated, a fact which suggests that amino groups react in the ionised rather than the un-ionised form.

Since the terminal amino group of SEP is probably
the more basic, it was considered possible that a preferential guanidination of this amino group might, therefore, be effected. In preliminary experiments it was found that at pH 9, 11 or 12, a guanidino compound behaving on chromatograms as natural lombricine was formed as the major product from the reaction of DL-SEP with O-methylisourea at room temperature. The rate of reaction and the yield were increased when two equivalents of reagent were used rather than one. The lower yield with one equivalent was no doubt due to loss of O-methylisourea by degradation in alkaline solution (Chapter I, Section II). Detectable amounts of several minor unidentified products were formed at pH 9 but only one of these appeared to be formed at pH 11, while at pH 12, in addition to the major product, only traces of 2-guanidinoethyl hydrogen phosphate were observed on chromatograms of the reaction mixture. Chromatographic examination of an acid hydrolysate of a crude product obtained by reaction at pH 11, indicated that the principal product was lombricine.

In larger scale preparations, DL-, L- or D-SEP were allowed to react with two equivalents of O-methylisourea in aqueous solution at pH 11 at room temperature for 8 hrs. The corresponding isomers of lombricine were obtained in yields of 54%, 72% and 62%, respectively.
The isolation of these products was accomplished by chromatography of the reaction mixture on a column of Dowex-50 (NH$_4^+$) resin with water as eluant, by which procedure the products were separated from small amounts of other ninhydrin- and α-naphthol/diacetyl-positive compounds.

The three synthetic products crystallised from aqueous methanol as colourless needles. Each chromatographed on paper in a variety of solvent systems as a single compound which gave colour tests for phosphorus, a free amino group and a monosubstituted guanidino group, and had $R_f$ values identical with those of natural lombricine in a number of solvent systems (see Experimental, Table II.2). Analytical figures for all three synthetic products were in excellent agreement with their formulation as XIV. The melting points (decomposition 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 the corresponding spectra of the D and L compounds and of natural lombricine, which were identical (see Fig. II.2), can be explained on the basis of differences in the structure of the crystal lattice (cf. the more marked
discrepancies between D- or L-SEP and the corresponding racemic compound).

Two \( pK_a \) values for each of the synthetic compounds and for natural lombricline were determined by potentiometric titration, but it was not possible to determine the \( pK_a \) values for the primary phosphoric acid and the guanidino group by this method. Comparison of the \( pK_a \) values obtained (ca. 2 and 8.9 in each case) indicated that it was the more strongly basic group, i.e., the terminal one, of SEP that had been guanidinated, thus supporting structure XIV for the compounds.

Acid hydrolysates of the synthetic compounds and of natural lombricine were examined by the use of ninhydrin, \( \alpha \)-naphthol/diacetyl and molybdate reagents, on paper chromatograms run in a variety of solvent systems. From all hydrolysates identical patterns were obtained, and the spots in these corresponded to markers of authentic serine, 2-guanidinoethanol and 2-guanidinoethyl hydrogen phosphate, thus confirming similar observations made by Thoai and Robin (1954), Rey (1956) and Nilsson (1957) in the case of natural lombricine, and extending them to the 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 the
compound (XIV) with nitrous acid in dilute acetic acid, i.e., under conditions not affecting the guanidino group (Hynd and Macfarlane, 1926), should lead to replacement of the α-amino group by a hydroxyl group (Levene and Schormüller, 1934; Neuberger, 1948), giving 2-carboxy-2-hydroxyethyl 2-guanidinoethyl hydrogen phosphate (XVII, Scheme II.2). In contrast to the parent compound hydrolysis of the product (XVII) would be expected to occur readily because of the presence in the latter compound of a hydroxyl group vicinal to a phosphodiester grouping. Under alkaline or acidic conditions, such a structure readily gives rise to a cyclic phosphodiester; a transesterification reaction occurs leading to the formation of a new ester bond involving the vicinal hydroxyl group, concomitant with loss of the second esterifying component. Opening of the ring of the labile cyclic phosphate then gives rise to a mixture of the two possible monoesters (see Brown and Todd, 1952b; 1953; Lipkin, Talbert and Cohn, 1954; Haake and Westheimer, 1961). It has been shown, for example, that alkyl esters of the 2'- and 3'-monoribonucleotides (partial formula represented by XXIIa and XXIIb) are readily hydrolysed to give an alcohol and a mixture of 2'- and 3'-monoribonucleotides (XXIVa and XXIVb), via the cyclic intermediate (XXIII) (Brown
SCHEME II.2. Degradation of Lombricine by Nitrous Acid
and Todd, 1952a, 1952b; Brown, Magrath, Neilson and Todd, 1956). This effect of vicinal hydroxyl groups also explains the lability of the phosphodiester bond of ribonucleic acids (Brown and Todd, 1952b), phosphatidic acids, e.g., α-glycerophosphorylcholine and α-glycerophosphorylethanolamine (Baer and Kates, 1948a, 1950a; Schmidt, Bessman and Thannhauser, 1953; cf. Brown and Todd, 1952b; Schmidt and Laskowski, 1961), and of 2-aminoethyl 2-hydroxyethyl hydrogen phosphate (Dekker and Lecocq, 1959).

Hydrolysis of the product (XVII) derived from lombricine would, therefore, be expected to give a mixture of 2-guanidinoethanol (XIX) and 2- and 3-phosphoglyceric acid (XX and XXI, respectively) via the intermediate formation of glyceric acid 2,3-cyclic phosphate (XVIII, Scheme II.2). On the other hand, a mixture of N-amidinoserine and 2-hydroxyethyl phosphate would be expected from the isomer of lombricine (XV).

Reaction of either the synthetic D isomer or natural lombricine with nitrous acid in 20% acetic acid, though incomplete, was shown by paper chromatographic examination of the reaction mixture to give almost exclusively a ninhydrin-negative, α-naphthol/diacetyl-positive, phosphorus-containing substance. This was shown to be the expected product (XVII) by its ready
hydrolysis (30 min., N HCl, 100°) to 2-guanidino-ethanol (XIX) and an α-naphthol/diacetyl-negative, phosphorus-containing spot behaving as 3-phosphoglyceric acid (XX) in a number of solvent systems. Under the acid conditions employed for the hydrolysis, phosphoglyceric acid is known to exist primarily in the form of the 3 isomer (Ballou and Fischer, 1954; Ballou, 1957). No clear evidence for the presence of the 2 isomer (XXI) was obtained from the chromatograms, though the solvent systems used included some that have been claimed (Bandurski and Axelrod, 1951; Mortimer, 1952; Cowgill, 1955) 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 by the isolation of D-serine from an acid hydrolysate of natural lombricine. That this did not arise by a racemisation of L-serine follows from the fact that, although serine is slowly decomposed by hot acid, no D isomer has been detected in such solutions (see Meister, 1957a; Neuberger, 1948). Indeed, Stein, Moore, Stamm, Chou and Bergmann (1942) isolated optically pure L-serine after hydrolysing silk
with boiling 20% hydrochloric acid, while Folch (1948) hydrolysed the natural phosphodiester, phosphatidyl-
serine, by refluxing the compound with 6 N hydrochloric acid with recovery of serine, characterised as the L isomer, in good yield.

A solution of natural lombricine was treated with 6 N sulphuric acid at 110° and the hydrolysate fractionated on a column of Dowex-50 (NH₄⁺) resin. Two of the products, serine and 2-guanidinoethyl hydrogen phosphate, were fully characterised, in confirmation of the earlier identification of these degradation products of lombricine by paper chromatography.

The former was characterised as D-serine on the basis of melting point and mixed melting points with authentic D- and L-serine, specific rotation, susceptibility to D-amino acid oxidase and infrared spectrum. In addition, the melting point of the dinitrophenyl derivative was unchanged by admixture with authentic dinitrophenyl D-serine.

The experiment designed to show that the isolated serine functioned as a substrate for the optically specific enzyme, D-amino acid oxidase, was carried out by Professor Ennor, who also showed (Beatty, Magrath and Ennor, 1959) that the presence of D-serine in the crude acid hydrolysate of lombricine could not be similarly demonstrated due to inhibition of the
enzyme under these conditions. Ennor and Rosenberg subsequently found (Beatty, Ennor, Rosenberg and Magrath, 1961) that this inhibition was not due to either 2-guanidinoethyl phosphate or 2-guanidinoethanol and that it could be partly overcome by the use of a more highly purified enzyme preparation. It was also demonstrated (Beatty, Magrath and Ennor, 1959; Beatty, Ennor, Rosenberg and Magrath, 1961) that D-amino acid oxidase was without effect on natural lombricine or synthetic D-SEP.

**Degradation of the copper complexes of SEP and lombricine**

As previously mentioned, the copper complexes of both SEP and lombricine were found to be 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 hydrogen 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 at any stage of the degradation; since the copper complex of serine was not appreciably
degraded under the conditions used, it may be concluded that serine was not formed. Furthermore, no ethanolamine was detectable in the SEP hydrolysate, nor was 2-guanidinoethanol detectable in the lombricine hydrolysate, which suggests that phosphoserine, if formed at all, could not arise by direct hydrolytic fission of the parent molecule.

A similar degradation of SEP when complexed with the copper chelate of 8-hydroxyquinoline-5-sulphonic acid or with cis-chloroaquotetraamino-Co(III) chloride was also observed.

Although the data at present are insufficient to permit of any definite conclusions regarding the mechanisms of degradation, the formation of phosphoethanolamine from SEP and of 2-guanidinoethyl hydrogen phosphate from lombricine without concomitant production of serine, is perhaps best explained in terms of an elimination reaction of the type undergone by \( \beta \)-acyloxy carbonyl compounds. The conversion of these compounds (represented by XXV) under alkaline conditions into \( \alpha-\beta \) unsaturated products (XXVI) was studied by Linstead, Owen and Webb (1953). Particularly relevant to the present discussion is the finding that the phosphoric acid esters, XXVII and XXIX, gave the unsaturated product, XXVIII, in 75% and 33% yield, respectively.
\[ \begin{align*}
XXV. & \quad \text{C-CH-C-OR} + \text{OH}^- \rightarrow \text{C=C=O} + \text{OR} + \text{H}_2\text{O} \\
XXVI. & \\
XXVII. & \quad \text{C}_2\text{H}_5\text{O-C-CH}_2\text{-CH-0-P}_3\text{H}_2 + \text{OH}^- \rightarrow \text{HOOC-CH=CH-CH}_3 + \text{H}_3\text{P}_4 \\
XXVIII. & \\
XXIX. & \quad \text{C}_2\text{H}_5\text{O-C-CH}_2\text{-CH-0-P}_3\text{(C}_6\text{H}_5\text{)}_2 + \text{OH}^- \rightarrow \text{HOOC-CH=CH-CH}_3 + \text{HOPO(C}_6\text{H}_5\text{)}_2 \\
XXX. & \quad \text{CHO-CH(OH)-CH}_2\text{-0-P}_3\text{H}_2 + \text{H}^+ \text{ or OH}^- \rightarrow \text{CHO-CH(OH)=CH}_2 + \text{H}_3\text{P}_4 \\
& \quad \text{CHO-CO-CH}_3 \rightarrow \text{HOOC-CH(OH)CH}_3 \\
XXXI. &
\end{align*} \]
The greater tendency of the diphenyl ester (XXIX) to undergo elimination was explained by the greater stability of the diphenyl phosphate anion.

Mechanisms involving elimination have been advanced to explain the degradation of other esters of phosphoric acid containing appropriately situated carbonyl groups. Whereas simple monoalkyl esters are resistant to alkali (see Bunton, Llewellyn, Oldham and Vernon, 1958), glyceraldehyde-3 phosphate (XXX) is converted into orthophosphoric acid and lactic acid (XXXI) under mildly alkaline conditions (Meyerhof and Lohmann, 1934; Baer and Fischer, 1943). Brown, Fried and Todd (1955) showed that, in contrast to ribonucleoside-5' phosphates (XXXII, $R = H$) and their benzyl esters (XXXII, $R = CH_2C_6H_5$), the corresponding dialdehyde derivatives (XXXIII, $R = H$ and $R = CH_2C_6H_5$) decompose rapidly in alkali at room temperature with the liberation of orthophosphoric acid and monobenzyl hydrogen phosphate, respectively. To account for the lability of phosphoryl groups of phosphoserine residues in proteins (Ferrel, Olcott and Fraenkel-Conrat, 1948; Mecham and Olcott, 1949) a β-elimination as represented by the change XXXIV to XXXV, has been postulated (Anderson and Kelley, 1959; Kalan and Talka, 1959).

Moreover, the instability of the fully protected
XXXII.

XXXIII.

XXXIV.

XXXV.

XXXVI.

XXXVII.

XXXVIII.

XXXIX.
diphenyl esters of phosphoserine (XXXVI) in alkali at room temperature, has been proved (Riley, Turnbull and Wilson, 1953, 1957; cf. Theodoropoulos, Gazopoulos and Souchleris, 1960) to be due to the rapid elimination of the acrylic acid derivative (XXXVII) with concomitant production of diphenyl hydrogen phosphate. On the other hand, the removal of the benzyloxycarbonyl group from XXXVI gives the triester (XXXVIII), which has no substituent on the nitrogen atom and shows little tendency to undergo a β-elimination reaction under similar conditions. When this ester (XXXVIII) is heated to 90° in the presence of alkali, however, β-elimination does occur (Riley, Turnbull and Wilson, 1957). This temperature effect is also significant in the alkaline degradation of SEP. Whereas SEP is stable to alkali at room temperature in the absence of copper, it is degraded in 0.1 N NH₄OH at 105° to phosphoethanolamine without the concomitant formation of serine, phosphoserine or ethanolamine (Roberts and Lowe, 1954), which suggests that a reaction pathway similar to that by which the copper complex is degraded at room temperature may be operating at the higher temperature.

The facile degradation of the copper chelates of SEP and lombricine by a mechanism involving elimination may be represented as shown in Scheme II.3. The strongly
SCHEME II.3. The Mechanism Postulated for the Degradation of SEP and Lombricline
The 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 (Ingold, 1953), with subsequent elimination of the phosphoryl residue.

The postulated role of the metal ion in the above scheme is similar to that advanced for the metal in the reactions of amino acids which are catalysed by pyridoxal and metal ions. Serine is degraded in aqueous solution (pH optimum, ca. 9) to pyruvic acid and ammonia, while phosphoserine yields orthophosphoric acid in addition to the above products but at a much faster rate (Metzler and Snell, 1952; Longenecker and Snell, 1957). It is significant that the reactions depend on the presence of both pyridoxal and metal ion, and that phosphoethanolamine does not undergo this type of reaction. According to the mechanism proposed for these reactions (see Scheme II.4), pyridoxal forms a Schiff base with serine or phosphoserine and in the corresponding chelate structure (XL) the metal ion provides an electron-attracting group that operates in the same direction as the nitrogen of the cyclic ring. The intensive electron displacement from the α-carbon
Scheme II. 4.

R = H or PO(OH)$_2$
atom results in labilisation of the α-hydrogen atom with release of the β-substituent as an anion, i.e., \( \text{OH}^- \) or \( \text{H}_2\text{PO}_4^- \). In each case hydrolysis of the Schiff base of the aminoacrylic acid (XL1) gives pyridoxal, pyruvic acid and ammonia as final decomposition products (Metzler, Ikawa and Snell, 1954; Snell, 1958).

The essential feature of the copper-catalysed degradation of SEP and lombricine to be taken into account by any proposed mechanism is the fact that serine is not formed. Thus, although metal-catalysed hydrolysis of phosphate esters by di- and tri-valent cations in alkaline solution has been reported by several groups of investigators (Helleiner and Butler, 1955; Westheimer, 1957; Butcher and Westheimer, 1955; Bamann, Riechl and Nicolai, 1956; Bamann and Trapmann, 1957), there seems to be little analogy between their findings and the present case where something other than simple hydrolysis is obviously involved.

Even though a mechanism such as is postulated above (Scheme II.3) seems to fit closely the facts relating to the degradation of lombricine and might reasonably be expected to apply equally to SEP degradation, the precise significance of the unidentified "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 II.2), suggests that the 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 regarding its structure is available.

Whatever the mechanism, the implications of this metal-catalysed degradation regarding the stability of other naturally-occurring phosphoric esters, e.g., phosphatidylserine, seems worthy of investigation.

SECTION III. THE BIOCHEMICAL SIGNIFICANCE
OF D-LOMBRICINE AND D-SEP OF EARTHWORMS
AND OF L-SEP IN REPTILIAN AND AVIAN SPECIES

The studies reported above show unequivocally that lombricine isolated from earthworms is D-2-amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate and that SEP from earthworms also contains a D-serine residue. On the other hand, SEP, isolated from river turtle, crocodile and chicken, is the L isomer.

The most interesting biochemical aspect of these naturally-occurring compounds is the presence of a D-amino acid residue in lombricine from the earthworm, in which organism the phosphorylated derivative of
lombricine acts as the phosphagen. Lombricine is the first compound from animal tissue which has been shown unequivocally to contain a D-amino acid residue.

The occurrence of D-amino acids and their derivatives in micro-organisms is now well established (Berg, 1953; Meister, 1957b; Ikawa, Snell and Lederer, 1960) and some products of microbiological origin containing D-amino acid residues have recently been recognised as functional structures of such cells, e.g., D-alanine residues in the teichoic acids of bacterial cell walls and in a nucleotide derivative suggested as a probable precursor in the biosynthesis of cell wall components (Ikawa, Snell and Lederer, 1960).

On the other hand, it seems that the occurrence of D-amino acids and their derivatives in animal tissues is a rarer event. In fact, the few reports which have appeared up to the present time have, for one reason or another, not met with general acceptance (Neuberger, 1948; Miller, 1950; Greenstein, 1954; Meister, 1957b).

It is not intended to record here details of the controversy over reports of the isolation of small amounts of D-amino acids from acid hydrolysates of various animal proteins, the first such report being that of Kögl and Erxleben (1939). The uncertainty as to whether or not a protein actually contains a D-amino acid component stems...
from difficulty in assessing the extent of racemisation occurring during hydrolysis of the protein (Desnuelle, 1953; Neuberger, 1948) and isolation of the amino acid in question, coupled with the fact that the D isomer is always isolated in small amount relative to that of the L isomer. Although the detection of small amounts of D-amino acids in protein hydrolysates cannot, therefore, be regarded as evidence for the occurrence of D-amino acid residues in protein, this possibility cannot be completely disregarded (cf. Meister, 1957b; Greenstein, 1954).

In a report by Fuchs (1937-1938) claiming the isolation of DL-alanine and DL-glutamic acid from goose and insect tissue the details are insufficient to exclude the possibility that these compounds arose by racemisation of the L isomer of these amino acids. Moreover, the characterisation of the isolated products must be regarded as unsatisfactory.

Evidence of the identification of a basic compound isolated from fish liver, as D-ornithine (Ackermann and Mohr, 1937-1938), rests solely on the sign of its optical rotation and an approximately correct elementary analysis of its picrate derivative.

Auclair and Patton (1950) detected D-alanine in the blood of the milkweed bug, Oncopeltis fasciatus, by
employing the enzyme, D-amino acid oxidase, in conjunction with paper chromatography, but reached no conclusion as to whether the origin of the compound was connected with intermediary metabolism, or was the result of bacterial activity in the digestive tract of this organism.

Each of these reports is subject to reservation on the grounds of inadequate structural characterisation, uncertainty as to the role played by racemisation during isolation, or the possibility that the compound may have originated from contaminating microorganisms. Furthermore, no functional importance has been ascribed to these compounds.

Substantial evidence has been advanced, however, for the allocation of the D configuration to the asymmetric carbon atom in the 'alanine' moiety (i.e., the \(\alpha\)-substituted propionic acid residue) of octopine (XLII), a compound isolated from scallop and octopus muscle (Morizawa, 1927). Since octopine is synthesised by the reaction of L-arginine with L-\(\alpha\)-bromopropionic acid in alkaline solution (Akasi, 1937; Ackermann and Mohr, 1937; Irvin and Wilson, 1939; Herbst and Swart, 1946), a reaction in which the bonds about the asymmetric carbon atom of L-arginine remain intact, an L configuration can be assigned with certainty to the groups about this
NH₂
| C=NH
| NH
| (CH₂)₃
| CH-NH₂ + Br-CH
| COOH COOH

Chemical synthesis

NH₂
| C=NH
| NH
| (CH₂)₃ CH₃
| CH-NH-CH₂
| COOH COOH

XLII.

NH₂
| C=NH
| NH
| (CH₂)₃
| CH-NH₂ + C=O
| COOH COOH

Enzymic synthesis

NAD
carbon atom. The configuration of the second asymmetric centre, i.e., that belonging to the \( \alpha \)-substituted propionic acid residue, depends on the mechanism of the reaction. Izumiya, Wade, Winitz, Otey, Birnbaum, Koegel and Greenstein (1957) demonstrated that the coupling reaction proceeded with second order kinetics indicative of an \( S_N^2 \) mechanism which is considered to proceed with inversion of stereochemical configuration. This established with reasonable certainty that octopine is an \( N \)-substituted derivative of D-alanine. Similar conclusions had earlier been reached on the basis of optical rotatory dispersion data (Herbst and Swart, 1946; confirmed by Izumiya et al., 1957), but the latter data is not generally considered to furnish an unequivocal answer, since the method of interpreting rotatory dispersion measurements is not valid for all amino acids (Neuberger, 1948; Djerassi, 1960).

L-Arginine has long been accepted as the precursor of octopine (Moore and Wilson, 1937; Irvin, 1938; Obata and Iimori, 1952). Recently, Thoai and Robin (1959, 1960) demonstrated that the biosynthesis of octopine involves the reductive condensation of L-arginine and pyruvic acid in the presence of an enzyme which is an NAD-dependent oxidoreductase and which is specific for a compound carrying both \( \alpha \)-amino and \( \omega \)-guanidino groups (Thoai and
Robin, 1961). The possibility that the same enzyme might catalyse the alternative condensation of D-alanine and α-keto-ω-guanidinovaleric acid therefore is excluded. Furthermore, there is no evidence for the occurrence of D-alanine in tissues containing octopine. Thus, octopine is metabolically related to L-arginine and not to D-alanine.

The isolation of small amounts of D-SEP from earthworms (Section I; Ennor, Rosenberg, Rossiter, Beatty and Gaffney, 1960) was consistent with the hypothesis that this compound might function as the biological precursor of lombricine (Ennor and Morrison, 1958). Additional evidence for this view was obtained from in vivo studies with earthworms. It was shown that the incorporation of $^{32P}$ orthophosphate, $^{14}C$ethanolamine, DL-$^{14}C$serine, L-$^{3}H$serine and D-$^{3}H$serine into SEP was considerably greater than into lombricine, as would be the case if SEP were a precursor of lombricine and not its degradation product (Gaffney, Rossiter, Rosenberg and Ennor, 1960; Rossiter, Gaffney, Rosenberg and Ennor, 1960a, 1960b). Finally, the oral administration of $^{14}C$-amidine labelled arginine to an earthworm resulted in the labelling of the amidine group of lombricine (Rossiter et al., 1960a, 1960b), which is consistent with the view that SEP acts as the
acceptor molecule in a transamidination reaction with arginine.

The finding of D-serine in both lombricine and SEP of earthworms and the in vivo incorporation of labelled D-serine into the compounds suggested that free D-serine or a D-serine derivative might occur as possible precursors of SEP in the earthworm. Subsequently, Rosenberg and Ennor (1960, 1961b) isolated D-serine as the racemate from perchloric acid extracts of earthworms. The possibility that the D-serine isolated, which amounted to 25% of the total serine extracted, could have arisen by racemisation of L-serine induced by procedures used in the extraction and isolation, was precluded by control experiments, e.g., a solution of L-serine, or a rabbit muscle extract, submitted to similar treatment, failed to yield any D-serine. Further, the possibility that D-serine may arise as a result of enzymic breakdown of lombricine or SEP was investigated, particularly as Robin (1954) had reported that complete breakdown of lombricine to serine and 2-guanidinoethanol occurred in the presence of insoluble particles of worm homogenates. Rosenberg and Ennor (1961b), however, were unable to confirm Robin's observations or to obtain evidence of a similar degradation of SEP, and concluded that the D-serine isolated formed part of the free amino acid
Following the discovery of D-SEP in earthworms and confirmation of the presence of L-SEP in turtles (Section I; Beatty, Ennor, Rosenberg and Magrath, 1961), a survey of the distribution and configuration of SEP in animals was made (Rosenberg and Ennor, 1961a), from which it would seem that the L isomer is confined to birds, fish, reptiles and amphibians, and the D isomer to earthworms. No trace of SEP was detected in thirteen other different groups of animals investigated.

The occurrence of two naturally-occurring isomeric forms of SEP has stimulated interest in the function and biosynthesis of these compounds. Whereas in earthworms the D isomer is the precursor of lombricine, the function of L-SEP in animals poses an intriguing question. It has been suggested (Rosenberg and Ennor, 1961) that in the chicken L-SEP may perform some specific function in the small intestine where its concentration is relatively high.

D-SEP appears to be synthesised from small molecular weight precursors since Gaffney et al. (1960) and Rossiter et al. (1960a, 1960b) demonstrated the in vivo incorporation of $[^{32}P]$orthophosphate and of isotopically-labelled ethanolamine, D-serine and L-serine, the rate of incorporation being similar to
that obtained in the earthworm phospholipids. The mechanism of incorporation of these compounds into D-SEP is not known.

In preliminary experiments, Rosenberg and Ennor (1961a) have demonstrated that \(^{32}\text{P}\)orthophosphate is incorporated into L-SEP in the presence of a chicken kidney homogenate. In addition, these preliminary experiments indicated that the omission of CTP resulted in about a 50\% diminution of \(^{32}\text{P}\) incorporation into L-SEP. This suggests that the biosynthesis of SEP may be related in some way to that of phosphatidylethanolamine and phosphatidylserine (Kennedy, 1957; Borkenhagen, Kennedy and Fielding, 1961).

Thus, the precise pathways by which D- and L-SEP are biosynthesised are not yet known. Further work must also establish whether the biosynthetic pathway is similar for both compounds. It is apparent, however, that considerations such as the sharply defined distribution of lombricine and the two isomers of SEP, the occurrence of D-serine in the two compounds found in the earthworm and the existence in animal tissue of the two isomeric forms of SEP, lend unusual interest to the problems which await solution.
EXPERIMENTAL

Paper chromatography was carried out at room temperature using the ascending technique and Whatman No. 1 paper unless otherwise stated. When the objective of chromatography was identification of a particular compound authentic marker substances were always included on the chromatograms and the latter run in several solvent systems. Amino compounds were located on the paper by spraying with 0.25% ninhydrin in water-saturated 1-butanol and then heating the paper for 5-10 min. at ca. 90°. Guanidino compounds were detected on the paper by the Sakaguchi (Archer and Crocker, 1952) or α-naphthol/diacetyl (Griffiths, Morrison and Ennor, 1957) spray reagents. The presence of phosphates was demonstrated by the method of Hanes and Isherwood (1949). Use was made of the following solvent systems:

A. Acetone-acetic acid-water (2:2:1 v/v) (Jones and Lipkin, 1956);
B. Methyl ethyl ketone-methyl cellosolve-acetic acid-water (40:15:6:24 v/v) (Porcellati, 1958);
C. 1-Butanol-acetic acid-water (50:20:30 v/v);
D. Phenol saturated with water (4:1 w/v);
E. Methyl ethyl ketone-methyl cellosolve-3 N ammonia (2:7:3 v/v) (Mortimer, 1952);
F. Methanol-aqueous ammonia (sp. gr. 0.91)-
water (60:10:30 v/v) (Bandurski and Axelrod,
1951);

G. 1-Propanol-aqueous ammonia (sp.gr. 0.91)-
water (60:30:10 v/v);

H. 1-Propanol-aqueous ammonia (sp.gr. 0.91)-
water (73:20:7 v/v);

J. Ethanol-ammonium acetate buffer, pH 7.2
(70:30);

K. 1-Butanol-acetic acid-water (8:20:20 v/v);

L. 1-Butanol-acetic acid-water (73:10:17 v/v).

Typical \( R_f \) values for SEP, lombricine and related
compounds, using freshly made-up solvents, are given in
Table II.2.

Column chromatography. Column size is specified
in terms of diameter and height. The ion-exchange
resins used included Dowex-50 x 4, 200-400 mesh and
Dowex-3 x 4, 20-50 mesh (The Dow Chemical Co., Midland,
Mich.) and Amberlite IRC-50 (Rohm and Haas Co.,
Philadelphia, Penna.). Cellulose powder was prepared as
described by Jones and Lipkin (1956). As a routine
procedure the elution of substances from chromatographic
columns was followed by spotting each fraction on
Whatman paper and spraying with the appropriate reagent,
The material in selected fractions giving positive tests
### TABLE II.2

**R<sub>f</sub> Values of Lombricine and Related Compounds**

<table>
<thead>
<tr>
<th>Compound</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolamine</td>
<td>0.75</td>
<td>0.58</td>
<td>0.57</td>
<td>0.66</td>
<td></td>
<td>0.73</td>
<td>0.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>0.53</td>
<td>0.37</td>
<td>0.41</td>
<td>0.35</td>
<td></td>
<td>0.54</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoethanolamine</td>
<td>0.47</td>
<td>0.30</td>
<td>0.33</td>
<td>0.35</td>
<td></td>
<td>0.30</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>0.35</td>
<td>0.23</td>
<td>0.26</td>
<td>0.10</td>
<td></td>
<td>0.26</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEP (DL, D or L)</td>
<td>0.19</td>
<td>0.12</td>
<td>0.22</td>
<td>0.28</td>
<td>0.34</td>
<td>0.65</td>
<td>0.37</td>
<td>0.12</td>
<td>0.21</td>
</tr>
<tr>
<td>N-Amidinoserine</td>
<td>0.67</td>
<td>0.47</td>
<td>0.49</td>
<td>0.56</td>
<td>0.45</td>
<td>0.51</td>
<td>0.43</td>
<td>0.27</td>
<td>0.49</td>
</tr>
<tr>
<td>2-Guanidinoethanol (hydrochloride)</td>
<td>0.77</td>
<td>0.66</td>
<td>0.63</td>
<td>0.78</td>
<td>0.69</td>
<td>0.42</td>
<td>0.50</td>
<td>0.34</td>
<td>0.74</td>
</tr>
<tr>
<td>(with streak to streak)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.66</td>
<td>0.62</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>2-Guanidinoethyl phosphate</td>
<td>0.57</td>
<td>0.36</td>
<td>0.40</td>
<td>0.57</td>
<td>0.11</td>
<td>0.49</td>
<td>0.21</td>
<td>0.06</td>
<td>0.20</td>
</tr>
<tr>
<td>Synthetic lombricine (DL, D or L)</td>
<td>0.30</td>
<td>0.15</td>
<td>0.26</td>
<td>0.51</td>
<td>0.29</td>
<td>0.58</td>
<td>0.32</td>
<td>0.11</td>
<td>0.26</td>
</tr>
<tr>
<td>Natural lombricine</td>
<td>0.30</td>
<td>0.15</td>
<td>0.27</td>
<td>0.50</td>
<td>0.30</td>
<td>0.60</td>
<td>0.32</td>
<td>0.11</td>
<td>0.26</td>
</tr>
</tbody>
</table>
was then examined by paper chromatography.

Concentration of solutions was normally carried out by evaporation under reduced pressure, using a rotating evaporator (Rinco Instrument Co., Greenville, Ill.).

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 and these melting points varied appreciably with rate of heating and state of subdivision. The melting points for such compounds were determined using unsealed capillary 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; SEP was heated at 80° (since it discoloured slightly above 100°), lombricine at 110°. L- and D-SEP were sampled and weighed out under anhydrous conditions because both compounds were hygroscopic. Vanadium pentoxide was added to lombricine and SEP in order to ensure complete combustion. Difficulty was sometimes experienced in
obtaining satisfactory hydrogen figures: this is attributed to the absorption of moisture during the subsequent addition of vanadium pentoxide to the sample. Nitrogen was determined by the Kjeldahl method and phosphorus colorimetrically (Berenblum and Chain, 1938) after wet ashing.

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

Optical rotations were measured using an instrument reading to 0.01° and small bore, 1 decimeter tubes (volume ca. 1 ml.). Solvent and concentration were as specified.

Ethanolamine was a commercial sample (British Drug Houses Ltd., Poole, England) and was distilled before use. DL-, L- and D-serine were commercial samples and were recrystallised where necessary from aqueous ethanol; DL-serine (British Drug Houses Ltd., Poole, England), m.p. 242-243° (decomp.); L-serine (Schwarz Laboratories, Inc., Mt. Vernon, N.Y., O.S. grade), $[\alpha]_D^{23} = -6.94$ (c = 4.04 g., H$_2$O), m.p. 220-221° (decomp.), with discolouration at about 210°; D-serine (California Corporation for Biochemical Research, Los Angeles, California, CfP grade), $[\alpha]_D^{23.5} = +7.4$ (c = 2.02 g., H$_2$O),
m.p. 221-222° (decomp.), with discolouration at about 210°.

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 (NH₄⁺) resin.

Cis-chloroaquotetra-amino-Co(III) chloride was a gift from Professor F.P. Dwyer, Unit of Biological Inorganic Chemistry, John Curtin School of Medical Research, Canberra.

The following three compounds were prepared by Dr. D.I. Magrath:-

0-Methylisourea hydrochloride was prepared by the method of Kurzer and Lawson (1954).

2-Guanidinoethanol hydrochloride was prepared by the reaction of 0-methylisourea hydrochloride with ethanolamine in dry ethanol. The product, m.p. 103-104° (cf. 107°, Mourgue, 1948), gave correct analytical figures for C, H and N.

N-Amidino-DL-serine was prepared by the guanidination
of DL-serine with \(\text{O-}\)methylisourea hydrochloride in aqueous solution at ca. pH 9. The reaction mixture was neutralised 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 eluant. The product gradually charred \(>210^\circ\), cf. m.p. 200° (decomp.) (Kapfhammer and Müller, 1934) and 205° (decomp.) (Mourgue, 1948), and gave correct analytical figures for C, H and N.

\textbf{2-Guanidinoethyl hydrogen phosphate.} Phosphoethanolamine (2 g.) was dissolved in sodium hydroxide (N, 16 ml.), \(\text{O-}\)methylisourea hydrochloride (1.6 g., 1 eq.) added, and the pH of the solution adjusted to between 10 and 11 by the addition of more alkali (6 ml.). The flask was stoppered and the reaction mixture set aside at room temperature for 3-4 days, the pH being checked night and morning and readjusted to approx. the original value where necessary. The reaction mixture was then applied to a column of Dowex-50 (H\(^+\)) resin and the column washed with water. The portion of the eluate containing the required product (730-1880 ml.) was evaporated to dryness under reduced pressure, giving a white, crystalline residue (2.192 g., 84.4%). Recrystallisation from aqueous methanol gave sheaves of fine needles, 1.94 g., m.p. 201-202° (decomp.), with shrinking above
195°. It was recrystallised once more for analysis, m.p. 202-204° (decomp.).

Analysis: Calcd. for C_{9}H_{10}N_{2}O_{4}P: 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 crystallised from aqueous ethanol as stout, colourless rods, m.p. 207-208° (decomp.) 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 hydrogen phosphate had previously been prepared in unstated yield (Robin, 1954; Thoai and Robin, 1954) by the action of phosphoryl chloride on 2-guanidinoethanol, but its characterisation rested solely on paper chromatographic data. Some time after the synthesis involving guanidination of phosphoethanolamine as described above was accomplished, Pant and Dubey (1960) described a similar reaction using S-methylthiourea as guanidinating reagent. Their product, obtained in 75% yield, was characterised by elementary analysis and the paper chromatographic behaviour of the compound and its acid hydrolysis products, and melted >290°.

Natural lombricine was isolated (Rosenberg and Emnor,
1959) from earthworms of mixed species, predominantly Allolobophora caliginosa and Octolasium cyaneum, and was made available for characterisation and comparison with the synthetic compounds.

Natural SEP isomers. Samples of SEP isolated from earthworms (mixed species as above), the skeletal muscle of river turtles (Chelodina longicollis), crocodile muscle and chicken muscle (Ennor, Rosenberg, Rossiter, Beatty and Gaffney, 1960; Beatty, Ennor, Rosenberg and Magrath, 1961; Morgan and Beatty, 1960) were provided for characterisation by comparison with the synthetic compounds.

Phenyl phosphorodichloridate was prepared from phosphorus oxychloride and phenol in the presence of zinc dust, according to the method of Zenftman and McGillivray (1951). The reagent was stored in a well-stoppered bottle.

50% Palladium on charcoal catalyst. This catalyst was prepared by a modification of the method of Linstead and Thomas (1940). Norit charcoal was treated as described in the method. The final heating was accomplished at 300-400°, 3 mm. pressure, in a flask immersed in a deep sand bath, the temperature of the charcoal being recorded by a thermometer in a jacket fitted into the neck of the flask. It is essential to
raise the temperature slowly and to reduce the pressure gradually in order to prevent excessive 'spurting' of the charcoal. After one hour, the flask was allowed to cool, and air slowly admitted through a drying tube. The prepared charcoal was stored in a desiccator.

Difficulty was experienced in dissolving the available sample of powdered palladium metal in dilute hydrochloric acid as described in the above method, but it dissolved readily in dilute acid through which a stream of chlorine was passed. Dissolved chlorine was removed when the solution was concentrated to the desired volume. The preparation was then completed according to the method described in the literature, and the catalyst stored in a desiccator.

**Platinum oxide catalyst** was prepared by the usual procedure (Adams, Voorhees and Shriner, 1941) and used directly.

**Benzylchloroformate** was prepared by the method of Carter, Frank and Johnston (1955) from 200 g. of phosgene in 500 ml. of toluene by the addition of 187 ml. (196 g., 1.814 moles) of benzyl alcohol. After the removal of a large proportion of the toluene, the weight of benzylchloroformate per ml. of solution was estimated by conversion of the benzylchloroformate in a small aliquot to benzylformamide which was filtered off, dried and
weighed (1.033 g./ml.). Yield, 280 g. (90%). The solution was stored at 0-5° in a well-stoppered bottle. A slight discolouration was apparent after several months, although the weight of chloride/ml. was not significantly altered (1.0 g./ml.).

\textbf{N-Benzylloxycarbonylethanalamine} was prepared by Dr. D.E. Griffiths.

\textbf{N-Benzylloxycarbonylserine.} The DL, D and L compounds were prepared by the method of Moore, Dice, Nicolaides, Westland and Wittle (1954).

\textbf{A. N-Benzylloxycarbonyl-DL-serine.} This compound was prepared from DL-serine (10 g.) and 17.5 ml. (18.06 g.) of the prepared solution of benzylchloroformate. Recrystallisation of the product from ethyl acetate gave 12.35 g., m.p. (uncorr.) 122.5-123.5°; 3.36 g. (second crop), m.p. 122-123°; 2.24 g. (third crop), m.p. 120.5-122°. Total yield, 79%. Melting points previously recorded for this compound prepared by this and other methods are in the range 120-122° to 124-125° (Skinner, McCord, Ravel and Shive, 1956; Jones and Lipkin, 1956; Baer and Maurukas, 1955; Bergmann and Zervas, 1932).

\textbf{B. N-Benzylloxycarbonyl-L-serine} was prepared similarly by Dr. D.I. Magrath.

\textbf{C. N-Benzylloxycarbonyl-D-serine.} This isomer
was prepared from D-serine (7.5 g.) as above in 85.6% yield; m.p. (uncorr.) 116-117°. Baer and Maurukas (1955) record m.p. 119° (cf. for the L isomer, 117-118° to 121°; Jones and Lipkin, 1956; Moore et al., 1954; Baer and Maurukas, 1955; Guttmann and Boissonnas, 1958; Fruton, 1942).

N-Benzylxycarbonylserine benzyl ester. The DL, D and L compounds were prepared from N-benzylxycarbonylserine by a modification of the method of Baer and Maurukas (1955) using benzyl chloride instead of benzyl bromide. The yields were higher than previously obtained and equal to that of a subsequent synthesis of the L compound from N-benzylxycarbonyl-L-serine and benzyl chloride, heated in anhydrous ethylamine at 75° for 1.5 hours (Baer, Buchnea and Stancer, 1959).

A. N-Benzylxycarbonyl-DL-serine benzyl ester. The sodium salt was prepared from 8 g. (0.03 moles) of the protected DL-serine derivative, dried and partly dissolved in dry distilled N,N-dimethylformamide, then heated at 70° with a five-fold excess of benzyl chloride (19.2 ml.) in a closed vessel for 28 hr. On working up the reaction mixture according to the procedure of Baer and Maurukas (1955), 7.95 g. (72% yield) of product, m.p. (uncorr.) 72.5-73.5°, was obtained. A yield of 80% of this ester, m.p. 72-73°,
was subsequently obtained (cf. m.p. 72.5 to 74.4° obtained by Bevan, Malkin and Tiplady, 1957; Fölsch and Mellander, 1957; Baer and Maurukas, 1955; Bergel and Wade, 1959; Skinner et al., 1956; Jones and Lipkin, 1956).

B. \text{n-Benzyloxycarbonyl-L-serine benzyl ester.}

This isomer, prepared similarly to the above in 85.5% yield, m.p. 82-83°, has been prepared by Baer and Maurukas (1955), Jones and Lipkin (1956), Fölsch and Mellander (1957), Bergel and Wade (1959) and Baer, Buchnea and Stancer (1959) (m.p. 82-83° to 84-85°).

C. \text{n-Benzyloxycarbonyl-D-serine benzyl ester.}

An analogous preparation gave 83% yield of product, m.p. 83-84°. Previous synthesis of this compound by Baer and Maurukas (1955) and by Fölsch and Mellander (1957) yielded products with m.p. 82.5-84.5° and 84-85°, respectively.

\text{DL-, D- and L-2-Amino-2-carboxyethyl 2-aminoethyl hydrogen phosphate (SEP).} The racemic and optically-active forms of SEP were synthesised according to the method of Jones and Lipkin (1956), slightly modified in the case of the D and L compounds.

A. \text{DL-SEP.} Phenyl phosphorodichloridate (5.49 g.) was transferred with 50 ml. of dry distilled chloroform into a three necked 250 ml. flask equipped with two
calcium chloride tubes and a sealed stirrer. The flask was placed in an ice-bath, and the solution stirred for a few minutes before replacing one drying tube with a small separating funnel containing N-benzyloxy carbonyl-DL-serine benzyl ester (8.24 g., 0.025 mole) dissolved in freshly distilled quinoline (3.5 ml.) and dry distilled chloroform (25 ml.), the drying tube being inserted in the neck of the funnel. This solution was added dropwise with stirring over a period of one hour. The ice-bath was removed and stirring continued for another hour, after which time the flask was immersed in a water-bath (ca. 20°). Following the addition of dry freshly distilled pyridine (10 ml.), N-benzyloxy carbonylethanolamine (4.88 g., 0.025 mole) dissolved in dry distilled chloroform (25 ml.) contained in a separating funnel was added dropwise with stirring during the course of one hour. The stirring was continued overnight (16 hours) and 100 ml. of ice-cold 6 N H$_2$SO$_4$ was then added. The chloroform layer was separated and washed twice with 100 ml. of 6 N H$_2$SO$_4$, then twice with water, once with 0.5 N NaHCO$_3$, and finally with water. The chloroform solution was dried over anhydrous magnesium sulphate and the chloroform removed by distillation under reduced pressure leaving a glassy residue of the mixed neutral phosphate esters.
The hydrogenation of the above material was carried out at atmospheric pressure in two stages. The first stage, using 50% palladium on charcoal catalyst, effected the removal of benzyloxycarbonyl and benzyl groups. To the mixture of neutral phosphate esters dissolved in 100 ml. of absolute ethanol and 4.2 ml. of 12 N HClO₄, the above catalyst (3 g.) was added as a slurry in absolute ethanol. No provision was made for the removal of carbon dioxide evolved during the hydrogenation. After 7 hours, when the hydrogen uptake appeared complete, the hydrogenation apparatus was emptied of carbon dioxide and residual hydrogen and refilled with hydrogen. No further uptake of hydrogen was observed. The catalyst was removed by filtration and washed successively with water (10 ml.), absolute ethanol (10 ml.) and two volumes of water (10 ml.). Platinum catalyst (1.5 g.) was added immediately to the combined filtrate and washings. This second stage of the hydrogenation involved removal of the phenyl groups with subsequent reduction of the benzene produced, as well as reduction of the toluene formed in the first stage. Approximately 80% of the total volume of hydrogen which was adsorbed in 17 hr. was taken up in 5 hr.

The perchloric acid was neutralised with sodium hydroxide and the catalyst removed by filtration and
quickly washed with water. The filtrate was concentrated under reduced pressure to remove alcohol, then extracted with ether and finally taken to dryness. The residue, after drying in vacuo, was an oil.

Chromatography (solvent A) showed that this oil contained several components giving a reaction with ninhydrin, the two components (Rf 0.19 and 0.09) giving the strongest reactions being distinguished from two other phosphorus-containing substances in that they gave only a faint or slowly developing colour with molybdate reagent.

The mixture was dissolved in 25 ml. of solvent A and chromatographed on a column (4.6 x 96 cm.) of prepared cellulose, by elution with the same solvent. The flow rate used was approximately 100 ml. per hour and 25 ml. fractions were collected. Ethanolamine, serine, phosphoethanolamine and phosphoserine, identified by comparison of Rf values (solvents A and C) with authentic specimens of those compounds, were eluted in fractions 49-111. Fractions 126-210 and fractions 246-360 (elution with water was commenced at fraction 320) contained single ninhydrin-positive substances of Rf 0.19 and 0.09 (solvent A), respectively, corresponding to the two components of the hydrogenated material which gave weak reaction to the molybdate spray. The substances were
assumed to be SEP and bis-DL-2-amino-2-carboxyethyl hydrogen phosphate, respectively, by comparison of their behaviour on the column and on paper chromatograms with that described by Jones and Lipkin (1956) for these compounds. Roberts and Lowe (1954), however, could not detect with the usual spray reagents the presence of phosphorus in SEP.

Fractions 126-190 were combined, concentrated under reduced pressure in a rotating evaporator in a bath at 30°, and then taken to dryness by distillation of the acetic acid at 0.2 mm Hg. The residue* dissolved in hot water (1-2 ml.), was obtained as an oil by the addition of hot ethanol (6 ml.). After storage at 5° for 3 days, the oil crystallised on scratching, giving colourless prisms which were filtered off and washed with ethanol. Recrystallisation from aqueous ethanol gave colourless prisms, 1.57 g. (28%), m.p. 193-194° (decomp.) (cf. 180-181° uncorr., decomp., quoted by Jones and Lipkin, 1956).

Analysis: Calcd. for C_{13}H_{28}N_{2}O_{6}P: 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.

*Some water insoluble material (possibly material extracted from plastic tubing used to feed solvent to the column) present in this residue was extracted with ether.
Figure II.1. Infrared Spectra of DL-L and D-2-amino-2-carboxylethyl 2-aminoethyl hydrogen phosphate (SEP) and of D-SEP isolated from Earthworms.
pK\textsubscript{a} values, ca. 2, 8.8 and 10.0; infrared absorption peaks (Figure II.1) 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, 752 cm\textsuperscript{-1}, in excellent agreement with the \( \lambda_{\text{max}} \) values reported by Jones and Lipkin (1956).

B. L-SEP. From N-benzyloxy carbonyl-L-serine benzyl ester (8.24 g.) and N-benzyloxycarbonylethanolamine (4.88 g.), the mixture of neutral phosphate esters was obtained as described in the preparation of the DL-SEP. The first stage of the hydrogenation was completed in 12 hr.; hydrogenation did not occur when the first batch of platinum catalyst was added, but after it had been filtered off and a second batch added, the second stage of the hydrogenation was effected.

The cellulose column fractionation of the hydrogenated material resulted in less efficient separation of the components than was obtained in A. Fractions containing SEP were selected by chromatographic examination, combined and concentrated as described above. After removal of the acetic acid a glassy residue was obtained which, when dissolved in water, gave a pale green solution, presumably indicative of the presence of a metal-SEP complex. Treatment of the solution with hydrogen sulphide gave a
precipitate and colourless filtrate. The residue from this filtrate failed to crystallise. Since chromatography (solvent A) showed that the SEP was now contaminated with other ninhydrin-positive substances*, further purification was necessary.

The impure product (1.9 g., 33.3%) was dissolved in water and applied to a column (3.2 x 7 cm.) of Dowex-50 (H+) resin. Elution of the resin with water (180 ml.), which removed one ninhydrin contaminant (phosphoserine) and then with N aqueous ammonia, furnished SEP contaminated with a little phosphoethanolamine. Crystallisation at this stage proved unsatisfactory and the material was re-chromatographed on a column of Dowex-50 (NH4+) resin of the same size, with water as eluant. Phosphoethanolamine and SEP were eluted successively. Fractions containing SEP were combined, evaporated to dryness and the residue so obtained was chromatographically pure with respect to ninhydrin and molybdate reagents. Crystallisation from aqueous methanol gave colourless, hygroscopic needles (microcrystalline), 1.34 g. (23.4%), m.p. 142-143° (decomp.) (cf. 139-141° uncorr., decomp., quoted by Jones and

*Cf. Alkaline degradation of metal complexes of SEP. Presumably acetic acid vapour had attacked certain metal parts of the rotating evaporator and led to the introduction of metal contaminants to the solution of SEP.
Lipkin, 1956).

Analysis: Calcd. for C_{5}H_{13}N_{2}O_{6}P: 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.

\[ \alpha_D^{23.5} = 14.8 \, (C = 0.811, H_2O); \] Jones and Lipkin quote \[ \alpha_D^{23.5} = 15.0 \, (C = 2.2, H_2O). \]

pK\text{a} values, ca. 2, 8.8 and 10.0; infrared absorption peaks (Figure II.1) 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\(^{-1}\) (cf. spectrum reproduced in the paper by Jones and Lipkin (1956) with which there appears to be good agreement.

C. D-SEP. Preparation of this compound was undertaken on the same scale as used in the previous preparations and also on a larger scale (using four-fold quantities). In the latter preparation, the inconvenience associated with the fractionation of the hydrogenated material on larger amounts of cellulose was avoided by the exploitation of the ion-exchange resin chromatographic techniques developed for the purification of crude DL- and L-SEP.

(a) Small-scale preparation. Using the amounts of reactants stated above, the mixture of the neutral phosphate esters was obtained and submitted to hydrogenation
during which it was necessary to make a further addition of platinum catalyst. Cellulose column chromatography did not separate all ninhydrin-positive contaminants from D-SEP, and all fractions containing D-SEP were combined and taken to dryness by distillation under reduced pressure. The crude product (2.36 g.) was chromatographed on a column (3.2 x 10 cm.) of Dowex-50 (H⁺) resin as described above, but this time the column was washed with water (ca. 500 ml.) until all ninhydrin-reacting impurities had been removed before stripping the column with N aqueous ammonia. SEP was obtained chromatographically pure from the column by this procedure. Concentration of these fractions containing SEP gave a product which crystallised from aqueous methanol as a hygroscopic, microcrystalline powder, 1.0 g. (20%), m.p. 143-144° (decomp.).

Analysis. Calcd. for C₁₃H₁₃N₂O₆P.₁/₂ CH₃OH:
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.

[α]₂₃°₊₁₈.₂ (C = 0.659 H₂O); pKₐ values, ca. 2, 8.8 and 10.0; the infrared spectrum was identical with that of the L isomer (Figure II.1).

(b) Larger scale preparation. From 32.96 g. (0.1 mole) of N-benzyloxycarbonyl-D-serine benzyl ester the neutral phosphate esters were obtained and submitted
to hydrogenation. The apparent uptake of hydrogen with palladium catalyst was 5.5 litres, and ca. 43 litres of hydrogen was absorbed in the presence of the platinum catalyst. After the hydrogenation the latter catalyst was filtered off, washed, and potassium hydroxide added to neutralise the perchloric acid. The precipitated potassium chlorate was removed by filtration and washed free of ninhydrin-positive material. The filtrate and washings were evaporated to dryness under reduced pressure. The residue was treated with cold water, undissolved material filtered off and washed with ice-cold water until free of ninhydrin-reacting material.

A concentrated solution of the water-soluble material was applied to a column (4.2 x 32.5 cm.) of Dowex-50 (H⁺) resin. Elution with water (3.5 litres) removed a number of ninhydrin-positive substances. Trace amounts of a substance having the chromatographic behaviour of the bis-2-amino-2-carboxyethyl hydrogen phosphate were 'leaking' from the column towards the end of the elution. The eluant was changed to 0.5 N aqueous ammonia and this gave some degree of fractionation of the symmetrical diester and SEP (the former being eluted preferentially) but traces of other ninhydrin-positive materials were also present in later fractions containing SEP. All the fractions containing SEP only
(with the exception of a few tubes accidentally lost) were combined (total volume 165 ml.) and concentrated to dryness. The residue was dissolved in 10 ml. of water and chromatographed on a column (6 x 40 cm.) of Dowex-50 (NH$_4^+$) resin with water as eluant. Fractions containing SEP were combined and concentrated under reduced pressure. The product crystallised from aqueous methanol in microcrystalline form, 6.36 g. (28%).

Characterisation of SEP from natural sources.

A. Earthworm tissue. The isolated material (400 mg.), which was chromatographically homogeneous as indicated by the ninhydrin reagent and had an $R_f$ identical to that of synthetic SEP, was recrystallised from aqueous methanol to yield 0.139 g. as a microcrystalline powder which was very hygroscopic. From the mother liquor a further yield of 0.159 g. of microcrystalline material was obtained by successive crystallisations. The first crop was fully characterised:— m.p. 144-145° (decomp.) (cf. simultaneous m.p. for synthetic D-SEP, 144°); $[\alpha]_D^{23.5} + 18.0$ (C = 0.71, H$_2$O).

Analysis: Calcd. for C$_5$H$_{13}$N$_2$O$_6$P. $\frac{1}{2}$ CH$_3$OH: C, 27.05; H, 6.19; N, 11.48; P, 12.69. Found: C, 26.73; H, 6.32; N, 11.17; P, 12.50.

The infrared spectrum (Figure II.1) was identical with that of synthetic D-SEP.
B. **Turtle muscle.** The isolated material was free from other ninhydrin-positive compounds and separated from aqueous methanol as colourless, hygroscopic microcrystals; m.p. 144-145° (decomp.); \([\alpha]_D^{23.5} = 12.1\) (C = 0.58, H₂O).

Analysis: Calcd. for \(C_5H_{13}N_2O_6P\cdot \frac{1}{2} CH_3OH \cdot \frac{1}{2} H_2O\): C, 26.09; H, 6.34; N, 11.07; P, 12.24. Found: C, 26.09; H, 6.69; N, 11.26; P, 12.37.

The infrared spectrum was identical with that of synthetic L- or D-SEP.

C. **Crocodile muscle.** An aqueous solution of the isolated material (0.332 mg.) was slightly discoloured but became colourless when treated with hydrogen sulphide which caused the precipitation of a small amount of a brown sulphide. (Presumably a metal contaminant had been introduced during the final stage of isolation). Attempts to crystallise the isolated material were unsuccessful and its aqueous solution was applied to a column (3.2 x 14.5 cm.) of Dowex-50 \((NH_4)^+\) resin. Elution of the column with water resulted in the separation of the bulk of the SEP from contaminating substances which included material behaving as phosphoethanolamine on paper chromatograms. Fractions containing SEP only were combined and evaporated to dryness under reduced pressure. The residue (0.174 g.) was crystallised from aqueous methanol
to yield colourless hygroscopic microcrystals, m.p. 144-145° (decomp.) (cf. synthetic L-SEP, 142-143°); 
\[\alpha\]_D^{23.5} = 16.6 (c = 0.78; H_2O).

Analysis: Calcd. for C_{13}H_{13}N_2O_6P.CH_3OH: C, 27.70; H, 6.59; N, 10.78; P, 11.91. Found: C, 27.73; H, 6.35; N, 10.99; P, 12.27.

The infrared spectrum was identical with that of synthetic D- or L-SEP.

D. Chicken muscle. The isolated material, crystallised as above, gave colourless hygroscopic microcrystals, m.p. 144-145° (decomp.) (cf. synthetic L-SEP, 144°); 
\[\alpha\]_D^{23.5} = 16.0.

Acid hydrolysis of synthetic DL-SEP and of natural SEP from turtle and crocodile*. Samples (10 mg.) of the natural compounds and of synthetic DL-SEP were dissolved in 6 N hydrochloric acid (0.2 ml.) and heated in sealed tubes for 4 hr. at 108°. The hydrolysates were transferred to watch glasses and evaporated to near dryness over a steam bath, the bulk of the remaining hydrochloric acid removed in a vacuum desiccator over potassium hydroxide pellets, and the residues each dissolved in 0.2 ml. of water containing 0.05 ml. of

*The SEP isolated from earthworms and synthetic SEP gave identical patterns of ninhydrin-positive and phosphorus-containing compounds when hydrolysed with 6 N hydrochloric acid (Rosenberg and Ennor, 1959).
conc. aqueous ammonia. Paper chromatographic examination of the hydrolysates after development in solvent systems B, C and H showed identical patterns of ninhydrin- and molybdate-positive spots; the stronger spots corresponded to markers of unchanged SEP, serine, ethanolamine, phosphoserine, phosphoethanolamine, orthophosphoric acid, and an unidentified material with \( R_f \) greater than ethanolamine which might have been \( \beta \)-chloroethylamine (cf. Rosenberg and Ennor, 1959).

2-Amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate (lombricine). In preliminary experiments, it was found that reaction of the copper complex of SEP with O-methylisourea at pH 10 or pH 8.5 and room temperature (see (a) and (b) below), gave a complex 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 \( R_f \) as natural lombricine in several solvent systems was readily formed at pH 9, 11 or 12 (see (c) and (d) below), the rate of reaction and the yield being increased when two, rather than one, equivalents of O-methylisourea were used.

(a) Reaction of the copper complex of SEP with O-methylisourea at pH 10. To a solution of the copper complex of DL-SEP (0.118 g.) formed as later (p. 141)
described, an equivalent amount of O-methylisourea (0.06 g.) was added. The solution was adjusted to pH 10 and maintained at this pH and at room temperature for 3.5 days, following the procedure described by Kurtz (1949). A sample was then taken and prepared for chromatography. The products included an α-naphthol/diacetyl-positive, phosphorus-containing substance with identical R<sub>f</sub> values in four solvent systems (A, C, H, L) to that of 2-guanidinoethyl phosphate, a substance with the same chromatographic behaviour as phosphoethanolamine run in two systems, A and C, and a ninhydrin-positive, phosphorus-containing substance which ran slightly slower than phosphoserine in the same solvents. (Solvent systems H and L gave little separation of the latter products).

(b) Reaction of the copper complex of SEP with O-methylisourea at pH 8.5. SEP (12 mg.) was complexed with copper, O-methylisourea (6 mg.) added to the solution and the pH adjusted to ca. 8.5. A sample was withdrawn after 1 hr. and subjected to chromatography in solvent C. In addition to SEP and O-methylisourea, a trace of an α-naphthol/diacetyl-positive material with the same R<sub>f</sub> as that of natural lombricine was observed on the chromatogram. After 24 hr. the slow-running degradation product of SEP (R<sub>f</sub> 0.12, cf. SEP, R<sub>f</sub> 0.19,
on this chromatogram) had formed, while after 27 hr. phosphoethanolamine was also detected. The substance with similar $R_f$ to that of lombricine was detected by both ninhydrin and $\alpha$-naphthol/diacetyl reagents but the increase in the amount of this substance formed during this period of time was slight.

(c) Guanidination of SEP at pH 9 and pH 12. A solution of SEP (5 mg., $2 \times 10^{-5}$ mole) and O-methyl-isourea hydrochloride (2.5 mg., $2 \times 10^{-5}$ mole) in 1 ml. of water, was adjusted with dilute sodium hydroxide to pH 9. A second solution of the same composition was adjusted to pH 12. Both solutions were kept at room temperature and slight changes in their pH were corrected by periodic addition of 0.1 N sodium hydroxide. Samples of both solutions were chromatographed after 32 hr. (solvents A, B and D). On the portions of the three chromatograms which were sprayed with $\alpha$-naphthol/diacetyl reagent a substance with the same $R_f$ as natural lombricine was observed from both solutions. The remaining portions of the chromatograms were developed with ninhydrin, which showed 'lombricine' and unreacted SEP in both solutions, and another compound in the reaction mixture which had been adjusted to pH 9.

A similar reaction mixture maintained at pH 9 and containing two equivalents of O-methylisourea gave a
better yield of the α-naphthol/diacetyl-reacting substance ('lombricine') in 24 hr. Addition of further quantities of O-methylisourea to this reaction mixture and to the reaction mixture kept at pH 12, caused almost complete conversion of SEP to the product behaving as lombricine, but the former reaction mixture gave more by-products.

(d) Guanidination of SEP at pH 11. A solution of O-methylisourea hydrochloride (0.221 g., 0.002 mole) was added to a solution of DL-SEP (0.228 g., 0.001 mole). The mixture was adjusted to ca. pH 11 by the addition of 2 N NaOH, the final volume being 5.5 ml. Chromatography (solvent B) showed that only a small amount of SEP was unreacted after 4 hr. and that the reaction was complete by 9.5 hr. The main product chromatographed as lombricine but an additional guanidino compound (Rf 0.21) was formed which did not react on the chromatogram with ninhydrin. The main product was isolated in a crude state by elution with water from paper chromatograms (Whatman No. 3 MM) run in solvent B (descending). This material chromatographed in solvents B, D and H as lombricine. Further, preliminary investigation of the products of acid hydrolysis (6 N H2SO4 at 110° for 8 hr.) indicated that this crude synthetic material was degraded to 2-guanidinoethyl phosphate, 2-guanidinoethanol and
serine.

A. DL-Lombricine. DL-SEP (0.228 g., 0.001 mole) and Q-methylisourea hydrochloride (0.221 g., 0.002 mole) were dissolved in water (2.5 ml.) and the solution adjusted to ca. pH 11 with 2 N sodium hydroxide. The solution was maintained at room temperature and readjusted to pH 11 by additions of dilute alkali at intervals over a period of 8 hr. At the end of this period the solution was neutralised with hydrochloric acid. Chromatography (solvent B) of the neutralised reaction mixture showed complete disappearance from the reaction mixture of SEP and formation of a product behaving on the chromatogram as lombricine (Rf 0.15) and of a small amount of another guanidino compound (Rf 0.21).

An attempt was made to obtain the product free from contaminating substances by descending chromatography on sheets of Whatman No. 3 MM paper with solvent B, which was allowed to drip off the bottom of the paper. Satisfactory separation of the two guanidino compounds was not effected. The crude product (0.260 g.) which had been eluted from these papers was redissolved in a minimum amount of water (ca. 2 ml.) and applied to a column (3.5 x 24 cm.) of Dowex-50 (NH₄⁺) resin. Traces of contaminating substances including 2-guanidinoethyl phosphate were eluted with water. After 50 ml. of the
aqueous eluant had been collected, the major product in a chromatographically pure state began to appear in the effluent. The second guanidino compound was subsequently eluted, but after evaporation of the effluent under reduced pressure, only 6 mg. of material was obtained. The fractions containing the main product (total volume 70 ml.) were evaporated to dryness under reduced pressure and yielded a crystalline residue (0.146 g., 54%). The compound recrystallised readily from aqueous methanol as colourless needles, 0.101 g., m.p. 217-218° (decomp.).

Analysis: Calcd. for C_{6}H_{15}N_{4}O_{6}P: 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 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\(^{-1}\). (For better resolution, the spectrum above 2400 cm\(^{-1}\) was determined with a lithium fluoride prism using a Perkin-Elmer Model 12-C instrument). The spectrum obtained using a Perkin-Elmer Model 21 is shown in Figure II.2.

B. L-Lombricine. L-SEP (0.254 g.) was reacted with O-methylisourea hydrochloride (0.246 g.) as described for the DL compound except that the final volume was
3.5 ml. After the prescribed time the neutralised reaction mixture was applied directly to a column (3.5 x 24 cm.) of Dowex-50 (NH₄⁺) resin. Water was used as eluant and 5 ml. fractions were collected. Chromatographic examination of the fractions (solvent B) indicated that the following compounds were successively eluted: trace amounts of 2-guanidinoethyl phosphate (fraction 18); SEP and an unidentified ninhydrin-positive, phosphorus-containing substance chromatographing slightly faster than lombricine (fractions 24-25); two α-naphthol/diacetyl-positive compounds (Rₖ values 0.27 and 0.21, fractions 49-54 and 72-100, respectively). Of these contaminants only the substance with Rₖ 0.21 was present in sufficient quantity to be detectable by chromatography of the reaction mixture, and it appears that the minor products formed here are those formed in greater quantity when SEP and 0-methylisourea are reacted in aqueous solution at pH 9. Fractions 28-48, which contained a single α-naphthol/diacetyl- and ninhydrin-positive, phosphorus-containing compound which behaved identically with natural lombricine on the chromatograms, were combined and evaporated to dryness under reduced pressure. After drying in a desiccator, the product weighed 0.216 g. (72%) and recrystallised readily from aqueous methanol as colourless needles (0.170 g.), m.p. 228-229° (decomp.).
Analysis: Calcd. for C_{6}H_{15}N_{4}O_{6}P: 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.

\[ \alpha \]_{D}^{23.5} = 13.0 (C = 0.81, H_{2}O); pK_{a} values, ca. 2, and 8.9; infrared absorption peaks (Figure II.2) 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\(^{-1}\) (peaks above 2400 cm\(^{-1}\) obtained as for the DL compound), which are not identical with those for DL-lombricine.

C. D-Lombricine. D-SEP (0.228 g.) and \(\text{O\text{-}methyl-}
\text{isourea \ hydrochloride (0.221 g.) were reacted as described in the preparation of the DL compound, the final volume of the reaction mixture being 3.2 ml. Slightly more Dowex-50 (NH\(_{4}\))\text{+} resin was used (column, 3.5 x 30.5 cm.) in the purification of the product. Minor products, including slightly more 2-guanidinoethyl phosphate than previously obtained, were separated from the main product (0.170 g., 62%) which recrystallised from aqueous methanol, giving colourless needles, m.p. 225-226\(^{\circ}\) (decomp.).

Analysis: Calcd. for C_{6}H_{15}N_{4}O_{6}P: C, 26.67; H, 5.60; N, 20.73; P, 11.47. Found: C, 26.62; N, 5.47; N, 20.68; P, 11.35.

\[ \alpha \]_{D}^{23.5} + 16.1 (C = 0.805, H_{2}O); pK_{a} values, ca. 2
and 8.8; infrared spectrum was identical with that of the L compound and natural lombricine (see Figure II.2).

**Characterisation of natural lombricine.** A sample of natural lombricine was recrystallised from aqueous methanol, giving colourless needles, m.p. 227-228° (decomp.); subsequent recrystallisation from aqueous ethanol raised this m.p. to 231-232° (decomp.) (cf. 223-224° (Thos and Robin, 1954) and 224° (decomp.) (Rosenberg and Ennor, 1959)). It had R<sub>f</sub> values in a number of solvent systems identical with those of the synthetic DL, L or D compounds (see Table II.2).

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

**The chromatographic examination of the acid hydrolysates of synthetic DL- and L-lombricine and of natural lombricine.**

The compounds (10 mg. of each) were dissolved in 0.2 ml. 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 each of the cold hydrolysates until no further precipitate was formed, the mixtures centrifuged, the supernatants retained and later combined with washings from their respective precipitates. Each of the hydrolysates was 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 lombricine, serine, 2-guanidinoethyl phosphate and 2-guanidinoethanol, was obtained for all three hydrolysates. In addition, there was also present in all four hydrolysates a trace of another ninhydrin-reacting material with \( R_f \) (0.46 in both systems) similar to that of alanine. Traces of 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 sulphuric acid at 110°. Though alanine is known to be formed from serine under alkaline conditions (Daft and Coghill, 1931; Wieland and Wirth, 1949), its formation under acid conditions does not appear to have been reported. (See, however, Damodaran and Ramachandran, 1941).

**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 ml. volumes of 20% acetic acid were halved and to each half, contained in 5 ml. 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 J. Solvent was removed from the remaining pair under reduced pressure and the gummy residues were kept under vacuum for 30 min. at room temperature. N HCl (0.3 ml.) was then added to each, the tubes stoppered and heated in a boiling water-bath for 30 min., under which conditions neither natural lombricine nor 2-guanidinoethyl phosphate are affected. After cooling, both solutions were carefully neutralised with concentrated ammonia solution and submitted to paper chromatography along with the unhydrolysed 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 with N HCl, 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*, one had $R_f$

*As it is known (Bettzieche, 1925) 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.
values in all systems identical with those of 2-guanidinoethyl phosphate; its concentration was not appreciably altered by hydrolysis. Another had $R_f$ values identical with those of 2-guanidinoethanol. The other two ($R_f$ values, 0.45 and 0.54, solvent C) were not identified; one appeared to have been completely, and the other partially, destroyed by hydrolysis.

The major product ($R_f$ values in the six solvent systems used, 0.37, 0.35 and 0.33, 0.59, 0.32 and 0.33, respectively), which contained phosphorus, was almost completely destroyed by hydrolysis. Its destruction was accompanied by the appearance on the chromatograms of a very strongly $\alpha$-naphthol/diacetyl-reacting substance with the same $R_f$ as 2-guanidinoethanol in systems C, B and J (systems E, F and G caused either streaking or double-spotting of the 2-guanidinoethanol marker), and of a ninhydrin- and $\alpha$-naphthol/diacetyl-negative, phosphorus-containing substance having $R_f$ values in all six systems (0.40, 0.35, 0.13, 0.52, 0.23, 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 was no substance observed on the chromatograms
which could be definitely identified as 2-phosphoglyceric acid. This may have been due to adverse effects of the ammonium chloride present in the hydrolysates on the chromatographic separation of 2- and 3-phosphoglyceric acids. However, it was not possible to separate clearly mixtures of the two authentic isomers in any of the solvent systems. Chromatograms of the hydrolysates using the system of Cowgill (1955) 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 consideraly displaced from those of authentic markers of these two compounds.

Isolation and characterisation of serine and 2-guanidinoethyl phosphate from the acid hydrolysate of natural lombricine. A solution of lombricine (580 mg.) in 6 N sulphuric acid (12 ml.) was heated in a sealed tube at 110° for 9 hr. After cooling, barium hydroxide solution (15% w/v) was added until the addition of a further drop of barium hydroxide solution produced no further precipitation of barium sulphate. The precipitate was collected by centrifugation and thoroughly washed with water. Washings and the original supernatant solution were combined,
concentrated to approximately 10 ml. and applied to a column (3.5 x 24 cm.) of Dowex-50 (NH₄⁺) resin, which was then washed with water. Examination of fractions (5 ml.) of the eluate by paper chromatography indicated the successive elution of 2-guanidinoethyl phosphate, traces of a ninhydrin-positive material (probably alanine, see previous paragraph), serine and lombricine. Serine and 'alanine' were incompletely separated as were serine and lombricine. All fractions containing serine were combined, concentrated to a small volume and applied as a narrow band to two sheets (16 x 42 cm.) of washed Whatman No. 3 MM paper which were then developed in solvent system H (descending). The papers were dried and the serine was located and eluted with water. The crystalline residue obtained on evaporation of the eluate was recrystallised from aqueous ethanol, yielding colourless bladelike crystals (22 mg.).

Since it was desirable to have more serine than the above amount, the fractions containing lombricine were evaporated to dryness, the residue (0.164 g.) dissolved in 6 N sulphuric acid (3.3 ml.) and heated for 24 hr. Variations were introduced in the procedure described above for the isolation of the serine formed. Firstly, sulphuric acid was removed by batch treatment with Dowex-3 (OH⁻) resin; secondly, the resulting solution was
applied to a column (1 x 7 cm.) of Dowex-50 (H⁺) resin and 2-guanidinoethyl phosphate eluted with water. The column was then washed with N aqueous ammonia and the eluate containing non-volatile ninhydrin-positive material was concentrated and applied to two sheets of paper which were developed as previously described. The serine thus obtained (20 mg.) was combined with the former amount and the whole recrystallised from aqueous ethanol, yielding 26 mg. of crystalline material and a crystalline residue of 12 mg. All analyses were carried out on the former and the dinitrophenyl derivative prepared from the latter.

The melting point of the crystalline material was 220-221° (decomp.); the mixed melting points (by solution and recrystallisation) with authentic D- and L-serine were 220-221° (decomp.) and 243-244° (decomp.), respectively; \([\alpha]_D^{23.5} + 7.4\ (C = 3.92, H_2O)\); cf. simultaneous m.p. (decomp.) for authentic D-, L- and DL-serine, 221-222°, 220-221° and 242-243°, respectively.

The infrared spectrum of the solid showed peaks at 3427, 3030, 2060, 1601, 1471, 1339, 1301, 1227, 1218, 1126, 1086, 1012, 967, 920, 855 and 805 cm⁻¹, and this spectrum was identical with that of D-serine.

The dinitrophenyl derivative was prepared by the method of Sanger (1945), m.p. 174-175° (decomp.), mixed melting point with the dinitrophenyl derivative of
authentic D-serine similarly prepared, 174-175° (decomp.).

A sample of the isolated serine was tested for its ability to act as a substrate for D-amino acid oxidase. Within experimental error, the oxygen uptake was close to that of an equivalent amount of authentic D-serine and to that theoretically expected. This test was carried out by Professor Ennor (see Beatty, Ennor, Rosenberg and Magrath, 1961).

The fractions from the two ion-exchange columns containing 2-guanidinoethyl phosphate were evaporated to dryness and the residue (75 mg.) was dissolved in water (5 ml.) and applied to a column (1 x 2.5 cm.) of Dowex-50 (H⁺) resin. The column was washed with water and those fractions containing 2-guanidinoethyl phosphate were evaporated to dryness, giving a crystalline residue. This was twice recrystallised from aqueous methanol and yielded 44 mg. of colourless rod-shaped crystals, m.p. 208°; authentic 2-guanidinoethyl phosphate, heated simultaneously, melted at 207-208° with decomposition.


The infrared spectrum was identical with that of the synthetic compound.

Alkaline degradation of metal complexes of SEP.

(a) The SEP-Cu(II) complex. 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 pH 10 with 0.1 N sodium hydroxide, the final volume of the solution being approx. 1.5 ml. A similar solution of SEP, but without the addition of copper carbonate, was prepared at the same time. The solutions were set aside at room temperature and maintained at ca. pH 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., acidified, treated with hydrogen sulphide to remove copper and submitted to paper chromatography in solvent A. At 3 hr. no degradation was apparent, but after 50 hr. little SEP remained in the solution to which copper carbonate had been added. No degradation of SEP in the absence of copper was observed. 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. The concentration of this material had greatly increased after 26 hr. but thereafter slowly decreased, although it was still present in appreciable amount after 94 hr. There was also present on chromatograms of samples taken after 19 hr. a trace of ninhydrin-positive, phosphorus-containing material with
the same $R_f$ as that of phosphoethanolamine. The concentration of this material relative to the other ninhydrin-positive materials gradually increased throughout the remaining period at pH 10. Since the concentration of the slow-running material was decreasing over this same period when the concentration of phosphoethanolamine was increasing, it appears that phosphoethanolamine was being formed at the expense of the material of low $R_f$. 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. At no time was phosphoric acid or material of a similar $R_f$ to serine or ethanolamine detected on the chromatograms.

(b) The mixed complex, SEP-Cu(II)-8-hydroxyquinoline-5-sulphonic acid. This was prepared by dissolving 8-hydroxyquinoline-5-sulphonic acid (7.4 mg.) in 1 ml. of water, adding 0.1 N sodium hydroxide until the solution was slightly alkaline and then dissolving cupric chloride (4.7 mg.) in the solution; SEP (5 mg.) was added to the resulting green solution; After adjusting the pH of the final solution to ca. 10, the volume was 2.6 ml.
Periodic adjustment of the pH of the solution and sampling of the solution at 3, 6, 19, 25, 43 and 73 hr. were carried out as described above. The chromatograms (solvent A) showed that at 6 hr. only the slow-running material could be detected in addition to SEP, while the substance behaving as phosphoethanolamine was again observed on chromatograms at 19 hr. Chromatographic evidence was identical to that described in (a). Additional solvent systems (B, D and K) were used to chromatograph the 73 hr. sample. In each of these solvents the substance behaving as phosphoethanolamine in solvent A had the same Rf as that of authentic phosphoethanolamine. The slow-running material, Rf 0.07 in A, had Rf values 0.04, 0.19 and 0.06 in solvents B, D and K, respectively.

(c) The mixed complex, SEP-cis-chloroaquotetra-amino-Co(III). cis-chloroaquotetra-amino-Co(III)chloride (2.5 mg., \(10^{-5}\) mole) was dissolved in 0.1 ml. water and 0.1 ml. of an aqueous solution of SEP (2.3 mg., \(10^{-5}\) mole) was added. A colour change in the solution was produced on warming and the pH was adjusted to ca. 10 with 0.1 N sodium hydroxide. Degradation of SEP was again observed. The apparent stability of serine and phosphoserine and their copper complexes at pH 10 at room temperature. Aqueous solutions of serine and phosphoserine (10 mg.) were treated with excess basic copper carbonate as described previously.
for SEP. The blue solutions were adjusted to pH 10 with 0.1 N sodium hydroxide and diluted to 2.0 ml. At the same time similar solutions of serine and phosphoserine, but without copper, and of SEP (10 mg.), with copper, were prepared. After standing at room temperature at pH 10 for intervals from 0 to 152 hr., each of the five solutions was chromatographed using solvent A. No decrease in concentration of either serine or phosphoserine was observed on the chromatograms developed either with ninhydrin or molybdate spray reagents, while SEP was rapidly degraded as described previously. Although these observations do not exclude the possibility that some degradation does occur, it was apparent that no appreciable degradation of these substances occurs comparable to that found for SEP.

Alkaline degradation of the copper complex of lombricine. Aqueous alkaline solutions (pH 10) of lombricine (5 mg.) and its copper complex (derived from 5 mg. lombricine) were prepared and examined by paper chromatography (systems A, B and C), after standing for 6, 18 and 42 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 appear to 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. reaction mixture in solvent C; it could still be seen after 18 hr. and 42 hr. on chromatograms developed in solvent 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-guanidino-ethanol was observed on any of the chromatograms.
CHAPTER III

SYNTHESIS OF D-2-AMINO-2-CARBOXYETHYL 2-(N'-PHOSPHORYLGUANIDINO)ETHYL HYDROGEN PHOSPHATE AND THE CORRESPONDING L ISOMER

The preparation of phosphagens by convenient synthetic methods is desirable because of the difficulties that are encountered in the extraction and purification of these compounds from natural sources. For example, the difficulty of obtaining sufficient of the appropriate organisms (marine annelids) precludes the isolation of phosphoguanidinoacetic acid and phosphotaurocyamine (Thoai and Thiem, 1957) on a preparative scale. As a general rule, enzymic dephosphorylation associated with muscle contraction, and the ease with which phosphagens undergo non-enzymic hydrolysis, contribute to the difficulties of the extraction of phosphagens. Furthermore, the purification procedures required for the isolation of a pure phosphagen from crude tissue extracts are tedious and exacting, and the yields realised (Meyerhof and Lohmann, 1928; Fiske and Subbarow, 1929; Thoai and Thiem, 1957) are generally too low to render such preparations worthwhile. An exceptional case is the isolation of phosphoarginine in reasonable amounts from the muscles of crayfish by the method of Ennor, Morrison and
Rosenberg (1956) in which the fact that phosphoarginine will form a copper salt-copper complex is exploited as a purification procedure.

The isolation of the earthworm phosphagen as a calcium salt was reported by Thoai and co-workers (Thoai, Roche, Robin and Thiem, 1953a; Thoai and Robin, 1954; Robin, 1954). Only a small amount of material (40 mg. from 800 g. of earthworms; Robin, 1954) was obtained and this was Sakaguchi-negative and yielded equimolar proportions of orthophosphoric acid and lombricine on mild acid hydrolysis. It was concluded on the basis of this evidence that the phosphagen had structure I.

![Structure I](image)

This chapter is concerned with the synthesis of 2-amino-2-carboxyethyl 2-\((N'\)-phosphorylguanidino)ethyl hydrogen phosphate (I). Before proceeding to a detailed description and discussion of the results, the methods presently available for the synthesis of phosphorylguanidines will be reviewed,
Synthesis of phosphorylguanidines

Until 1958 the only preparative method for the synthesis of phosphorylguanidines consisted in phosphorylation of the parent guanidine derivative with phosphoryl chloride in strongly alkaline solution (Method 1 (a)). Phosphocreatine and monophosphoryl derivatives of guanidinoacetic acid and taurocyamine had been synthesised, but attempts to prepare the phosphoryl derivative of arginine had been unsuccessful (see Ennor and Morrison, 1958; discussion below). Subsequently, a monophosphoryl derivative with a free ε-amino group was prepared from arginine by the above procedure (Thoai, Thiem and Roche, 1960).

The first approach to more elegant procedures for synthesising phosphorylguanidines was made by Cramer and Vollmar (1958a) and involved the use of other phosphorylating agents which had replaced phosphoryl chloride as a reagent for phosphorylating amines. In principle these procedures (see Method 1 (b)) are similar to that using phosphoryl chloride in that the parent guanidine is phosphorylated directly.

The second approach involves the direct transfer of the phosphorylated amidino group from O-methyl-N-phosphorylisourea (XIX, Scheme III.4) or diphenyl esters of S-methyl-N-phosphorylisothiourea (XVI, Scheme III.5) to an amino compound (Method 2). The former reagent was
first used for the synthesis of the phosphoryl derivative of lombricine (I) which is described in this chapter. Independently, Cramer and Vollmar (1958b) synthesised phosphoryl derivatives of a number of simple alkyl- and aryl-guanidines via the corresponding diphenylphosphorylguanidines, formed by the reaction of the diphenyl ester of S-methyl-N-phosphorylisothiourea with alkyl or aryl amines. This procedure was subsequently applied to the synthesis of phosphocreatine and N'-phosphorylguanidinoacetic acid.

There are thus two distinct methods for the synthesis of phosphorylguanidines. It has been generally accepted, although the evidence advanced was not unequivocal, that direct phosphorylation of the guanidino group of a monoalkylguanidine leads to the formation of an N-alkyl-N'-phosphorylguanidine (II) rather than an N-alkyl-N-phosphorylguanidine (III). On the other hand, phosphoryl derivatives of monoalkylguanidines prepared by direct transfer of the phosphorylamidino group from O-methyl-N-phosphorylisourea or from the derivatives of the S-analogue to a monoalkylamine, must possess structure (II). The naturally-occurring monoalkyl-phosphorylguanidines, e.g., phosphoguanidinoacetic acid, also have been formulated as N-alkyl-N'-phosphorylguanidines (cf. the N-N-dialkyl-N'-phosphorylguanidine,
II.

III.

IV. Phosphocreatine
phosphocreatine (IV), but again this formulation, although perhaps correct, was based on little more than pure supposition. The general question of the precise location of the phosphoryl group in these phosphagens and in the synthetic products derived by phosphorylation of monoalkylguanidines, and of the identity of the natural compounds and the synthetic preparations, will be discussed later. For the purposes of the following discussion concerning the synthetic methods, it will be assumed that these synthetic products are, in fact, identical with the compounds isolated from natural sources, and therefore may be designated according to the accepted nomenclature for phosphagens, e.g., phosphoguanidinoacetic acid.

**Method 1. Direct phosphorylation of the parent guanidine.**

(a) Use of phosphoryl chloride. The first of such syntheses followed attempts by Zeile (1935) to prepare phosphocreatine by reacting creatine and phosphoryl chloride in pyridine, or by heating creatine and phosphoryl chloride in the absence of a solvent. The former reaction gave an unidentified product containing two phosphoryl residues, while the latter gave "creatinine phosphoric acid" (1-methyl-$N^2$-phosphorylglucoseimid; Zeile and Meyer, 1938). Zeile and Fawaz (1938) were able to prepare phosphocreatine using the conditions for the
Schotten-Baumann acylation of amines, i.e., treatment of an alkaline solution of creatine cooled in an ice-bath with alternate portions of sodium hydroxide solution and phosphoryl chloride (3 moles). The product was isolated in low yield (20%) as the calcium salt after the removal of inorganic salts and unreacted creatine by fractionation with ethanol.

Similar preparations of phosphocreatine and other phosphorylguanidines reported subsequently have been carried out under essentially the same reaction conditions but with significant modifications in the original isolation procedure. It is difficult to assess whether slight variations in temperature and differences in the molar ratios of reagents affect the course of the reaction because appropriate quantitative studies have not been reported. The increased yields claimed by Thoai and co-workers (Pradel, Thiem, Pin and Thoai, 1959; Thiem, Thoai and Roche, 1960) could be attributed solely to improvements in isolation and purification procedures. Pradel et al. (1959) recommended that the reaction mixture be cooled in an ice-bath and efficiently stirred during dropwise addition of the reagents, with 5-10 minute intervals between alternate portions. This procedure would seem equivalent to maintaining the reaction mixture at 0-5° as described by Ennor and Stocken (1948) for the
preparation of phosphocreatine. There is no evidence to suggest that temperatures down to -5° (Morrison, Ennor and Griffiths, 1958; Peanasky, Kuby and Lardy, 1957) lead to lower yields. However, Thiem et al. (1960) have stated that the yield of phosphocreatine is lower when the phosphorylation is carried out at -10°. This result would not be expected since phosphoryl chloride solidifies at -10°. The molecular proportions of phosphoryl chloride to the guanidino compound vary from 4 to 10 and therefore the amounts of sodium hydroxide, added either as 17, 16 or, more conveniently, 10 N solutions, also vary widely, but presumably the general requirement that the reaction mixture should remain alkaline has been met in all syntheses. Peanasky et al. (1957) and Pradel et al. (1959) specifically mention that the pH of the reaction mixture should be maintained between 12 and 14. The use of ten (Thoai and co-workers) rather than four (Morrison et al., 1958) molar proportions of phosphoryl chloride would not necessarily be advantageous in securing a higher final yield of product (see below).

The introduction of new techniques in the purification and isolation procedures has, however, practical significance. The original procedure described by Zeile and Fawaz (1938) for the isolation of phospho-
creatine and subsequently adopted by Fawaz and Zeile (1940) and Fawaz and Seraidarian (1946) in the preparation of phosphorylguanidine and phosphoguanidinoacetic acid, was superseded by the method introduced by Ennor and Stocken (1948). Peanasky et al. (1957) introduced some modifications in the latter method but in both procedures crystalline sodium phosphate formed during the reaction was removed by filtration and the addition of ethanol (3 volumes) to the filtrate precipitated a further quantity of inorganic salts. The barium salt of phosphocreatine was then precipitated by the addition of barium chloride, barium removed by the addition of sodium sulphate and the crystalline sodium salt of phosphocreatine obtained by the addition of ethanol.

Two laboratories independently introduced purification and isolation procedures employing ion-exchange resins (Thoai and Thiem, 1957; Morrison et al., 1958). In these methods, it is still general practice to remove inorganic salts by precipitation aided by the addition of ethanol (see, also, Pradel et al., 1959). Free guanidine base contaminating the salt may be fixed on appropriate cationic resins through which the phosphorylguanidine passes, or the phosphorylguanidine may be absorbed on an anion exchange resin which does not retain the free guanidino compound. Mixtures of phosphorylated
derivatives (see below) may also be fractionated by the use of resins (Morrison et al., 1958; Thiem et al., 1960). In addition, one salt form may be converted to another by passage through a suitable cation-exchanger (Morrison et al., 1958; Pradel et al., 1959; Thiem, Thoai and Roche, 1960).

Although the ready availability of creatine, guanidinoacetic acid, taurocyamine and arginine makes the phosphoryl chloride method a practical means of obtaining phosphorylated derivatives of these bases, in other respects the method has serious limitations. The yields obtained are consistently low (<20%). Since phosphoryl chloride is rapidly hydrolysed in alkaline solution, this reagent must necessarily be added in considerable excess together with sodium hydroxide to preserve the alkalinity of the reaction mixture. The amount of phosphoryl chloride and sodium hydroxide that may be added in order to secure more phosphorylation is limited, however, by the quantity of precipitated salts because the procedures for removal of inorganic salts are tedious and result in considerable loss of the labile phosphorylguanidino derivative (see Morrison et al., 1958).

A further disadvantage of this method is the formation of more than one phosphorylguanidine. Morrison et al. (1958) isolated, in addition to phosphotaurocyamine,
a more acidic derivative of taurocyamine with two phosphorus atoms for each taurocyamine residue. This product, which constituted at least 30% of the total taurocyamine phosphorylated during the reaction, was isolated as a barium salt for which the elementary analysis did not entirely agree with a formulation such as \( \text{BaPO}_3 \text{N} = \text{C(NHPO}_3 \text{Ba)} \text{NH(CH}_2)_2 \text{SO}_3 \text{H} \). Thiem et al. (1960) have indicated recently that two phosphorylguanidines, separable by paper chromatography, are formed in the reaction of phosphoryl chloride with taurocyamine and with guanidinoacetic acid, but they have not characterised the products which are formed in addition to phospho-taurocyamine and phosphoguanidinoacetic acid. In the preparation of phosphoarginine using phosphoryl chloride, "a number of mono- or poly-phosphate derivatives" have been observed among the reaction products (Thiem et al., 1960) but the nature of the additional products has not been determined.

A precedent is known for the formation of a derivative with two acyl substituents on the guanidino group as a result of the reaction of a guanidino compound with an acid chloride under Schotten-Baumann conditions. In most instances the reaction of an acyl chloride with a guanidino compound in the presence of sodium hydroxide has yielded a product with only one acyl substituent on the
guanidino group and no further substitution of this group appeared to take place. Thus, guanidine, methylguanidine, and asym. dimethylguanidine yielded monobenzenesulphonyl derivatives, and arginine gave $N^\alpha,N^\omega$-dibenzenesulphonyl-arginine (Clark and Gillespie, 1932). Similarly, arginine yielded only $N^\alpha,N^\omega$-disubstituted derivatives with benzoyl chloride (Zervas and Bergmann, 1928; Felix and Dirr, 1928) and p-nitrobenzyloxy carbonyl chloride (Gish and Carpenter, 1953). On the other hand, arginine reacts in the presence of two or four equivalents of benzyl chloroformate to form a mixture of two isomeric tribenzyloxy carbonyl derivatives of arginine, one of which is "alcohol susceptible" undergoing preferential cleavage of one of the two $N$-benzyloxy carbonyl substituents of the guanidino group to give $N^\alpha,N^\omega$-dibenzyloxy carbonylarginine (Zervas, Winitz and Greenstein, 1957; Zervas, Otani, Winitz and Greenstein, 1959; cf. deacetylation of tri- and di-acetylguanidine by repeated crystallisation from alcohol as observed by Greenhalgh and Bannard, 1959). Zervas, Winitz and Greenstein (1961) have shown that "tribenzyloxy carbonylarginine" has the structure V. It is not clear, however, whether this structure (V) represents both isomers of tribenzyloxy carbonylarginine and thus whether cis-trans- (syn-anti) isomers of V (Va and Vb) exist.

It is possible, by analogy with tribenzyloxy carbonylarginine, that the "diphosphoryltaurocyamine" of
Morrison et al. (1958) could be a mixture of isomeric diphosphoryl derivatives of taurocyamine, one of which might be expected to be particularly labile in the presence of alcohol, decomposing to phosphotaurocyamine. In this connection, it is worthy of note that Morrison et al. (1958) believed, on the basis of enzymic studies, that the di-phosphorylated product became contaminated with phosphotaurocyamine as a result of breakdown of the original 'compound' as first obtained from the ion-exchange resin. Such breakdown may have occurred during the isolation of the 'compound' by precipitation as the barium salt in the presence of alcohol.

There is thus some evidence that phosphorylation of a monoalkylguanidine can give rise to a diphosphoryl derivative in which the two acyl substituents are on different nitrogen atoms. The question as to which nitrogen atom carries the phosphoryl residue in the monophosphoryl derivative obtained by the reaction of phosphoryl chloride is discussed in connection with the identification of the product obtained by direct phosphorylation of lombricine.

Acylation of arginine with phosphoryl chloride might be expected to lead to phosphorylation of the α-amino group as well as the guanidino group in view of the fact that the α-amino group of arginine is readily acylated.
by acid chlorides such as benzenesulphonyl chloride and benzyl chloroformate in the presence of sodium hydroxide (as indicated above) or even in the presence of sodium carbonate, under which conditions $N^\alpha$-benzenesulphonyl-arginine (Clark and Gillespie, 1932) and $N^\alpha$-benzyloxy-carbonylarginine (Zervas, Winitz and Greenstein, 1957) are formed. Because it was expected that such an acylation might occur with phosphoryl chloride in the presence of sodium hydroxide, Ennor, Morrison and Rosenberg (1956) attempted the synthesis of phosphoarginine using copper to protect the $\alpha$-amino group. However, while it is known that glycine and alanine react with phosphoryl chloride in the presence of magnesium oxide suspensions to form derivatives which have approximately correct compositions for monosubstituted derivatives of orthophosphoric acid (Neuberg and Oertel, 1914; Winnick and Scott, 1947; Sciarini and Fruton, 1949; Zervas and Katsoyannis, 1955), the phosphorylation of an $\alpha$-amino acid with phosphoryl chloride in the presence of sodium hydroxide has been described only in the case of arginine. Apart from the fact that Thiem et al. (1960) isolated phosphoarginine in unstated yield, the other products of the reaction (see above) were not identified but may possibly have included an $N^\alpha$-phosphoryl derivative.

Finally, acylation with phosphoryl chloride may be
further complicated by the fact that two or three of the chlorine atoms may be replaceable by amino groups. Such a reaction, giving di- and tri-substituted derivatives of orthophosphoric acid in the presence of 10% or 25% sodium hydroxide is known in the case of the reaction of primary aromatic amines with phosphoryl chloride (Autenrieth and Rudolph, 1900; see Kosolapoff, 1950a).

(b) Use of protected phosphorylating agents. Tetrabenzyl pyrophosphate and diesters of phosphoro-chloridic acid (phosphorochloridates), which have been used successfully for the acylation of amines and alcohols, have been found (Cramer and Vollmar, 1958a) to react readily with guanidino compounds with the formation of diesters of phosphorylguanidines from which the ester groups could be readily removed. It was presumed that the products were \( \text{N-,N'} \)-disubstituted guanidines. Evidence was later advanced (Cramer and Vollmar, 1958b) which is consistent with this allocation of structure (see later discussion).

The reaction of phenylguanidine with tetrabenzyl pyrophosphate (VI) in dimethylformamide, followed by hydrogenation in the presence of a palladium catalyst, gave a moderate yield of \( \text{N-phenyl-N'} \)-phosphorylguanidine (VIII), via the dibenzyl ester (VII, see Scheme III.1). Excellent yields of the diphenylphosphoryl derivatives (X)
SCHEME III.1. Synthesis of N-Phenyl-N'-phosphoryl-guanidine using tetrabenzyl pyrophosphate
of a number of simple aliphatic and aromatic guanidino derivatives were obtained using diphenyl phosphorochloridate (IX, see Scheme III.2). Hydrogenation of the diphenyl esters (X), smoothly accomplished with platinum oxide catalyst in methanol, furnished crystalline phosphoryl-guanidines (XI) as the free acids. These methods are attractive because of the good yields obtained, the simplicity of the reaction procedures and the ease of isolation of the products. The free guanidino bases are allowed to react with the phosphorylating agent in ethanolic solution for 2 hours, when the product is extracted into benzene; alternatively, the free bases are first liberated from their salts by dissolution in dilute aqueous alkali and the solution then stirred with a benzene solution of diphenyl phosphorochloridate, when reaction in the organic phase ensues. In either case crystalline diesters of phosphorylguanidines are readily obtained from the benzene solution.

The elegant and simple "phosphite-carbon tetrachloride" method used by Todd and co-workers for the preparation of phosphorylated amines (Atherton and Todd, 1947; Atherton, Openshaw and Todd, 1945) was also applied by Cramer and Vollmar (1958a) to the synthesis of phosphoryl derivatives of simple guanidines (see Scheme III.3). Thus, a solution of dibenzyl phosphite (XII) in carbon
SCHEME III.2. Synthesis of Phosphorylguanidines using diphenyl phosphorochloridate
SCHEME 111. Synthesis of Phosphorylguanidines using the "dibenzyl phosphate-carbon tetrachloride" method
tetrachloride is stirred with an aqueous solution of the guanidinium salt from which the base is liberated \textit{in situ} by the addition of a solution of sodium hydroxide. The guanidino compound immediately reacts in the organic phase with dibenzyl phosphorochloridate (XIII), which is formed as an intermediate in the reaction (Atherton and Todd, 1947; Steinberg, 1950). Debenzylation of the readily isolated product (XIV) by catalytic hydrogenation with palladium catalyst gives excellent yields of crystalline phosphorylguanidines.

In order to achieve phosphorylation of a compound by these methods, it must possess some solubility in organic solvents in which the phosphorylating agents, tetrabenzyl pyrophosphate (VI) and the phosphorochloridates (IX and XIII), are soluble and in which these reagents do not hydrolyse. Compounds which are not thus soluble must be converted to suitable soluble derivatives. An attempt to phosphorylate \(\alpha\)-amino acids with diphenyl phosphorochloridate in aqueous solution led only to the formation of the diphenylphosphoric acid salt of the \(\alpha\)-amino acid (Bernton, 1922). Similarly, the dibenzylphosphoric acid salt of guanidine was obtained by Deutsch and Fernö (1945) instead of dibenzylphosphorylguanidine (see Cramer and Vollmar, 1958a). On the other hand, Sciarini and Fruton (1947) phosphorylated esters of \(\alpha\)-amino acids with diphenyl
phosphorochloridate in ethyl acetate solution after liberating the α-amino acid ester from its salt. Similar syntheses using other diesters of phosphorochloridic acid are described in the literature (Zervas and Katsoyannis, 1955; Plapinger and Wagner-Jauregg, 1953).

Cramer and Vollmar (1959) have pointed out that these phosphorylation methods cannot be applied to the acylation of α-guanidino carboxylic acid esters, such as creatine and guanidinoacetic acid esters, which when liberated from their salts cyclise rapidly to creatinine and glycocyamidine, respectively (Lempert, 1959; Mold, Gore, Lynch and Schantz, 1955; Failey and Brand, 1933; see p. 29). The benzyl ester of creatine seems to possess greater stability than the methyl or ethyl ester (Abderhalden and Suzuki, 1928), but the feasibility of phosphorylating the former has not been investigated.

Cramer, Vollmar and Scheiffele (1960) have reported a synthesis of phosphoarginine in 55% yield by the reaction of the benzyl ester of \( N^\alpha \)-benzyloxy carbonylarginine with di-\( p \)-nitrobenzyl phosphorochloridate, followed by hydrogenation of the product with palladium charcoal catalyst in methanol. The yield obtained clearly indicates that this method of phosphorylation is more satisfactory than that using phosphoryl chloride.

(c) Use of \( N^1, N^3 \)-diphosphorylimidazole. The
formation of a phosphorylated derivative by a transphosphorylation reaction between diphosphorylimidazole and creatine at pH 13-14 was observed by Rathlev and Rosenberg, (1936). The product had the chromatographic behaviour of phosphocreatine and furthermore, acted as a phosphoryl donor to ADP in the presence of a rabbit muscle extract. Although the reaction has not been used as a preparative procedure for obtaining phosphorylguanidines, it is, nevertheless, interesting, since it bears a formal analogy to the enzymic transfer of unesterified phosphoryl groups to acceptor molecules, e.g., from ATP to creatine and similar guanidines, with the formation of phosphagens (see Chapter I).

Method 2. Transfer of the phosphorylated amidino group from O-methyl-N-phosphorylisourea and from diphenyl esters of S-methyl-N-phosphorylisothiourea. Zeile (1935) made the original suggestion that it should be possible to synthesise phosphocreatine from sarcosine and a phosphorylated derivative of S-methylisothiourea as a logical extension of the guanidination of an amine with S-methylisothiourea. Zeile, however, could not obtain a phosphorylated guanidinating reagent; unidentifiable phosphorus-containing material was isolated from the reaction of S-methylisothiourea (XV) with phosphoryl chloride and the product obtained by the phosphorylation of XIV with diphenyl
\[
\begin{align*}
\text{XV.} & \quad \text{XVI.} \\
\text{XVII.} & \quad \text{XIII.} \\
& \quad \text{XVIII.} \\
& \quad \text{XIX.}
\end{align*}
\]
phosphorochloridate in alkaline solution was the diphenyl-
phosphoric acid salt of \(\text{S-}\text{methylisothiourea}\) and not
diphenylphosphoryl-\(\text{S-}\)methylisothiourea (XVI).

In this laboratory, Dr. D.I. Magrath applied the
"phosphite-carbon tetrachloride" method of phosphorylating
guanidino compounds (Cramer and Vollmar, 1958a; see Scheme
III.3), described to him prior to publication in a personal
communication from Cramer, to the synthesis of the dibenzyl
ester of \(\text{O-}\text{methyl-}\text{N-}\)phosphorylisourea (XVIII) from
\(\text{O-}\text{methylisourea}\) (XVII). Catalytic hydrogenation of the
dibenzy1 ester (XVIII) readily afforded crystalline \(\text{O-}\text{methyl-}\text{N-}\)
phosphorylisourea (XIX) in high yield. It was proposed
to employ the phosphorylated derivative of \(\text{O-}\text{methylisourea}\)
(XIX) in the synthesis of phosphorylguanidines from the
corresponding amines, as shown in Scheme III.4.

Preliminary experiments in this laboratory showed
that \(\text{O-}\text{methyl-}\text{N-}\)phosphorylisourea reacted in aqueous alkali
with taurine and phosphoethanolamine to give phospho-
taurocyamine and 2-(\(\text{N'}\)-phosphorylguanidino)ethyl phosphate,
respectively. At the same time it was shown that a product
with the chromatographic behaviour of phospholombricine was
obtained from DL-SEP by an analogous reaction. A brief
reference to these preliminary experiments was made by
Ennor and Morrison (1958). While these preliminary studies
were in progress a publication by Cramer and Vollmar (1958b)
\[ \text{(XIX.)} \]

**SCHEME III.4.** Synthesis of \( \text{N-alkyl-} \text{N'}-\text{phosphoryl-} \text{guanidines using O-methyl-N-phosphorylisourea} \]

\[
\begin{align*}
\text{(C}_6\text{H}_5\text{O})_2\text{P}=\text{N} &= \text{C-SCH}_3 + \text{RNH}_2 \\
&\rightarrow \text{CH}_3\text{SH} + \text{N}^+\text{H}_3\text{N}
\end{align*}
\]

\[ \text{XX.} \]

\[
\begin{align*}
\text{(OH)}_2\text{P}=\text{N} &= \text{C-NHR} \\
&\xrightarrow{\text{Pt/H}_2} \text{(C}_6\text{H}_5\text{O})_2\text{P}=\text{N} &= \text{C-NHR}
\end{align*}
\]

\[ \text{XXII.} \]

**SCHEME III.5.** Synthesis of \( \text{N-alkyl-(or N-aryl-)} \text{N'}-\text{phosphorylguanidines by the method of Cramer and Vollmar (1958b)} \]
appeared which described not only the preparation of O-methyl-N-phosphorylisourea and its dibenzyl ester, but also that of the diphenyl, di-p-nitrobenzyl and dibenzyl esters of S-methyl-N-phosphorylisothiourea, which were synthesised in high yields (80%) from S-methylisothiourea using diphenyl phosphorochloridate, di-p-nitrobenzyl phosphorochloridate and dibenzyl phosphorochloridate, respectively. Cramer and Vollmar (1958b) investigated the reaction of the latter compounds, but not of O-methyl-N-phosphorylisourea or the corresponding dibenzyl ester, with simple amines.

N-diphenylphosphoryl-S-methylisothiourea (XVI, Scheme III.5) reacted readily when refluxed in ethanol or p-chlorotoluene with a strongly basic amine, namely, cyclohexylamine, to form the cyanamide salt (XX, R = C$_6$H$_{11}$) which gave the diphenyl ester of the corresponding phosphorylguanidine (XXI, R = C$_6$H$_{11}$) by isomerisation when heated at 150°. Weaker bases, such as aniline, showed less tendency for the formation of the cyanamide salt but on the other hand, their cyanamide salts isomerised more readily than those of strong bases, e.g., the anilinium salt (XX, R = C$_6$H$_5$) isomerised at 78°. The reaction of weak bases was facilitated, however, by the addition of mercuric oxide which accelerated the elimination of mercaptan necessary for the formation of the cyanamide salt. Thus
while no methyl mercaptan was evolved when aniline was refluxed with XVI in ethanolic solution, the addition of mercuric oxide led to the formation of $N$-diphenylphosphoryl-$N'$-phenylguanidine (XXI, $R = C_6H_5$).

The heavy metal oxide was used routinely to facilitate the reaction with amines, thus avoiding the use of high temperatures. However, since the cyanamide salts of strong bases formed more readily than those of weaker bases, Cramer and Vollmar (1958b) found it practical to prepare phosphorylated derivatives of a guanidino compound corresponding to a weakly basic amine by adding a salt of the weakly basic amine to the cyanamide salt of a stronger base, e.g., the triethylamine or sodium salt. The final stage in the synthesis of the phosphorylguanidine (XXII), the removal of the phenyl ester groups, was accomplished by catalytic hydrogenation (see also below).

Results with the dibenzyl ester of $S$-methyl-$N$-phosphorylisothiourea were less satisfactory. The formation of the cyanamide salt of very weak aromatic amines, and the isomerisation of the cyanamide salt of aliphatic amines, were not accomplished successfully (Cramer and Vollmar, 1958b).

Shortly after the results of these studies were published another report came from Cramer and Vollmar (1959) describing the application of these reactions to the
preparation of $N'$-phosphorylguanidinoacetic acid and phosphocreatine from glycine and sarcosine benzyl esters, respectively (Scheme III.6). An ethanolic solution of the sodium or triethylamine salt of diphenylphosphorylcyanamide, prepared by heating an ethanolic solution of $N$-diphenylphosphoryl-$S$-methylisothiourea in the presence of mercuric oxide and either sodium carbonate or triethylamine, was treated with the hydrochloride salt of glycine or sarcosine benzyl ester. The resulting cyanamide salts (XXIII, $R = H$ and $R = CH_3$) isomerised when refluxed in isobutyl alcohol to yield $N'$-diphenylphosphorylguanidinoacetic acid benzyl ester (XXIV, $R = H$) and diphenylphosphorylcreatine benzyl ester (XXIV, $R = CH_3$), respectively. Hydrogenation of these derivatives in the presence of palladium/charcoal and platinum oxide catalysts removed the ester groupings and furnished $N'$-phosphorylguanidinoacetic acid (XXV, $R = H$) and phosphocreatine (XXV, $R = CH_3$) as the crystalline-free acids in 43 and 38% yields, respectively, based on the $\alpha$-amino acid esters.

Some observations concerning the utility of Cramer and Vollmar's method should be made. For reasons of solubility it is necessary to use esters of amino acids. However, the methyl or ethyl ester of glycine or sarcosine cannot be utilised in the above preparations because the removal of
SCHEME III.6. Synthesis of N'-phosphorylguanidinoacetic acid (R = H) and phosphocreatine (R = CH₃) by the method of Cramer and Vollmar (1959)
these ester groupings by saponification results in the formation of cyclic lactam derivatives (glycocyamidine or creatinine derivatives, see above). It is likely that similar difficulties would be experienced in the synthesis of phosphoarginine, since methyl and ethyl esters of arginine are also unstable (see p. 29). Use of the benzyl ester of ornithine would, of course, eliminate this difficulty as the benzyl group is readily removed by catalytic hydrogenation as above.

The synthesis of phosphoarginine from ornithine is further complicated by the presence of two amino groups in the latter. Although preferential reaction with the ω-amino group might be expected to occur on the basis of an analogy with the reaction of ornithine and S-methylisothiourea (Odo, 1953; see Chapter I), the possibility that the α-amino group may react readily (cf. reaction with cyanamide) cannot be excluded. Nα-benzyloxy carboxylornithine benzyl ester would be a suitably protected derivative for selectively synthesising phosphoarginine.

As a consequence of the necessity to remove protecting groups by catalytic hydrogenation, exhaustive purification of the intermediates is necessary to exclude all traces of S-containing compounds which would poison the catalysts.

On the other hand, the method using O-methyl-N-phosphoryl isourea (Scheme III.4), by which the phosphoryl
derivative of lombricine (i.e., compound I) has been prepared from SEP (described below), is simpler than that of Cramer and Vollmar (1959). Thus, the use of O-methyl-N-phosphorylisourea leads to the direct formation of the phosphorylguanidine at room temperature in aqueous alkali. The recent brief report by Cramer, Vollmar and Scheiffele (1960) that phosphoarginine has been synthesised in 60% yield by the reaction of O-methyl-N-phosphorylisourea with ornithine may be taken as indicative of the usefulness of this simpler method.

As mentioned previously, a fact of considerable interest which distinguishes the method of synthesis which uses either O-methyl-N-phosphorylisourea or the diphenyl ester of S-methyl-N-phosphorylisothiourea from that involving acylation of the parent guanidine, is that the precise position of the phosphoryl residue on the guanidino group of the product is known with certainty. The implications of this will be discussed in detail below.

Finally, an extension of this synthetic method may be envisaged by the use of N-alkyl-O-methyl-N'-phosphorylisourea and S-analogues. N-methyl-N'-phosphorylguanidinoacetic acid, an isomer of phosphocreatine, is one interesting compound which might be synthesised in this way.
The synthesis of 2-amino-2-carboxyethyl 2-(N'-phosphorylguanidino)ethyl hydrogen phosphate (I)

The phosphoryl derivative of lombricine (I) was synthesised by two routes (Scheme III.7); firstly, by the reaction of SEP (XXVI) with 0-methyl-N-phosphorylisourea (XIX) and, secondly, by direct phosphorylation of lombricine (XXVII) with phosphoryl chloride in strongly alkaline solution.

A product resulting from the reaction of L-SEP and 0-methyl-N-phosphorylisourea in alkaline solution at pH 12 and room temperature was purified by fractionation of the reaction mixture on Dowex-50 (H⁺) resin at 0-5°. Immediately after elution, fractions containing the desired material were made alkaline with conc. aqueous ammonia and evaporated to give the amorphous ammonium salt of I. This was converted to a microcrystalline powder by recrystallisation from aqueous methanol. Titration and elementary analytical data indicated that this material was a mixture of the mono- and di-ammonium salts of L-2-amino-2-carboxyethyl 2-(N'-phosphorylguanidino)ethyl hydrogen phosphate (I). The amorphous ammonium salt was obtained in a yield of 79% calculated on the assumption that it was the di-ammonium salt.

The product gave a positive ninhydrin reaction, liberated orthophosphoric acid on treatment with molybdate reagent and did not react with Sakaguchi or α-naphthol/
SCHEME III.7. Synthesis of 2-amino-2-carboxyethyl 2-\(N'\)-phosphorylguanidino)ethyl hydrogen phosphate (I)
diacetyl reagents. On paper chromatography in a number of solvent systems it was homogeneous with respect to the former two reagents.

Hydrolysis of the material in 0.1 N hydrochloric acid for 1 minute at 100° caused complete conversion to orthophosphoric acid and a guanidino compound behaving as lombricine on paper chromatography in six solvent systems. The guanidino base was further characterised as lombricine by its behaviour on nitrous acid degradation (see Chapter II).

By potentiometric titration, pKₐ values of ca. 2, 4.6 and ca. 8.8 were obtained, corresponding to the ionisation of the carboxylic acid group, the secondary hydroxyl group of the phosphoryl residue and the α-amino group, respectively (cf. pKₐ values of ca. 2 and 8.9 for lombricine and ca. 2, 8.9 and 10.0 for SEP; see Chapter II).

Thus, guanidination with O-methyl-N-phosphorylisourea occurs preferentially, as it does with O-methylisourea, at the terminal amino group of SEP. Paper chromatographic examination of the reaction mixture and of the eluate from the column of Dowex-50 (H⁺) resin indicated, however, that a second product was formed during the reaction. This was ninhydrin-negative and was eluted from the resin before compound I. Mild acid hydrolysis of the material liberated orthophosphoric acid and gave a product which reacted with α-naphthol/diacetyl reagent. This evidence is consistent
with the compound being 2-carboxy-2-(N'-phosphorylguanidino)-
ethyl 2-(N'-phosphorylguanidino)ethyl hydrogen phosphate.

Finally, the infrared spectrum of the main product was
identical with that of the ammonium salt of the compound
formed by the direct acylation of lombricine with phosphoryl
chloride (see Figure III.1).

The reaction of natural lombricine with phosphoryl
chloride (6.6 moles, added in five portions) under alkaline
conditions (pH 13-14) resulted in the conversion of ca. 20%
of the lombricine to a material which did not react with
α-naphthol/diacetyl reagent unless treated with acid.
Ethanol fractionation of the reaction mixture gave an oil
which contained all the acid-labile material and some free
lombricine. This oil was redissolved in a small volume of
water and the solution applied to a column of Dowex-50 (H+)
resin. A ninhydrin-positive, α-naphthol/diacetyl-negative,
phosphorus-containing substance was isolated as an amorphous
ammonium salt in low yield (7-14%). Recrystallisation of
this material from aqueous methanol gave a microcrystalline
powder which, from titration and elementary analytical data,
was formulated as a mixture of the mono- and di-ammonium
salts of I. The $R_f$ and $pK_a$ values as well as the infrared
spectrum (Figure III.1) were identical with those of the
product obtained from the reaction of SEP with Q-methyl-N-
phosphorylisourea.
The method of synthesis of the product obtained from SEP establishes that the phosphoryl residue is located on one of the two unsubstituted nitrogen atoms of the guanidino group of lombricine. These two positions are equivalent because of resonance within the zwitterionic guanidino group (see, also, the Appendix, p. 179) and it is possible, therefore, to assign unambiguously structure I to the compound. The identity of this compound with the product obtained by the phosphorylation of lombricine established that the phosphoryl residue in the latter occupies a similar position.

The formulation, I, is in agreement with the generally accepted formulation for the structure of phosphagens derived from monoalkylguanidines, and for the corresponding synthetic products derived by phosphorylation of the parent guanidine. Theoretically, however, there are two possible positions for the phosphoryl residue in phosphorylated derivatives of monoalkylguanidines and thus two possible structures (see Appendix, p. 179), that of an \( \text{N-alkyl-N'} \)-phosphorylguanidine (\text{sym} \text{-di-substituted}, II) or of an \( \text{N-alkyl-N-phosphorylguanidine} \) (\text{asym} \text{-di-substituted}, III). By analogy with the structure of phosphocreatine, for which there is only one possible formulation (IV), there has doubtless been a tendency to assume that the same orientation of the phosphoryl group to alkyl substituents
would be found in the other phosphagens. However, with the exception of the two synthetic preparations described above, and the phosphoryl derivatives of guanidinoacetic acid and arginine recently prepared by Cramer and co-workers (Cramer and Vollmar, 1959; Cramer, Vollmar and Scheiffele, 1960) via N-diphenylphosphoryl-β-methylisothiourea and O-methyl-N-phosphorylisourea, respectively, the accepted formulation of monoalkylphosphorylguanidines isolated from natural sources and of the corresponding synthetic products, although perhaps correct, remains unproven. Thus it appears that the evidence cited in support of structure II, rather than III, is based primarily on the fact that the compounds do not give a colour reaction with the α-naphthol/diacetyl reagents, and on the supposition that phosphorylguanidines will show the same specificity towards this reagent as do simple alkylguanidines, of which only the mono- and asymmetric di-substituted react (see the Appendix, p. 182). This supposition is incorrect, however, for in the course of the present work it has been found that phosphorylguanidine gives no colour reaction with this reagent. That phosphoryl derivatives of monoalkylguanidines do not give a colour reaction does not, therefore, exclude their formulation as asymmetric di-substituted guanidines. The α-naphthol/diacetyl reaction is thus valueless for defining the precise position of the phosphoryl residue, and the claims made in the case of
the natural compounds, phosphoguanidinoacetic acid, phosphotaurocyamine and phosphoarginine, and the products obtained by phosphorylation of guanidinoacetic acid, taurocyamine and arginine with phosphoryl chloride, should be disregarded (Thoai and Thiem, 1957; Thiem, Thoai and Roche, 1960).

The only other evidence that could have any bearing on the position of the phosphoryl residue concerns the structure of analogous acyl derivatives of guanidino compounds. Little attention has been paid to the precise structure of such compounds. Felix and Dirr (1928), however, prepared dibenzoylarginine by acylation of arginine with benzoyl chloride in strongly alkaline solution, and determined the position of the benzoyl groups by conversion of the product to dibenzoylacetylanhydro-arginine (XXX), which on hydrolysis gave β-benzoylamino-α-piperidone (XXXII) and acetylbenzoylurea (XXXI). One benzoyl group was therefore on the α-amino group and the other on an unsubstituted nitrogen of the guanidino group of arginine, and thus the dibenzoyl derivative has the structure shown (XXVIII). By similar methods, Zervas, Otani, Winitz and Greenstein (1959) determined the structure (XXIX) for the dibenzyloxycarbonyl derivative of arginine, prepared by the degradation of tribenzyloxycarbonylarginine. As it is not possible, however, to
prepare this diacyl derivative directly from arginine (Zervas, Winitz and Greenstein, 1957), proof of its structure does not afford unequivocal evidence that the guanidino group is first acylated at the unsubstituted guanidinium nitrogen of arginine (see "tribenzylloxycarbonyl-arginine", V).

The identity of the infrared spectra of the product prepared by phosphorylation of lombricine with that obtained by unambiguous synthesis of compound (1) using O-methyl-N-phosphorylisourea, constitutes the first occasion on which proof has been deduced for the structure of the product obtained by phosphorylation of a naturally-occurring monoalkylguanidine. Although, as mentioned above, N'-phosphorylguanidinoacetic acid has been synthesised (Cramer and Vollmar, 1959) by the unambiguous route via N-diphenylphosphoryl-S-methylisothiourea, no comparison of this product with that prepared by the phosphorylation of guanidinoacetic acid with phosphoryl chloride has yet been made. However, products obtained by Cramer and Vollmar's phosphorylation methods (Method 1 (b)) from a number of simple alkyl- and arylguanidines have melting points similar to analogous products prepared by ambiguous synthesis via diester derivatives of S-methyl-N-phosphorylisothiourea, which is consistent with - but not proof of - the former preparations
possessing a symmetrical substitution. Mixed melting points and a comparison of infrared spectra of these products would provide proof of the identity — or otherwise — of these compounds. The two synthetic preparations of phosphoarginine prepared by Cramer et al. (1960) have not yet been fully described.

By analogy with the structure of the phosphorylation product of lombricine, it is reasonable to expect that, in the cases of the similarly prepared phosphoryl derivatives of guanidinoacetic acid (Fawaz and Seraidarian, 1946; Thoai and Thiem, 1957), taurocyamine (Morrison, Ennor and Griffiths, 1958; Thoai and Thiem, 1957) and arginine (Thiem, Thoai and Roche, 1960), the phosphoryl group would occupy an analogous position. Furthermore, if these synthetic products are identical with the corresponding natural compounds, as has been claimed, it would follow that the latter compounds, also, would be expected to possess a structure analogous to I.

The evidence of the identity of natural phosphagens and the corresponding synthetic preparations, however, seems insufficient to warrant any definite conclusions on this point. Thus, it has been shown that a synthetic preparation of the monophosphoryl derivative of taurocyamine acts as a substrate for the enzyme, ADP:phospho-taurocyamine transferase (Morrison, Ennor and Griffiths,
178.

1958), and while this finding suggests that the synthetic material might be identical with the natural compound, it does not constitute proof, as the specificity of the enzyme for the groups surrounding the N—P bond (i.e., for the structures, \((\text{HO})_2\text{PO—N}(\text{R})—\text{C} (=\text{NH})\text{NH}_2\) and \((\text{HO})_2\text{PO—NH—C} (=\text{NH})\text{NHR}\)), is not known. Again, chemical evidence for the identity of natural phosphoarginine and the product prepared by the direct phosphorylation of arginine rests solely on similarity of behaviour on paper chromatography (Thiem, Thoai and Roche, 1960). Similar synthetic preparations of phosphoryl derivatives of guanidinoacetic acid and taurocyamine and the corresponding natural compounds have been assigned identical structures on the basis of comparable melting points of the ammonium salts (Thoai and Thiem, 1957). Such criteria afford no definite proof of the identity of the compounds. Finally, no direct comparison of the phosphoryl derivatives of guanidinoacetic acid and arginine prepared by Cramer and co-workers (Cramer and Vollmar, 1958b; Cramer, Vollmar and Scheiffele, 1960) was made with the natural compounds.

It would, therefore, be of considerable interest to compare the infrared spectra of the synthetic phosphoryl derivative of lombricine with the natural compound isolated from earthworms. In any event, from the above discussion, the potential value of the synthetic method
involving O-methyl-N-phosphorylisourea in orientating the phosphoryl residue throughout the group of naturally-occurring compounds is clear.

Appendix

Guanidine is unique among organic bases because of its high basicity which approaches that of sodium or potassium hydroxide. In the neutral molecule (XXXIII) a small amount of stabilisation by resonance involving dipolar forms (XXXIIIb and XXXIIIc) is to be expected; on the other hand, in the cation, which has been shown (see, e.g., Theilacker, 1935; Drenth, Drenth, Vos and Wiebenga, 1953; Curtis and Pasternak, 1955; Taylor and Baker, 1937c) to possess structure XXXIV, the resonance involves three equivalent structures, XXXIVa, XXXIVb and XXXIVc, and hence is considerably greater. Because of this difference in resonance stabilisation, cation formation is greatly favoured, i.e., guanidine is a considerably stronger base than might otherwise be expected (Wheland, 1955).
XXXIIIa.  

XXXIIIb.  

XXXIIIc.  

XXXIVa.  

XXXIVb.  

XXXIVc.  

XXXVa.  

XXXVb.  

XXXVc.
A monoalkylguanidine (e.g., lombricine) may be represented by either of the two tautomeric structures, XXXVa or XXXVb, and for all practical purposes may therefore be regarded as an equilibrium mixture of these two readily interconvertible forms. Both, of course, would be stabilised by resonance and on the addition of a proton would give rise to the common cation XXXVc. It is therefore to be expected, and this is borne out in practice, that for any particular monoalkylguanidine only one substance is known. In the case of a guanidine substituted with an appreciably electronegative group, e.g., a nitro group, the imine form would be expected to be more stable and the behaviour of this type of guanidine derivative is consistent with the structure, XXXVb (Kumler, 1955).

Replacement of two hydrogen atoms of the guanidine molecule by similar or dissimilar substitution gives in each case two distinct disubstituted guanidines (represented by XXXVI and XXXVII or XXXVIIIa and XXXIX, respectively). Both XXXVI and XXXVIIIa are defined as being asymmetrically disubstituted derivatives, in contrast to XXXVII and XXXIX, which are defined as symmetrically disubstituted derivatives. As in the case of mono-substituted derivatives, only one sym. dialkylguanidine corresponds to the structures XXXVIIa and XXXVIIb, and
Similar Substitution

\[
\begin{align*}
\text{NR}_2 & \\
\text{C} & \\
\text{HN} & \text{NH}_2 \\
\end{align*}
\]

\[
\begin{align*}
\text{NHR} & \\
\text{C} & \\
\text{RN} & \text{NH}_2 \\
\end{align*}
\]

XXXVI. (asym.)

XXXVIIa. (sym.)

XXXVIIb. (sym.)

Dissimilar Substitution

\[
\begin{align*}
\text{R-N-R'} & \\
\text{C} & \\
\text{HN} & \text{NH}_2 \\
\end{align*}
\]

\[
\begin{align*}
\text{NHR'} & \\
\text{C} & \\
\text{RN} & \text{NH}_2 \\
\end{align*}
\]

XXXVIIIa. (asym.)

XXXIXa. (sym.)

XXXIXb. (sym.)

XXXIXc. (sym.)

\[
\begin{align*}
\text{R-N-PO}_3\text{H}^- & \\
\text{C} & \\
\text{H}_2\text{N} & \text{NH}_2 \\
\end{align*}
\]

\[
\begin{align*}
\text{NHPO}_3\text{H}^- & \\
\text{C} & \\
\text{RHN} & \text{NH}_2 \\
\end{align*}
\]

XXXVIIIb. XXXIXd. XXXIXe. XXXIXf.
to XXXIXa, XXXIXb and XXXIXc.

Two monoalkylphosphorylguanidines are possible and may be represented by XXXVIII and XXXIX (R = alkyl group and R' = phosphoryl residue), cf. II and III. Although the basicity of the guanidino group is known to be diminished when a phosphoryl group is introduced, the effect is not appreciable. For example, arginine and phosphoarginine have pK\textsubscript{a} values 12.84 (Albert, 1952; cf. 12.48, Schmidt, Kirk and Appleman, 1930) and 11.2 (Kumler and Eiler, 1943), respectively. Therefore, the structures, XXXIXa, XXXIXb and XXXIXc (R = alkyl group, R' = phosphoryl group) would be regarded as non-separable tautomers. In any event, the phosphorylguanidino group is zwitterionic (cf. Cramer and Vollmar, 1958a) and thus a \textit{sym.} monoalkylphosphorylguanidine (XXXIX) is a resonance hybrid of the structures, XXXIXd, XXXIXe and XXXIXf (R = alkyl group), and the \textit{asym.} isomer is a resonance hybrid of structures of the type XXXVIIIb (R = alkyl group).

The extension of the accepted usage of the terms \textit{asym.} and \textit{sym.} to the description of trisubstituted guanidines is illustrated in formulae XL-XLIII. It is obvious, however, that it is difficult to describe compounds XLIV and XLV by this system of nomenclature and a more specific approach must be adopted in naming these
asym. trimethylguanidine

sym. trimethylguanidine

asym. (dimethyl)ethylguanidine

sym. dimethylcylethylguanidine

XLIV

XLV
compounds, e.g., XLIV as $N$-ethyl-$N,N'$-dimethylguanidine, and XLV as $N$-ethyl-$N'$-methyl-$N$-propylguanidine.

It is possible to distinguish chemically between guanidine, a monoalkylguanidine and a sym. or asym. dialkylguanidine on the basis of certain colour reactions. Sakaguchi (1925) observed that an alkaline solution of arginine containing $\alpha$-naphthol gave a red colour on treatment with sodium hypochlorite. The colour reaction is not specific for arginine, but is characteristic of particular guanidino compounds. Formerly it was thought that mono- and sym. di- and sym. tri-substituted guanidines, but not guanidine or its asym. substituted derivatives, were capable of giving this reaction (Poller, 1926; cf. Robin, 1960). However, Mold, Ladino and Schantz (1953) unequivocally synthesised sym. dimethylguanidine and sym. triethylguanidine and found that these compounds, in addition to guanidine and its asym. alkyl substituted derivatives, failed to yield a positive colour reaction. It is now generally accepted (see, e.g., Ennor and Morrison, 1958) that only mono-substituted guanidines will react with the Sakaguchi reagent (see, however, below).

Barritt (1936) showed that guanidine and guanidino derivatives which reacted with diacetyl in alkaline solution to form a pink or salmon fluorescence (the
Voges-Proskauer reaction, Harden and Norris, 1911; O'Meara, 1931; Lang, 1932) gave a red colour of greater intensity when α-naphthol was also present. The reaction with α-naphthol/diacetyl reagent is less specific than the Sakaguchi reaction. Thus, Eggleton, Elsden and Gough (1943) reported that guanidine, the monosubstituted guanidines, arginine and glycocyamine, and the asym dialkylguanidine, creatine, yielded a colour reaction with the reagent. These results were confirmed and extended to the simple alkylguanidines, methyl- and asym dimethylguanidine, both of which gave a positive reaction (Ennor and Stocken, 1953). On the other hand, sym dimethylguanidine or trisubstituted guanidines failed to react (Mold, Ladino and Schantz, 1953).

Both reactions are used for the detection of guanidino derivatives on paper chromatograms and for their estimation (see Ennor and Morrison, 1958; Robin, 1960). The α-naphthol/diacetyl method (Eggleton, Elsden and Gough, 1943; Ennor and Stocken, 1953) for the determination of creatine gives only one-tenth of the colour (on a molar basis) with monosubstituted guanidines but by increasing the concentration of α-naphthol and adding n-propanol, Rosenberg, Ennor and Morrison (1956) obtained the same sensitivity with monosubstituted guanidines as for the asym disubstituted guanidines,
The known specificity of both colour reactions is summarised in Table III.1, from which it is readily seen how guanidine, monoalkylguanidines and sym. and asym. dialkylguanidines differ in their behaviour towards the two reagents.

On the basis of these findings it has been generally accepted that a colour reaction with the α-naphthol/diacetyl reagent is characteristic of a guanidino derivative "possessing a free guanidino-NH₂ group" (Rosenberg, Ennor and Morrison, 1956) and is not given by compounds "with a substituent on an amidino nitrogen atom" (e.g., Thoai and Thiem, 1957). Thus, the fact that phosphagens do not give a colour reaction with α-naphthol/diacetyl has been interpreted to mean that these compounds are sym. substituted guanidines. However, generalisations regarding the specificity of both the Sakaguchi and α-naphthol/diacetyl reactions are based on the reactions of alkyl substituted guanidines. As far as can be ascertained from the literature, the only monosubstituted guanidine which has been tested, other than alkyl guanidines, is aminoguanidine which failed to give a reaction with α-naphthol/diacetyl reagent (Ennor and Stocken, 1953). It cannot be assumed that other monosubstituted guanidines, such as
### TABLE III.1

**Specificity of the Sakaguchi and α-naphthol/diacetyl reactions for alkyl substituted guanidines**

<table>
<thead>
<tr>
<th>Guanidine derivative ( R = \text{alkyl group} )</th>
<th>Sakaguchi reaction</th>
<th>α-naphthol/diacetyl reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{NH}_2-\text{C}(=\text{NH})\text{NH}_2 )</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>( \text{NH}_2-\text{C}(=\text{NH})\text{NHR} )</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>( \text{NH}-\text{C}(=\text{NH})\text{NR}_2 )</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>( \text{RNH}-\text{C}(=\text{NH})\text{NHR} )</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
phosphoryl-, formyl-, or acetyl-guanidine, would show the same specificity towards either of the reagents as do alkyl guanidines, and, in fact, it has now been found (Chapter III) that phosphorylguanidine does not give a colour reaction with the α-naphthol/diacetyl reagent. Therefore, in the case of phosphagens derived from monoalkylguanidines, other evidence is required to determine whether these compounds are sym. or asym. disubstituted guanidines. Such evidence is provided by the unambiguous synthesis of N-alkyl-N'-phosphorylguanidines (sym. substituted) which is described in this chapter.

EXPERIMENTAL

Paper chromatography and the location of guanidino, amino and phosphorus-containing compounds on paper chromatograms was carried out as described previously. Phosphorylguanidino compounds were detected on paper chromatograms with the Sakaguchi or α-naphthol/diacetyl spray reagents after the compounds had been hydrolysed
by exposure to an atmosphere of hydrochloric acid vapour for 3-4 hr. at room temperature. Use was made of the following solvent systems:

A. Pyridine-isoamyl alcohol-water (80:40:70);
B. Methyl ethyl ketone-methyl cellosolve-3N ammonia (2:7:3);
C. Ethanol-ammonium acetate buffer, pH 7.2 (70:30);
D. Methanol-aqueous ammonia (sp.gr. 0.91)-water (60:10:30);
E. Methyl ethyl ketone-methyl cellosolve-acetic acid-water (40:15:6:24);
F. Acetone-acetic acid-water (2:2:1);
G. Butanol-acetic acid-water (50:20:30);
H. Phenol saturated with water (4:1 w/v);
J. Propanol-ammonium acetate buffer, pH 4.1 (7:5).

Dowex-50 (H⁺) resin and Amberlite IRC-50 were as previously described. Elution of substances from Dowex-50 (H⁺) resin was followed by spotting each fraction on Whatman paper and spraying with the appropriate reagent (before and after hydrolysis in the case of α-naphthol/diacetyl reagent) and then examining selected fractions by paper chromatography.

Lombricine and "total lombricine" (lombricine + phosphoryl derivatives of lombricine, i.e., free + bound lombricine) were estimated in solution before and
after hydrolysis (0.1 N hydrochloric acid for 9 min. at 65°), respectively, by the α-naphthol/diacetyl method of Rosenberg, Ennor and Morrison (1956).

Samples were prepared for analysis by drying at 0.1-0.5 mm. over phosphorus pentoxide for 60 hr. at room temperature and were weighed under anhydrous conditions. Nitrogen and phosphorus were determined as previously described.

\( pK_a \) values and infrared spectra were obtained as previously stated.

Natural lombricine (D-lombricine) was supplied by Professor A.H. Ennor and Dr. H. Rosenberg of the Department of Biochemistry, John Curtin School of Medical Research, Canberra.

L-SEP was a synthetic sample as previously described.

O-Methyl-N-phosphorylisourea was prepared by Dr. D.I. Magrath by the reaction of O-methylisourea with dibenzyl phosphite in carbon tetrachloride, followed by catalytic hydrogenation (cf. Cramer and Vollmar, 1958b).

Phosphoryl chloride was a commercial sample (The British Drug Houses Ltd., Poole, England) and was distilled before use.

The reaction of DL-SEP with O-methyl-N-phosphorylisourea. In preliminary experiments the reaction of DL-SEP with two molecular proportions of O-methyl-N-
phosphorylisoura at pH 12 and pH 10 was followed by paper chromatography using solvent A. The disappearance of SEP was faster at pH 12 than at pH 10 but apparently slower than in the reaction of SEP with O-methylisourea. The main product formed at both pH values was a ninhydrin-positive, phosphorus-containing substance which reacted with α-naphthol/diacetyl spray reagent only after acid treatment. These properties were similar to those expected for compound I. A small amount of material with a lower R_f value than the main product in solvents B, C and D was detected in the reaction mixtures; this material was ninhydrin-negative, liberated orthophosphoric acid when treated with molybdate reagent, reacted with α-naphthol/diacetyl only after acid treatment, and was assumed to be the disubstituted derivative of SEP, 2-carboxy-2-(N'-phosphorylguanidino)ethyl 2-(N'-phosphorylguanidino)ethyl hydrogen phosphate. These two products were fractionated by elution with water from a column of Dowex-50 (H^+) resin equilibrated at 0-5°C, the latter being eluted first. The main product when hydrolysed liberated a guanidino derivative which chromatographed as lombricine in solvent systems B, C, E, F, G and H. The base was further characterised as lombricine by comparison of its behaviour with that of an authentic sample of lombricine on nitrous acid degradation (see
Experimental, Chapter II) as ascertained by paper chromatography in solvents C and E.

L-2-Amino-2-carboxyethyl 2-(N'-phosphorylguanidino)ethyl hydrogen phosphate. A mixture of L-SEP (0.399 g., 1.75 x 10^-3 mole) and O-methyl-N-phosphorylisourea (0.170 g.) was moistened with a few drops of water and dissolved by the addition of 5 N sodium hydroxide. The pH of the solution was adjusted to ca. 12 with 2 N sodium hydroxide, the final volume of solution being 4.4 ml. The reaction mixture was kept at room temperature. After 12 hr. and again after 54 hr. additional amounts of O-methyl-N-phosphorylisourea (0.170 g. and 0.080 g., respectively) dissolved in 5 N sodium hydroxide were added and the pH of the solution was readjusted to 12 after each addition. (A total of 2.7 x 10^-3 mole of the reagent was added). After 72 hr. the reaction mixture was cooled in an ice-bath, brought to pH 7 by the addition of Dowex-50 (H^+) resin and the resin then filtered off and washed with cold water. The combined filtrate and washings were applied immediately to a column (4.2 x 31.7 cm.) of Dowex-50 (H^+) resin which had been set up in a cold room (2°). When the column had been washed with 130 ml. of water the eluate became acidic and all subsequent fractions (5 ml.) were collected in sufficient conc. aqueous ammonia to render each fraction distinctly
alkaline. The elution pattern was determined in the usual manner and selected fractions examined by paper chromatography in solvent B. The first acidic fractions (fractions 27-42) contained orthophosphoric acid and a small amount of material with phosphoryl-guanidino groups. The material behaving as the disubstituted derivative of SEP was detected in fractions 48-60, while fractions 65-110 contained a single compound corresponding to the main product in the preliminary experiments.

Fractions 65-110 were combined and freeze-dried, and the amorphous residue thus obtained dried at 0.1 mm. over potassium hydroxide (0.530 mg., 79% yield, assuming the material was in the form of a di-ammonium salt). The amorphous material was crystallised from aqueous methanol as a microcrystalline powder of which 0.120 g. was collected and dried as a sample for analysis.

Analysis: Calcd. for 1 part of the mono-ammonium salt, $\text{C}_6\text{H}_{15}0_9\text{N}_4\text{P}_2\cdot\text{NH}_4\cdot\frac{1}{2}\text{CH}_3\text{OH}$ and 4 parts of the di-ammonium salt, $\text{C}_6\text{H}_{14}0_9\text{N}_4\text{P}_2(\text{NH}_4)_2\cdot\frac{1}{2}\text{CH}_3\text{OH}$: C, 19.67; H, 5.94; N, 20.47; P, 15.61. Found: C, 19.55; H, 5.96; N, 20.10; P, 15.54.

Potentiometric titration indicated that the material was a mixture of the mono- and di-ammonium salts in the ratio of 1:4, and $pK_a$ values of ca. 2, 4.6 and ca. 8.8.
were obtained.

The $pK_a$ values of the guanidino group and the primary hydroxyl of the phosphoric acid residues were not determined.

The main infrared absorption peaks were at 777, 969, 1056, 1084, 1220, 1309, 1349, 1413, 1458, 1628, 3020 and 3180 cm$^{-1}$ (Figure III.1).

The product did not react with Sakaguchi or 6-naphthol/diacetyl reagents but did react with ninhydrin and with molybdate reagent. On paper chromatograms in solvents A, B, C and D it was homogeneous when tested with the latter two reagents. Hydrolysis in 0.1 N hydrochloric acid for 1 min. at 100$^\circ$, or for 9 min. at 65$^\circ$, caused complete conversion to a guanidino derivative with liberation of orthophosphoric acid.

**Phosphorylation of lombricine with phosphoryl chloride.**

**Preliminary experiments:**

(1) Natural lombricine (0.27 g., 0.001 mole) was phosphorylated with phosphoryl chloride (0.004 mole) in alkaline solution using the conditions and procedure described by Morrison, Ennor and Griffiths (1958) for the phosphorylation of taurocyamine. The reaction mixture was filtered and the filtrate treated with Amberlite IRC-50 (H$^+$) resin until the pH of the solution had fallen to 7.4. Paper chromatographic examination of
the resin-treated solution showed the presence of a
ninhydrin-positive, phosphorus-containing material
which did not react with the α-naphthol/diacetyl reagent
unless the paper chromatograms had been exposed to an
HCl atmosphere. In the two solvents, A and J, the
material ran in a discrete crescent-shaped area behind
other α-naphthol/diacetyl-positive material.

(ii) Using the conditions and procedure outlined
for the preparation and isolation of phosphocreatine by
Pradel, Thiem, Pin and Thoai, (1959), an attempt was
made to isolate the barium salt of a phosphorylated
derivative of lombricine, starting with 2.7 g. of
natural lombricine. After the removal of inorganic salts
by precipitation with 50% ethanol, barium bromide
solution was added to the filtrate and the precipitate
thus formed extracted with water. The solution was
treated with 60% ethanol and the precipitate collected.
The procedure was unsatisfactory as estimation of
lombricine in the precipitate before and after mild
hydrolysis (0.1 N hydrochloric acid at 65° for 9 min.)
showed that only 20% of the "total lombricine" was
acid-labile.

(iii) A solution of lombricine (0.297 g., 1.1 x 10⁻³
mole) in 3 ml. of water and 0.6 ml. of sodium hydroxide
(10 N) was cooled in an ice-bath and stirred as vigorously
as possible throughout the following procedure. Sodium hydroxide solution (10 N, 0.72 ml.) was added, then after 5 min. a small volume of water (1 ml.) and freshly distilled phosphoryl chloride (ca. 0.12 ml.), each addition being made dropwise. After an interval of 10 min. these additions were repeated until five portions of phosphoryl chloride (6.8 x $10^{-3}$ mole) had been added. The pH of the solution was checked after the addition of each portion of phosphoryl chloride and was ca. 14. The reaction mixture was stirred for 30 min., then filtered. The precipitate was washed with two small portions of ice-cold water and the washings combined with the filtrate (ca. 15 ml.). Estimation of the α-naphthol/ diacetyl-reacting material in this filtrate before and after mild acid hydrolysis indicated that 23% of the lombricine was present as a phosphorylated derivative. The presence of a phosphorylated derivative was confirmed by paper chromatography as in (i). Ethanol fractionation of the product and lombricine was not successful.

**D-2-Amino-2-carboxyethyl 2-(N'-phosphorylguanidino)ethyl hydrogen phosphate.** D-Lombricine (4.05 g., 1.5 x $10^{-2}$ mole) was added to a three-necked flask (250 ml.) containing 47 ml. of water and 8.8 ml. of 10 N sodium hydroxide. The flask was immersed in an ice-bath and during the following additions (made dropwise) stirring was maintained as
vigorously as possible in order to effect rapid cooling of the contents of the flask and efficient mixing of the added reagents in the slurry that was produced. A check was made to ensure that the reaction mixture was always strongly alkaline (ca. pH 14). In order, 10.8 ml. of 10 N sodium hydroxide, 15.4 ml. of water and, after 5 min. equilibration, 1.75 ml. of phosphoryl chloride were added. These additions were repeated after an interval of 10 min. until five portions of phosphoryl chloride (9.1 x 10^{-2} mole) had reacted. The reaction mixture was then cooled to -5° and maintained at this temperature for 30 min. The precipitated salt was removed by filtration and the solid residue washed with two portions (10 ml.) of ice-cold water. The precipitate, containing ca. 30 mg. total lombricine of which only 5% was bound lombricine, was discarded. The washings were combined with the filtrate and kept cold. Chromatographic examination of the solution in solvents A and C showed the presence of a ninhydrin-positive, phosphorus-containing material which reacted with α-naphthol/diacetyl reagent only after the paper chromatogram had been exposed to an HCl atmosphere. Analysis indicated that ca. 17% of the total lombricine present in the solution was liberated by acid hydrolysis (0.1 N hydrochloric acid at 65° for 9 min.).

The cold solution (230 ml.) was adjusted to pH 7.6
with cold conc. hydrochloric acid (ca. 4.5 m.), added slowly with stirring. The addition of 60 ml. of ethanol produced a crystalline precipitate which was removed by filtration and discarded, since it contained a negligible amount of total lombricine. A second precipitate was formed after the addition of 100 ml. of ethanol and was removed by centrifugation and washed with 30% ethanol. This precipitate contained less than 6 mg. of bound lombricine and was discarded. The corresponding supernatant (364 ml.) was analysed and found to contain ca. 516 mg. of bound lombricine. On the addition of a further 50 ml. of ethanol to the supernatant an oil was deposited. This was allowed to settle for 1 hr. at 5°, and then centrifuged. The supernatant liquid was removed by decantation and the oil dissolved in 25 ml. of water. Analysis showed that the oil contained 279 mg. of bound lombricine and 787 mg. of lombricine, while the supernatant liquid* contained ca. 275 mg. of bound lombricine and 3.18 g. of lombricine.

*This liquid was discarded accidentally. In a subsequent phosphorylation of lombricine (2.7 g.) in which 21.4% of the lombricine was phosphorylated during the reaction, it was found that two volumes, rather than one volume, of ethanol were required to remove all the bound lombricine from the supernatant. The oil thus obtained was applied to a column (4.2 x 31.7 cm.) of Dowex-50 (H⁺) resin (as above). The yield of the amorphous ammonium salt (as above) was 14%. 
The aqueous solution of the oil was applied to a column (5.2 x 25 cm.) of Dowex-50 (H⁺) resin which had been equilibrated at 0-5°. The column was eluted with water at the same temperature and 5 ml. fractions were collected. When the pH of the eluate indicated that acidic material was being eluted from the column, subsequent fractions were collected in sufficient conc. aqueous ammonia to make the solution in each tube distinctly alkaline. Fractions 24-50 gave tests for chloride and orthophosphoric acid and fractions 34-36 contained a small amount of a bound guanidino derivative. Fractions 52-93, which were free of chloride and orthophosphoric acid and contained a single ninhydrin- and molybdate-positive compound which reacted with α-naphthol/diacetyl reagent only after mild acid hydrolysis, were combined and the solution freeze-dried. After drying in a desiccator over potassium hydroxide at 0.1 mm., the amorphous material weighed 0.410 g. (7.1% yield) and was obtained as a microcrystalline powder from aqueous methanol. It had Rf values in a number of solvent systems identical with those of the compound derived from the reaction of L-SEP with α-methyl-N-phosphorylisourea.

Titration and elementary analytical data were in agreement with this material being a mixture of the mono- and di-ammonium salts of D-2-amino-2-carboxyethyl
2-(N'-phosphorylguanidino)ethyl hydrogen phosphate.

Analysis: Calcd. for 1 part of the mono-ammonium salt, \( C_{6}H_{15}O^{9}N_{4}P_{2} \cdot (NH_{4}) \cdot CH_{3}OH \) and 3.35 parts of the di-ammonium salt, \( C_{6}H_{14}O^{9}N_{4}P_{2} \cdot (NH_{4})_{2} \cdot CH_{3}OH \): C, 20.39; H, 6.19; N, 19.60; P, 15.02. Found: C, 20.19; H, 6.15; N, 19.70; P, 14.76.

\( pK_{a} \) values ca. 2, 4.6 and ca. 8.8.

The infrared spectrum was identical with that of L-2-amino-2-carboxyethyl 2-(N'-phosphorylguanidino)ethyl hydrogen phosphate (Figure III.1).


Ennor, A.H. and Rosenberg, H. personal communication.


p. 1127.
chem. Soc. **76**, 2871.
**225**, 409.


218.


Paris, 145, 1674.
Acta, 14, 76.
Acta, 25, 446.
Thoai, N.V. and Robin, Y. (1960). In Biochimie
Comparee des Acides AminesBasiques, p. 353. Ed. du
Centre National de la Recherche Scientifique, 15.
Qua: Anatole, France, Paris, VII.
Acta, 52, 221.
Biochim. biophys. Acta, 14, 448.
Thoai, N.V., Roche, J., Robin, Y. and Thiem, N.V.
1389.
Publishers Inc.


