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THE METABOLISM OF LOMBRICINE

A THESIS

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by

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This thesis embodies the results of research carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, from February, 1959 to February, 1962, during the tenure of a Australian National University Research Scholarship.

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. H. Rosenberg, to the Registrar of the University:

".....Some of the work was carried out in collaboration with Professor R.J.Rossiter, Professor A.H.Ennor and myself, and the candidate's contribution may be described in the following terms:

The earlier part of the work, namely that dealt with in Chapter I and part of that dealt with in Chapter II, was planned in collaboration with the workers mentioned above, and carried out in close collaboration with Professor Rossiter. The candidate's contribution to that portion of the work was that of a junior worker.

In the work described in Appendix I the candidate's contribution was that of a co-worker. The work described in Chapters III and IV, and that described in Appendix II, is entirely the candidate's own contribution"

Candidate's signature:



ACKNOWLEDGEMENTS

It is a pleasure to express my sincere thanks to my supervisor, Dr. H. Rosenberg, for his helpful advice and guidance throughout this work. I would also like to thank Professor A. H. Ennor for his many useful suggestions and encouragement, and Professor R. J. Rossiter for generously devoting much of his time in discussion and practical assistance during the earlier phases of these studies.

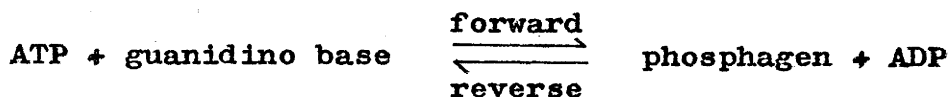
The skilful technical assistance of Messrs. B. Thorpe, P. Allen and M. De Smet is gratefully acknowledged. My thanks are also due to Mr. R. Adams for carrying out the electrophoretic analyses, as well as for ready cooperation in many other ways. Mr. V. Paral and Mr. R. Westen of the photographic department have been more than helpful at all times.

I am also grateful to my many Departmental colleagues who have generously given of their time and specialised knowledge, and in particular to Mr. W. O'Sullivan for his unselfish assistance during the kinetic investigations. My especial thanks are also due to Miss B. Nightingale for her cheerful assistance with the typing of draft copies, and to Mrs. P. Lanigan and Mrs. W. Schenk for the final typing of this thesis.

PREFACE

It has been suggested (Ennor and Morrison, 1958) 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". Throughout this thesis the term phosphagen will be used in accordance with the above definition.

The Report of the Commission on Enzymes of the International Union of Biochemistry (I.U.B. Symposium, 1961) recommended that the phosphotransferases with a nitrogenous group as acceptor (2.7.3) be referred to as ATP:guanidinoacetate phosphotransferase (2.7.3.1), ATP:creatine phosphotransferase (2.7.3.2), etc. for systematic nomenclature. Accordingly the term "forward reaction" will be applied to phosphoryl group transfer from ATP to the guanidino base, and the term "reverse reaction" to phosphoryl group transfer from the phosphagen to ADP, as shown by the reaction:



Temperatures are expressed in °C.

Figures and tables are presented on separate pages, a particular figure or table following immediately the page on which first reference to it has been made. Analytical methods and experimental procedures are described in detail in Methods, and will not be quoted in figures or tables.

In addition to standard abbreviations adopted by the Biochemical Journal the following will be used:

AGA	Acetyl glutamic acid
cpm	Counts per min.
HMB	<u>p</u> -hydroxymercuribenzoate
NEM	N-ethyl morpholine
PA	phosphoarginine
PC	phosphocreatine
PG	phosphoguanidinoacetate
PL	phospholombricine
PT	phosphotaurocyamine
P ₁	inorganic orthophosphate
PP ₁	inorganic pyrophosphate
SEP	Serine ethanolamine phosphodiester
TCA	Trichloroacetic acid

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GENERAL INTRODUCTION

A. THE BIOSYNTHESIS OF PHOSPHAGENS

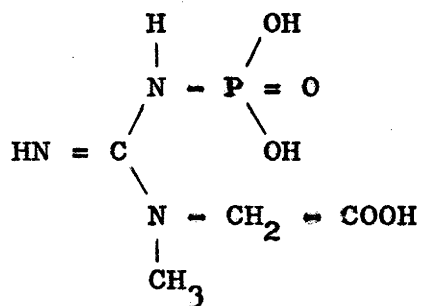
B. THE DISCOVERY OF LOMBRICINE AND
SERINE ETHANOLAMINE PHOSPHODIESTER
IN THE EARTHWORM

GENERAL INTRODUCTION

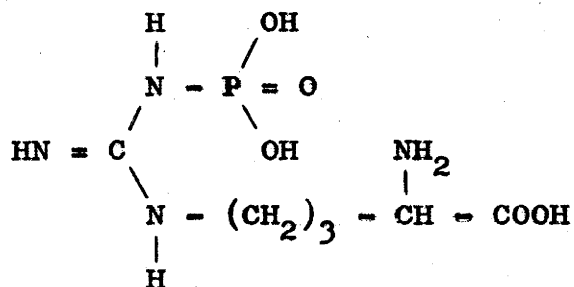
A. THE BIOSYNTHESIS OF PHOSPHAGENS

Until recently the only recognised phosphagens were phosphocreatine (PC,i) and phosphoarginine (PA,ii) which had been isolated by Fiske and Subbarow (1929) and Meyerhof and Lohmann (1928) respectively. Indirect evidence for the presence of other phosphagens in the Phylum Annelida, however, had been adduced by Arnold and Luck (1933), Kurtz and Luck (1937-38) and Baldwin and Yudkin (1950), but these were not identified. During the last decade, French workers in Roche's laboratory have utilised paper and ion exchange chromatographic techniques to isolate these Annelid phosphagens and identify them as phosphoguanidinoacetate (PG,iii) in Nereis diversicolor, phosphotaurocyamine (PT,iv) in Arenicola marina (Thoai, Roche, Robin and Thiem, 1953a), phospholombricine (PL,v) in Lumbricus terrestris (Thoai and Robin, 1954; Ennor and Rosenberg, 1962) as well as the guanidino base hirudonine (vi) in the leech, Hirudo medicinalis (Roche, Thoai, Robin and Pradel, 1956; Robin and Thoai, 1961).

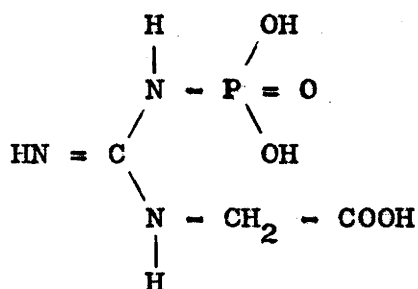
The guanidino base of PL, the phosphagen of the earthworm, was shown to be a mixed phosphodiester of 2-guanidinoethanol and serine (Thoai and Robin, 1954).



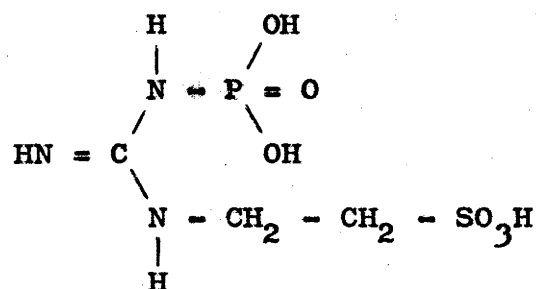
(i) phosphocreatine



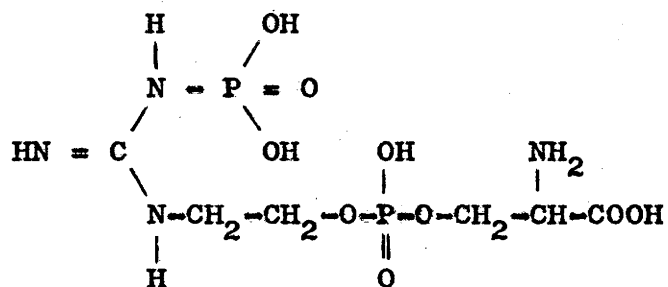
(ii) phosphoarginine



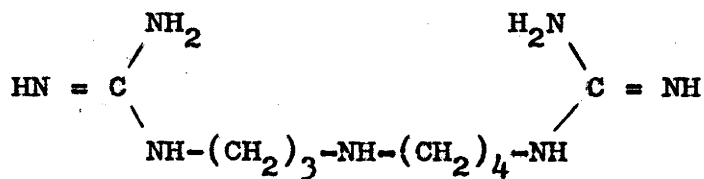
(iii) phosphoguanidinoacetate



(iv) phosphotaurocyamine



(v) phospholombricine



(vi) hirudonine

These findings provided the starting point for the studies presented in this thesis, which deals with the metabolism of lombricine with regard to its biosynthesis and enzymic phosphorylation. Before dealing with lombricine in particular it is proposed to discuss the biosynthesis of phosphagens in general. The biochemistry of phosphagens which has been fully covered in recent reviews (Ennor and Morrison, 1958; Morrison and Ennor, 1960) will not be dealt with in detail in this dissertation.

At the present time the complete biosynthetic pathways for only two phosphagens, PA and PC, are fully known, those of the more recently discovered phosphagens having not as yet been studied in great detail. This is due in part to physical difficulties, which are encountered in working with the small invertebrates in which PG, PT, PL and hirudonine occur; and also because the precise chemical structures of some of the parent bases e.g. D-lombricine and hirudonine, have been determined only recently (Beatty et al, 1959; Robin and Thoai, 1961). Nevertheless the extensive investigations concerning PA and PC biosynthesis have revealed a basic pattern for the formation of phosphagens which will be considered in some detail. Preliminary studies with respect to PG, PT and PL biosynthesis will also be

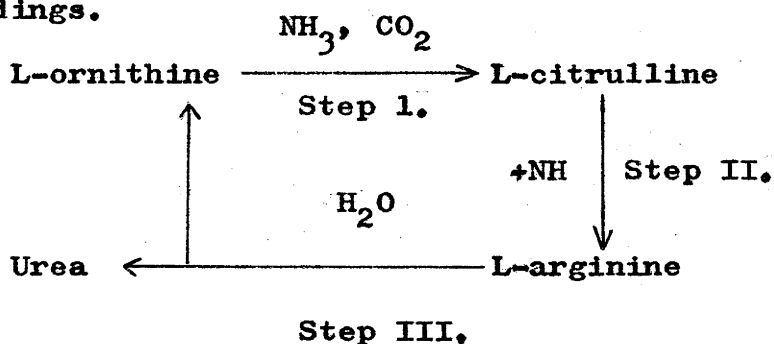
discussed to assess their conformity with, or divergence from, this pattern.

1. The biosynthesis of the guanidino base.

The guanidino base may be considered as consisting of two main moieties - the amidino group common to all phosphagens, and the side chain, the structure of which differs in various species. The biosynthesis of the two portions will be discussed separately below.

(a) The formation of the amidino group.

It is recognised that the primary biological source of the amidino group is L-arginine (Ratner, 1954). Knowledge of the overall reactions involved in the synthesis of L-arginine stemmed from the original observations of Krebs and Henseleit (1932) who demonstrated that L-ornithine and L-citrulline caused a marked enhancement of urea production from CO_2 and NH_3 in respiring rat liver slices. As the arginase activity in mammalian liver was known to be high, they proposed their now classic "ornithine-urea" cycle to account for their findings.



In the decade that followed isotopically labelled compounds were administered in the diet to various animals and the subsequent metabolic fate of these compounds determined. The results obtained from these studies were consistent with the operation of the "ornithine-urea" cycle in intact animals (see Schoenheimer, 1942). The conversion of L-citrulline to L-arginine (Step II) was investigated in kidney slices (Borsook and Dubnoff, 1941b), respiring liver homogenates (Cohen and Hayano, 1946) and in soluble extracts of acetone-dried beef liver (Ratner, 1947). It was concluded from these experiments that the immediate nitrogen donor in Step II was not NH_3 or L-glutamate as originally supposed, but L-aspartic acid, for although NH_3 and L-glutamate were active nitrogen donors in intact liver or in respiring liver homogenates, only L-aspartate met this requirement in soluble liver extracts.

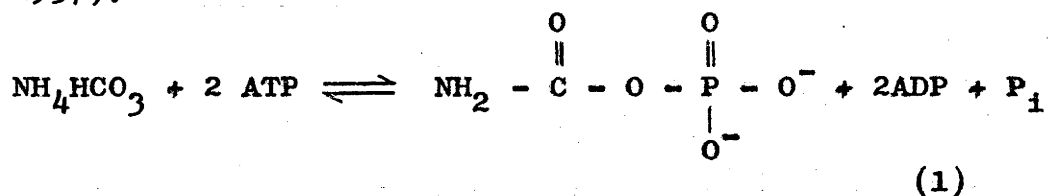
Similar investigations of Step I demonstrated that the conversion of NH_3 , CO_2 and L-ornithine to L-citrulline by soluble nonrespiring rat liver preparations (Grisolia and Cohen, 1951) required ATP, Mg^{2+} , and catalytic amounts of N-acylglutamic acid. It was apparent therefore that the synthesis of L-arginine was a complex multistep process.

In the last decade the individual enzymes catalysing each step have been isolated and purified, and their properties investigated in many laboratories. Before dealing with each of these reactions more fully it should be emphasised that the enzymes catalysing many of these steps are widely distributed in nature. Thus they have been demonstrated in mammalian liver and kidney and in yeast (Ratner, Anslow, and Petrack, 1953) ; in pea seeds and heart (Davison and Elliott, 1952); in Chlorella (Walker, 1952); in mammalian brain, testes, thymus, spleen, pancreas, heart, and skeletal muscle (Walker, 1958). Their presence has been inferred in Penicillium (Bonner, 1946); in E. coli (Abelson, Bolton and Aldous, 1952); in Tetrahymena gelii (Wu and Hogg, 1952); in Lactic acid bacteria (Volcani and Snell, 1948) and in Neurospora (Srb and Horowitz, 1944) from studies of the effects of various intermediates of the cycle on the growth of all these organisms. Thus these reactions serve not merely as a means of nitrogenous excretion, as has been stressed in the past, but are also vitally important in providing L-arginine for protein synthesis and as a source of the amidino group.

It is now recognised that the synthesis of L-arginine involves the following sequential steps:

The formation of carbamoyl phosphate from CO_2 , NH_3 and ATP.

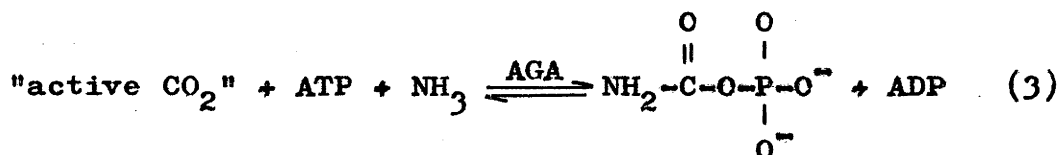
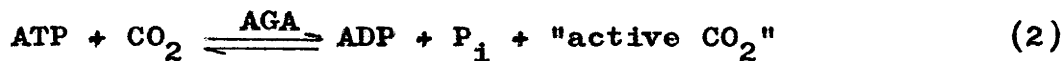
In the livers of mammals, amphibians, chelonian reptiles and certain primitive fish e.g. sharks and lungfish (Brown and Cohen, 1960), and in mammalian intestinal mucosa (Hall, Johnson and Cohen, 1960) carbamoyl phosphate is formed from NH_3 , CO_2 and ATP, the stoichiometry of the reaction being expressed by the equation: (Metzenberg, Hall, Marshall and Cohen, 1957).



This reaction, catalysed by carbamoyl phosphate synthetase, was initially described in soluble rat liver systems (Grisolia and Cohen, 1952) and later the enzyme was purified from frog liver (Marshall, Metzenberg, and Cohen, 1958). The reaction is only partially reversible (Metzenberg, Marshall, and Cohen, 1958; Jones, 1959) and has an absolute requirement for catalytic amounts of N-acetylglutamic acid (AGA) (Grisolia and Cohen, 1953) a naturally occurring cofactor which has been isolated from liver and yeast (Hall, Metzenberg and Cohen, 1956). Isotope studies with deuterium-labelled AGA (Grisolia, Burris and Cohen, 1954),

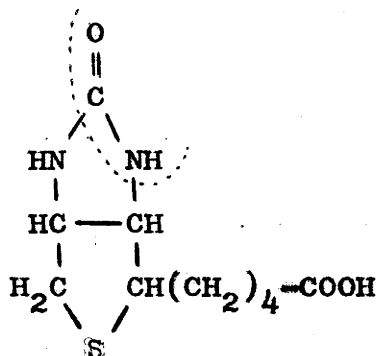
AGA labelled with ^{18}O in the carboxy oxygens (Reichard, 1958) and in the acetyl oxygen (Jones and Spector, 1960) have shown that neither the carbon-bonded hydrogens nor the labelled oxygens were displaced during reaction (1).

Although carbamoyl phosphate synthetase has been purified to homogeneity (Marshall *et al.*, 1958; Marshall and Cohen, 1961), the reaction which it catalyses can be subdivided on the evidence of tracer studies. Reichard (1958) and Metznerberg, Marshall, Cohen and Miller (1959) demonstrated that one atom of H_2^{18}O appeared in the orthophosphate and one in the carbamoyl phosphate in reaction (1) when the experiment was conducted at pH 8.0 in H_2^{18}O . As two ATP-molecules were used in reaction (1) the isotope labelling was interpreted as being consistent with the following mechanism:

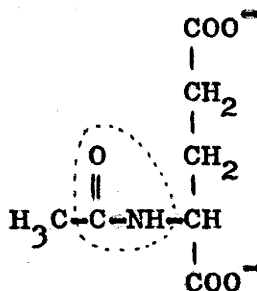
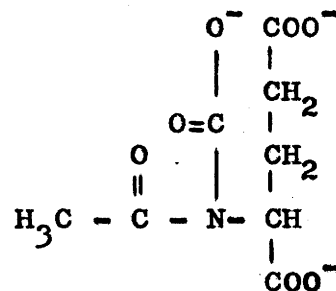


However Jones and Spector (1960), by conducting the same type of experiment under conditions in which the equilibration between H_2O and HCO_3^- was controlled, were

able to demonstrate that the direct donor of ^{18}O was HC^{18}O_3 , and that H_2^{18}O was only an indirect participant by virtue of its rapid equilibration with bicarbonate. Jones and Spector (1960) have proposed a mechanism similar to reaction (2) and (3), but, in addition, postulated the formation of an active carbonic acid derivative of AGA, similar to the biotin- CO_2 complex isolated during studies of methylcrotonoyl-CoA carboxylase (Lynen, Knappe, Lorch, Jütting and Ringelmann, 1959). Biotin and AGA both have a secondary amide group (outlined below) and Jones (1959) proposed the following formulation for the carboxy-AGA derivative.



Biotin

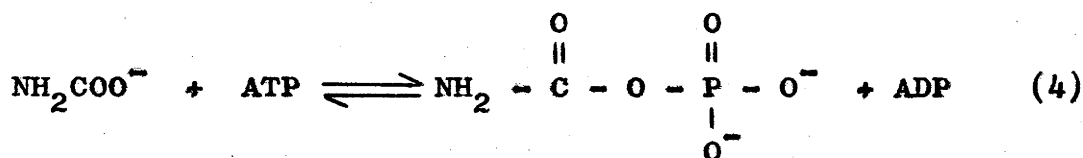
N-Acetylglutamic
Acid

Carboxy-AGA

Grassl and Bach (1960) synthesised various analogues of AGA and demonstrated that the reactivity of AGA, as judged by its catalytic efficiency in reaction (1), resided in its substituted amino group, a finding which lends support to the AGA- CO_2 complex proposed by Jones and Spector (1960).

Feldott and Lardy (1951) reported that liver homogenates from biotin-deficient rats formed citrulline from ornithine at diminished rates, and similar studies with biotin-deficient microorganisms (Estes, Ravel and Shive, 1956; Ravel, Grona, Humphreys and Shive, 1958) indicated that the carbamylation of ornithine and aspartate was markedly reduced, and suggested that biotin was necessary for the synthesis of carbamyl-transferase enzymes. However, recent studies (Grillo and Mistry, 1960) have shown that the carbamylation of aspartate in biotin-deficient rats is not reduced, which suggests that the observed results with biotin-deficient microorganisms may be due to indirect effects (Ravel and Shive, 1960; Mistry and Grillo, 1960).

In contrast with carbamoyl phosphate synthesis in higher animals, as described above, the corresponding reaction in microorganisms is endergonic, reversible, and involves the direct phosphorylation of carbamate by ATP (Jones and Lipmann, 1960). The stoichiometry of the reaction is expressed by the equation:



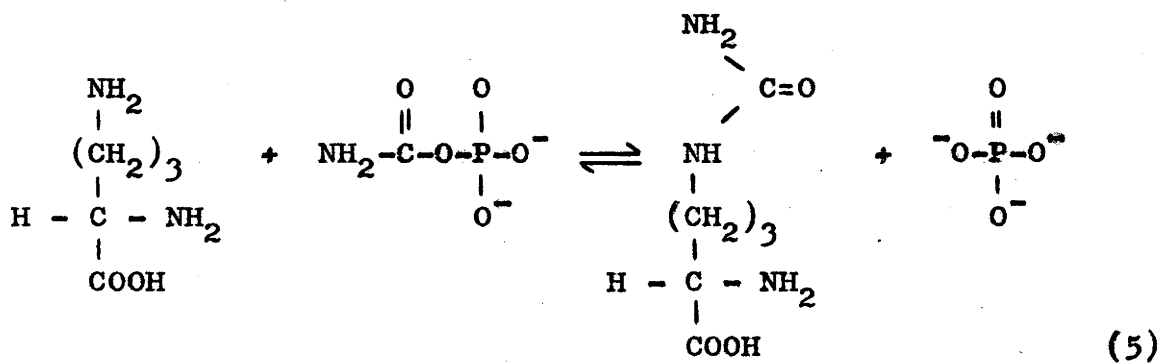
Carbamate kinase which catalyses reaction (4) has been purified (Jones, Spector and Lipmann, 1955; Glasziou, 1956; Jones and Lipmann, 1960; Mokrasch, Caravaca and

Grisolia, 1959; Caravaca and Grisolia, 1960) and shown to require no cofactors.

The intermediate carbamoyl donor, formerly known as "compound x" (Grisolia and Cohen, 1952) which had been isolated previously from mammalian liver incubation systems (Grisolia, Wallach and Grady, 1955; Marshall, Hall and Cohen, 1955) was shown to be identical with carbamoyl phosphate (Hall and Cohen, 1957) which had been synthesised by Jones et al. (1955) and Jones and Lipmann (1960). Although the quantity of carbamoyl phosphate formed by microbial systems has been too small to isolate, Caravaca and Grisolia (1960) have established its identity more firmly by coupling the exergonic reaction (1) to the endergonic reaction (4), thereby demonstrating that an intermediate, namely carbamoyl phosphate, was common to both reactions.

The formation of citrulline from ornithine and carbamoyl phosphate

It is now well established that the final step in the synthesis of citrulline from L-ornithine and carbamoyl phosphate may be represented by the equation:

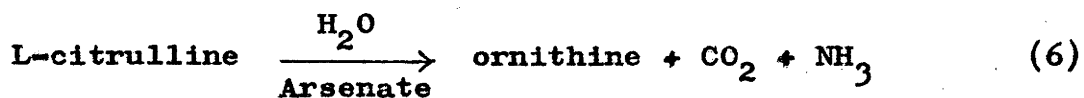


L-ornithine + carbamoyl
phosphate

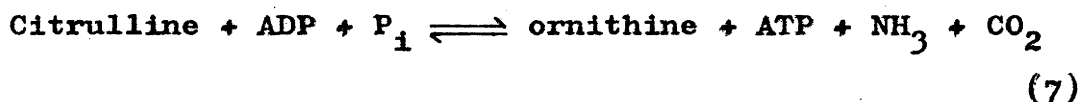
L-citrulline + orthophosphate

The equilibrium of this reaction, catalysed by ornithine carbamoyltransferase strongly favours the synthesis of citrulline, and L-ornithine is the specific acceptor of the carbamoyl group (Reichard, 1957). Ornithine carbamoyltransferase has been purified from mammalian and amphibian liver (Grisolia and Cohen, 1952; Reichard, 1957; Burnett and Cohen, 1957; Brown and Cohen, 1959), from microorganisms (Krebs, Eggleston and Knivett, 1955; Glasziou, 1956; Grisolia, Wallach and Grady, 1955) and was also shown to be present in small quantities in the mammalian gastrointestinal tract and in bile (Reichard, 1959). No cofactor requirement was demonstrated.

This enzyme is also responsible for the observed arsenolysis and phosphorolysis of citrulline by bacterial extracts (Oginsky and Gehrig, 1953; Knivett, 1954) and liver preparations (Krebs *et al.*, 1955), as shown by the equation:



It has been demonstrated also that the anaerobic degradation of citrulline by bacterial extracts could be linked to the esterification of phosphate in the presence of ADP (Slade, 1953; Slade, Doughty and Slamp, 1954; Knivett, 1954; Korzenovsky and Werkman, 1954).

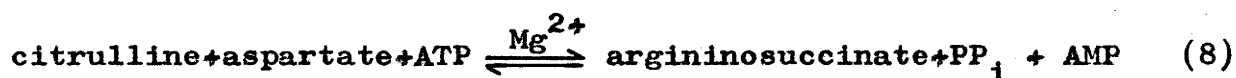


It is now recognised that reactions (6) and (7) represent the reverse of reaction (5) with either the decomposition of the carbamoyl phosphate so formed, or the transfer of its phosphoryl moiety to ADP (Krebs *et.al.* 1955; Smith and Reichard, 1956; Reichard and Reichard, 1958).

The synthesis of L-arginine from L-citrulline and L-aspartic acid

The synthesis of L-arginine from L-citrulline and L-aspartate was shown to be catalysed by liver (Ratner and Pappas, 1949) and kidney extracts (Ratner and Petrack, 1953) in a two-step reaction requiring ATP and Mg^{2+} , with the formation of an intermediate compound. Partial purification of this system into "condensing" and "splitting" enzymes allowed this intermediate to be

isolated (Ratner and Petrack, 1951) and identified as argininosuccinate (Ratner, Petrack and Rochovansky, 1953).



Argininosuccinate synthetase which catalyses reaction (8) was purified from beef liver and kidney (Petrack and Ratner, 1958) until free of adenylate kinase and inorganic pyrophosphatase, and, in the absence of these interfering enzymes it was possible to demonstrate that the stoichiometry of the reaction was as expressed above.

Ratner and Petrack (1953) originally considered argininosuccinate synthesis to be catalysed by two enzymes, one of which they isolated from liver and the other from yeast. However it became evident that the yeast enzyme was inorganic pyrophosphatase which greatly facilitated the rate of argininosuccinate formation by removal of PP_i . It was shown that PP_i inhibited the forward reaction both directly, as well as by a mass-action effect (Petrack and Ratner, 1958).

Studies with L-citrulline labelled exclusively in the ureido-oxygen with ^{18}O have conclusively demonstrated that L-citrulline is the amino acid which is activated by ATP, with splitting out of AMP and PP_i .

However, unlike other mechanisms of this type, $^{32}\text{PP}_i$ -ATP and $[8-^{14}\text{C}]\text{AMP}$ -ATP exchange did not occur unless all three substrates of argininosuccinate synthetase were present (Rochovansky and Ratner, 1961). These workers proposed that either an adenylo-citrulline complex and PP_i were formed and that neither of these compounds dissociated from the enzyme; or that a concerted action mechanism occurred in which the CN bond of argininosuccinate was formed as the PP bond of ATP was broken. However, as the authors point out, it is premature to draw a final conclusion about the mechanism involved on the basis of the evidence available.

Argininosuccinate is cleaved to L-arginine and fumarate (Reaction 9) by argininosuccinate lyase. This enzyme has been purified from ox liver until free of arginase and fumarate hydratase (Ratner, Anslow and Petrack, 1953). The possibility that the above reaction was reversible was suggested by the work of Davison and Elliott (1952) who demonstrated that pea seed extracts catalysed the formation of a ninhydrin-reacting compound, later identified as argininosuccinate, from L-arginine and fumarate. It also provided an explanation of the earlier reports (Ratner and Pappas, 1949) that L-arginine and L-malate were the products of reaction (9) since fumarate hydratase present in crude preparations converted fumarate to L-malate (Ratner et al., 1953).



In the reverse direction of reaction (9) no other compound could replace fumarate, but Walker (1953) showed that L-canavanine could substitute for L-arginine with the formation of L-canavaninosuccinate.

Until recently the liver was the only organ known to possess the full complement of urea cycle enzymes. This has been investigated more thoroughly following the discovery of a new inborn error of metabolism (Allan, Cusworth, Dent and Wilson, 1958) in which gross mental retardation was associated with the excretion of large amounts of argininosuccinate in the urine (Westall, 1960). As the concentration of argininosuccinate was higher in the cerebrospinal fluid than in the plasma, it was suggested that another probable site of synthesis was the brain (Allan et al., 1958; Dent, 1959), where argininosuccinate accumulated due to lack of argininosuccinate lyase (Dent, 1959; Ratner, Morell and Carvahlo, 1959). With the use of more sensitive techniques it has now been demonstrated by in vivo (Sporn, Dingman, De Falco and Davies, 1959) as well as by in vitro studies (Ratner et al., 1959; Tomlinson and Westall, 1960) that mammalian brain synthesises urea.

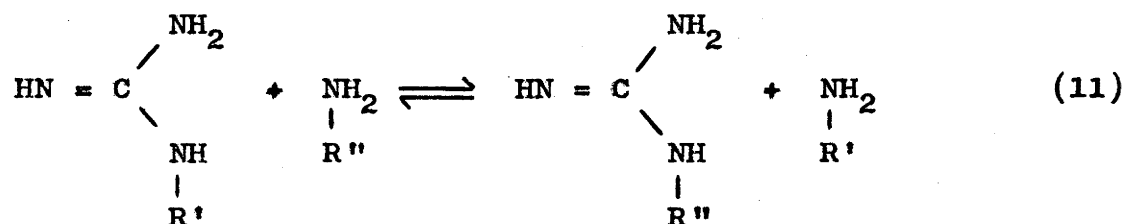
(b) The formation of the side chain.

In the synthesis of creatine, glycine is the acceptor of the amidino group from L-arginine to form guanidino acetate (Borsook and Dubnoff, 1941a; Bloch and Schoenheimer, 1941). The latter compound is subsequently methylated by S-adenosylmethionine to form creatine (Borsook and Dubnoff, 1940; Cantoni and Vignos, 1954) in a reaction catalysed by methionine adenosyltransferase. Whether glycine taurine and SEP are acceptor compounds for the formation of the annelid phosphagen bases, guanidinoacetate, taurocyamine and lombricine is at present in dispute, while an amidino group acceptor for the recently identified hirudonine has not been proffered so far. In animal tissues glycine arises predominantly from serine (Shemin, 1946). In animals, higher plants and microbial systems two-carbon compounds are converted to glycine via glyoxylic acid (Weinhouse and Friedmann, 1951; Tolbert and Cohan, 1953; Campbell, 1956) but in animals this represents only a minor pathway. The origin of taurine, which occurs in the free state in invertebrates, is well known. It is derived from L-cysteine, either via L-cysteine sulphinic acid and hypotaurine or via L-cysteine sulphinic acid and L-cysteic acid, the former pathway being of the more significance (Awapara and Wingo, 1953)

The formation of SEP will be discussed in Chapter I.

(c) Amidino group transfer to the side chain.

Enzymes which catalyse the transfer of an amidino group from a donor to an acceptor molecule are termed transamidinases. The reactions catalysed by these enzymes may be represented by the equation:



in which R' and R'' represent the remainder of the donor or acceptor molecules.

In vertebrates the formation of guanidinoacetate by transamidination on the pathway of creatine biosynthesis is well established, and the same mechanism suggests itself as the means for the formation of guanidinoacetate and the other phosphagen bases, taurocyamine and D-lombricine in invertebrates by the interaction of L-arginine with glycine, taurine, or D-SEP respectively (Ennor and Morrison, 1958). However, attempts to demonstrate this reaction conclusively with glycine or taurine as amidino-group acceptors have been uniformly negative (Robin, 1954).

(1) Historical

Present knowledge of transamidination reactions stemmed largely from the study of creatine formation,

complemented by the discovery that transamidinations played a major role in the formation of other biologically important guanidino compounds, such as L-canavanine and γ -guanidinobutyrate.

Many years prior to the demonstration of transamidination reactions in biological systems, Bergmann and Zervas (1927) had shown that triacetyl-anhydroarginine under anhydrous conditions easily yielded a diacetyl-amidino group to the ethyl ester of glycine or sarcosine to form guanidinoacetate and creatine respectively. They suggested that biological amidino transfer was implicated in the formation of creatine in vivo. Support for this contention was provided by Shapiro and Zwarenstein (1932) who demonstrated an increased output of urinary creatinine or creatine after the injection of arginine or guanidinoacetate to rabbits. Similarly Takahashi (1933) showed that injection of arginine increased the creatinine level in fertile eggs. These findings lent support to the original suggestion of Czenecki (1905) that arginine was a precursor of creatine. These balance studies however did not constitute conclusive evidence, although the demonstration of increased urinary creatine after glycine or guanidinoacetate ingestion in patients with muscular dystrophy (Brand, Harris and Ringer, 1929) and

of the conversion of L-arginine to creatine in perfused rat hearts (Fischer and Wilhelmi, 1937) gave much stronger indications that L-arginine and glycine were creatine precursors.

Final proof was provided by Bloch and Schoenheimer (1940) who demonstrated that the amidino-group of creatine was derived from the amidino-group of L-arginine and that glycine was the source of the remaining portion of the molecule. These conclusions were based on the incorporation of L-[amidino- ^{15}N]-arginine and [^{15}N]-glycine into carcass creatine of rats. With the finding that mammalian liver slices could methylate guanidinoacetate to form creatine, the final step in creatine biosynthesis had been established (Borsook and Dubnoff, 1940). As methionine was the sole amino acid to enhance this methylation process, it was considered to be the methyl group donor. This was confirmed by the isolation of labelled creatine from animals after the administration of methionine labelled specifically by deuterium in the methyl group (Du Vigneaud, Chandler, Cohn and Brown, 1940).

Borsook and Dubnoff (1941a) then demonstrated that rat kidney slices and cell-free extracts catalysed the transfer of the amidino-group of L-arginine to glycine with the formation of guanidinoacetate. The

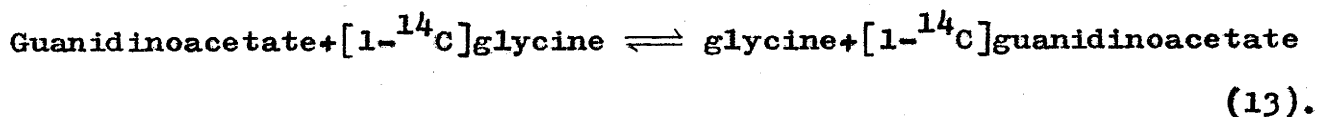
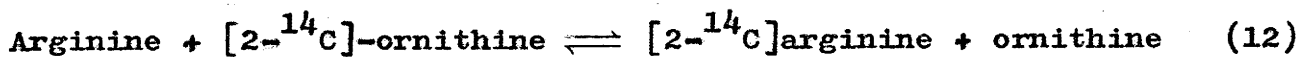
transfer was insensitive to cyanide or anaerobiosis. This new biochemical reaction was called transamidination, and the enzyme that catalysed it "glycine transamidinase".

Inhibition of glycine transamidinase by ornithine and heavy metals was reported for the first time in 1953 (Sorm, Sebesta and Tursky). In the following year Fuld (1954) showed that transamidination was a reversible reaction. This worker employed manometric techniques with specific bacterial decarboxylases to identify reactants and products. The reversibility of transamidination in vivo was demonstrated by the isolation of [amidino-¹⁴C]-arginine after the administration of [amidino-¹⁴C]-guanidinoacetate to rats (Horner, Siegel and Bruton, 1956).

(ii) Distribution and specificity of transamidinase

Transamidinase is confined largely to the kidney and pancreas in mammals, although weak activity has also been detected in thymus and testes. In birds transamidinase activity resides chiefly in the liver, but very low levels were reported in heart, skeletal muscle and kidney as well. Recently transamidinase activity has been demonstrated in Streptomyces griseus and in amphibian liver (Borsook and Dubnoff, 1941; Walker and Walker, 1959^{a, b}).

Arginine, canavanine, guanidinoacetate and γ -guanidinobutyrate were found to be amidino-group donors, whereas ornithine, canaline, glycine, β -alanine, γ -aminobutyrate and hydroxylamine were acceptors (Borsook and Dubnoff, 1941a; Fuld, 1954, 1956; Ratner and Rochovansky, 1956a; Walker, 1957, 1958b; Pisano, Mitoma and Udenfriend, 1957). Some transfers which were not accompanied by any net change in either of the reactants were explored with [^{14}C]-labelled acceptors, e.g.



It was concluded from these investigations that any donor could transfer to any acceptor, but the rates at which amidino-group transfer occurred differed markedly between various donor-acceptor pairs e.g. arginine-glycine transfer proved ten times faster than guanidinoacetate-glycine transfer, but was not as rapid as canavanine-glycine transfer (Ratner and Rochovansky, 1956b).

Fuld (1956) reported that lysine was an acceptor with the formation of homoarginine, but Walker (1957) was unable to confirm this. Ratner and Rochovansky (1956a) observed that the formation of lysine with homoarginine as donor proceeded at a rate which was 1% of that of arginine

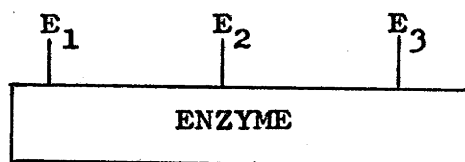
as donor. Thus some of the discrepancies reported with different donor-acceptor pairs are probably explained by the fact that some of these interactions proceed at rates which are difficult to detect. No transamidination was detected with creatine, guanidine or agmatine as donors, or sarcosine, taurine, alanine, β -alanine, 2:4-diaminobutyrate, lysine, serine, phosphoethanolamine, NH_3 , aspartate, glutamate or histidine as acceptors (Walker, 1957).

Hydroxylamine in high concentration accepted the amidino-group to form hydroxyguanidine. This reaction has been successfully used by Walker (1958b) to detect "arginine-x transamidinases" where the physiological acceptor was unknown e.g. in the transamidination reaction catalysed by S. griseus. It was shown that this mould catalysed arginine-ornithine, canavanine-ornithine and arginine-hydroxylamine transfers, but was unable to utilise glycine as an acceptor.

(iii) Postulated mechanisms of amidino-group transfer

Ratner and Rochovansky (1956a) purified hog-kidney transamidinase, and showed that the equilibrium constant of the reaction was close to unity, which indicated that the high energy of the amidino-group was retained on transfer. Kinetic data and inhibition studies of the arginine-glycine transamidinase reaction

were consistent with the transfer of the amidino-group by a single displacement mechanism. These workers visualised donor and acceptor molecules as being simultaneously present on the enzyme surface at separate sites, represented schematically by E_1 , E_2 , E_3 .

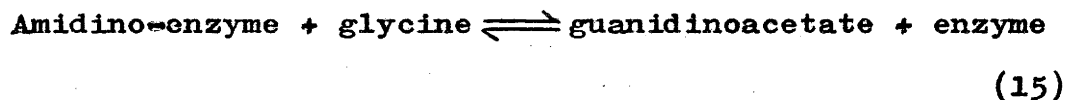
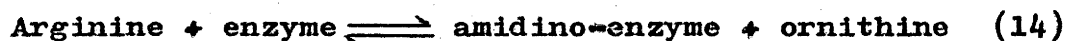


In this scheme acceptors occupy E_1 or E_3 and donors E_1 - E_2 or E_3 - E_2 , E_2 being the site of amidino-group attachment. Thus transfer occurred by a single substitution reaction, with the bond attaching the amidino-group to the donor site breaking as a new bond was formed at the acceptor site.

Mutual inhibitions between all donors, all acceptors, and donor-acceptor pairs were found, and these proved to be of the competitive type. Affinity constant determinations for donors and acceptors showed that ornithine displayed a high affinity for two sites (E_1 and E_3), a finding which was consistent with its proven marked inhibitory effect on all transamidination reactions. No competition was evident between guanidinoacetate and glycine. The importance of this observation lay in the fact that with arginine occupying

either of two sites (E_1-E_2 or E_3-E_2) only one site (E_1 or E_3) was left for guanidinoacetate and glycine. If effective competition existed between these, then two amidino-group donors would simultaneously have to occupy the catalytic area (E_2). This would not be compatible with the concept that only one amidino site was present on the enzyme surface.

There exists another possibility, strongly favoured by Walker (1956) that amidino-group transfer is a two stage process, with the formation of an amidino-enzyme complex.



This concept was consistent with the observed arginine-ornithine and canavanine-ornithine exchange reactions. These exchanges were likewise catalysed by S. griseus extracts, and since ornithine was structurally quite different from glycine or any of the likely acceptors which serve as precursors of streptomycin, Walker (1958b) considered that these results were more compatible with a two-step mechanism.

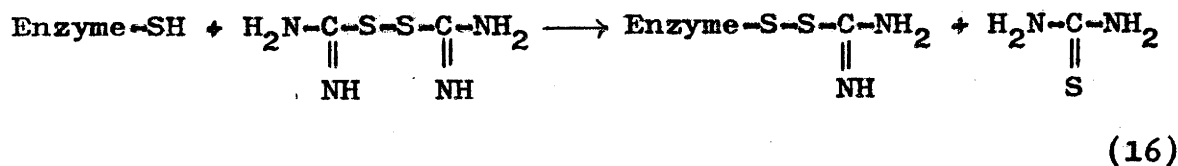
As the amidino-group was also transferred to hydroxylamine with the formation of hydroxyguanidine,

Walker (1958b) interpreted this as indicating that hydroxylamine trapped the postulated enzyme-amidino intermediate.

In an attempt to exclude a two stage transfer mechanism Ratner and Rochovansky (1956b) incubated [L-amidino-¹⁴C]-arginine with the purified hog-kidney transamidinase. After multiple precipitations the residual counts found in the enzyme were insignificant, and it was therefore assumed that either an enzyme intermediate was not formed, or if it were formed, that it was highly labile.

Stimulation of transamidinase activity by chelating agents and cysteine, with inhibition by heavy metals and HMB suggested that a free sulphydryl group was essential for enzymic activity (Walker, 1957; Van Pilsum, Berman and Wolin, 1957). It was suggested that the role of the -SH groups in transamidinase reactions was to link the enzyme to the amidino group, forming an enzyme-amidino complex which could be regarded as a macromolecular S-substituted isothiourea, analogous to S-methyl-isothiourea used in the chemical synthesis of guanidino derivatives (Walker, 1957). It was later found that sulphydryl agents containing an amidino group, which presumably attached the sulphydryl agent to the active centre of the enzyme, were powerful inhibitors.

Walker (1957) postulated that disulphide bridges were formed between an -SH group at the active site and the inhibitory sulphhydryl agents, as illustrated by the potent transamidinase inhibitor formamidino disulphide (Walker and Walker, 1960).



Transamidinase + formamidino disulphide → inactive enzyme + thiourea

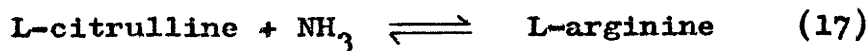
Diminished levels of transamidinase activity have been reported in Vitamin E deficiency and thyrotoxicosis (Fitch, Hsu and Dinning, 1960, 1961) and after the addition of creatine to the diet of mammals and birds (Walker, 1960; Fitch et al., 1960). The one factor common to all these conditions was a raised level of creatine, unlike the transamidinase depression observed in mercury poisoning and starvation (Van Pilsum, Berman and Wolin, 1957) in which toxic factors and protein depletion were implicated.

Other related enzymes such as arginase or S-adenosylmethylferase were not affected by creatine administration, and creatinine caused no fall in transamidinase levels. It was concluded therefore that this was an example of end-product depression of enzyme formation (Walker, 1960; Fitch et al., 1960). In addition to this, Walker (1960) demonstrated a hormonal control, for in prepubertal rats the

levels of kidney transamidinase in males and females was equal whereas after puberty transamidinase levels in the males were double those in the females.

Alternative pathways of amidino group formation

The formation of the amidino group via the synthesis of L-arginine as described in the preceding sections has been substantiated at each step by direct experimental evidence. Other pathways have however been postulated, but these have not as yet met with general acceptance. The formation of L-arginine from L-citrulline and NH_3 by extracts of river crab muscle was reported by Szorenyi, Elodi and Deutsch (1954).



Subsequently from the river and lake crabs (Potambius astacus and leptodactylus) Szorenyi (1955) crystallised citrulliniminase, the enzyme which catalysed the above reaction. This enzyme had a maximal activity at pH 9.1, was activated by pyridoxal phosphate and was inhibited by L-aspartate. It was noted that this was the mechanism of L-arginine synthesis in ammoniotelic species.

Petrack, Sullivan and Ratner (1957) purified arginine deiminase from extracts of Streptococcus faecalis, and showed that it catalysed the degradation of L-arginine to L-citrulline and NH_3 i.e. the reverse

of that catalysed by citrulliniminase. Since arginine deiminase was not activated by metals or pyridoxal phosphate and no reverse reaction was demonstrable, it appeared that this enzyme was not identical with citrulliniminase.

On thermodynamic grounds Petrack et al., (1957) considered it difficult to conceive reaction (17) proceeding in the absence of an energy donor. In support of this contention the thermodynamic aspects of the synthesis of L-arginine from L-citrulline and L-aspartate have been investigated by Schuegraf, Warner and Ratner (1960). It was found that the standard free energy change for this reaction at 37° at pH 7.5 was +8.2 kilocalories. However the splitting of argininosuccinate to L-arginine and fumarate is known to be largely endergonic, having a standard free energy value of -2.8 kilocalories (Ratner and Rochovansky, 1956,a). Furthermore the hydrolysis of arginine to NH₃ and L-citrulline involved a standard free energy change of -8.6 kilocalories (Schuegraf et al., 1960). Thus the thermodynamics of reaction (17) appear unfavourable to the synthesis of L-arginine from citrulline and NH₃ without the participation of an energy donor, and as a consequence the findings of Szorenyi et al. (1954) must be treated with reservation until confirmed.

It has been suggested (Thoai, 1960) that creatine may be formed, not only by the well known pathways, but also by another pathway involving sarcosine (N-methyl-glycine) or carbamoylsarcosine. This speculation was based on the finding that the production of creatine in perfused rabbit liver was stimulated by the addition of sarcosine or carbamoylsarcosine to the perfusate. However it is known that sarcosine leads to creatine formation by a process involving demethylation to glycine, followed by creatine formation by the usual pathway (Bloch and Schoenheimer, 1941). Thoai (1960) considered that the stimulation observed with carbamoylsarcosine was too marked and rapid to follow a pathway similar to that of sarcosine. In vitro experiments failed to confirm the above findings. It would seem presumptive therefore to lay too much stress upon them at this stage.

Thoai (1960) also proposed that taurocyamine was formed by reactions analogous to those involved in the biosynthesis of arginine. Supporting evidence for this was the demonstration of carbamoyltaurine and taurocyamine in mammalian urine after the ingestion of taurine (Thoai, Roche and Olumucki, 1948) and the demonstration of ureido compounds in extracts of Arenicola marina, in which taurocyamine occurs naturally.

The inability to demonstrate any transamidination of taurine to taurocyamine, either by extracts of A. marina or by purified rat kidney transamininase (Robin, 1954) was taken as further support for this view. Thoai (1960) concluded that taurine was not the precursor of taurocyamine and stated that the precursor was in the process of isolation and identification. The claim of Abbott and Awapara (1960) in connection with the transamidination of [³⁵S]taurine by arginine in vivo in A. cristata does not stand critical analysis, since calculation of the net counting rate which would have been observed in order to obtain the reported incorporation of [³⁵S] yields a figure of 1 cpm.

2. Phosphorylation of the guanidino base to form the phosphagen.

The final step in phosphagen biosynthesis involves the phosphorylation of the guanidino base by ATP, in a reversible reaction catalysed by the specific guanidino kinase corresponding to the guanidino base, e.g.

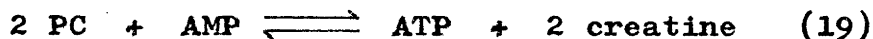


It is outside the scope of this section to detail the complete literature concerning the guanidino kinases which has accumulated over the last thirty years.

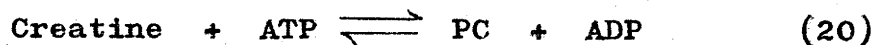
Furthermore, as the general properties of guanidino

kinases are very similar in many respects, the description of lombricine kinase presented in Chapters 3 and 4 will serve to illustrate their properties. This section is limited, therefore, to a brief historical account of the guanidino kinases studied so far.

The enzymic hydrolysis of PC by muscle extracts was shown by Meyerhof and Suranyi (1927). Meyerhof and Lohmann (1928) demonstrated that this activity was lost when extracts were kept for several hours, but it was later found (Lohmann, 1934) that it was restored on the addition of adenine nucleotides. It was concluded, therefore, that PC was hydrolysed indirectly by participation of the adenylic acid system, as shown:



Lehmann (1935) demonstrated that this reaction was reversible and that Mg^{2+} ions were necessary for activity. Banga (1943) partially purified extracts of rabbit muscle and demonstrated the presence of an enzyme which catalysed the reaction between creatine and ATP



In addition this worker claimed that a second enzyme was present which catalysed the reaction between creatine and ADP



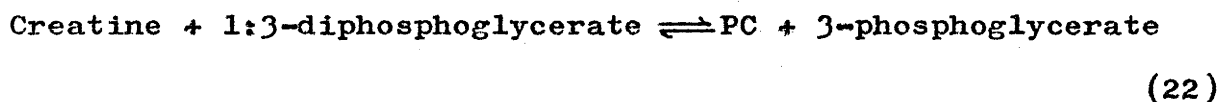
However, it has now been demonstrated that this reaction (21) was due to the integrated action of creatine kinase and myokinase (Ennor and Rosenberg, 1954a; Chappell and Perry, 1954). Askonas (1951) and Ennor and Rosenberg (1954a) further purified creatine kinase, and the latter authors conclusively demonstrated that AMP was not implicated in the creatine kinase reaction.

Subsequently, crystalline creatine kinase was prepared by ethanol fractionation of extracts of rabbit skeletal muscle, in which advantage was taken of the extraordinary stability of creatine kinase to ethanol, even at room temperature, and of the sharp separation effected by ethanol in the presence of certain cations such as Mg^{2+} , Mn^{2+} and Zn^{2+} . The enzyme obtained by this procedure was homogenous to numerous physico-chemical criteria and detailed kinetic and equilibrium studies were presented (Kuby, Noda and Lardy, 1954, a,b; Noda, Kuby and Lardy, 1954, a,b). These authors investigated the relationship between total Mg^{2+} ion, total nucleotide and enzymic activity, and concluded that the true substrate for the reaction was the Mg^{2+} -nucleotide complex.

The interaction between creatine kinase and the substrates Mg^{2+} and ATP has also been studied by

equilibrium binding techniques (Kuby and Mahowald, 1958) as a preliminary to elucidation of their mechanism of action. Similarly, as a prelude to sequence studies of creatine kinase, variations in its physico-chemical properties with pH have been investigated (Da Costa and Friedberg, 1960). More recently, Morrison, O'Sullivan and Ogston (1961) have re-investigated the role of the metal ion, Mg^{2+} , in the activation of creatine kinase, taking into consideration all the complexes which would be involved. This aspect is treated fully in Chapter 4.

There have been several claims that creatine could be phosphorylated by phosphoryl group donors without the participation of the adenylate system. Thus, Cori, Abarca, Frenkel and Traverso-Cori (1956) postulated that extracts of rabbit skeletal muscle catalysed the following reaction



Later, Cori, Traverso-Cori, Lagarrigue and Marcus (1958) indicated that the above reaction was catalysed by a single enzyme. Recently, Morrison and Doherty (1961) investigated this reaction and demonstrated that more than one enzyme was involved and that the actual phosphorylation of creatine was catalysed by creatine

kinase. The ATP required for this was shown to arise, by a series of reactions, from the pyridine nucleotides which Cori et al., (1958) included as an essential component of this reaction system. It was shown that NAD was converted non-enzymically to ADP-ribose (or this compound was present as a contaminant) and subsequently ADP-ribose was enzymically converted to AMP. It was demonstrated that charcoal treated skeletal-muscle preparations converted AMP to ATP in the presence of PC or phosphoenolpyruvate. In addition, ATP was also formed when AMP and PC were incubated in the presence of creatine kinase and myokinase (see reaction 19 above).

It has also been claimed (Bresler, Rubina and Vinokurov, 1957) that creatine kinase catalysed the transfer of phosphoryl groups from phosphorylated-RNA to creatine. However, this work could not be confirmed (Rosenberg, personal communication). It would appear, therefore, that the above reactions do not represent exceptions to the rule that the guanidino bases are phosphorylated only by ATP.

Arginine kinase activity was demonstrated in crude extracts of muscle of the crab, octopus, and

echinoid (Lohmann, 1935, 1936; Baldwin and Needham, 1937). Later the enzyme was crystallised (Szorenyi, Dvornikova and Degtyar, 1949) from aqueous extracts of freshwater crab muscle, but few properties of the enzyme were described. Elödi and Szorenyi (1956) subsequently published a modified procedure for obtaining crystalline arginine kinase, which was homogenous as judged by ultracentrifugal and electrophoretic studies. Although Morrison, Griffiths and Ennor (1957) were unable to obtain crystalline enzyme by the method of Szorenyi et al., (1949), they obtained a highly purified preparation and studied the kinetics of the forward and reverse reactions in relation to the concentration of free Mg^{2+} ion. It was concluded that the role of the metal ion was to form a metal enzyme complex, and that free nucleotide was the true substrate for the reaction. Thus this concept of the function of the metal ion was opposite to that proposed by Kuby et al., (1954b) for the reaction catalysed by creatine kinase. This conflict of opinion has stimulated a reappraisal of the role of the metal ion in guanidino kinase reactions (Morrison et al. 1961; Noda, Nihei and Morales, 1960; Nihei, Noda and Morales, 1961). This work will be discussed in detail in Chapter 4.

Taurocyamine kinase was identified in extracts of the annelid, Arenicola marina, (Hobson, 1955) and partially purified from the muscle extracts of this animal by Thoai (1957) by ethanol fractionation. From extracts of the whole animal, Arenicola assimilis, Griffiths (1958) obtained a partially purified, relatively stable preparation. The general properties and kinetic behaviour shown by taurocyamine kinase were similar to those of other guanidino kinases.

Guanidinoacetate kinase was prepared by ethanol fractionation of extracts of Nereis diversicolor by Thoai (1957), using the whole animal as starting material. However, the difficulties encountered during purification, and the very low stability of the preparations, have precluded extensive investigation of this enzyme.

B. THE DISCOVERY OF LOMBRICINE AND SERINE
ETHANOLAMINE PHOSPHODIESTER IN THE EARTHWORM

1. The isolation and characterisation of lombricine

(a) Historical

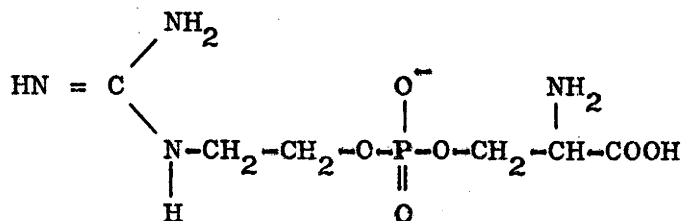
On the grounds that the L-arginine content of the muscle of the earthworm, Lumbricus terrestris was low and that no PA was detectable, Arnold and Luck (1933) predicted that "probably new phosphagens of annelids and closely related phyla may exist". Kutscher and Ackermann (1931) and Baldwin and Yudkin (1950) had also noted the paucity of L-arginine and the absence of PC or PA in Lumbricus sp.

Thoai, Roche, Robin and Thiem (1953b) extracted a hitherto unknown phosphagen from earthworms, and demonstrated by paper chromatography that its parent base, lombricine, released by acid hydrolysis, was different from any of the other known phosphagen bases.

In the following year Thoai and Robin (1954) isolated lombricine from earthworm extracts by a procedure which involved the separation of the basic compounds, lombricine and L-arginine, from other acidic and neutral components by chromatography on cation exchange resin. Further purification was effected by subsequent passages through anion exchange resins of varying anionic strength,

which permitted separation of L-arginine from lombricine. The latter compound was crystallised from aqueous ethanol in low yield (60 mg./kg.). Using the same procedure Pant (1959) obtained a ten-fold improvement in yield; more recently, however, simplified methods have become available whereby lombricine may be recovered quantitatively (1200 mg./kg.) from earthworms. (Rosenberg and Ennor, 1959; Ennor et al., 1960; see also Appendix 1).

From a knowledge of the elementary analysis, the nature of the functional groups and paper chromatographic identification of the acid hydrolysis products as serine, 2-guanidinoethanol and 2-guanidinoethylphosphate, Thoai and Robin (1954) proposed the following structure.



Lombricine

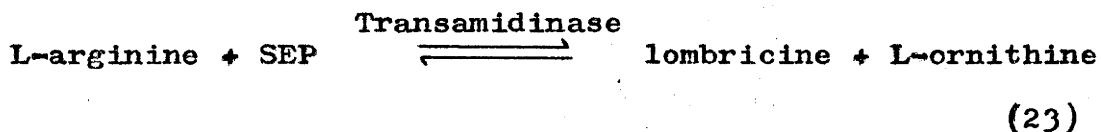
The configuration of the serine moiety of lombricine was not determined by these workers. This aspect of the structure of lombricine was investigated when, as a result of the isolation procedures referred to above (Rosenberg and Ennor, 1959; Ennor et al., 1960) pure crystalline lombricine became more readily available.

Natural lombricine was degraded by acid hydrolysis, and serine was isolated from the hydrolysis products by ion exchange and paper chromatography. The crystalline serine thus obtained was shown unequivocally to be identical with authentic D-serine. The evidence included a comparison of the physical properties such as melting point, specific optical rotation, dinitrophenyl derivatives and infrared spectra of the isolated serine with authentic D-serine. In addition a sample of the isolated serine was shown to be oxidised by sheep kidney D-amino acid oxidase with the utilization of the required amount of oxygen. (Beatty et al., 1959; Beatty et al., 1961). Confirmation of the structure allocated above was provided by Beatty and Magrath (1959, 1960) who synthesised L-, DL-, and D-lombricine by the selective guanylation with O-methylisourea of the terminal amino group of L-, DL-, and D-SEP, prepared by unequivocal synthetic routes. The D-isomer was shown to be identical with natural lombricine, as judged by an investigation of its physical properties similar to that applied to its serine component.

(b) The Biosynthesis and degradation of D-lombricine

The biosynthesis of D-lombricine has as yet received little experimental attention. Ennor and Morrison (1958) suggested that lombricine may be formed in earthworms in a manner analogous to L-arginine, or

alternatively by a transamidination reaction involving L-arginine and SEP, according to reaction (23):



Support for the latter pathway was provided by the identification of SEP in earthworms (Rosenberg and Ennor, 1959) and its subsequent isolation and complete characterisation as the D-isomer (Ennor *et al.*, 1960). Thoai (1960) stated that lombricine was not formed according to reaction (23) on the grounds that SEP was not present in earthworms, and that in turtles, in which L-SEP was abundant, no lombricine was found. However D-SEP has now been positively demonstrated in earthworms (see above). The fact that its optical configuration is different from the SEP found in reptiles makes it unlikely that the latter compound would have a similar function to D-SEP. These findings invalidate the argument of Thoai (1960) and indicate the danger of postulating alternate pathways on the strength of negative evidence alone.

Robin (1954) demonstrated that natural lombricine was partially degraded by acid hydrolysis to serine, 2-guanidinoethanol, and 2-guanidinoethylphosphate, when heated at 110° in a sealed tube with 6N-sulphuric acid for 8 hr. By paper chromatographic techniques it was shown that serine was the first residue liberated, while

the phosphoric acid moiety remained attached to the 2-guanidinoethanol group. With more vigorous hydrolysis (15 hr. at 115°) the degradation products were 2-guanidinoethanol, serine and phosphoric acid.

Confirmation of these findings was provided by Beatty and Magrath (1960), who extended these observations to synthetic L, D, and DL-lombricine and showed that synthetic and natural lombricine yielded identical hydrolysis products. In addition these latter authors demonstrated the presence of another degradation product in trace amounts, which by paper chromatography was tentatively identified as alanine. Conclusive evidence of the identity of the acid hydrolysis products was provided by the isolation of two of the products, serine and 2-guanidinoethylphosphate from a large scale acid degradation of natural lombricine (Beatty et al., 1961). Both serine and 2-guanidinoethylphosphate were purified from the reaction products, crystallised, and shown to be identical with authentic D-serine and 2-guanidinoethyl phosphate respectively.

A metal-catalysed degradation of lombricine in alkaline solution was reported by Beatty and Magrath, (1959, 1960). These authors found that although lombricine was stable for at least 90 hr. at pH 10 at 23° it was rapidly degraded under these conditions if

copper or cobalt were present. Thus within 42 hr. a copper complex of lombricine was completely degraded to 2-guanidinoethylphosphate, without the concomitant appearance of either 2-guanidinoethanol and phosphoric acid, or of serine, which was presumably destroyed.

Robin (1954) reported the biological degradation of lombricine by earthworm tissues. Thus in the presence of insoluble particles of whole worm homogenates at pH 8.0 or 6.5, lombricine was completely degraded to 2-guanidinoethanol and serine within 3 to 6 hr. respectively at 37°, as judged by paper chromatography of the reaction products. However no conclusive evidence was adduced to show that this degradation was, in fact, an enzyme catalysed reaction, nor were any precautions reported to be taken to exclude the possibility of bacterial degradation of lombricine.

A non-enzymic metal-catalysed degradation of lombricine, as discussed earlier, could not account for these findings as the products of the reactions are quite different. A study of the metabolism of guanidinoacetate and taurocyamine (guanidinoethylsulphonic acid) has revealed that these compounds are not catabolised by tissues in which they occur naturally as phosphagen bases (Robin, 1954). However since these compounds have a simple carbon side chain they may not

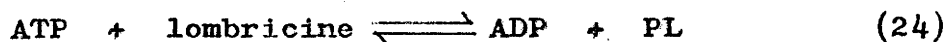
be as susceptible to cleavage as lombricine, which has a phosphodiester linkage.

It would appear therefore that a definitive answer to the question of lombricine biodegradation must await further experimental investigation.

(c) The distribution and function of D-lombricine

Apart from an unconfirmed report (Robin, Thoai, Pradel and Roche, 1956) that lombricine was detected on paper chromatograms of extracts of Audouinia tentaculata eggs in association with taurocyamine, arginine and guanidinoacetate, D-lombricine appears to be confined to oligochaetes (Robin, 1954). Morgan and Beatty (1960), who investigated numerous animal groups, detected no lombricine in species other than earthworms.

The primary function of lombricine is to serve as a parent base for the phosphagen, PL. Evidence for this role was provided by the isolation of PL from earthworms (Thoai et al., 1953b; Thoai and Robin, 1954; Ennor and Rosenberg, 1962). Final proof was furnished with the demonstration of lombricine kinase activity in homogenates and acetone powder extracts of Lumbricus terrestris (Pant, 1959) and partially purified extracts of Megascolides cameroni muscle (Rosenberg, Rossiter, Gaffney and Ennor, 1960). In this reaction ATP serves as a phosphoryl group donor:



In view of the restricted distribution of D-lombricine in nature and of the presence of a D-serine moiety in its structure, it is tempting to speculate that D-lombricine may have some evolutionary significance. However as pointed out (Ennor and Morrison, 1958) a critical assessment of the relationship between the phylogenetic classification of animals and the distribution of the guanidino bases revealed very little correlation between the two. If therefore D-lombricine has some evolutionary significance its nature at the present time is obscure.

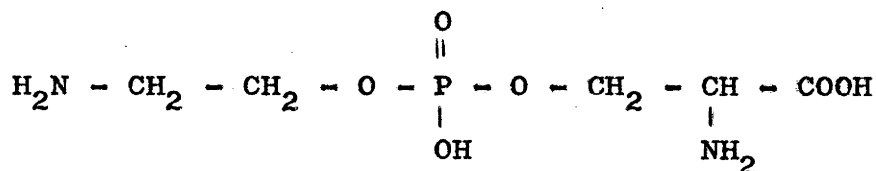
2. The isolation and characterisation of serine ethanolamine phosphodiester.

(a) Historical

Both isomers of SEP have recently been described in Nature. Roberts and Lowe (1954) reported the presence of large amounts of a new ninhydrin-reacting material on paper chromatograms of alcoholic extracts of river turtles and alligators. In the young river turtle (Pseudemys elegans) this material was found mainly in heart and muscle, from which it was isolated by preparative paper chromatographic techniques. On mild acid hydrolysis this purified material yielded serine, phosphoserine, ethanolamine and phosphoethanolamine; on mild alkaline

hydrolysis only phosphoethanolamine was formed. These degradation products were identified by paper chromatography and electrophoresis.

Enzymic degradation by the concerted action of Crotalus adamanteus phosphodiesterase and potato alkaline phosphatase yielded serine, ethanolamine, and orthophosphate in the molar ratio 1:1:1. As the serine moiety was not destroyed by sheep kidney D-amino acid oxidase, it was assumed to be the L-isomer. Roberts and Lowe (1954) concluded that the new compound was a phosphodiester of L-serine and ethanolamine and suggested the following formula:



L-SEP has subsequently been identified in snakes and alligators (Ayengar and Roberts, 1957) birds, fish, amphibia and reptiles (Morgan and Beatty, 1960; Rosenberg and Ennor, 1961b).

Rosenberg and Ennor (1959) isolated a substance from earthworm extract which was identified as SEP on the basis of its identity in chromatographic behaviour, acid hydrolysis products and guanylation products with synthetic SEP. Later Ennor et al., (1960) reported the isolation of 300 mg. of crystalline SEP from 9.6 kg. of

earthworms. Chemical and physical analyses and comparison with synthetic D-SEP confirmed the identity of the compound isolated from earthworms as SEP, and showed its serine moiety to possess the D-configuration.

(b) The synthesis and degradation of serine ethanolamine phosphodiester.

The chemical synthesis of L-, DL-, and D-SEP by techniques which lead to unequivocal structures for the products was reported by Jones and Lipkin (1956) and Beatty and Magrath (1959, 1960). However, studies in connection with the biosynthesis of SEP are at the present time only in the preliminary stages, and will be discussed in Chapter 1.

The chemical degradation of SEP is similar to that already discussed in relation to the metal-catalysed destruction of lombricine. At room temperature a copper complex of SEP in alkaline solution is rapidly degraded to phosphoethanolamine, without the concomitant appearance of serine or phosphoric acid. Attempts by Rosenberg and Ennor (1961a) to demonstrate an enzymic degradation of D-SEP by extracts of earthworm tissues, under the experimental conditions used to study the enzymic degradation of lombricine (see above) were unsuccessful. However L-SEP is known to be attacked by a phosphodiesterase present in snake venom, with the

formation of serine and phosphoethanolamine (Roberts and Lowe, 1954).

(c) The distribution and function of serine ethanolamine phosphodiester.

The D-isomer of SEP occurs only in earthworms, whereas the L-isomer has a wider distribution, being found in birds, fish, reptiles and amphibians (Rosenberg and Ennor, 1961b). One interesting feature is that the groups of animals in which L-SEP is present are closely related on the evolutionary tree.

It is considered that the function of the D-isomer is to act as a precursor of D-lombricine (Ennor et al., 1960) and further evidence in support of this contention will be presented in Chapter 1. So far no definite function has been ascribed to L-SEP. At the time of its discovery Roberts and Lowe (1954) considered that it might serve as a donor of serine, ethanolamine, phosphoserine or phosphoethanolamine moieties for the synthesis of phospholipids or phosphoproteins. Later, when the results of tracer studies in turtles indicated that the turnover of SEP was relatively slow (Ayengar and Roberts, 1957) it was suggested that SEP was a degradation product of a larger molecule. More recently Rosenberg and Ennor (1961b) have demonstrated active incorporation of [^{32}P]orthophosphate into L-SEP in chicken kidney (see Chapter 1). In addition it was felt that the peculiar distribution of L-SEP in the

phylogenetic tree might indicate some evolutionary significance. It may thus be concluded that while the function of D-SEP is probably that of a precursor of lombricine (see subsequent chapters) the elucidation of the physiological role of L-SEP still requires further elucidation.

CHAPTER 1

THE BIOSYNTHESIS OF D-SERINE ETHANOLAMINE
PHOSPHODIESTER AND D-LOMBRICINE IN VIVO IN
THE EARTHWORM

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INTRODUCTION

Previous reports dealing with the biosynthesis of SEP have been concerned solely with the L-isomer. In vivo studies by Ayengar and Roberts (1957, 1960) demonstrated that in river turtles [^{32}P]orthophosphate was rapidly incorporated into phosphoethanolamine, phosphoprotein and phospholipids, followed by a much slower appearance of label in L-SEP. As judged by autoradiography [^{32}P] appeared earliest in phosphoethanolamine and L-SEP isolated from kidney, liver and heart, whereas the labelling of L-SEP was much slower in muscle, in which tissue the L-SEP concentration was highest (> 500 mg./kg.).

In vitro experiments with a variety of preparations such as isolated beating hearts, tissues slices, red blood cells and haemolysates showed a slight degree of incorporation of [^{32}P] into L-SEP only with intact nucleated red blood cells after prolonged incubation (12 hr.). With the same preparation no incorporation into L-SEP of [^{14}C] labelled acetate, glycine or glucose was observed. Ayengar

and Roberts (1957) considered that these tracer studies were consistent with L-SEP being a degradation product of some larger molecule.

After it was discovered that L-SEP was present not only in reptiles but also in birds, fish and amphibians, Rosenberg and Ennor (1961b) reinvestigated the biosynthesis of L-SEP in these animals. It was demonstrated that rapid incorporation of [^{32}P] into L-SEP occurred in the chicken. Quantitative determination of the distribution of L-SEP in chicken showed that its concentration was highest in kidney and small intestine, whereas after [^{32}P] administration the pattern of labelling was such that in all the organs L-SEP had much the same specific radioactivity with the exception of L-SEP in kidney, blood and ileum, in which tissues the specific radioactivities were higher. In vitro studies of the incorporation of [^{32}P] into L-SEP by homogenates of chicken kidney, liver and small intestine, in the presence of L-serine, ethanolamine, CTP, and an ATP generating system, indicated that L-SEP was synthesised in the kidney, but no in vitro synthesis was demonstrable in liver or small intestine. The formation of L-SEP proved energy dependent, and since synthesis was diminished when CTP was omitted, it was suggested that this nucleotide was involved in the biosynthetic pathway.

The experiments reported in this chapter are concerned with the biosynthesis of D-SEP and D-lombricine.

The purpose of these investigations was to identify some of the possible precursors of these compounds, and to establish more firmly the relationship between D-SEP and D-lombricine. In particular, the experiments were designed to test the hypothesis (Ennor et al., 1960) that D-SEP was the direct biological precursor of D-lombricine.

Consideration of the chemical structure of D-SEP and D-lombricine suggested that orthophosphate, serine, and ethanolamine might serve as precursors. Accordingly, experiments were designed to trace the incorporation of [^{32}P]orthophosphate, DL-[$3\text{-}^{14}\text{C}$]serine, D-[^3H]serine, L-[^3H]serine and [$1:2\text{-}^{14}\text{C}_2$] ethanolamine into D-SEP and D-lombricine, as well as into certain free amino acids under in vivo conditions.

MATERIALS AND METHODS

Reagents

All reagents were analytical grade. Ethanol was purified by fractional distillation, after refluxing over KOH and aluminium powder. Butanol was purified by fractional distillation.

Reference Compounds

L-SEP was synthetic material kindly donated by Mrs. I. M. Beatty and Dr. D. I. Magrath. D-SEP and D-lombricine were obtained from natural sources, as described by Rosenberg and Ennor (1959) and Ennor et al., (1960). Amino acids, unless otherwise stated, were obtained from

California Corporation for Biochemical Research, Los Angeles, California, U.S.A.

Radioactive Precursors

[³²P]Orthophosphate (carrier free) was obtained from the Atomic Energy Research Establishment, Harwell, England. [1;2-¹⁴C₂]ethanolamine and DL-[3-¹⁴C]serine were obtained from The Radiochemical Centre, Amersham, Bucks., and were used at specific radioactivities of 5 and 2.7 μC/μmole respectively.

L-Serine and D-serine were tritiated by The Radiochemical Centre according to the method of Wilzbach (1957). A sample of each isomer (100 mg.) was dissolved in water (20 ml.) and applied to a column (10 cm. x 1 cm.) of Amberlite CG-120 (mesh 100-200, H⁺ form) ion exchange resin. In order to remove exchangeable tritium and decomposition products, the resin was washed with several litres of water, and the eluate collected in 50 ml. fractions. Those fractions containing serine were pooled, and the serine adsorbed onto a column (10 cm. x 2 cm.) of Zeo-Karb 225 (mesh 20-50, H⁺) resin. The adsorbed material was eluted with aq. 2.5N-NH₃ soln. and taken to dryness under reduced pressure at 40°. Sufficient water and carrier L- or D-serine were added to make a solution with a final concentration of 200 μmoles/ml. and a specific radioactivity of 13.5 μC/μmole.

Animals

Giant earthworms (Megascolides cameroni) of average weight 8 g. were collected in the Australian Capital Territory. The worms were kept in cardboard cartons containing about one pound of moist earth. Usually 3 worms were placed in each carton, and, when maintained at 10° the worms remained in good condition for several months.

Analytical Methods

Lombricine was estimated by the procedure described by Rosenberg, Ennor and Morrison (1956) for the estimation of arginine. Orthophosphate was estimated as described by Ennor and Stocken (1950), and the specific radioactivity of orthophosphate as described by Ennor and Rosenberg (1952a).

In order to estimate the amounts and specific radioactivities of phosphate in SEP and lombricine, isolated by paper chromatography, the areas containing these compounds were cut out and the compounds eluted with water. The eluates were taken to dryness, and the residue ashed in a micro-Kjeldahl digestion flask with 0.5 ml. of a mixture of 2 volumes of 70% HClO_4 (w/v) and 3 volumes of conc. H_2SO_4 . The ashed residue was diluted with 2 ml. of water, heated to 100° for 1 hr. to convert any pyrophosphate into orthophosphate, and the specific radioactivity determined as described above. Similar digestion procedures were applied to phospholipids and phosphoproteins for the estimation of orthophosphate.

Chromatographic Procedures

Descending paper chromatography on Whatman No.3 paper (56 cm. x 56 cm.) was employed throughout, using the following solvent systems: (1) Ethanol-formic acid-water (7:1:2, by vol.); (2) Phenol-water (4:1, w/v); (3) Butanol-water-acetic acid (5:3:2, by vol.). Prior to use the papers were washed for at least three days with distilled water, air dried, and stored flat in dust-free polythene containers.

Unless otherwise specified two-dimensional chromatograms were run in ethanol-formic acid-water (first solvent) and phenol-water (second solvent). The optimum amount (see below) of material for chromatography was applied to the corner of the paper for two-dimensional chromatography, or between marker guide strips for unidimensional chromatography. In all instances either solvent systems (1) or (3) were employed in the first run lasting 13 hours, as these solvents were easily removed at the end of this period by heating in a current of air at 50°. When phenol-water was used, however, as the second solvent, care was taken to ensure that the phenol vapours were removed under mild conditions. Accordingly the papers were dried at 23° in a stream of air overnight to remove as much phenol as possible, prior to heating at 50° until the final traces of phenol were removed.

Amino compounds were visualised by spraying the paper chromatograms with a 0.2% solution of ninhydrin in acetone, followed by heating at 80° for 10 min. In experiments with [³H]serine, the strength of the ninhydrin was reduced to 0.05% to minimise interference with subsequent scintillation counting of [³H].

Guanidino compounds were detected on chromatograms by the diacetyl reagent as described by Rosenberg (1959).

Elution of Compounds from Paper Chromatograms

After location of compounds on the chromatograms (see below) a triangular shaped area containing each spot was cut out. This paper was then suspended by the apex of the triangle on a platinum loop, so that the lower end of the paper just dipped into a container of water. After a few hours, when the compound was concentrated at the tip, the paper was inverted with subsequent elution of the compound in a few drops of water (Reith, 1957).

Identification of Amino Acids

The free amino acids of viscera and muscle were identified by comparison of their distribution pattern with authentic marker amino acids in numerous unidimensional and two-dimensional chromatograms in solvent systems 1, 2, and 3. The identity of the amino acids was confirmed by chromatography with marker amino acids. In instances where doubt still existed after application of the above procedures, the

amino acid was eluted and re-chromatographed in other standard solvent systems used for identification of amino acids.

Isolation of SEP, Lombricine and Free Amino Acids

Four isolation methods were employed to separate SEP, lombricine or amino acids, the choice in any particular instance being dependent upon the nature of the isotope being studied. For example, in experiments in which [^{32}P] orthophosphate was administered, two-dimensional chromatograms were run, and after visualisation of SEP and lombricine with ninhydrin, the appropriate areas were cut out and the material eluted. The [^{31}P] and [^{32}P] content of the material was then determined as described previously.

With [^{14}C] precursors the extracts were chromatographed uni-dimensionally in solvent system (1) between guide strips, with SEP and lombricine markers. By this procedure SEP and lombricine, with approx. R_F 0.2 and 0.3 respectively, were located and easily separated from other amino compounds, which in this solvent system have $R_F > 0.4$. Separation between SEP and lombricine, however, was not always discrete, necessitating elution and unidimensional re-chromatography in solvent system (2), which separated SEP (R_F 0.3) from lombricine (R_F 0.55) completely. These compounds were identified and eluted as before.

[^{14}C] labelled free amino acids, and in some instances [^{32}P] labelled and [^{14}C] labelled SEP and

lombricine were separated by two-dimensional paper chromatography, and located by radioautography. The areas on the chromatograms corresponding to darkened areas on the x-ray films were cut out and the material was eluted.

When [^3H] labelled precursors were employed the extracts were chromatographed two-dimensionally, and after detecting the amino compounds by spraying with 0.05% ninhydrin, the appropriate areas were cut out and the material was eluted. This method combined rapidity and precision of identification of the amino acids with the added advantage that the faint colour developed by the eluates did not seriously interfere with scintillation counting of the samples.

Quantitative Determination of Amino Compounds

SEP, lombricine and certain amino acids were determined quantitatively by the ninhydrin method of Moore and Stein (1954). In many instances since the amount of amino compound being measured was quite small (0.05 μmoles) and so significant errors in these determinations could be introduced by extraneous ninhydrin-reacting compounds,

In order to reduce accidental contamination during the handling of chromatograms, all manipulation was carried out with plastic forceps. Also the absorption onto filter paper of NH_3 vapour from the atmosphere was considerably lessened by conducting the chromatography and subsequent operations in a laboratory as free as possible from NH_3 vapours.

Another source of error may be introduced by NH_3 present in ordinary distilled water and in paper spot eluates. To overcome these difficulties the distilled water used in these determinations was rendered NH_3 -free by passage through a column (60 cm. x 5 cm.) of Zeo-Karb 225 (mesh 20-50, H^+) ion exchange resin immediately prior to use. In addition NH_3 was removed from paper eluates by taking the samples to dryness in the presence of alkaline buffer. To each sample 0.1 ml. of borate buffer in methanol prepared as described by Connell, Dixon and Hanes (1955) was added and the samples were left overnight under reduced pressure in a dessicator containing conc. H_2SO_4 .

Prior to the development of the ninhydrin colour 0.5 ml. of 30% (v/v) acetic acid was added to the sample to adjust the pH to 5, which was found optimum for colour development (Moore and Stein, 1954). With muscle extracts sufficient material was available to run duplicate chromatograms of each sample, which provided a further check on the reliability of the procedure. Reference standards of SEP, lombricine and serine were included with every determination.

Hydrolysis of Lombricine

In certain experiments the distribution of label throughout the lombricine molecule was determined, by degrading lombricine eluates to 2-guanidinoethanol, 2-guanidinoethylphosphate and serine by acid hydrolysis in

6N-HCl at 110° for 24 hr. in a sealed pyrex tube. HCl was removed from the hydrolysis products under reduced pressure in a dessicator over KOH. The hydrolysis products were dissolved in 0.1 ml. of water and separated by paper chromatography in solvent system (2), with appropriate marker guide strips. This permitted serine (R_F 0.35) and a combined 2-guanidinoethanol and 2-guanidinoethylphosphate (R_F 0.8 and 0.7 respectively) to be located, eluted, and their specific radioactivities determined as described.

Measurement of Radioactivity

The radioactivity of [^{32}P] labelled compounds was estimated in a M6 liquid counter (20th Century Electronics) and the counting rate was corrected for background counting rate and decay.

The radioactivity of [^{14}C] and [3H] labelled compound was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer model 314 (Packard Instrument Co., La Grange, Illinois, U. S. A.) in the following manner. The compound was eluted from the chromatogram as stated, and after a portion of the eluate was pipetted into a 25 ml. glass vial the water was evaporated at a temperature below 50°. The residue was dissolved in 0.2 ml. of 0.5M-p-N-(diisobutylcresoxyethoxyethyl)-NN-dimethyl-N-benzylammonium hydroxide (hydroxide of Hyamine 10-X, Rohm

and Haas Co., Philadelphia, Pa., U. S. A.) in dry methanol as described by Passmann, Radin and Cooper (1956) and Vaughan, Steinberg and Logan (1957). To this was added 10 ml. of solvent-scintillator containing 4 g. of 2:5-diphenyloxazole and 100 mg. of 1:4-di-(2:5'-phenyloxazolyl)-benzene/1. of toluene. The radioactivity was determined with a 10-50 v. window and high-voltage tap 4 for [^{14}C] labelled precursors and tap 8 for [^3H] labelled precursors. Whenever the materials were located on the chromatogram with the ninhydrin-detection spray, an internal standard of either [^{14}C] serine or [^3H]serine was used to correct for quenching. Specific radioactivities were expressed as counts/min./ μmole .

EXPERIMENTAL

In any experimental series each worm received the same amount of labelled precursor expressed as number of cpm/g. body weight. The amount of isotopically labelled compound given in each particular experiment is listed in the tables. In preliminary experiments attempts were made to administer the radioisotopes by injection into the earthworm's coelomic cavity. This method did not prove successful as the worms invariably died after a few days, probably as a result of the trauma caused by injection. However, it was found that radioisotopes could be administered orally to the giant earthworm (Megascolides cameroni)

with very little apparent disturbance of the normal physiology. Accordingly the labelled compounds were delivered accurately from a microsyringe through a fine polythene tube (0.8 mm external diameter) inserted into the upper end of the earthworm's gastrointestinal tract for a distance of at least 3 cm. In most instances this procedure was quite satisfactory, but occasionally the earthworm regurgitated some of the compound. After intubation the worms were returned to the cold room (10°), and kept in separate containers in moist earth for periods ranging from 1 to 16 days.

After suitable time intervals the worms were killed, and rapidly divided into "viscera" and "muscle" by dissection of the whole of the gastrointestinal tract and appendages free from the muscular body wall. From this stage onwards these two tissues were treated separately. The "viscera" and "muscle" samples were weighed, and disintegrated in the micro cup of Nalco homogeniser (MSE, Birmingham) with two volumes of $1.5N-HClO_4$. The suspensions were centrifuged, the supernatants neutralised with $2.5N-KOH$ and after cooling to 0° on an ice bath the precipitated potassium perchlorate was removed by centrifugation and discarded.

One volume of ethanol was added to the supernatant and after cooling to 3° for one hour the precipitated glycogen was removed by centrifugation. In experiments in

which [^{32}P]orthophosphate was given, a portion of the supernatant was taken at this stage for the estimation of the specific radioactivity of the orthophosphate.

The supernatant fluid was applied to a column (6 cm. x 1 cm.) of Zeo-Karb (mesh 20-50, H^+ form) ion exchange resin at the rate of 1 ml./min. The resin was washed with water until the effluent was approximately pH 6.0 and the effluent was discarded. In those experiments in which [^{32}P] was used, the resin was treated with 0.01N-phosphoric acid followed by 0.01N-HCl prior to washing with water, in order to remove traces of [^{32}P] remaining on the resin. Adsorbed materials were eluted from the resin with aq. 2.5N- NH_3 soln. at a rate of 3 ml./min. and the eluate (50 ml.) taken to dryness under reduced pressure at 40° . The brown residue so obtained was dissolved in 0.1 ml. and 0.3 ml. of water for viscera and muscle respectively. In the following sections these solutions are referred to as viscera and muscle extracts.

Some experiments were designed to permit comparison of the relative rates of incorporation of [^{32}P] into the labile phosphates of ATP and PL, into the diester phosphate of SEP, lombricine and phospholipids, as well as into the phosphate of phosphoprotein. Accordingly, 2 and 4 days respectively after the administration of [^{32}P], the entire worms were frozen in liquid air, ground to a fine powder in

a cooled mortar, and extracted with two volumes of cold 1.5N-HClO₄ as described above. The homogenates was centrifuged at 0° and the acid-insoluble precipitate retained for the estimation of phosphoprotein and phospholipid phosphorus (see below).

The supernatant was immediately adjusted to pH 7.2 by the addition of 2.5N-KOH, and, after removal of the precipitated KClO₄ by centrifugation at 0°, portions of the supernatant were taken for the estimation of the specific radioactivities of SEP and lombricine. Another portion of the supernatant was taken for analysis of phosphorylated intermediates as described by Le Page (1951).

Thus the pH of the solution was adjusted to 8.2 with 0.1N-KOH, excess of 25% barium acetate added, and the precipitate removed by centrifugation at 0°. The "barium soluble" material was retained, and the "barium insoluble" material redissolved in 0.1N-HCl, and then precipitated as before. After centrifugation the supernatant was added to the "barium soluble" material, while the precipitate containing the "barium insoluble" material was taken to dryness at 40° under reduced pressure. The latter fraction was taken up in a small volume of water and converted to its sodium salt by stirring with Zeo-Karb 225 (mesh 20-50, Na⁺ form). After this procedure the solution was Ba²⁺ free, as judged by a negative test with Na-rhodizonate.

The resin was filtered off, washed with water, and the filtrate and washings combined. Portions of this solution were taken for the determination of the specific radioactivity of orthophosphate and the labile phosphates of ADP and ATP. The latter were determined as the phosphate released by the hydrolysis in 1N-HCl at 100° for 7 min. (Le Page, 1951). Determinations of the [³¹P]orthophosphate were carried out on 1 ml. samples as described by Ennor and Stocken (1950). The radioactivity of the orthophosphate was determined by the method of Ennor and Rosenberg (1952a).

After the removal of the "insoluble" fraction, the "barium soluble" salts were precipitated by the addition of four volumes of 95% ethanol, and the precipitate washed twice by suspending it in ethanol and once in acetone, and finally dried under reduced pressure in a dessicator over P₂O₅. The dried material was dissolved in water and reprecipitated from ethanol as before. This precipitate was dissolved in water and rendered Ba²⁺ free by passage through a column (5 cm. x 1 cm.) of Zeo-Karb 225 (mesh 20-50, Na form). Portions of the effluent were taken for estimation of the specific radioactivities of the residual orthophosphate and the labile phosphate of PL. The procedure adopted for the hydrolysis of PL was similar to that used by Ennor and Rosenberg (1952b) for the hydrolysis of PC, namely heating the sample at 65° for 9 min. in 0.1N-HCl. This method

released phosphate and lombricine in a molar ratio varying between 0.97 to 1.01, and thus the orthophosphate released may be assumed to have its origin in the labile phosphate of PL.

The acid-insoluble precipitate was washed repeatedly with $N-HClO_4$ at 0° until the wash was no longer radioactive (Ennor and Rosenberg, 1954b) and then washed once with water. The pellet was then extracted with lipid solvents as described by Schmidt and Thannhauser (1945) by suspending in 40 vols. of an alcohol-ether mixture (75:25 v/v). After boiling for a few min. the suspension was centrifuged and the precipitate washed with ether and dried. The dry residue was ground in a mortar, and refluxed for 30 min. with 40 vols. of a boiling mixture of methanol-chloroform (50:50 v/v).

After filtration and washing of the residue with ether all the lipid extracts were combined, while the dry fat-free material was retained for estimation of phosphoprotein phosphorus. The combined lipid extracts were dried under reduced pressure at 40° , and re-extracted with light petroleum (boiling range: $40-60^\circ$). This extract was reduced to a small volume (2 ml.) under reduced pressure at 40° , and ten volumes of acetone added, followed by two drops of $Mg Cl_2$ in ethanol. The precipitated phospholipids were removed by centrifugation, dried under reduced pressure, and wet-ashed (see Methods).

The phosphate of the phosphoproteins was determined by hydrolysing the fat-free material in 1N-KOH at 37° for 16 hr., and the orthophosphate released was precipitated according to the method of Delory (1938). The specific radioactivities of the phosphate of the phosphoproteins and phospholipids were determined as described previously.

In order to evaluate the extent of re-ingestion of excreted radioactive material, one worm was given a quantity of [^{14}C]serine equal to that given to the experimental animals, and was placed in a box filled with moist earth (400 g.). Another worm, which received no isotope, was placed in the same box, and the two worms were left in the earth for 8 days. They were then both killed and each worm was treated as described above. The earth from the box was extracted with perchloric acid and the extract was desalted and reduced in volume. The total [^{14}C] present in both worms and in the soil was assessed. Of the total radioactivity present, the injected worm was found to contain 86.0%, the earth 13.6% and the control worm 0.5%. Complications through re-ingestion of excreted radioactive material were therefore not considered of importance in the present experiments.

RESULTS

An approximate estimate of the pool sizes of D-SEP and D-lombricine was obtained by comparing the amounts of these compounds present in two-dimensional paper chromatograms of viscera and muscle extracts. Typical chromatograms of viscera extract (Fig.1A) and muscle extract (Fig.2A) reveal that the distribution of D-SEP and D-lombricine conforms to a regular pattern. The lombricine content of muscle is always very much greater than that of viscera, whereas the amounts of D-SEP in these two tissues are approximately equal to each other, and are of the same order as the lombricine content of the viscera.

Quantitative estimations of the concentrations of SEP and lombricine in viscera and muscle substantiate the qualitative findings just discussed. The values (Table 1) are representative of a typical experiment, and show that the lombricine content of muscle is about eight times that in viscera. The concentrations of SEP in both tissues, as well as the lombricine content of viscera, are of the same order.

The incorporation of [^{32}P] into SEP and lombricine

After the administration of [^{32}P]orthophosphate, label was incorporated comparatively rapidly into SEP but slower into lombricine. This is apparent from inspection

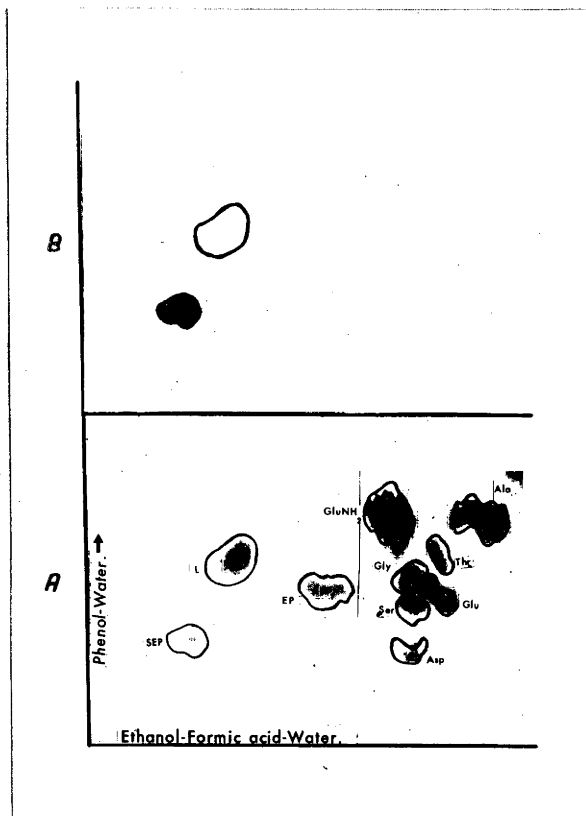


Fig.1(A). Chromatogram of extract of earthworm viscera prepared 8 days after the administration of [^{32}P] orthophosphate (1.1×10^6 counts/min.)/g. body weight. Abbreviations: SEP, serine ethanolamine phosphodiester, L, lombricine, Glu-NH₂, glutamine, Ala, alanine, Thr, threonine, Glu, glutamic acid, Ser, serine, Gly, glycine, Asp, aspartic acid, EP, phosphoethanolamine.

Fig.1(B). Radioautograph of chromatogram shown in 1(A). The lines encircling the ninhydrin-stained areas of SEP and lombricine (L) on the chromatogram were superimposed on the radioautograph.

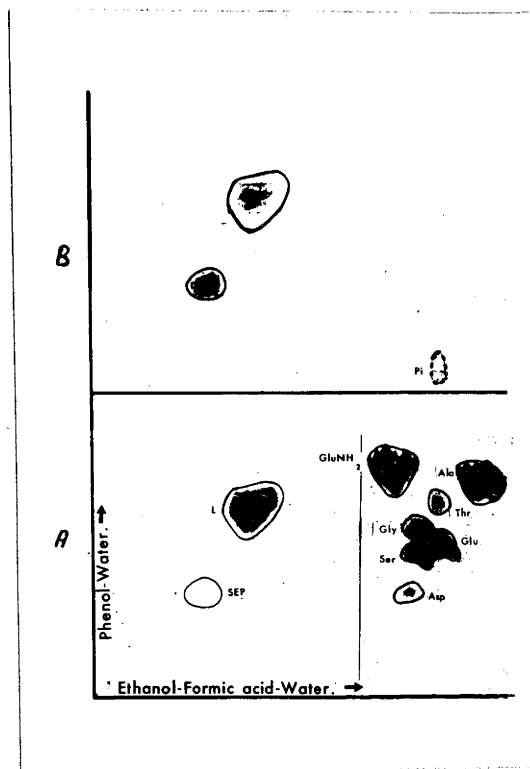


Fig.2(A). Chromatogram of extract of earthworm muscle prepared 8 days after the administration of $[^{32}\text{P}]$ orthophosphate (1.1×10^6 counts/min.)/g. body weight.

Fig.2(B). Radioautograph of chromatogram shown in 2(A). Abbreviations: P_i , orthophosphate. Others as in Fig.1(A). The lines encircling the ninhydrin-stained areas of SEP and lombricine (L) on the chromatogram were superimposed on the radioautograph.

TABLE 1.

Concentration of serine ethanolamine phosphodiester
and lombricine in muscle and viscera of the earthworm

(μ moles/100 g. tissue)

MUSCLE		VISCERA	
SEP	Lombricine	SEP	Lombricine
15.3	176	10.6	23.8

of the radioautographs (Figs. 1B and 2B) corresponding to the chromatograms (Figs. 1A and 2A). As judged from the darkening of the x-ray film the most intense radioactivity was in the SEP of viscera, with diminishing levels in SEP in muscle, and lombricine of viscera and muscle. In the reproduction of the radioautograph of visceral tissues the labelling of the lombricine area is not shown, but in the original x-ray film a definite darkening could be seen. A comparison of these radioactivities, taking into account the known pool sizes of SEP and lombricine, clearly shows that the specific radioactivities of SEP in both tissues were much higher than those of lombricine. Furthermore, the specific radioactivities of SEP and lombricine of viscera were greater than their counterparts in muscle.

Table 2 expresses these findings quantitatively. In the viscera the specific radioactivity of orthophosphate was highest, and this declined steadily from injection time onwards. In contrast the specific radioactivity of orthophosphate in muscle was relatively unchanged from the second day onwards which indicates that orthophosphate was continually being absorbed from the gastrointestinal tract over the experimental period. The quantitative data substantiate the fact that the specific radioactivities of SEP both in viscera and in muscle were at all time intervals much greater than those of lombricine in either tissue.

TABLE 2.

Incorporation of [³²P] orthophosphate into serine
ethanolamine phosphodiester and lombricine of
muscle and viscera of the earthworm

Radioactivity administered (1.1×10^6 counts/min.)/g. body weight

Time after [³² P] (days)	Specific radioactivity (counts/min./ μ mole)					
	MUSCLE			VISCERA		
	P _i	SEP	Lombricine	P _i	SEP	Lombricine
1	17,000	270	11	56,700	2,190	40
2	11,700	560	20	29,500	4,310	160
4		840	97		4,990	100
9	12,900	2,140	140	13,400	10,500	470
16	13,000	2,640	250	10,600	7,160	310

It is also apparent that the specific radioactivities of SEP in viscera reached a peak around the ninth day, but were always much higher than the corresponding muscle values, which were still rising at the end of the experimental period (16 days). Similarly, the specific radioactivities of lombricine in viscera also reached a maximum value at about the ninth day, and were always somewhat higher than the corresponding muscle values, which showed a progressive rise with time throughout the course of the experiment.

Some estimate of the rate of [^{32}P] incorporation into SEP and lombricine was obtained by comparing the labelling of these compounds with that of other phosphorylated derivatives (Table 3). The specific radioactivities of these compounds were compared at two time intervals (2 days and 4 days) with the specific radioactivities of orthophosphate, and expressed as relative specific radioactivities.

While the value of the specific activities of ATP and PL were of the same order as those of orthophosphate, the specific activity of phosphoprotein was approximately an order below, those of SEP and phospholipid two orders, and those of lombricine three orders below those of ATP and PL (see Discussion).

TABLE 3.

Incorporation of [^{32}P] orthophosphate into certain
phosphorus-containing compounds of the earthworm

Radioactivity administered (1.1×10^6 counts/min.)/g. body weight

Relative specific radioactivity (based on $\text{P}_i = 100$)

Time after [^{32}P] (days)	ATP	PL	SEP	Lombricine	Phospho- protein	Phospho- -lipid
2	23	-	0.44	0.012	4.5	0.8
2	78	53	0.86	0.073	-	-
2	44.5	36.2	3.98	0.19	-	-

The incorporation of [^{14}C] labelled serine into SEP,

Lombricine and Amino-acids

After the administration of DL-[3- ^{14}C]serine, label was incorporated into SEP and lombricine, as well as into several amino acids. Figs. 3 and 4 show typical chromatograms with their corresponding radioautographs, and it is evident that the pattern of labelling of SEP and lombricine was essentially similar to that obtained with [^{32}P]orthophosphate. Thus the specific radioactivities of SEP viscera and muscle were much higher than the corresponding lombricine values. Also the specific radioactivities of SEP and lombricine in viscera were greater than the corresponding specific radioactivities of SEP and lombricine in muscle. The darkening in the lombricine area (Fig. 3B) was quite visible on the original x-ray film, but it has not been reproduced in the photograph. It is also evident that serine was incorporated into many of the free amino acids, in particular into glutamic acid, glutamine, alanine and aspartic acid. In addition, as will be appreciated from the quantitative data below, serine, glycine and threonine were also radioactive, but as these were present in very small quantities with low radioactivities no darkening of the x-ray film was detectable in these areas.

Quantitative aspects of the incorporation of

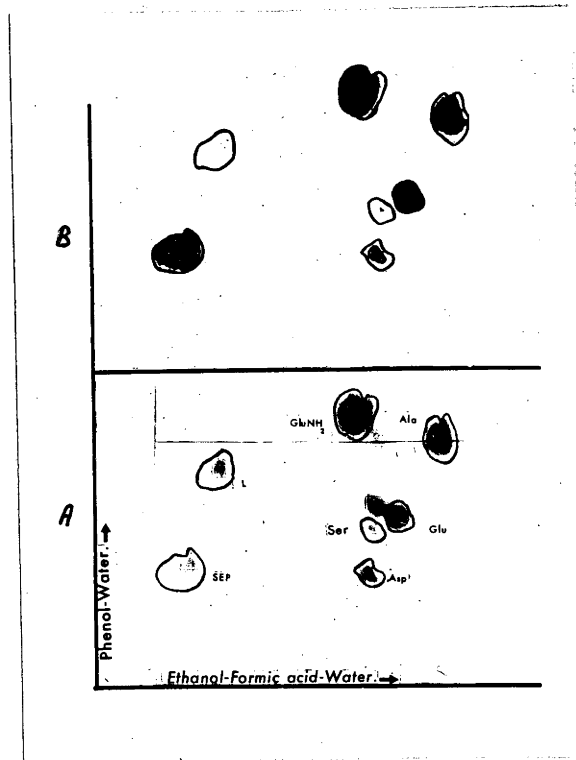


Fig.3(A). Chromatogram of extract of earthworm viscera prepared 8 days after the administration of DL-[3-¹⁴C] serine (10^7 counts/min.)/g. body weight. Abbreviations as in Fig.1(A).

Fig.3(B). Radioautograph of chromatogram shown in 3(A). Abbreviations as in Fig.1(A). The lines encircling the ninhydrin-stained areas on the chromatogram were superimposed on the radioautograph.

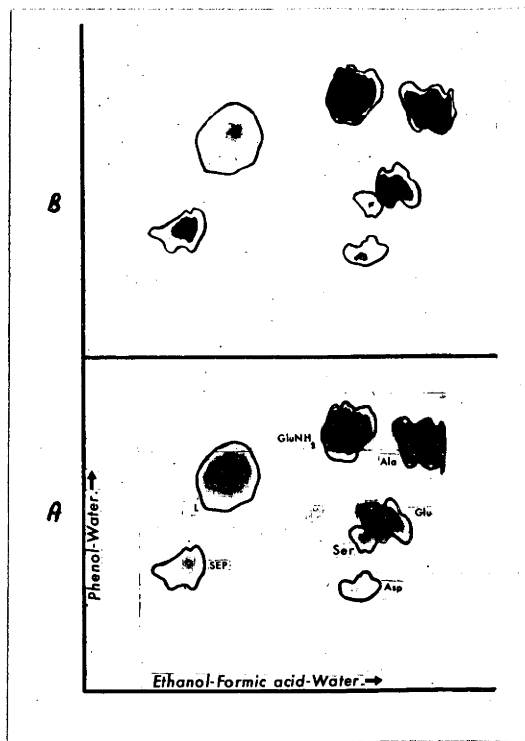


Fig.4(A). Chromatogram of extract of earthworm muscle prepared 8 days after the administration of DL-[3-¹⁴C] serine (10^7 counts/min.)/g. body weight. Abbreviations as in Fig.1(A).

Fig.4(B). Radioautograph of chromatogram shown in 4(A). Abbreviations as in Fig.1(A). The lines encircling the ninhydrin-stained areas on the chromatogram were superimposed on the radioautograph.

DL-[3-¹⁴C]serine are presented in Table 4. These results confirm the pattern of labelling of SEP and lombricine in viscera and muscle, as revealed by Figs. 3 and 4 and show the time course of labelling. The specific radioactivities of both SEP and lombricine in viscera and muscle rose progressively throughout the experimental period (9 days). Initially the specific radioactivities of SEP in both tissues were considerably greater than the corresponding lombricine values, being some 58 and 35 times higher in viscera and muscle respectively on the second day. With the passage of time the values of specific radioactivities of both SEP and lombricine converged. This is evident from the ratios of the specific radioactivities of SEP and lombricine (Table 4) which showed a progressive fall during the experimental period.

The high concentration of lombricine in muscle permitted the isolation of sufficient labelled lombricine from chromatograms for the purpose of acid degradation, as described in the Methods section. Fig. 5 shows a unidimensional chromatogram developed in phenol-water (4:1 w/v) of the hydrolysis products of lombricine. It is evident that the serine moiety can readily be separated by this method from the other hydrolysis products (*g*-guanidinoethanol and 2-guanidinoethylphosphate), and thus the distribution of label within the relevant portions of the lombricine molecule can be assessed.

TABLE 4.

Incorporation of radioactivity from DL-[3-¹⁴C] serine into
serine ethanolamine phosphodiester and lombricine of the
muscle and viscera of the earthworm

Each worm was given 2 μ moles of DL-[3-¹⁴C] serine (equivalent to 10^7 counts/min.)/g. body weight, and kept at 10°. Figures in parentheses represent the percentage of the total radioactivity in the relevant portion of the lombricine molecule.

<u>MUSCLE</u>	Specific radioactivity (Counts/min./ μ mole)		
	(2 days)	(8 days)	(9 days)
SEP	31,500	41,700	70,200
Lombricine (total)	900	6,270	15,700
Lombricine (serine moiety)	680 (76%)	4,450 (71%)	9,340 (60%)
Lombricine (guanidino- ethanol moiety)	225 (24%)	1,820 (29%)	6,360 (40%)
<u>VISCERA</u>			
SEP	100,000	222,000	422,000
Lombricine	1,730	9,900	27,500
Ratios of specific radioactivities (SEP:lombricine)			
<u>MUSCLE</u>	35	7	5
<u>VISCERA</u>	58	22	15

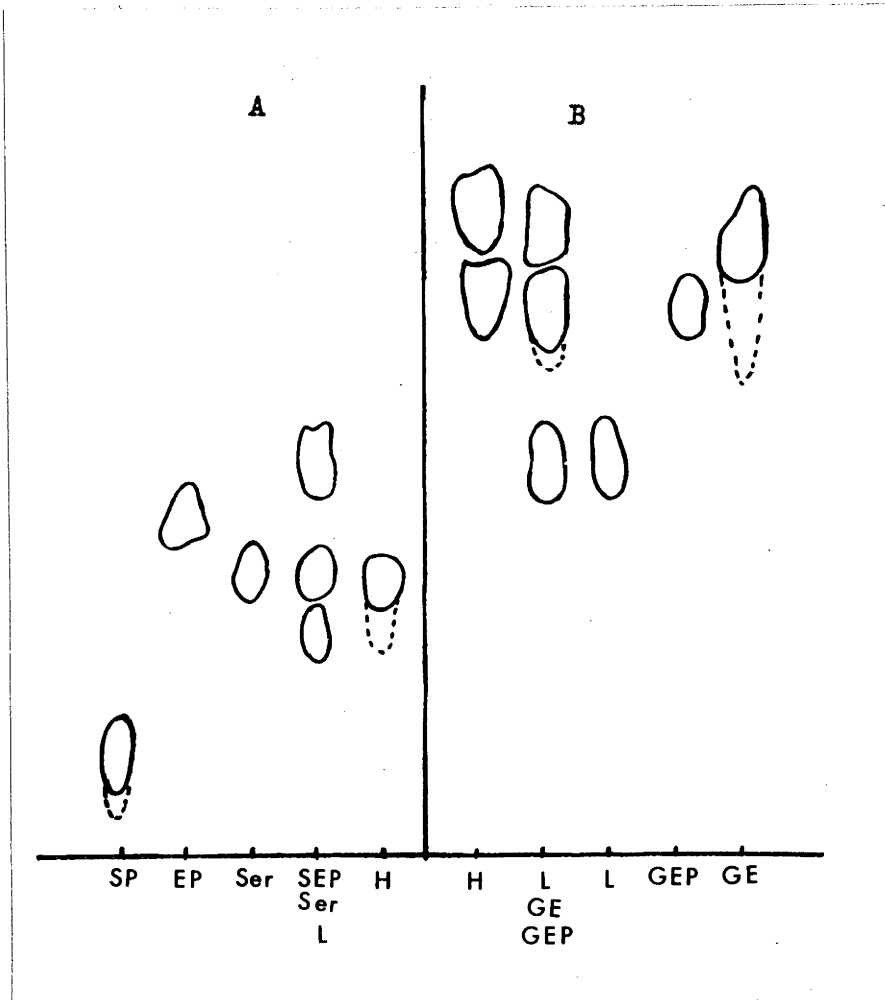


Fig. 5. Chromatogram of the acid hydrolysis products of lombricine in phenol-water (4:1, w/v). Portion A has been developed with ninhydrin, and portion B with diacetyl reagent. Abbreviations: SP, phosphoserine, H, hydrolysis products of lombricine, GE, 2-guanidinoethanol, GEP, 2-guanidinoethylphosphate. Others as in Fig. 1(A).

As would be anticipated, after the administration of DL-[3-¹⁴C] serine, the major portion of the label in the muscle lombricine was in the serine portion of the molecule. However, a significant proportion of the label was in the 2-guanidinoethanol moiety of lombricine (Table 4). This accounted for some 24% of the incorporated isotope in 2 days, and up to 40% in nine days.

The specific radioactivities of the free amino acids of earthworm muscle, after the administration of DL-[3-¹⁴C]serine, are presented in Table 5. Glutamic acid and glutamine showed the highest incorporation, with lesser degrees of labelling of aspartic acid, alanine, glycine and threonine. These quantitative data were in accord with the findings obtained from the chromatograms and radioautograph of muscle extract (Fig.4).

The incorporation of [³H] labelled Serine into SEP,

Lombricine and Amino-acids

With the knowledge that serine was a precursor of SEP and lombricine, some experiments were carried out with [³H] labelled D- and L-serine, in order to ascertain if there was any preferential incorporation of a particular isomer into these compounds. The results of a series of these tracer experiments of 2 and 8 days duration are presented in Tables 6 and 7. Within each experiment the

TABLE 5.

Incorporation of radioactivity from DL-[3-¹⁴C] serine
into the free amino acids of earthworm muscle

Each worm was given 2 μ moles DL-[3-¹⁴C] serine (equivalent to 10^7 counts/min.)/g. body weight, and kept at 10°.

<u>Free amino acids</u>	Specific radioactivity (counts/min./ μ mole)	
	(8 days)	(9 days)
Glutamic acid	71,000	173,000
Glutamine	62,700	243,000
Aspartic acid	27,800	63,900
Alanine	32,800	57,400
Glycine	10,500	28,700
Threonine	6,700	28,400
Serine	12,800	57,400

TABLE 6.

Incorporation of radioactivity from L-[³H] serine or
D-[³H] serine into serine ethanolamine phosphodiester and
lombricine of the muscle and viscera of the earthworm

Each worm was given 10 μ moles L-[³H] serine or D-[³H] serine (equivalent to 4×10^7 counts/min.)/body weight, and kept for 2 days at 10°. Figures in parentheses represent the percentage of the total radioactivity in the relevant portion of the lombricine molecule.

	Specific radioactivity (counts/min./ μ mole)				
	from L-[³ H] serine			from D-[³ H] serine	
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
<u>MUSCLE</u>					
SEP	41,300	35,200	9,190	8,800	24,900
Lombricine (total)	1,670	1,270	1,300	220	550
Lombricine (serine moiety)	575 (34%)	430 (33%)	300 (23%)	140 (64%)	220 (40%)
Lombricine (guanidino-ethanol moiety)	1,095 (66%)	840 (67%)	1,000 (77%)	80 (36%)	330 (60%)
<u>VISCERA</u>					
SEP	101,000	70,000	181,000	28,600	199,000
Lombricine (total)	5,470	2,910	9,230	860	8,660
Lombricine (serine moiety)	920 (17%)	640 (22%)	2,310 (25%)	315 (37%)	1,660 (19%)
Lombricine (guanidino-ethanol moiety)	4,550 (83%)	2,270 (78%)	6,920 (75%)	545 (63%)	7,000 (81%)

TABLE 7.

Incorporation of radioactivity from L-[³H] serine or D-[³H] serine into the serine ethanolamine phosphodiester and lombricine of the muscle and viscera of the earthworm

Each worm was given 10 μ moles L-[³H] serine or D-[³H] serine (equivalent to 4×10^7 counts/min.)/g. body weight, and kept for 8 days at 10°. Figures in parentheses represent the percentage of the total radioactivity in the relevant portion of the lombricine molecule.

	Specific radioactivity (counts/min./ μ mole)					
	from L-[³ H] serine			from D-[³ H] serine		
	Expt.6	Expt.7	Expt.8	Expt.9	Expt.10	Expt.11
<u>MUSCLE</u>						
SEP	13,400	30,900	52,600	70,000	33,200	23,900
Lombricine (total)	1,560	1,360	6,500	5,260	2,910	2,600
Lombricine (serine moiety)	680 (44%)	460 (34%)	-	2,750 (52%)	1,140 (39%)	-
Lombricine (guanidinoethanol moiety)	880 (56%)	900 (66%)	-	2,510 (48%)	1,770 (61%)	-
<u>VISCERA</u>						
SEP	80,600	30,300	64,000	191,000	122,000	22,100
Lombricine (total)	-	-	15,000	7,670	14,700	8,000
Lombricine (serine moiety)	-	-	-	3,900 (51%)	2,800 (24%)	-
Lombricine (guanidinoethanol moiety)	-	-	-	3,770 (49%)	11,900 (76%)	-

magnitude of the specific radioactivities of SEP and lombricine are consistent with results obtained with [^{32}P]orthophosphate and DL-[3- ^{14}C]serine as precursors, in that the specific radioactivities of both SEP and lombricine were greater in viscera than in muscle, and in each of these tissues the specific radioactivity of SEP was greater than that of lombricine. However, over the whole range of individual experiments there was a marked scatter in the degree of isotope incorporation. No preference was indicated for any particular isomer, and taken as a whole, L-[^3H]serine was incorporated just as readily as the D-isomer.

The distribution of label within the lombricine molecule showed a quantitative but not a qualitative difference from that seen with DL-[3- ^{14}C]serine as the precursor. Thus the amount of radioactivity in the guanidinoethanol portion at 2 days was greater than 60% of the total radioactivity of the molecule, as compared with 24% when DL-[3- ^{14}C]serine was administered.

The incorporation of [^3H]serine into the free amino acids of muscle after two days is shown in Table 8. These results demonstrated no consistent differences between the L- or D-isomer. It will be noted that the specific activities of glycine were of the same order as other amino acids, such as glutamic acid or glutamine.

TABLE 8.

Incorporation of radioactivity from L-[³H] serine or
D-[³H] serine into the free amino acids of the muscle
of the earthworm

Each worm was given 10 μ moles L-[³H] serine or D-[³H] serine (equivalent to 4×10^7 counts/min.)/g. body weight, and kept for 2 days at 10°.

Specific radioactivity (counts/min./ μ mole)

<u>Free Amino Acids</u>	From L-[³ H] serine	From D-[³ H] serine
Glutamic acid	35,900	44,700
Glutamine	53,400	27,000
Aspartic acid	27,900	33,100
Alanine	22,400	15,900
Glycine	42,600	48,600
Serine	262,000	267,000

This contrasts with the much lower value obtained when DL-[3-¹⁴C]serine was the precursor (cf. Table 5).

The incorporation of [1:2-¹⁴C₂] ethanolamine into SEP,

Lombricine and Amino-acids

The chromatogram and corresponding radioautograph obtained with muscle extract 4 days after the administration of [1:2-¹⁴C₂]ethanolamine are presented in Fig.6. The original radioautograph showed incorporation of label into SEP and lombricine as well as into glutamine, glutamate and alanine, but in the reproduction the darkening of the x-ray film in the lombricine area was not apparent. While there was no darkening in the area corresponding to phosphoethanolamine, the area corresponding to free ethanolamine was intensely dark, which indicated that the administered ethanolamine still had a high residual activity even after 4 days.

Table 9 shows that 3 days after the administration of [1:2-¹⁴C₂]ethanolamine the specific radioactivity of SEP in the whole worm was considerably greater than that of lombricine. In another experiment of 4 days duration the specific radioactivity of SEP was greater than that of lombricine, and the specific radioactivities of each compound in the viscera were higher than those of the corresponding compounds in muscle. The distribution of label within the lombricine molecule in muscle was such that 94% of the radioactivity resided in the 2-guanidino-

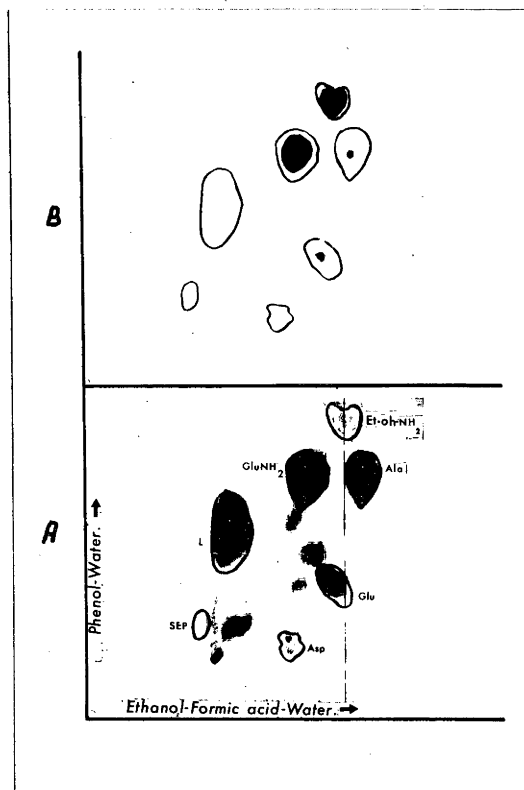


Fig.6(A). Chromatogram of extract of earthworm muscle prepared 4 days after the administration of [1:2-¹⁴C₂] ethanolamine (7.5×10^6 counts/min.)/g. body weight. Abbreviations: ET-OH-NH₂, ethanolamine. Others as in Fig.1(A).

Fig.6(B). Radioautograph of chromatogram shown in 6(A). The lines encircling the ninhydrin-stained areas on the chromatograms were superimposed on the radioautograph.

TABLE 9.

Incorporation of radioactivity from $[1:2-^{14}\text{C}_2]$ ethanolamine into serine ethanolamine phosphodiester and lombricine of the earthworm

Each worm was given 3.7 μmoles of $[1:2-^{14}\text{C}_2]$ ethanolamine (equivalent to 7.5×10^6 counts/min.)/g. body weight, and kept at 10° . Figures in parentheses represent the percentage of total radioactivity in the relevant portion of the lombricine molecule.

	Specific radioactivity (counts/min./ μmole)		
	(3 days)	(4 days)	
	Whole worm	Muscle	Viscera
SEP	231,000	67,900	126,000
Lombricine (total)	2,650	1,080	2,580
Lombricine (serine moiety)	-	60 (6%)	-
Lombricine (guanidinoethanol moiety)	-	1,020 (94%)	-

ethanol moiety, with only 6% in the serine portion.

Of the free amino acids of muscle, radioactivity was incorporated mainly into glutamic acid and glutamine, with lesser amounts into aspartic acid, alanine, glycine, threonine and serine (Table 10). Muscle ethanolamine had a specific radioactivity of 0.4×10^6 cpm/ μ mole, which represented approximately one-fifth that of the ethanolamine administered four days previously. The low specific radioactivity of free serine and the slight labelling of the serine portion of the lombricine molecule indicated that carboxylation of ethanolamine to produce serine did not occur to any appreciable extent.

DISCUSSION

Ayengar and Roberts (1957) concluded from isotope incorporation studies that L-SEP was not synthesised in turtles from small molecular weight precursors. It was conceivable therefore that the same might be true of D-SEP. The obvious structural similarity of SEP and lombricine may prompt the speculation that D-SEP of earthworms might arise by degradation of D-lombricine. Similar types of cleavage of phosphagen bases are known, e.g. the conversion of arginine to ornithine and urea by arginase in mammalian and other tissue, or the splitting of creatine to sarcosine and urea catalysed by microorganisms (Appleyard and Woods, 1956).

TABLE 10.

Incorporation of radioactivity from $[1:2-^{14}\text{C}_2]$ ethanolamine
into the free amino acids of the earthworm

Each worm was given 3.7 μmoles of $[1:2-^{14}\text{C}_2]$ ethanolamine (equivalent to 7.5×10^6 counts/min.)/g. body weight, and kept at 10° .

	Specific radioactivity (counts/min./ μmole)	
	(3 days)	(4 days)
	Whole worm	Muscle
Glutamic acid	156,000	38,900
Glutamine	72,000	48,500
Aspartic acid	21,800	7,400
Alanine	18,900	4,100
Glycine	5,900	3,400
Threonine	4,800	6,300
Serine	3,800	3,600
Ethanolamine	-	409,000

On the other hand, by inference from the biosynthesis of some other guanidines, SEP may be considered, and has been predicted (Ennor and Morrison, 1958) to be the possible precursor of lombricine.

The present experiments provide conclusive evidence that D-SEP cannot be a degradation product of D-lombricine. Thus, irrespective of the isotope administered or of the time period studied the specific radioactivity of SEP was consistently much higher than that of lombricine. Indeed, the rapid labelling of SEP followed by the progressive increase with time in the labelling of lombricine lends weight to the suggestion that SEP is the direct biological precursor of lombricine. Additional studies substantiating this viewpoint will be presented in the following chapter.

The size of the internal organs of the earthworm has precluded more than a rough division into muscle and viscera, the latter comprising gastrointestinal tract, gonads, pseudo-hearts, etc. Nevertheless, large differences in the specific radioactivities of SEP in muscle and in viscera have been observed, and in all instances the higher values were found in SEP isolated from the viscera. It may thus be concluded that SEP is synthesised in some portion of the viscera. Again it is probable that lombricine is also formed in the viscera, because in this

tissue the specific activities of lombricine were somewhat greater than in muscle. However, this conclusion cannot be drawn with certainty from the evidence available.

Studies with [^{32}P] revealed that this isotope was incorporated most rapidly into ATP and PL, and less rapidly into SEP, lombricine, phospholipids and phosphoprotein. The finding that the specific radioactivities of ATP and PL were of the same order was in keeping with the fact that the equilibrium between these compounds is catalysed by a guanidino kinase. The results also demonstrated that the specific radioactivities of SEP and phospholipids were of the same order. Although this may be fortuitous, it is of interest because of the structural similarity between SEP and certain phospholipids. Indeed Weiss, Smith and Kennedy (1958) have drawn attention to the fact that, besides the polynucleotides, the phospholipids are the only compounds with a few rare exceptions which possess a monophosphate in diester linkage. The results (Table 3) do not permit any conclusions to be drawn as to the speed of equilibration of ATP and inorganic phosphate. The observed specific radioactivities of ATP were well below those of P_i for several days after the oral administration of [^{32}P]. However, these results were obtained from experiments using the whole earthworm, and as a consequence the estimated P_i comprised not only absorbed

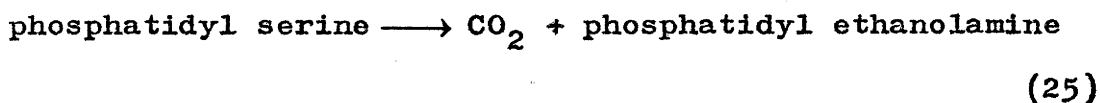
P_i , but also administered P_i which had not as yet been excreted from the earthworms gastrointestinal tract. Thus from the nature of the experiment it was not possible to obtain a true value of the inorganic phosphate with which ATP was in equilibrium.

According to Ayengar and Roberts (1957) after [^{32}P] administration reptilian SEP contained no detectable label at a time when the phospholipids and phosphoproteins attained considerable radioactivity. By comparison, the specific radioactivities of SEP and phospholipids in earthworms, as shown in Table 3, were similar. It would be tempting, therefore, to speculate that D-SEP of earthworms is metabolically more active than L-SEP of reptiles. However, this difference in activity may be more apparent than real, for in reptiles the large pool of L-SEP (>500 mg./kg.) may have caused marked isotope dilution, whereas with D-SEP (30 mg./kg.) this dilution effect may not be as pronounced.

After the administration of DL-[3- ^{14}C] serine activity was detected in several free amino acids (Table 5). The activity in serine and glycine was low in comparison with that of other amino acids. In contrast after [3H] serine, in which presumably the H atoms attached to carbons 2 and 3 were labelled, the specific radioactivity of the serine in the free amino acid pool remained high, and the specific radioactivity of glycine, while below

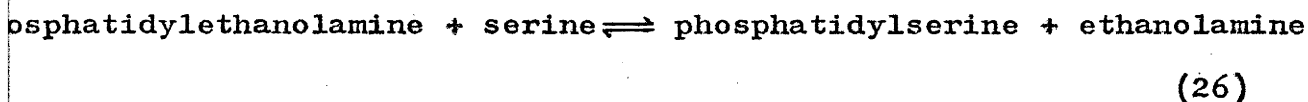
that of serine, was still of the same order as that of other amino acids (Table 8). These findings are compatible with the conversion of serine to glycine and a one carbon fragment.

The administration of radioactive serine was followed by the appearance of label in the serine moiety of lombricine. It is assumed that the same applied to SEP, but the low concentration of SEP in earthworms, precluded the isolation of sufficient material for acid degradation studies of this compound. It was observed (Tables, 4, 6, 7) that after the administration of radioactive serine the guanidinoethanol portion of the lombricine molecule also became labelled. Such a finding indicates that some of the serine had been decarboxylated to ethanolamine. This reaction has been well established from in vivo studies (Stetten, 1942; Weissbach, Elwyn and Sprinson, 1950; Elwyn, Weissbach and Sprinson, 1951; Arnstein, 1951). More recently however it has become apparent that it is not free serine which is decarboxylated but phosphatidyl serine (Hubscher, Dils and Pover, 1959; Bremer, Figard and Greenberg, 1960; Wilson, Gibson and Udenfriend, 1960). This has been confirmed by Borkenhagen, Kennedy and Fielding (1961) who demonstrated that rat liver and other tissues decarboxylated natural and synthetic phosphatidyl-L-serine.

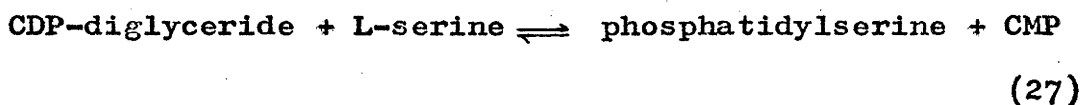


This being the case, and with the further proviso that one may extrapolate from rats to earthworms, it follows that administered serine is incorporated into phosphatidyl serine prior to its decarboxylation. The labelling of the ethanolamine portion of SEP (and subsequently lombricine) could then be considered to involve either a direct incorporation from phosphatidyl ethanolamine, or from labelled free ethanolamine (see below).

Two pathways have been elucidated for the incorporation of serine into phosphatidyl serine. In mammals it has been shown (Borkenhagen et al. 1961) that phosphatidyl serine may be formed by an exchange reaction:



In E.coli it has been demonstrated (Kanfer and Kennedy, 1962) that the biosynthesis of phosphatidyl serine follows a pathway similar to that of inositol monophosphate, the final reaction of which may be written:

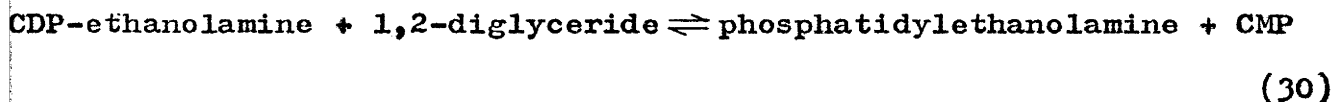
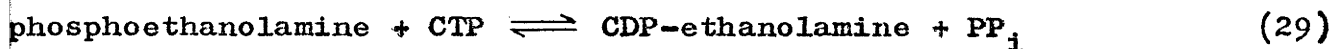


After administration of DL-[3-¹⁴C] serine no labelled free ethanolamine was detected (Figs. 3 and 4). Experiments with mammalian tissues have led to similar

findings. For example, Brady, Formica and Koval (1958) noted a marked discrepancy between the high levels of $^{14}\text{CO}_2$ production and the low rates of free ethanolamine synthesis in rat brain microsomes incubated with uniformly labelled serine. Again other workers (Wilson et al. 1960; Bremer et al. 1960) have reported on the paucity of label in free ethanolamine in in vivo and in vitro studies with radioactive serine. The explanation of these findings probably lies in the fact that free ethanolamine is not formed directly from serine, but indirectly from phosphatidyl serine (Borkenhagen et al. 1961), via reaction (26). However, in the present experiments, although phosphatidyl ethanolamine and free ethanolamine would be poorly labelled initially, one would anticipate that over a period (2 to 9 days) that both these compounds would acquire label progressively.

It was anticipated that D- ^3H serine would be built directly into the serine moiety of lombricine molecule, whereas L- ^3H serine would be incorporated indirectly after conversion to D-serine. The finding of small amounts of D-serine in the free amino acid pool of earthworms (Rosenberg and Ennor, 1961a) was in keeping with this viewpoint. However, in the present experiments no particular preference for the D-isomer was evident (Tables 6 and 7).

After the administration of radioactive ethanolamine the guanidinoethanol portion of the lombricine molecule became labelled almost exclusively. The results obtained from the administration of radioactive serine suggested that the guanidinoethanol portion of lombricine acquired label either directly from phosphatidylethanolamine, or indirectly from free ethanolamine, which in turn was derived from phosphatidyl ethanolamine. The possibility arises therefore that administered ethanolamine might require incorporation into phosphatidyl ethanolamine prior to its incorporation into SEP. If this is the case then ethanolamine would be incorporated via the pathways described by Kennedy and Weiss (1956).



Alternatively one could postulate that CDP-ethanolamine (or "activated" ethanolamine) might react with free serine to form SEP. It seems improbable that serine would be phosphorylated prior to its incorporation into SEP for in the absence of any known serine kinase (Nemer and Elwyn, 1957) the formation of phosphoserine would involve a circuitous pathway via carbohydrate (Ichihara and Greenberg, 1957).

This would lead to gross isotope dilution, which would not be in accord with the experimental findings.

The tracer studies have thus demonstrated that both SEP and lombricine may be biosynthesised from small molecular weight precursors such as serine, phosphate, and ethanolamine. Moreover, the rate of incorporation definitely excluded the possibility that SEP is a degradation product of lombricine. The intriguing possibility that phospholipids may be intimately involved in SEP biosynthesis cannot be resolved in the present experiments, and must await more definitive information which should ultimately become available from in vitro studies.

SUMMARY

1. After the oral administration of [^{32}P]orthophosphate, DL-[3- ^{14}C]serine D- and L-[^3H]serine and [1:2 $^{14}\text{C}_2$]ethanolamine to earthworms, each of these compounds was incorporated into SEP and lombricine of viscera and muscle tissues.
2. The specific radioactivities of SEP in viscera and muscle were always considerably in excess of those of lombricine at time intervals ranging from one to sixteen days.
3. The specific radioactivities of SEP and lombricine in viscera were much higher than those of the corresponding compounds in muscle.
4. The distribution of label within the lombricine molecule was such that after the administration of radioactive serine both the serine and the guanidinoethanol moieties became labelled. With radioactive ethanolamine only the guanidinoethanol portion of the lombricine molecule contained significant label.
5. Comparison of the rates of incorporation of [^{32}P]orthophosphate showed a fairly rapid labelling of ATP and PL with much slower labelling of SEP, lombricine, phospholipids and phosphoproteins.
6. It is concluded that serine, ethanolamine and phosphate are precursors of SEP and lombricine, and that SEP is not a degradation product of lombricine.

CHAPTER 2.

TRANSAMIDINATION IN THE EARTHWORM

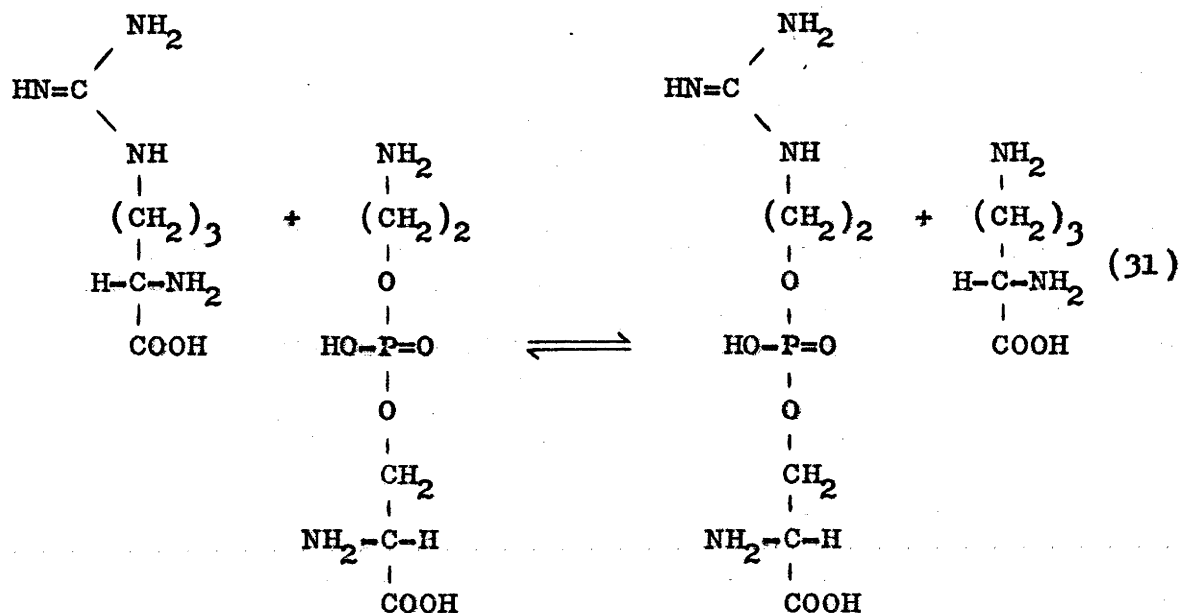
CHAPTER 2.

TRANSAMIDINATION IN THE EARTHWORM

INTRODUCTION

Several lines of evidence suggest that SEP is the biological precursor of lombricine. In addition to its obvious structural relationship to lombricine SEP is present in low concentration (30 mg./kg.) in earthworm tissue, as might be expected of a biological intermediate; also, as is the case with lombricine, the serine moiety of SEP possesses the unusual D-configuration. Furthermore, SEP acquires label from administered isotopes at a rate consistent with its being the precursor of lombricine.

It was therefore considered worthwhile to investigate the existence in earthworms of a pathway for the conversion of SEP to lombricine by a transamidinase catalysed reaction involving arginine as shown below.



L-arginine

D-SEP

D-lombricine

L-ornithine

The studies presented in this chapter are mainly concerned with attempts to demonstrate that lombricine is biosynthesised according to the above reaction.

While the results obtained from in vivo studies have provided convincing evidence that transamidation does in fact occur in earthworms, the results of in vitro studies have been uniformly negative. However, this was not entirely unexpected for the high arginase activity in earthworms (Cohen and Lewis, 1950; Robin, 1954) poses special problems in demonstrating transamidation reactions.

Some experiments were designed, therefore, to test the ability of purified mammalian transamidinase, which has a low arginase activity, to utilise SEP as an

amidino group acceptor.

In addition, some consideration was given to the possibility that lombricine could be formed in a manner analogous to arginine biosynthesis. It would be anticipated that the final step in such a pathway would involve the reversible cleavage of lombricinosuccinate to lombricine and fumarate, similar to the reversible splitting of argininosuccinate to arginine and fumarate. If such a mechanism did occur it should be possible to demonstrate the formation of lombricinosuccinate from fumarate and lombricine by crude earthworm extracts (cf. Davison and Elliott, 1952).

MATERIALS

Animals

The earthworms were as described in Chapter 1.

Substrates

L-SEP was obtained from alligator muscle as described by Beatty et al. (1961) and was kindly donated by these workers.

D-SEP and D-lombricine were obtained from earthworms as described by Ennor et al. (1960).

Glycine, L-ornithine-HCl, L-canavanine-sulphate, L-arginine-HCl were obtained from California Corporation for Biochemical Research.

Taurocyamine (2-guanidinoethyl sulphonic acid) and 2-guanidinoethanol were synthesised in this laboratory according to the method of Schutte (1943).

D-Arginine was a gift of the late Dr. J. Greenstein.

L- [Amidino- ^{14}C] arginine was prepared from $\text{Na } ^{14}\text{CN}$ (1 mC) and L-ornithine monohydrochloride as described by Stetten and Bloom (1956). The specific radioactivity of the arginine thus obtained was 0.042 $\mu\text{C}/\mu\text{mole}$.

Chelating Agents

EDTA was a commercial preparation. 8-Hydroxyquinoline-5-sulphonic acid was a gift from Professor A. Albert.

Buffer Components

N-Ethylmorpholine (Eastman Kodak) was distilled twice under reduced pressure (35°) and diluted with water to 1 M. It was stored at -10° .

All other reagents used were analytical grade.

METHODS

The intubation procedure, method of isolation of lombricine, SEP and other amino acids from viscera and muscle, and the technique for the determination of

specific radioactivity of these compounds after paper chromatography, were as described in Chapter 1. Dosages and time intervals were as noted in the tables.

Protein estimation

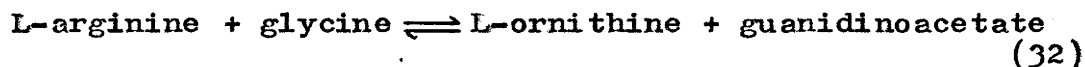
Protein was estimated colorimetrically by the biuret method of Gornall, Bardawill and David (1949).

Estimation of ornithine

Ornithine was estimated colorimetrically by the method of Chinard (1952). Buffers, amino acids, and especially SEP, have the effect of depressing ornithine colour development. This was allowed for by supplementing each set of standards (0.04 - 0.2 μ moles ornithine) with a solution identical with the incubation mixture.

Transamidinase assay

In the forward reaction the transamidinase activity of hog kidney and earthworm preparations were assayed by measuring the rate of L-ornithine release from L-arginine as illustrated:



The reaction mixture (1 ml.) contained 0.1M-NEM-HCl buffer, pH 7.5, 0.02M-L-arginine, 0.02M-glycine and enzyme preparation. The reaction was started by the addition of enzyme preparation, and after 20 min. at 30° was stopped

with 2 ml. of 10% TCA. Arginase activity of the enzyme preparations was assessed by the inclusion of control tubes from which amidino group acceptors were omitted during the incubation. Protein was removed by centrifugation and samples were taken for the estimation of ornithine. The amount of enzyme extract added to each reaction mixture was adjusted so that no more than 0.2 μ mole of ornithine was formed in the control tubes. Transamidinase activity was assessed as the difference between the ornithine released in the complete system and that released in the control.

One unit is defined as the amount of enzyme which catalyses the formation of 1 μ mole of ornithine per min. at 30° under the conditions stated in the assay procedure.

Specific activity is expressed in units per mg. of protein.

Purified mammalian transamidinase

An active transamidinase preparation was obtained from hog kidneys by the procedure described by Ratner and Rochovansky (1956a). The preparation (15 mg. of protein per ml.) had a specific activity of 0.15 which represented an overall 20-fold purification. The preparation was stored at -5°.

RESULTS

(a) Transamidination in vivo

In order to demonstrate possible transamidination from arginine to SEP in vivo the earthworms were given appropriate amounts of L-[amidino- ^{14}C] arginine, and the distribution of the label was checked after two days. Within that time the worms were grossly oedematous. Approximately 60% of the body weight was fluid, a rise of 40% above normal values. Two days from administration of [^{14}C] arginine (Table 11) lombricine in both muscle and viscera had incorporated radioactivity (540 and 120 cpm/g. body weight respectively). In lombricine of muscle this label was located in the guanidinoethanol portion of the molecule, with negligible radioactivity in the serine moiety. No label was detected in SEP either in muscle or viscera. In these experiments free ornithine and free arginine, both of which were not normally identified on chromatograms, were found in considerable quantities in earthworm muscle. Whereas the ornithine contained negligible counts the arginine contained 12,400 cpm/g. body weight.

In another experiment (Table 11) the specific radioactivities of SEP, lombricine and amino acids were determined. Radioactivity in SEP in muscle and viscera,

TABLE 11.

Incorporation of radioactivity from L-[amidino-¹⁴C] arginine into the serine ethanolamine phosphodiester, lombricine and certain free amino acids of the muscle and viscera of the earthworm

Each worm was given 20 μ moles L-[amidino-¹⁴C] arginine (equivalent to 10^6 counts/min.)/g. body weight in experiment 1, and twice that amount in experiment 2. They were killed 2 days later. Figures in parentheses represent the percentage of total radioactivity in the relevant portion of the lombricine molecule. "Negligible" indicates that the net counting rate was less than one half of the background counting rate.

	Experiment 1.		Experiment 2.	
	Radioactivity (counts/min./g.worm)		Specific radioactivity (counts/min./ μ mole)	
	Muscle	Viscera	Muscle	Viscera
SEP	negligible	negligible	negligible	negligible
Lombricine (total)	540	120	329	382
Lombricine (serine moiety)	negligible	-	negligible	negligible
Lombricine (guanidino- ethanol moiety)	525 (97%)	-	318 (97%)	348 (91%)
Ornithine	negligible	-	negligible	-
Glutamine; Alanine Aspartate	-	-	negligible	-
Arginine	12,400	-	38,700	-

and in the free ornithine, glutamine, aspartate and alanine in muscle was negligible. Considerable counts were observed however, in lombricine in muscle and viscera, and almost all of these counts were in the guanidinoethanol portion of the molecule. The free arginine in muscle had a specific radioactivity of 0.38×10^5 cpm/ μ mole, which was not greatly different from the specific radioactivity (0.5×10^5 cpm/ μ mole) of the arginine that had been given two days previously.

(b) Transamidination in vitro

In preliminary experiments a variety of extraction procedures were utilised: homogenisation in a Micro Waring Blendor, disintegration with a Potter Elvehjem all-glass homogeniser, freezing in liquid air followed by powdering with pestle and mortar, grinding with silica, etc.

Also a variety of extracting mediums were used: 0.01M-KCl, pH 7.0, 0.02M-potassium phosphate buffer, pH 7.4, 0.02M-NEM-HCl, pH 7.4. To some of these buffers chelating agents, 0.001M-EDTA, pH 7.0, or 0.0001M-8-hydroxyquinoline-5-sulphonic acid, pH 7.0, were added. To others cetyl-trimethyl ammonium bromide (0.1%) was added to assist solubilisation of particulate enzymes.

Since neither the extraction procedure nor the type of buffer, chelating agent, or detergent used made

any difference to the results it would be pointless to elaborate these procedures in detail. However, a typical enzyme preparation will be briefly described as representative of the procedure employed.

Preliminary experiments with crude earthworm extracts, using the transamidinase assay (see Methods) showed that the arginase activity of such preparations was sufficiently high to preclude the detection of any transamidinase activity. Fractionation of the viscera extracts with ammonium sulphate was therefore carried out in the hope of achieving some separation of arginase from transamidinase.

Preparation of earthworm extracts

All operations were carried out in the cold room at 4°, and centrifugations were at 15,000 g. for 15 min. Precipitates were taken up in minimal quantities of 0.02M-NEM-HCl buffer, pH 7.5.

Eight live earthworms (Megascolides cameroni) were rapidly dissected into viscera and muscle. The pooled viscera (16 gm.) were homogenised for 3 min. in a Micro Waring Blendor with 3 vol. of 0.01M-NEM-HCl buffer, pH 7.5 (containing 0.001M-EDTA). The frothy homogenate was centrifuged, and the turbid supernatant filtered through glass wool. The precipitate consisting mainly

of soil and cell debris was suspended in buffer (Fraction I).

To the supernatant (Fraction II, 70 ml.) saturated ammonium sulphate solution, adjusted to pH 7.4 with 1 N-NH₄OH, was added dropwise with efficient stirring over a 20 min. period until 0.3 saturation was attained. After standing for 30 min. the solution was centrifuged. The precipitate was suspended in buffer as stated above, (Fraction III), and the supernatant brought to 0.45 saturation by the further addition of ammonium sulphate solution. After centrifugation the precipitate was suspended in buffer (Fraction IV). In a similar fashion 0.45 - 0.6 (NH₄)₂SO₄ (Fraction V) and 0.6 - 0.8 (NH₄)₂SO₄ (Fraction VI) saturation were attained by the slow addition of solid ammonium sulphate.

All resuspended precipitates were dialysed for 12 hr. against 20 vol. of 0.02M-NEM-HCl buffer, pH 7.5, with three changes. It was found that the dialysis sacs had to be changed every 6 hr. to prevent minute perforation developing. Since these perforations only developed when worm viscera extract was dialysed, it seems reasonable to suppose that they were caused by earthworm intestinal cellulases.

Extracts were also made of whole earthworms and earthworm muscle by procedures similar to those described

for earthworm viscera.

Attempts to demonstrate transamidination in the forward reaction

The transamidinase assay (see Methods) was employed as described. In addition 0.02M-D-arginine was tried as a donor, and 0.02M-D-SEP or L-SEP were tried as acceptors in numerous experiments.

Table 12 shows the distribution of arginase activity in muscle and viscera tissues. It is evident that crude viscera extracts (Fraction II) and the 0.6 - 0.8 $(\text{NH}_4)_2\text{SO}_4$ fraction (VI) contain the highest arginase activities, with specific activities of 4 and 1 respectively. The arginase activities of the other $(\text{NH}_4)_2\text{SO}_4$ fractions (III, IV, and V) are much lower. Negligible arginase activity was found in earthworm muscle. Assay systems to detect transamidinase activity based on ornithine release proved too insensitive in worm extracts in which arginase activity was high, such as in Fractions II and VI. The ornithine released in the complete system in 20 min. incubations frequently differed from that in the control by comparatively minute amounts which did not exceed the limits of experimental error. No transamidination was observed with Fractions III, IV, and V in which arginase activity was low, or with muscle extracts.

In certain experiments D-arginine, which was not attacked by arginase, was tried as an amidino group donor, but no transamidination was detected.

Since arginase is a metal enzyme which requires when impure either Mn^{2+} , Co^{2+} or Ni^{2+} for activation (Greenberg, 1960), it seemed feasible to reduce its activity by removal of metal ions by dialysis against chelating agents. Therefore Fraction II was dialysed against 20 vol. of 0.02M-NEM-HCl buffer, pH 7.0, containing either 0.001M-EDTA or 0.0001M-8-hydroxy quinoline-5-sulphonic acid, for three days with three changes of buffer. However, as is apparent from Table 12, no appreciable decrease in arginase activity was obtained.

The effect of thermal denaturation and isoelectric precipitation on the arginase activity of crude viscera extracts was also determined. Thus Fraction II was heated rapidly with stirring to 55° in a water bath, and maintained at this temperature for 7 min. After cooling to 2° , denatured protein was removed by centrifugation. The supernatant was retained for enzyme assay. Another portion of Fraction II was taken to pH 5.4 with stirring by the cautious addition of 0.1 N-HCl. Precipitated protein was removed by centrifugation and the pH of the supernatant readjusted to pH 7.5 with 0.1 N-NaOH. The supernatant was retained for enzyme assay.

TABLE 12.

Summary of the L-arginase activities of various preparations used in attempts to demonstrate transamidination in vitro in the earthworm

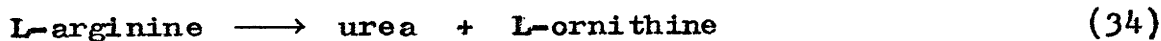
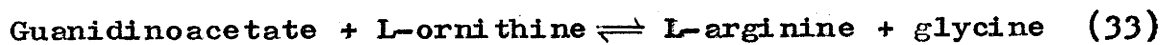
			L-arginase activity (units)	Trans-amidase activity
<u>Extracts of whole worm</u>				
	Isoelectric precipitation step		0.005	0
	Thermal denaturation step		0.003	0
<u>Extracts of worm viscera</u>				
	Fraction I, resuspended particles		-	0
	Fraction II, crude extract		4.0	0
	Fraction III	0 -0.3	0.17	0
	Fraction IV	0.3 -0.45	0.06	0
Ammonium Sulphate Fractionation	Fraction V	0.45-0.6	0.05	0
	Fraction VI	0.6 -0.8	1.0	0
	Fraction II, dialysed against chelating agents		1.0	0
<u>Extracts of worm muscle</u>				
	Crude muscle extract		0	0

The units of arginase activity are expressed as μ moles of ornithine/min./mg. protein.

The effects of the above procedures are shown in Table 12. Arginase activity was reduced by isoelectric precipitation but transamidinase was not demonstrable. Attempts to selectively destroy arginase activity by thermal denaturation were equally unsuccessful, for despite the lowering of arginase activity no transamidinase was detected (Table 12).

Attempts to demonstrate transamidination in the reverse reaction

Because high arginase activities of earthworm viscera extracts interfered with the detection of transamidinase activity in the forward reaction, attempts were made to utilise arginase activity by assisting the reverse reaction, as illustrated by the following equations:



The net result would be the disappearance of guanidinoacetate with ornithine acting in a catalytic fashion. Thus the ornithine-dependent disappearance of guanidinoacetate would be a measure of transamidinase activity and could be followed colorimetrically.

The reaction mixture (2 ml.) contained 0.1M-buffer (imidazole-HCl, potassium phosphate, NEM-HCl,

or sodium pyrophosphate) to cover a range of pH values 6.3 to 8.4, L-ornithine (0.01M, 0.001M, or 2×10^{-4} M), amidino compounds such as D-lombricine, taurocyamine, or guanidinoacetate, at one of three concentrations (0.016M, 0.005M, or 5×10^{-4} M) and the enzyme preparation. The reactions were started by the addition of 1 ml. of worm extract and stopped after time periods ranging from 1 hr. to 24 hr. at 30° by the addition of 2 ml. of 10% HClO₄. Control tubes contained no ornithine during the incubations. A crystal of thymol was added to mixtures incubated for periods longer than 3 hr. After removal of protein by centrifugation, suitable portions were taken for the estimation of guanidino-reacting compounds. Transamidation was assessed as the difference between guanidino-reacting compounds in the complete incubation system and the control.

Attempts to demonstrate transamidation in the forward reaction using L-canavanine as an amidino group donor

Canavanine does not form a coloured complex with diacetyl reagent (Rosenberg et al., 1956) used to detect guanidino-reacting compounds. Therefore, during the transfer of the amidino group from canavanine to form another guanidino-compound as illustrated below,

L-canavanine + glycine → guanidinoacetate + L-canaline

the reaction can be followed by the appearance of a compound reacting with the diacetyl reagent.

This is the basis of the present assay procedure which was designed to demonstrate amidino group transfer between L-canavanine and either glycine or L-SEP.

The reaction mixture (1 ml.) contained 0.1M-NEM-HCl buffer, pH 7.5, 0.02M-L-canavanine, 0.02M-glycine (or D-SEP or L-SEP) and enzyme extract. The reaction was started by the addition of enzyme, and after 3 hr. at 30° was stopped with 2 ml. 10% HClO₄. After removal of protein by centrifugation suitable portions were taken for the estimation of guanidino-reacting compounds. Transamidination was assessed by the appearance of a guanidino-reacting compound in the complete incubation system as compared with the control tubes from which acceptors were omitted.

Attempts to demonstrate transamidination by a chromatographic procedure

Hydroxylamine in high concentration accepts the amidino group of L-arginine in the presence of transamidinase to form hydroxy-guanidine (Walker, 1957). This latter compound may be detected on paper chromatograms with the diacetyl reagent. The reaction mixture (1 ml.) contained 0.1M-potassium phosphate buffer, pH 7.5, 0.1M-L-arginine, 0.4M-NH₂OH, and enzyme preparation. After

4 hr. incubation at 30°, the reaction was stopped with 0.5 ml. of 30% TCA, and after removal of protein by centrifugation, and of TCA by ether extraction, suitable portions were taken for chromatography as described by Walker (1957). Transamidation was assessed by the appearance of hydroxy-guanidine on the chromatograms.

A similar chromatographic procedure was used in attempts to demonstrate a transamidation reaction between guanidinoacetate and D-SEP. The expected reaction products would be D-lombricine and glycine.

The results with assay systems based on the disappearance of amidino group donors were uniformly negative. In systems where canavanine was tested as a donor, no compounds reacting with 1-naphthol-diacetyl reagent were formed. Attempts to demonstrate chromatographically the appearance of hydroxyguanidine through amidino group transfer from arginine to NH_2OH , or the appearance of D-lombricine by transfer from guanidinoacetate to D-SEP, were equally unsuccessful.

Transamidation studies with purified hog kidney transamidinase

An active transamidinase from hog kidney was prepared as described by Ratner and Rochovansky (1956a). As transamidinases from various sources evidence a relative lack of specificity with regard to donors and

acceptors (see Introduction) it was considered that SEP might act as an acceptor of the amidino group from arginine in the presence of purified hog kidney transamidinase. Although hog kidney transamidinase catalysed the conversion of 0.15 μ mole of glycine/min./mg. of protein to guanidinoacetate with L-arginine as the donor (Table 13) under the same experimental conditions, neither isomer of SEP acted as an acceptor of the amidino group.

Attempts to demonstrate the formation of lombricino-succinate

The assay system employed was similar to that described by Davison and Elliott (1952) and Ratner (1957) for the formation of argininosuccinate.

The reaction mixture (5 ml.) contained 0.1M-potassium phosphate buffer, pH 7.5, 0.1M-potassium fumarate, and 0.1M-D-lombricine. A crystal of thymol was added, and the incubation started by the addition of 1 ml. of crude whole worm homogenate, prepared as described previously. Samples (1.0 ml.) were removed into tubes containing 1 ml. of 10% HClO_4 at zero time, 1, 3, 6 and 15 hr. The protein was removed by centrifugation, and the pH adjusted to 7 by addition of 10 N-KOH. After standing for 30 min. at 0° the precipitated KClO_4 was removed by centrifugation, and a

TABLE 13.

Studies of hog kidney transamidinase with
L-arginine as donor

Amidino group acceptor	Transamidinase activity		
		(units)	
	5 min.	10 min.	15 min.
Glycine	0.78	1.55	2.4
D-SEP	0	0	0
L-SEP	0	0	0

The units of transamidinase activity are expressed in terms of μ moles of ornithine released per mg. protein in the time intervals indicated.

sample of the neutralised extract (0.02 ml.) was chromatographed directly as described in Chapter I, while the remainder was heated for 30 min. at 100° to form the cyclic anhydride (analogous to the formation of the cyclic anhydride of argininosuccinate). A sample of the heated extract (0.02 ml.) was also chromatographed as above. The chromatograms were developed with 0.2% ninhydrin and with Jaffe reagents respectively. However, no compound was identified on any of the chromatograms which would have corresponded to lombricosuccinate.

DISCUSSION

The administration of L- [amidino-¹⁴C] arginine to earthworms was followed by the slow incorporation of label into lombricine, both in viscera and in muscle. No label was detected in either SEP or ornithine. These findings were consistent with the occurrence of a transamidination reaction involving SEP and L-arginine, as formulated in equation

Degradation experiments revealed that the label incorporated into lombricine was restricted to the guanidinoethanol portion of the molecule, which indicated that at least one carbon of this portion was derived from the amidino group of arginine. No significant label was detected in the serine portion of lombricine. It follows

that there would be no label in the ethanolamine portion of the guanidinoethanol moiety of lombricine, since ethanolamine is derived from serine (cf. Chapter 1). It may be concluded, therefore, that the label in the guanidinoethanol portion of lombricine was located in the amidino carbon, and that therefore transamidation must be the final step in lombricine biosynthesis.

Some attention was given to the possibility that the label found in the amidino group of lombricine could have been incorporated from $^{14}\text{CO}_2$ for the latter might have been formed by the concerted action of arginase and bacterial urease on the administered arginine. However, since none of the free amino acids other than arginine were radioactive, there was no likelihood of label being incorporated into lombricine from $^{14}\text{CO}_2$.

It has been observed previously that labelled precursors were incorporated rapidly into SEP and slower into lombricine (Chapter 1) from which it could be inferred that transamidation was the rate controlling step in lombricine biosynthesis. The tardy incorporation of label from arginine reported herein also lends support to this viewpoint. It is of interest that creatine biosynthesis is regulated by repression and restoration of the arginine-glycine transamidinase enzyme in which the ultimate end product (creatinine) exerts a negative

feedback control on the first enzyme of the biosynthetic sequence (transamidinase). By analogy the control of lombricine biosynthesis might also be at the transamidinase level.

However there are additional factors which could contribute to the slow incorporation of L- [amidino-¹⁴C] arginine into lombricine. Firstly the administration of arginine proved toxic to the earthworms, as judged by the gross oedema which subsequently developed. In this connection Cohen and Lewis (1949) have also noted the toxic effects of high arginine levels in earthworms. Van Pilsum et al. (1957) have reported that toxic factors depressed transamidinase levels in other animals, and a comparable effect might also be expected to occur with transamidinase in earthworms.

Secondly, although ornithine was not normally detected on chromatograms of earthworm extracts, large quantities were found after the administration of arginine. Almost all this ornithine would have arisen from the hydrolysis of arginine by arginase. Since ornithine is a potent inhibitor of all known transamidinases, it would be anticipated that the high ornithine concentration would similarly inhibit transamidination reactions in earthworms (see below).

Despite the fact that in vivo studies provided

convincing evidence for the existence of transamidination attempts to confirm this in vitro were unsuccessful. The main reason for this failure may be attributed to the high arginase activity encountered in viscera extracts. Arginase rapidly destroys the physiological amidino group donor, arginine, with the concomitant production of ornithine, which, as stated earlier, is a powerful transamidinase inhibitor. In addition, in the forward reaction, any assay system based on ornithine release as a measure of transamidination is rendered too insensitive to be of value. The inability to demonstrate arginine-SEP or arginine-NH₂OH transfers may be due to the above factors. The same considerations apply to canavanine-SEP transfer, for arginase hydrolyses canavanine to urea and canaline, and the latter compound is also an inhibitor of transamidination reactions.

The obvious solution to the problem raised by high arginase activities is to find some means of separating arginase from transamidinase. In larger animals this poses no real difficulty for these enzymes are located in different organs. In ureotelic animals, for example, arginase is found chiefly in the liver and mammary gland (Greenberg, 1960), whilst transamidinase occurs mainly in the pancreas and kidney (Walker, 1958a). On the other hand, in uricotelic animals transamidinase

is located in the liver, whereas arginase is absent from the livers of these animals (Clementi, 1919; Banerova and Sorm, 1956). In earthworms arginase is confined to the viscera (Table 12), and although arginase and transamidinase may well be separated from one another at the cellular level, the small size of the viscera precludes any separation of these enzymes by method relying on gross anatomical differentiation.

Earthworm muscle contained negligible arginase, but no transamidinase was detected. This finding was not unexpected for the only report of transamidinase activity in muscle extracts has been that of Borsook and Dubnoff (1941a), who found very low levels of transamidinase in heart and skeletal muscle of the pigeon.

The effect of ammonium sulphate fractionation on the distribution of arginase activity of viscera extracts is presented in Table 12. Although this activity has been considerably reduced in Fractions III, IV and V, no transamidinase was demonstrable.

There is no known specific inhibitor of arginase. Attempts were made therefore to reduce its activity by removal of activating metal ions with chelating agents. However, despite prolonged dialysis against EDTA or 8-hydroxyquinoline for the arginase specific activities only fell from 4 to 1, which was still too high to

demonstrate transamidination reactions. Considerable reduction in arginase activity was effected by thermal denaturation and isoelectric precipitation, but once again no transamidinase was demonstrable. Presumably the latter enzyme was inactivated or removed together with the arginase.

In some assay systems attempts were made to utilise arginase activity by studying the transamidination reaction in the reverse direction. Since any arginine formed in this manner is rapidly hydrolysed to urea and ornithine, only catalytic amounts of ornithine are required, and inhibition of transamidinase by ornithine should be circumvented. However, although no ornithine-dependent disappearance of guanidinoacetate, taurocyamine or lombricine was evident, there are reasons, other than lack of transamidinase, which could be advanced as an explanation of the failure to demonstrate the above reaction. Thus, although in mammals and in S. griseus guanidinoacetate has been shown to be an amidino group donor, it does not follow that taurocyamine and lombricine can serve in this manner. The only possibility indicated is the known reversibility of all transamidination reactions reported so far.

In addition, the rate of transamidination

reactions in the reverse direction is much slower than in the forward direction. It has been demonstrated (Ratner and Rochovansky, 1956a) that arginine-glycine transfer catalysed by mammalian transamidinase was about six times faster in the forward direction. If similar slow rates occurred with earthworm transamidinase, it would prove extremely difficult to detect a small disappearance of either guanidinoacetate, taurocyamine or lombricine. Furthermore if prolonged incubation periods are necessary for significant amounts of these compounds to disappear, this could well provide sufficient time for intestinal peptide hydrolases to destroy transamidinase (see below).

Purified transamidinase from hog kidney catalysed the formation of guanidinoacetate from arginine and glycine at a rate of 0.155 μ moles/min. (Table 13). Under the same conditions neither isomer of SEP served as an acceptor. While it is recognised that, in general, transamidinases are relatively non-specific as regards donors and acceptors, it is known that the transamidinase of S. griseus is one exception to this, for the latter enzyme will not utilise glycine as an acceptor. The inability of mammalian transamidinase to catalyse arginine-SEP transfer, as reported herein, may reflect a similar type of specificity.

In the following chapter evidence is adduced that the peptide hydrolase activity of earthworm visceral extracts is high. Attempts to purify lombricine kinase from preparations in which viscera extracts were present met with failure, whereas when muscle extracts were used as starting material it was possible to prepare a stable lombricine kinase. It seems highly probable that peptide hydrolases could also destroy earthworm transamidinase, and contribute to the already formidable difficulties of demonstrating the latter enzyme in in vitro experiments.

Crude earthworm extracts failed to catalyse the formation of lombricinosuccinate from lombricine and fumarate. Under similar experimental conditions crude pea extracts catalysed rapid formation of considerable quantities of argininosuccinate (Davison and Elliott, 1952). The lack of lombricinosuccinate formation suggests that lombricine is not synthesised by a pathway analogous to arginine.

SUMMARY

1. Following the administration of L- [amidino-¹⁴C]- arginine to earthworms, label was incorporated into lombricine, but no radioactivity was detected in SEP or ornithine.
2. The results of degradation studies of the isolated lombricine were consistent with the label being located in the amidino carbon.
3. It is concluded that the final step in lombricine biosynthesis is a transamidination reaction involving L-arginine and D-SEP.
4. In vitro studies with earthworm extracts failed to demonstrate transamidination; the possible causes for this failure are discussed.
5. An active mammalian transamidinase did not catalyse transamidination reactions between L-arginine and SEP.
6. Crude earthworm extracts failed to catalyse the formation of lombricinosuccinate from lombricine and fumarate.

CHAPTER 3.

PURIFICATION AND PHYSICAL PROPERTIES

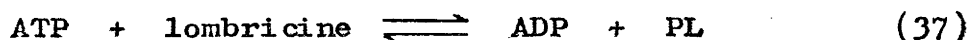
OF LOMBRICINE KINASE

CHAPTER 3

PURIFICATION AND PHYSICAL PROPERTIES
OF LOMBRICINE KINASE

INTRODUCTION

The previous chapters dealt with the biosynthesis of lombricine. The work described in this, and the following chapter, is concerned with the further metabolism of lombricine, namely with the reaction catalysed by lombricine kinase:



This enzyme has not been hitherto described in any detail. Pant (1959) studied this reaction in the forward direction with crude homogenates and acetone powder extracts of whole earthworms, Lumbricus terrestris. At pH 9.0 these extracts showed some activity, with minor stimulation of the reaction by Mg^{2+} ions. In addition to lombricine two other guanidino compounds possessing acidic moieties, taurocyamine (2-guanidinoethyl sulphonic acid) and 2-guanidinoethyl phosphate, acted as phosphoryl group acceptors in the reaction. It was noted that lombricine kinase activity was lost on prolonged dialysis, and that

addition of concentrated dialysate to the enzyme system did not restore activity. It was suggested that this loss of enzymic activity was due to the removal of a labile cofactor by dialysis.

In a preliminary note Rosenberg et al. (1960) described the partial purification of lombricine kinase isolated from the muscle of Megascolides cameroni. This enzyme preparation rapidly phosphorylated L-lombricine, as well as D-lombricine and taurocyamine, and was activated by Mn^{2+} and Co^{2+} ions, in addition to Mg^{2+} ions.

The experimental work reported in this chapter provides an extension of these studies, and is concerned with the purification and physical properties of lombricine kinase.

MATERIALS

All reagents were analytical grade. Glass distilled water was used for the preparation of solutions of the various reaction components. An aqueous solution of $MgCl_2 \cdot 6H_2O$ (British Drug Houses) was prepared as a 0.1M stock solution and stored at 2°. From this 2.5×10^{-2} M solutions were made up as required.

Animals

Small earthworms, Octolasion cyaneum and

Allolobophora caliginosa, were obtained locally, and after freezing in liquid air, were stored at -20° .

Large earthworms, Megascolides cameroni, were obtained from the Australian Capital Territory and southern New South Wales. Each live worm was divided into muscle and viscera by dissection. The muscle was washed in cold water, dropped into liquid air, and stored at -18° .

Phospholombricine

PL was isolated from natural sources as described by Ennor and Rosenberg (1962), or was prepared enzymically from ATP and D-lombricine (see Appendix 2 for details). For routine assays 100 mg. of PL (Mg^{2+} salt) was converted prior to use to the Na^{+} form by passage through a column (15 cm. x 2 cm.) of Dowex-50 (200-400 mesh, Na^{+}) ion exchange resin. The effluent was collected in 2 ml. fractions, and those containing PL were pooled. The concentration of the PL solution was assayed (see Methods), adjusted to the desired volume and stored at -10° .

D-lombricine

D-lombricine was prepared from natural sources as described by Ennor et al. (1960), and was recrystallised four times from aqueous ethanol. It was dried in air and stored at room temperature. Elementary analysis

showed the product to be pure. Solutions (0.05 M) were adjusted to pH 8.6 with 1 N-KOH and stored at -10° .

L-lombricine

L-lombricine was synthesised as described by Beatty and Magrath (1960) and was a kind gift from these workers.

Adenosine-5'-diphosphate, sodium salt

ADP was obtained from Sigma Chemical Co. As chromatography in isobutyric acid/ NH_3 sp.gr. 0.88/water (66:1:33, v/v) showed only trace amounts of ATP and AMP, no further purification was considered necessary. ADP was stored at -10° over CaSO_4 . Solutions were standardised by measurement of ADP concentration at 259 $\text{m}\mu$, and after dilution to 1×10^{-2} M stored at -10° .

Adenosine-5'-triphosphate, disodium salt

Crystalline ATP obtained from Sigma Chemical Co. was recrystallised twice from aqueous ethanol (Berger, 1956). The free orthophosphate was less than 0.1% after the recrystallisation procedure. The dried ATP was stored at -10° over CaSO_4 . Solutions were adjusted to pH 8.6 with 1 N-KOH, standardised by measurement at 259 $\text{m}\mu$ and, after dilution to 2.5×10^{-2} M, stored at -10° .

Nucleotides

CDP, UDP, GDP, IDP and deoxyadenosine diphosphate were obtained from Sigma Chemical Co.

Buffer systems

Triethanolamine, purified after the method of Germann and Knight (1933) was kindly supplied by Mr. W.J. O'Sullivan.

N-Ethylmorpholine (NEM) was obtained from Eastman-Kodak and was twice distilled under reduced pressure at 38°. It was stored at -10° as a 1M stock solution. For routine preparative work stock NEM was adjusted to the required pH with 5 N-HCl and diluted to a 0.5M solution.

The components of other buffer systems, glycine and imidazole were obtained from California Corporation for Biochemical Research.

Guanidino compounds

Taurocyamine, γ -guanidino-propionic acid, γ -guanidino-n-valeric acid, 2-guanidinoethanol-HCl and 2-guanidinoethyl phosphate were synthesised in this laboratory by the method of Schütte (1943).

L-Arginine-HCl, guanidinoacetate and L-canavanine sulphate were obtained from California Corporation for Biochemical Research.

Creatine was a thrice recrystallised commercial preparation.

Sulphydryl reagents

p-Hydroxy mercuribenzoate (HMB) was obtained

from Sigma Chemical Co. Other sulphhydryl reagents such as o-iodosobenzoic acid, N-ethylmaleimide, phenyliodoacetate, were available as departmental material.

Inhibitors

Arsenite and dinitrophenol were obtained from British Drug Houses.

METHODS

Estimation of lombricine and PL

Lombricine was estimated colorimetrically by the method described by Rosenberg et al. (1956) and Morrison, Griffiths and Ennor (1957) as follows: To 1 ml. of reaction mixture was added 1 ml. of NaOH-EDTA solution (3N-NaOH containing 0.04M-EDTA) and 2 ml. of 1-naphthol-diacetyl reagent. The volume was adjusted to 10 ml. with water, and after allowing 30 min. at room temperature (usually 25°) for colour development, the extinction of the coloured complex was measured in a Hilger Spekker absorbiometer with an Ilford Spectrum green filter. In a cell with a 1 cm. light path 0.5 μ mole of lombricine gave an extinction of 0.4.

PL was estimated as lombricine, after the PL had been quantitatively hydrolysed to lombricine and orthophosphate by heating for 1 min. in the presence of

0.1 ml. of 5 N HCl at 100°. After cooling to room temperature 0.1 ml. of 5 N-NaOH was added, and the lombricine then estimated as described above. Authentic samples of PL under these conditions were quantitatively hydrolysed, without any destruction of the lombricine released. When significant amounts of free lombricine were present in addition to PL, this method of estimating PL concentration proved too insensitive, and it became necessary in such cases to estimate PL as orthophosphate released under certain conditions (see below).

Cysteine, glycine-KOH buffer and metals present in high concentrations in certain reaction mixtures caused interference with the colorimetric estimation of lombricine. Cysteine inhibition was overcome by the addition of 1×10^{-4} M-HMB (1 ml.) to the usual reagents used for colour development. The inhibition by glycine-KOH buffer was corrected with the aid of appropriate internal controls.

Many metal salts interfere in the above procedure for the estimation of lombricine by forming precipitates (occasionally coloured) on addition of the alkaline reagents used for colour development. To obviate this, reaction mixtures containing metal salts were incubated in 10 ml. centrifuge tubes, and the reaction

stopped by the addition of 0.5 ml. of 5 N-NaOH. After centrifugation at 5000 g for 15 min. a sample (0.5 ml.) of the clear supernatant fluid was taken for the estimation of lombricine as described above. The protein concentration (5-10 μ g of protein/ml.) was too low to cause any interference with colour development.

Estimation of orthophosphate

Orthophosphate was estimated colorimetrically by the method of King (1932) as follows: To the sample (usually 2 ml.) were added 2 ml. of the acid molybdate followed by 0.5 ml. of reducing agent. After mixing and diluting to 10 ml. with water, the colour was allowed to develop for exactly 10 min. at room temperature, and the extinction determined in a Spekker absorbiometer using an Ilford spectrum red filter. In a cell with a 1 cm. light path 0.33 μ mole of orthophosphate gave an extinction of 0.110.

Estimation of protein

Protein was determined colorimetrically by the biuret method of Gornall, Bardawill and David (1949) or by the method of Lowry, Rosebrough, Farr and Randall (1951). Crystalline bovine serum albumin was used as a standard.

Measurement of enzymic activity

The forward reaction



was followed by measuring the rate of PL formation. The latter was estimated colorimetrically as orthophosphate after acid hydrolysis of the PL formed. This assay takes advantage of the high acid lability of PL compared with that of ATP.

The reaction mixture (1 ml.) contained 0.1M-glycine-KOH buffer, pH 8.6, 0.01M-D-lombricine, 0.005M-ATP, 0.005M-MgCl₂, and enzyme (5-10 µg protein). In practice a stock mixture of appropriate concentrations of buffer, lombricine, MgCl₂, and ATP was prepared, and portions (0.8 ml.) pipetted into five graduated (10 ml.), glass stoppered pyrex tubes.

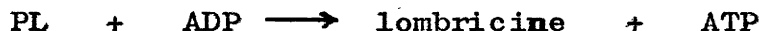
After equilibration for 3 min. at 30° the reaction was started by the addition of 0.2 ml. of appropriately diluted enzyme. Enzyme dilutions were made with cold 0.001M-glycine-KOH buffer, pH 8.6, before use to give a solution containing 50 µg/ml. protein. After incubation the reaction was stopped by the addition of 1 ml. of 0.2M-TCA, and the PL formed during the reaction was quantitatively hydrolysed to lombricine and orthophosphate by heating at 100° for 1 min. After hydrolysis the tubes were cooled rapidly to 0° in an

ice bath, and the total orthophosphate present in the reaction mixtures was estimated by the method of King (1932).

The total orthophosphate present is derived mainly from orthophosphate liberated by hydrolysis of PL, with a minor contribution from ATP, whether originally present as a contaminant in the ATP preparation or liberated to a limited degree during the hydrolysis procedure and colour development. The amount of orthophosphate derived from ATP was calculated by plotting total orthophosphate liberated in the reaction as a function of time. The extrapolation of this plot to zero time provides the correction value as the ordinate intercept.

In some experiments in which the reaction was not followed for varying time periods, the correction value for ATP was determined by inclusion of a zero time control containing the complete incubation system.

The reverse reaction



was followed by measuring the rate of lombricine release from PL.

The reaction mixture (1 ml.) contained 0.1M-NEM-HCl buffer, pH 7.0, 0.005M-PL, 0.002M-ADP, 0.005M-

MgCl₂ and enzyme (1-5 μg). In practice a stock mixture of appropriate concentrations of buffer, PL, MgCl₂ and ADP was prepared, and portions (0.8 ml.) pipetted into five graduated (10 ml.) glass stoppered pyrex tubes.

After equilibration for 3 min. at 30° the reaction was started by the addition of 0.2 ml. of appropriately diluted enzyme solution. Enzyme dilutions were made with cold 0.001M-NEM-HCl buffer, pH 7.0, before use to give a solution containing 20 μg of protein/ml. After incubation the reactions were stopped with NaOH-EDTA solution (1 ml.) and the lombricine released estimated as described above.

Definition of activity unit

One unit of lombricine kinase activity is defined as that amount of enzyme which catalyses the formation of 1 μmole of PL from ATP and lombricine in 1 min. under the conditions described in the assay procedure.

Specific activity

Specific activity is expressed in terms of units per mg. of protein.

Electrophoretic studies

(a) Purified lombricine kinase was analysed by free boundary electrophoresis at pH values ranging from 6.0 to 9.8 in potassium phosphate and veronal-HCl

buffers, using an Antweiler Microelectrophoresis Apparatus. Ionic strength of 0.1M and protein concentrations of 0.5%-1% were employed.

(b) Purified lombricine kinase was analysed by zone electrophoresis on OXOID cellulose acetate strips (25 cm. x 9 cm.) using a Shandon paper electrophoresis apparatus and a voltage gradient of 25 v/cm. The pH values employed ranged from 6 to 9.8 with the buffers described above. At the conclusion of the run the strips were dried in air and stained with Coomassie Blue by the method described by Fazekas de St. Groth, Webster and Dagtner (1962) as follows: the strips were fixed for 2 min. in 20% sulphosalicylic acid, and stained with a 0.25% aqueous solution of Coomassie Blue for 5 min. The excess dye was removed by washing five times in distilled water, each wash lasting for 2 min., and the strips were dried in air.

When it was desired to identify the enzymically active bands on the cellulose acetate strip, electrophoresis was conducted at 0° and the protein bands were located by cutting two longitudinal strips off either edge of the main strip and staining as described above. The protein bands were cut out and eluted with 0.1M-NEM- HCl buffer, pH 7.0, and samples tested for lombricine kinase, adenylate kinase, and

phosphoamidase activity.

Chromatography on ion exchange cellulose

N,N-diethylaminoethyl-cellulose (DEAE-cellulose) with an anion exchange capacity of 0.5 m-equiv/g. (Eastman Organic Chemicals) was converted to triethylaminoethyl-cellulose (TEAE-cellulose) as described by Porath (1957).

The dry TEAE-cellulose (30 g.) was stirred in 2 l. of 0.01M-NEM-HCl buffer, pH 8.6, and the pH readjusted to 8.6 with 1M-NEM (free base). Stirring was continued for 30 min., after which the cellulose was freed from as much liquid as possible on a Buchner funnel. The moist material was resuspended in buffer as before, stirred for another 30 min., filtered again, and resuspended in buffer. At this stage the pH was found to be stable at 8.6, indicating that the TEAE-cellulose was equilibrated with the buffer. The suspension was poured into a column 3 cm. in diameter and allowed to settle under gravity to a height of 40 cm. Approximately 10-bed volumes of 0.01M-NEM-HCl buffer, pH 8.6, was then passed through the cellulose. The column was cooled to 2° prior to use, and all subsequent operations were carried out at that temperature.

Carboxymethyl cellulose (Bio-Rad) with cation exchange capacity of 0.5 m-equiv/g. was equilibrated

against 0.01M-NEM-HCl, pH 7.0, in the same manner as described above for TEAE-cellulose.

Concentration of dilute protein solutions

Dilute protein solutions such as the eluates from TEAE-columns were concentrated by the pressure dialysis procedure described by Hofsten and Falkbring(1960).

Rapid desalting of protein solutions

In certain experiments Sephadex G-25 (Pharmacia, Uppsala, Sweden) was used to remove ammonium sulphate from protein solutions. The gel was equilibrated at 2° with 0.01M-NEM-HCl buffer, pH 8.6, and poured into a column (30 cm. x 3 cm.). The protein solution (70 ml. of a 0.45-0.9 ammonium sulphate fraction of whole earthworm extract) was passed slowly into the gel, and washed through with the above buffer over a 2 hr. period. The desalted protein solution was collected in a volume of 100 ml.

RESULTS

ISOLATION PROCEDURE

Unless otherwise stated all purification steps were carried out in a cold room at 2-4°. The pH was determined with a glass electrode. All centrifugations were carried out at -5°.

Fraction I

Earthworm muscle (100 g.) was dropped in liquid air, ground to a fine powder in a cooled mortar and suspended in 300 ml. of 2.5×10^{-2} M-buffer, pH 7.2, (containing 0.003M-EDTA). The suspension was stirred for 1 hr., disintegrated in a Waring Blendor for 3 min., and the thick frothy homogenate centrifuged at 1,500 g for 10 min. using the large capacity (858) head of an International centrifuge. The supernatant fluid was filtered through glass wool to remove fat particles, and the precipitate again extracted with a further 200 ml. of buffer.

The extract was centrifuged for 1 hr. at 1,500 g and the supernatant, after filtering as above, combined with the first supernatant. The third extract of the residue contained only 3.3 mg. of protein per ml. and was discarded. The combined supernatants (360 ml.) had a protein concentration of 11.6 mg./ml., which represented a 4% (w/w) extraction of protein from worm muscle.

Fraction II

Fraction I was brought to 0.45 saturation by dropwise addition of saturated $(\text{NH}_4)_2\text{SO}_4$ (adjusted to pH 7.4 with 1 N-NaOH). The mixture was stirred during the addition, and then allowed to stand for 1 hr., after

which time it was centrifuged at 15,000 g for 15 min. The precipitate containing 10-20% of the total activity was discarded. The supernatant fluid was brought to 0.9 saturation by the slow addition of solid $(\text{NH}_4)_2\text{SO}_4$ with rapid mechanical stirring, and the mixture allowed to stand for 30 min. After centrifugation as before the precipitate was taken up in 70 ml. of extracting buffer. The recovery of activity in the 0.45-0.9 $(\text{NH}_4)_2\text{SO}_4$ fraction was usually 80%.

Fraction III

Fraction II was dialysed against 20 volumes of 0.01M-NEM-HCl buffer, pH 7.4, for 40 hr. with gentle rocking. Four changes of the buffer were made at 8 hr. intervals. The clear red extract (about 7 mg. protein per ml.) was checked with Nessler's reagent to ensure that the $(\text{NH}_4)_2\text{SO}_4$ was completely removed, concentrated by pressure dialysis until its protein concentration reached about 30 mg./ml. and then dialysed against 20 volumes of 0.01M-NEM-HCl buffer, pH 8.6, with two changes. A small precipitate which formed in the dialysis bag was removed by centrifugation at 15,000 g for 10 min. The pH was adjusted to 8.6 with a few drops of 1M-NEM (free base), the extract (approximately 50 ml.) was carefully layered on top of a TEAE-cellulose column (40 cm. x 3 cm.) and allowed to pass slowly into the

upper portion of the column.

After washing the cellulose with 2 bed-volumes of 0.01M-NEM-HCl buffer, pH 8.6, the enzyme was eluted with the same buffer at a higher molarity (0.5M). All the red-coloured components of the extract remained adsorbed to the cellulose, while other proteins moved down the column as a discrete brown band. The eluate was collected in 10 ml. fractions and the protein and lombricine kinase activity estimated in each fraction. A typical elution pattern is shown in Fig. 7 from which it may be seen that about 90% of the activity applied to the column emerged between 250 and 290 ml. eluate volume. These fractions were pooled while the less active fractions on either side of the main peak were discarded.

The initial recovery of activity after TEAE-cellulose chromatography was 80-90%. However, during the subsequent concentration and dialysis procedures, activity was gradually lost, and the overall recovery fell to 50-70%.

Fraction IV

Fraction III was concentrated by pressure dialysis until its protein concentration reached approximately 60 mg./ml. The solution was dialysed overnight against 20 volumes of 0.01M-NEM-HCl buffer,

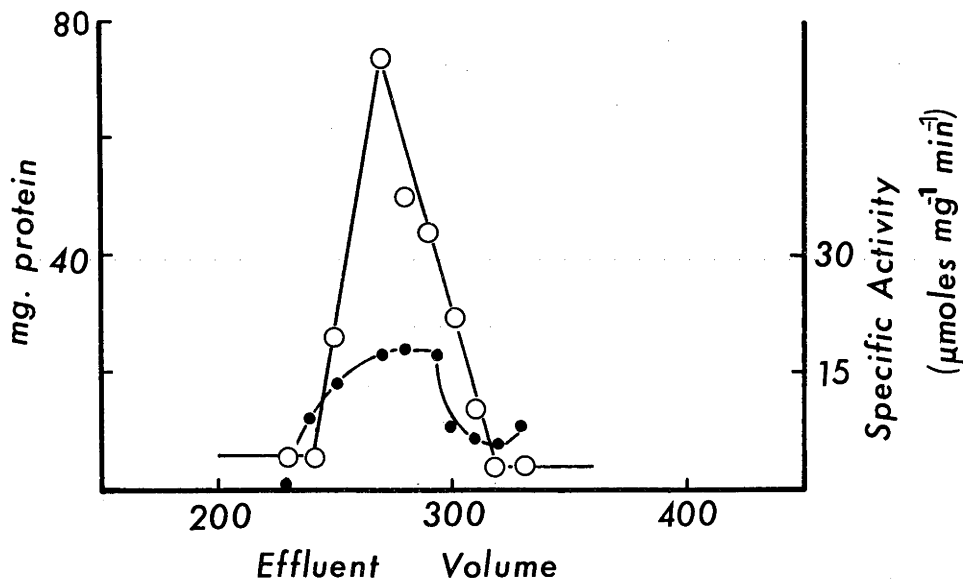


Fig.7. Chromatography of lombricine kinase on TEAE-cellulose. The fraction was applied in 0.01M-NEM-HCl buffer pH 8.6, and eluted with 0.5M-NEM-HCl buffer pH 8.6.

- , total protein per 10 ml. fraction.
- , specific activity of lombricine kinase (units/mg. protein).

pH 8.6, with two changes of buffer. The small precipitate which formed in the dialysis bag was removed by centrifugation at 15,000 g for 10 min. The extract was applied to another TEAE-cellulose column of smaller size (15 cm. x 1 cm.) and the procedure described for Fraction III was repeated. The column eluates were collected in 1 ml. fractions, analysed for protein and activity, and the most active fractions pooled.

Fraction V

Fraction IV was concentrated by pressure dialysis to 30 mg./ml., dialysed against 20 volumes of 0.01M-NEM-HCl buffer, pH 7, overnight with two changes of dialysis fluid, and stirred for 15 min. with 1 g. of CM-cellulose which had been equilibrated with 0.01M-NEM-HCl buffer, pH 7. The cellulose was centrifuged at 15,000 g for 10 min. and the supernatant decanted. Protein estimation of the supernatant indicated that approximately 20% of the protein was adsorbed onto the CM-cellulose. To elute the enzyme the cellulose was resuspended in 5 ml. 0.5M-NEM-HCl buffer, pH 7, and the suspension stirred for a further 15 min., after which time it was centrifuged as before. The supernatant (Fraction V) was dialysed for 12 hr. against 20 volumes of 0.005M-NEM-HCl buffer, pH 7.5, (containing 0.001M-

EDTA) and then for a further 12 hr. against 20 volumes 0.005M-NEM-HCl buffer, pH 7.5. The enzyme solution was mixed with an equal volume of cold glycerol and stored at 2°.

The progress of a typical purification procedure, showing yields and specific activities of the various fractions, is presented in Table 14.

Assay for other enzymic activities

Mg²⁺ activated ATPase was determined in routine assays of the forward reaction, but with lombricine omitted. The orthophosphate released by ATPase activity was less than 3% of that transferred by lombricine kinase activity in a comparable complete incubation mixture using a Fraction I enzyme preparation. No ATPase activity was detected with Fraction V enzyme.

Adenylate kinase activity was determined by substituting ADP for ATP in routine assay of the forward reaction. No significant formation of PL was detected with a Fraction I enzyme preparation, and with Fraction V enzyme no adenylate kinase activity was observed.

Phosphoamidase activity was tested in the reverse reaction by following the release of lombricine from PL in a routine assay system from which ADP was omitted. No phosphoamidase activity was detected with a Fraction V enzyme.

TABLE 14.

Summary of yields and specific activities of fractions
obtained during the purification of lombricine kinase
from 100g. earthworm muscle

Fraction	Total Protein (mg.)	Total Activity units	Specific Activity units/mg. protein	Purification Overall	Recovery Overall (%)
I Crude muscle extract	4100	13300	3.2	1.0	100
II 0.45-0.9 (NH ₄) ₂ SO ₄ precipitate	1830	10600	5.8	1.8	80
III TEAE-cellulose chromatography	298	4655	15.6	4.9	35
IV TEAE-cellulose chromatography	133	3192	24	7.5	24
V CM-cellulose treatment	24	798	33	10.3	6

Electrophoretic studies

The free boundary electrophoretic patterns of a 0.5% lombricine kinase preparation (Fraction V, specific activity 36) are presented in Fig. 8. In the pH range 6-9.8 the protein moved as a single component towards the anode. However its rate of migration was slow, and at the conclusion of the run the effect of diffusion was apparent, with some broadening of the base of the protein peaks.

With a more concentrated enzyme solution (1%) a more sharply defined peak was obtained, and again only one component was visible. It would seem therefore that at the Fraction V stage of purification lombricine kinase is electrophoretically homogenous.

The mobility of lombricine kinase at the various pH values indicated that its isoelectric point was in the vicinity of pH 6.

Zone electrophoresis on cellulose acetate strips using the same buffers and enzyme as above confirmed that only one protein component was detectable. The pattern obtained at pH 8.6 is shown in Fig. 9. It will be noted that a single protein band is present, which has moved slightly towards the cathode. This cathodic migration was presumably caused by electro-osmotic flow, which was sufficient to overcome the

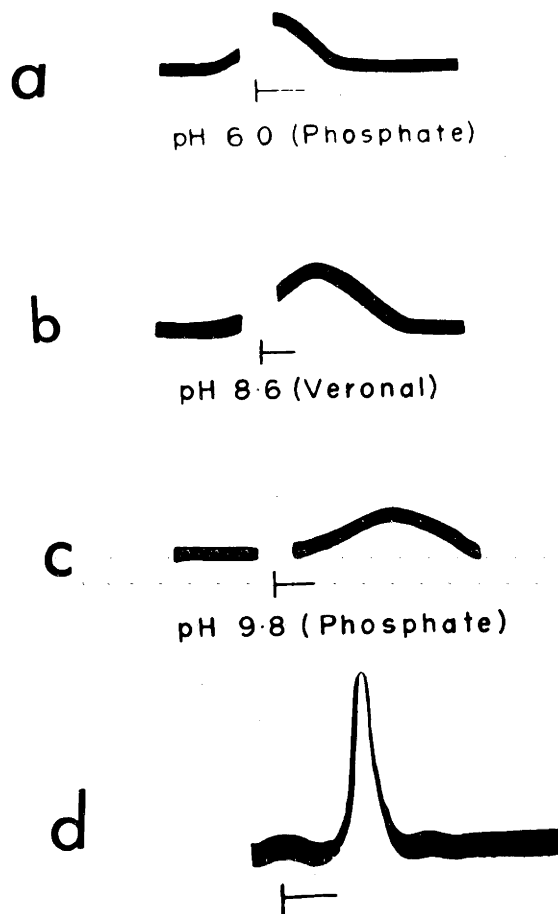


Fig.8. Free boundary electrophoresis of purified lombricine kinase (Antweiler microelectrophoresis apparatus). The buffers were (a) potassium-phosphate pH 6.0, (b) and (d) veronal-HCl pH 8.6 and (c) potassium-phosphate pH 9.8 at ionic strength 0.1M. Protein concentration 5 mg./ml. for (a), (b) and (c) and 10 mg./ml. for (d). Time 20 min. Temperature 1°.

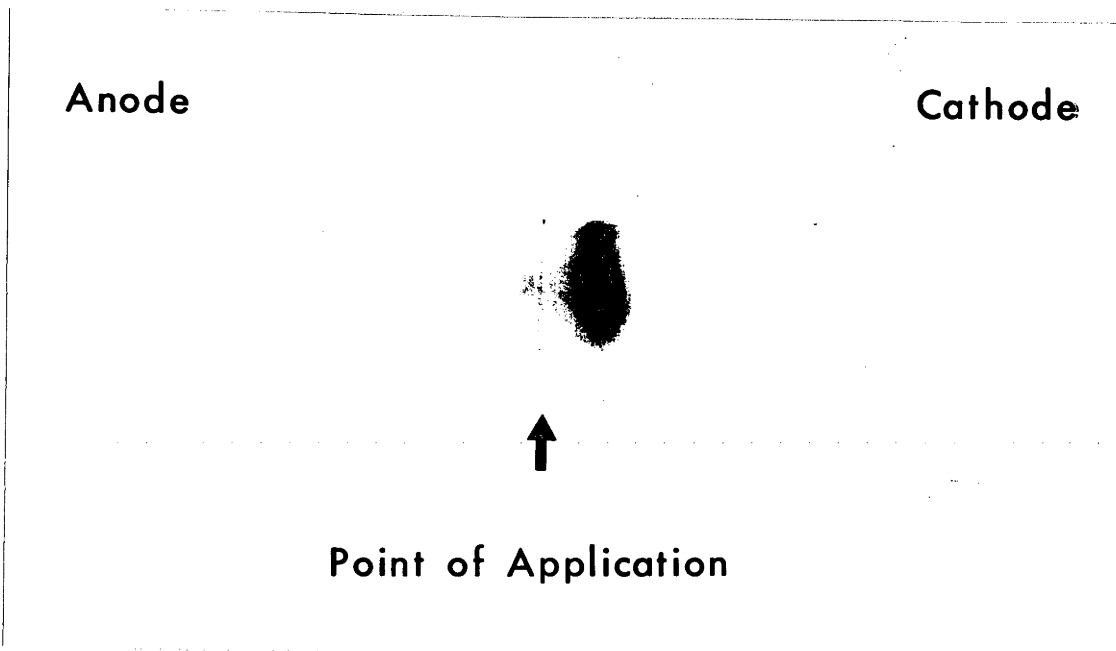


Fig.9. Zone-electrophoresis of purified lombricine kinase (Fraction V, specific activity 36 units /mg. protein) on cellulose acetate strips (OXOID) in 0.1M-veronal-HCl buffer pH 8.6. The bridge gap was 13 cm. and the voltage 25v/cm. Temperature 1°. The protein (5 μ g in 10 μ l) was applied as a line at the origin. The run lasted for 4 hr. after which the protein was stained by the Commassie Blue technique.

slow anodic migration observed with free boundary electrophoresis.

Attempts to isolate lombricine kinase from whole earthworms

Because small earthworms were readily available, and evisceration of large earthworms was a laborious procedure, in initial experiments attempts were made to isolate lombricine kinase from whole earthworms, using the extraction procedure described above. Lombricine kinase was found in Fraction I (crude extract) which had a specific activity of 0.7. This specific activity was increased to 2.0 in Fraction II, 0.45-0.9 $(\text{NH}_2)_2\text{SO}_4$ saturation. However, although this activity remained as long as Fraction I was frozen at -18° at pH 7.4, or while Fraction II kept at high $(\text{NH}_4)_2\text{SO}_4$ concentration, it rapidly disappeared on thawing Fraction I or on dialysing Fraction II. No protection against inactivation by dialysis was afforded by the addition of 0.01M cysteine to the dialysis fluid. As lombricine kinase prepared from worm muscle is stable to both thawing and dialysis, it was considered that the loss of enzymic activity in whole worm extracts probably reflected its destruction by proteolytic enzymes of visceral tissues.

Attempts were made to separate lombricine

kinase and proteolytic enzymes from each other rapidly, before the inactivation of lombricine kinase became marked. Sephadex G-25 (see Methods) was used to remove $(\text{NH}_4)_2\text{SO}_4$ and equilibrate the enzyme extract prior to chromatography on TEAE-cellulose. This reduced the time required for desalting and equilibration to 3 hr. The enzyme extract was chromatographed on TEAE-cellulose as described previously, and this effected an overall 15-fold purification. Although the specific activity of the lombricine kinase had risen to 10.2 the stability of the enzyme was not increased, and its isolation from whole earthworm was abandoned.

PROPERTIES OF LOMBRICINE KINASE

Effect of enzyme concentration on reaction velocity

The initial velocity was directly proportional to the enzyme concentration for both the forward and reverse reaction over the range shown in Figs. 10 and 11. The maximum rate of the reverse reaction was approximately five times that of the forward reaction.

Effect of pH on reaction velocity

The effects of pH and buffer species on the initial velocity in the forward and reverse reactions are shown in Figs. 12 and 13. It is evident that the pH optimum for the forward reaction is 8.6, and that at the pH optimum the reaction rate was slightly higher in

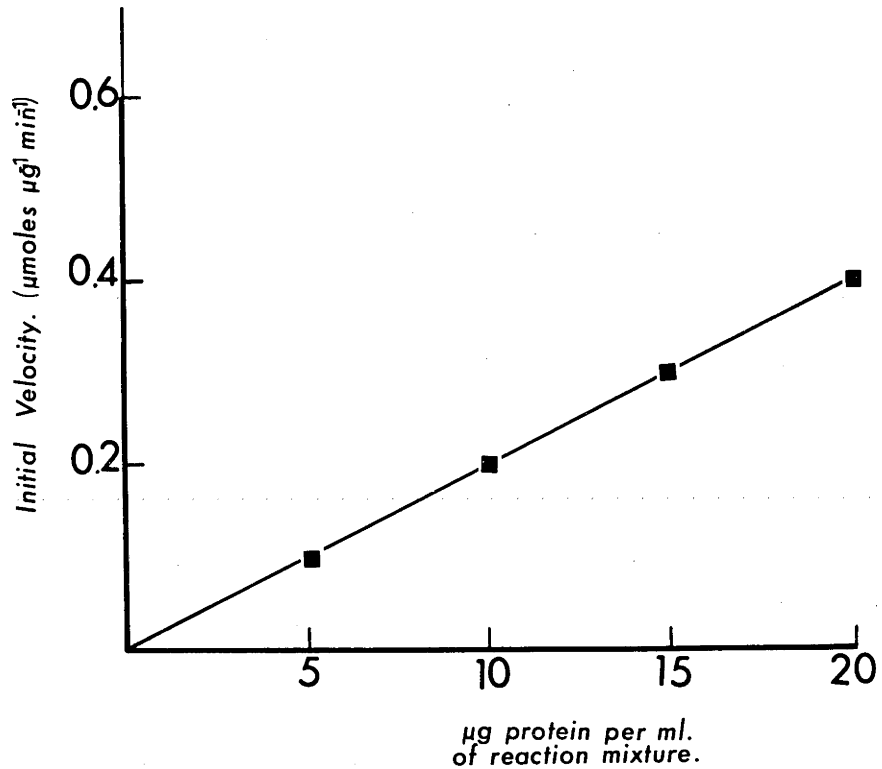


Fig.10. Effect of lombricine kinase concentration on the initial velocity in the forward reaction. The reaction mixture (1 ml.) contained 0.1M-glycine-KOH buffer pH 8.6, 0.010M-D-lombricine, 0.005M-MgCl₂, 0.005M-ATP, and 0.2 ml. of enzyme solution (50µg protein/ml., specific activity 20 units/mg. protein). Temperature 30°. Incubation time 1 min.

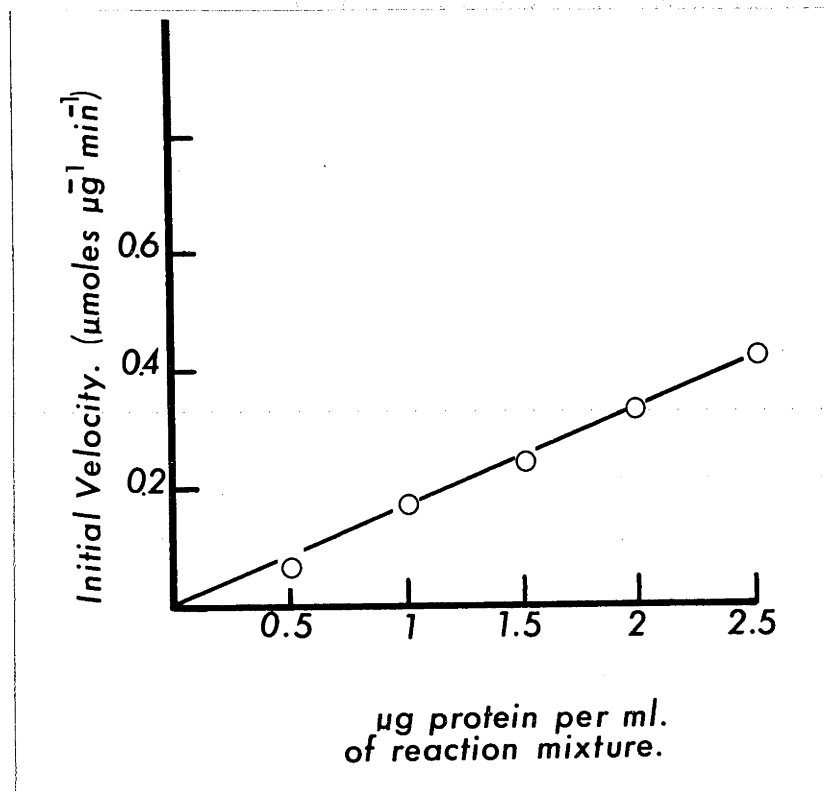


Fig. 11. Effect of lombricine kinase concentration on the initial velocity in the reverse reaction. The reaction mixture (1 ml.) contained 0.1M-NEM-HCl buffer pH 7.0, 0.0025M-PL(Na⁺ salt), 0.01M-MgCl₂, 0.0025M-ADP and 0.2 ml. of enzyme solution (20 μg protein/ml., specific activity 33 units/mg. of protein). Temperature 30°. Incubation time 1 min.

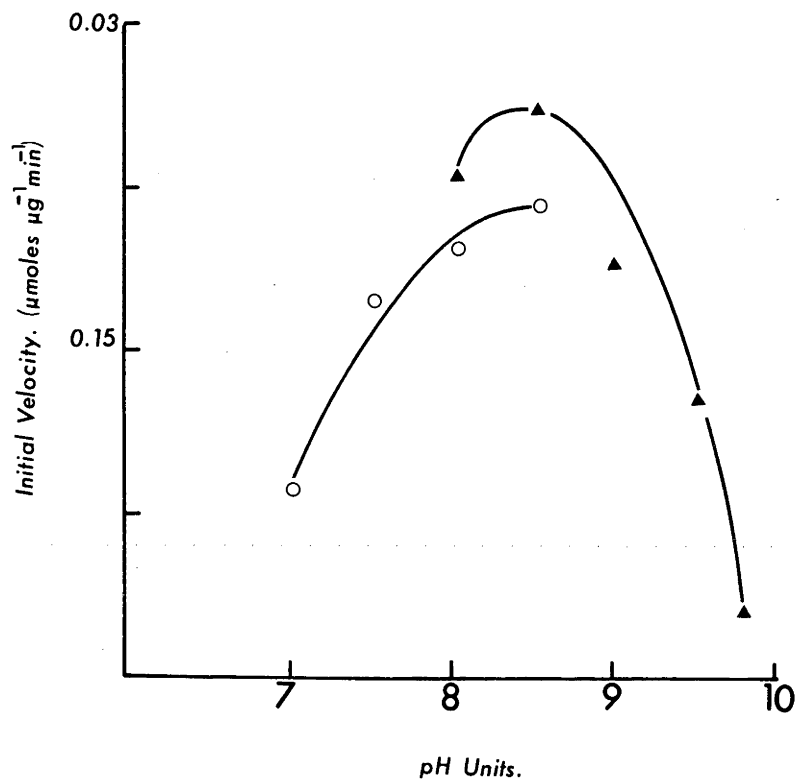


Fig.12. Effect of pH and buffer species on the initial velocity in the forward reaction. The reaction mixture (1 ml.) contained 0.1M-glycine-KOH or triethanolamine-HCl buffers at the pH values indicated, 0.010M-D-lombricine, 0.005M-MgCl₂, 0.005M-ATP, and 0.2 ml. of enzyme solution (50 μg protein/ml., specific activity 20 units/mg. protein). Temperature 30°. Incubation time 1 min. The pH was measured with a glass microelectrode in a duplicate set of reaction mixtures, at the conclusion of the reaction period.

▲, 0.1M-glycine-KOH buffer.

○, 0.1M-triethanolamine-HCl buffer.

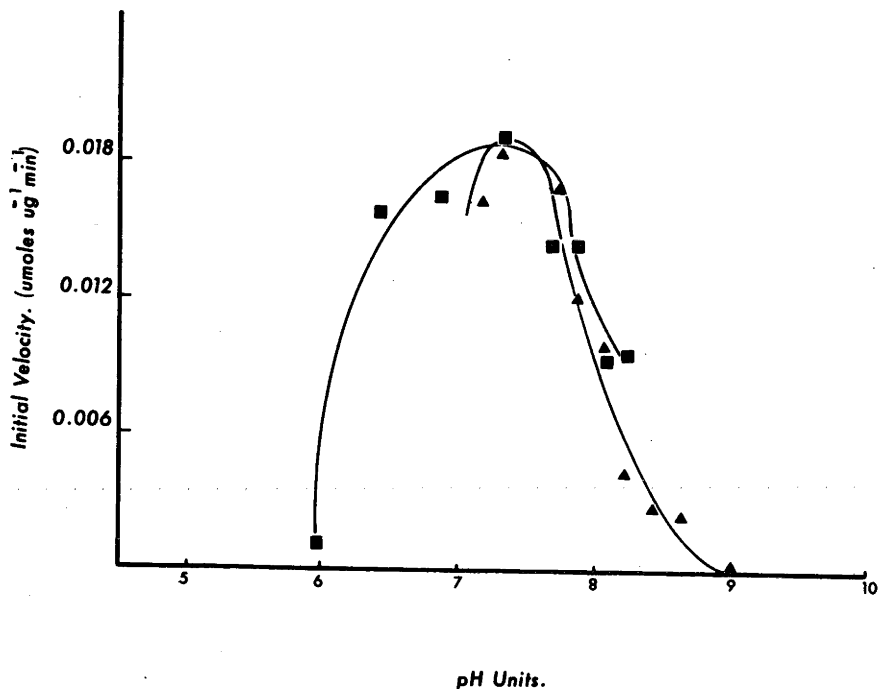


Fig.13. Effect of pH and buffer species on the initial velocity in the reverse reaction. The reaction mixture (1 ml.) contained 0.1M-imidazole-HCl or NEM-HCl buffers at the pH values indicated, 0.0025M-PL(Na⁺ salt), 0.01M-MgCl₂, 0.0025M-ADP and 0.1 ml. of enzyme solution (20 μg protein/ml., specific activity 33 units/mg. of protein). Temperature 30°. Incubation time 1 min. The pH was measured with a glass microelectrode in a duplicate set of reaction mixtures at the conclusion of the reaction period.

■ , Imidazole-HCl buffer.

▲ , NEM-HCl buffer.

glycine-KOH than in triethanolamine-HCl buffer.

In the reverse reaction the pH optimum was 7.2, the maximum rates being equal in imidazole-HCl and NEM-HCl buffers.

Effect of metals on the reaction velocity

As shown in Table 15 lombricine kinase was activated by Mn^{2+} , Mg^{2+} , Co^{2+} and Ca^{2+} in both the forward and reverse reactions, although the degree of activation by Co^{2+} and Ca^{2+} was much less than that by Mn^{2+} or Mg^{2+} . No activation was detected with Ni^{2+} , Cu^{2+} , Fe^{3+} , Al^{3+} , Sn^{2+} , Ba^{2+} , Cd^{2+} , Zn^{2+} and Be^{2+} , when these metals were tested in both the forward and reverse reactions.

Substrate specificity of lombricine kinase

The specificity of lombricine kinase for other guanidino compounds was investigated in the forward reaction. It is evident from Table 16 that, in addition to D-lombricine, L-lombricine, taurocyamine, and 2-guanidinoethylphosphate also serve as phosphoryl group acceptors from ATP, although the degree of phosphorylation of taurocyamine and 2-guanidinoethylphosphate is much less than that observed with either isomer of lombricine. Creatine, arginine, guanidinoacetate, guanidinoethanol, guanidinobutyrate, guanidinopropionate, guanidino-n-valerate and L-canavanine were not phosphorylated.

TABLE 15.

Metal activation of lombricine kinase

The values given refer to the initial velocities with the corresponding metal ion relative to that with Mn^{2+} (100%).

Metal	Reverse Reaction	Forward Reaction
Mn^{2+}	100%	100%
Mg^{2+}	90%	95%
Co^{2+}	18%	21%
Ca^{2+}	8%	27%

For the reverse reaction the mixture (1 ml.) contained 0.05M-NEM-HCl buffer pH 7.0, 0.0025M-PL(Na^+ salt), 0.001M-ADP, 0.005M-metal ion, and 0.1 ml. lombricine kinase solution (50 μ g. of protein/ml., specific activity 40 units per mg. protein).

Incubation time 3 min. at 23°.

For the forward reaction the mixture (1 ml.) contained 0.1M-glycine-KOH buffer pH 8.6, 0.01M-D-lombricine, 0.005M-ATP, 0.005M-metal ion, and 0.1 ml. of lombricine kinase solution (50 μ g. of protein/ml., specific activity 40 units per mg. protein).

Incubation time 5 min. at 30°.

TABLE 16.

Specificity of lombricine kinase for guanidino-compounds

The values given refer to the degree of phosphorylation of the particular guanidino compound relative to that with D-lombricine (100%).

	%
L-lombricine	100
D-lombricine	100
Taurocyamine	67
2-Guanidinoethylphosphate	28
2-Guanidinoethanol	0
Creatine	0
L-Arginine	0
Guanidinoacetate	0
Guanidinopropionate	0
Guanidinobutyrate	0
Guanidino-n-valerate	0
L-Canavanine	0

The incubation mixtures (1 ml.) contained 0.1M glycine-KOH buffer, pH 8.6, 0.005M ATP, 0.01M appropriate guanidino compound, and 0.1 ml. lombricine kinase (200 µg/ml; specific activity of 40 units per mg. of protein). Reaction time 10 min. at 30°.

During the purification of lombricine kinase the initial rates of phosphorylation of D-lombricine and taurocyamine were compared at each stage of the purification procedure. It is evident (Table 17) that the ratios of the specific activities with lombricine and taurocyamine as acceptors of the phosphoryl group are the same at each fractionation step. This was consistent with one enzyme using both compounds as substrates.

The specificity of lombricine kinase for nucleotides (Table 18) was also investigated. No significant reaction occurred with CDP, GDP, IDP and UDP. However deoxyADP was phosphorylated, but to a much lesser degree than ADP.

Stability of lombricine kinase

The stability of lombricine kinase was markedly dependent upon its degree of purity. Thus a Fraction II enzyme preparation withstood dialysis against 0.02M-NEM-HCl buffer, pH 7.5, for a week with a minimal loss of activity, whereas a Fraction IV preparation became opalescent with marked loss of activity after five days dialysis.

Fractionation with ethanol at -5° of a Fraction II preparation, in the absence of added cations, caused very little loss of activity, but achieved no

TABLE 17.

Relative lombricine kinase and taurocyamine kinase
activities in the fractionation of lombricine kinase
preparation

Fraction	Specific Activity (units/mg. of protein)		Ratios of Specific Activities
	lombricine kinase	taurocyamine kinase	
I Crude Muscle Extract	3.2	0.72	0.23
II 0.45-0.9 (NH ₄) ₂ SO ₄ precipitate	5.8	1.2	0.21
III TEAE-cellulose chromatography	15.6	3.2	0.21
IV TEAE-cellulose chromatography	24	-	-
V CM-cellulose treatment	33	8.2	0.25

TABLE 18.

Specificity of lombricine kinase for nucleotides

Nucleotide	Results expressed relative to phosphorylation of ADP.
	%
ADP	100
deoxy-ADP	16
CDP	2.9
GDP	2.4
IDP	3.5
UDP	1.8

The reaction mixture (1 ml.) contained 0.1M-NEM-HCl buffer pH 7.0, 0.001M-nucleotide as indicated, 0.0025M-PL(Na⁺ salt), 0.010M-MgCl₂, and 0.020 ml. of lombricine kinase (200 µg. of protein/ml., specific activity 40 units per mg. of protein). Reaction time 6 min. at 30°.

worthwhile purification. There was as much activity in the 0.3-0.45 ethanol fraction as there was in the 0.45-0.6 ethanol fraction, while the overall specific activities rose only from 4 to 6. However when the preparation was made 0.1M with respect to Mg^{2+} prior to ethanol fractionation (cf. Kuby et al., 1954a) lombricine kinase was completely inactivated. Ethanol fractionation at room temperature in the absence of Mg^{2+} ions also resulted in complete loss of activity.

During initial attempts at purification the thermal stability of lombricine kinase was tested on a Fraction II preparation. It was found that when the preparation was heated at 40° for 10 min. marked inactivation occurred at pH 6.0, 7.0 and 8.6. Lombricine and Mg^{2+} afforded no protection against the inactivation. Heating at 50° for 5 min. at pH 7.0 caused complete loss of activity.

A fraction II preparation could be stored for at least two months at -18° at pH 7.5, but repeated freezing and thawing caused loss of activity. At Fraction III stage freezing at pH 8.6 caused complete inactivation, while freeze drying at pH 7.5 in non-volatile buffers, e.g., glycine-KOH, caused a 36% loss of activity.

Purified lombricine kinase stored at 2° in

0.025M-NEM-HCl buffer, pH 7.5, became cloudy after a few days, and the specific activity of the enzyme rapidly declined. It was found that glycerol had a marked stabilising effect upon the enzyme, for the addition of an equal volume of glycerol (final concentration 50% v/v) to a purified lombricine kinase preparation (protein concentration 20-30 mg./ml.) permitted storage for four months at 2° without loss of activity.

Effect of sulphhydryl reagents and other compounds on lombricine kinase activity

Lombricine kinase was inhibited by compounds (Table 19) which are reactive with -SH groups, namely p-hydroxy mercuribenzoate, phenyliodoacetate, o-iodosobenzoate and N-ethyl maleimide. These results indicate that a free -SH grouping is essential for lombricine kinase activity. The enzyme proved extremely sensitive to p-HMB inhibition, showing complete inhibition at 1×10^{-7} M and no inhibition at 1×10^{-8} M. The inhibition at 1×10^{-7} M p-HMB was not reversed by a twenty-fold excess of cysteine. Other metabolic inhibitors, arsenite and dinitrophenol were without effect on lombricine kinase activity.

DISCUSSION

The fractionation procedure for the isolation of lombricine kinase described above yielded enzyme

TABLE 19.

The effect of sulphhydryl reagents and metabolic inhibitors on lombricine kinase activity

	Final concentration (M)	Inhibition %
p-hydroxymercuribenzoate	1×10^{-8}	0
	5×10^{-8}	20
	1×10^{-7}	100
p-HMB (1×10^{-7} M) + cysteine	1×10^{-6}	100
p-HMB (1×10^{-7} M) + cysteine	2×10^{-6}	100
phenyliodoacetate	3×10^{-3}	100
	1×10^{-3}	100
O-iodosobenzoate	3×10^{-4}	100
N-ethylmaleimide	3×10^{-4}	100
arsenite	1×10^{-3}	0
2:4-dinitrophenol	1×10^{-3}	0

The reaction mixture (1 ml.) contained 0.1M-NEM-HCl buffer pH 7.0, 0.0025M-PL(Na⁺ salt), 0.001M-ADP, 0.01M-MgCl₂, inhibitors at concentrations indicated above, and 0.02 ml. of lombricine kinase (250 µg. of protein/ml., specific activity 40 units per mg. of protein). The reaction was started with ADP after preincubating all the other components for 5 min. at 30°, and stopped as described previously 5 min. after the addition of ADP. The reaction mixtures containing cysteine were incubated for 10 min. after the addition of ADP.

preparations which were homogenous as judged by electrophoresis studies, with specific activities varying between 30 and 40. Earthworm muscle as starting material was found to possess several advantages over whole earthworms, in that initial specific activities were higher (3.2 as contrasted with 0.7 for whole worm) and the enzyme was stable during the fractionation procedure. For these reasons it was considered unprofitable to persist with attempts at the isolation of lombricine kinase from whole earthworms. Pant (1959) considered that the disappearance of activity during dialysis was due to loss of a labile cofactor. This conclusion cannot be justified since only whole worm extracts lose activity during dialysis, presumably due to peptide hydrolases destroying lombricine kinase. Further proof is afforded by the stability of enzyme preparations from muscle to exhaustive dialysis, and the lack of any demonstrable cofactor, apart from Mg^{2+} ions, with purified enzymes. Moreover, whole worm extracts retained activity provided storage conditions were such that all enzymic activities were minimal, e.g., freezing at -18° or maintenance in high $(NH_4)_2SO_4$ concentrations. However, once the preparation was thawed or the salt removed, lombricine kinase activity rapidly disappeared. Rapid purification procedures aimed at separating proteolytic enzymes from

lombricine kinase resulted in a fifteen-fold purification of whole worm extracts, but the enzyme preparation was still unstable, and rapidly lost activity.

The initial extract of worm muscle provided a 4% (w/w) yield of protein. From this lombricine kinase was obtained in an homogenous state after a ten-fold overall purification. These findings were similar to those reported by Kuby et al. (1954a) for the purification of creatine kinase of rabbit muscle. The Mg^{2+} activated ATPase and adenylate kinase activities of Fraction I were responsible for less than 3% of the phosphorus liberated or transferred and no correction for the activities of these enzymes was applied. In the first purification step the usual recovery of activity was 70-80% in the 0.45-0.9 $(NH_4)_2SO_4$ fraction.

In preliminary experiments it was shown that lombricine kinase was eluted from TEAE-cellulose at 0.2M-NEM-HCl buffer, pH 8.6. However, when gradient elution was employed in order to obtain high resolution of lombricine kinase from other proteins (cf. Sober and Peterson, 1958) it was found that lombricine kinase tended to trail over a large number of fractions. This required prolonged assay and yielded a very dilute enzyme solution. Since lombricine kinase in dilute solution is unstable to high pH at this stage of purification, marked loss of

activity occurred. It was found advantageous therefore to sacrifice resolution for speed of operation. The concentration of the eluant buffer was raised sharply to 0.5M-NEM-HCl, pH 8.6, and purified lombricine kinase was obtained with minor loss. Further resolution was then obtained by the use of another TEAE-cellulose column of much smaller size. Each of these fractionation procedures gave recoveries of 50-70% of the activity applied to the cellulose. Many enzyme preparations were terminated at this stage (Fraction IV) with overall recoveries of 40% and with a purity of approximately 80%.

The final purification procedure, namely elution from CM-cellulose, resulted in a very pure enzyme in low yield. Lombricine kinase was found to be very unstable in acid pH. Thus it was impossible to use CM-cellulose at pH values which favoured absorption of the enzyme, and only about 20% of the enzyme was adsorbed.

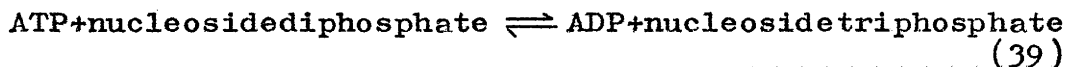
The pH optima in the forward and reverse reactions were 8.6 in triethanolamine-HCl and glycine-KOH buffers and 7.2 in NEM-HCl and imidazole-HCl buffers respectively. These findings were in conformity with corresponding pH optima determined for other guanidino kinases. In the reverse reaction at pH 7.2 the maximum velocities in NEM-HCl and imidazole-HCl buffers were equal, whereas at pH 8.6 in the forward reaction the

rates in glycine-KOH buffer, which complexes metals, were slightly higher than those in triethanolamine, which is a non-complexing buffer. It is generally recognised that buffers which complex metals depress reaction velocities by reducing the effective concentration of metal activator (usually Mg^{2+}) available for the reaction. The elevated velocities observed in glycine-KOH buffer may have been due to removal of heavy metal inhibitors as glycine complexes, for the guanidino kinases, being -SH enzymes, are susceptible to heavy metal inhibition.

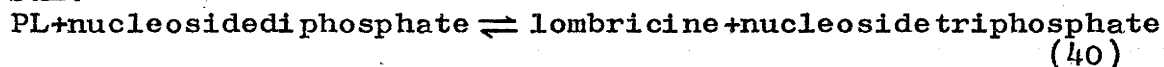
A comparison of the rates in the forward and reverse reactions showed that the latter is approximately five times as rapid as the former, a finding which was in agreement with that found for creatine and arginine kinases and was consonant with the necessity for the rapid resynthesis of ATP from ADP during muscular contraction.

Table 18 shows that with lombricine kinase only ADP and deoxy-ADP, of the nucleotides tested, served as phosphoryl group acceptors in the reverse reaction and that the degree of phosphorylation with deoxy-ADP was only 0.16 that with ADP. The phosphorylation of the other nucleotides may be regarded as insignificant, for although the nucleotides differed widely in base

composition the same low level of activity (0.03 that of ADP) was found with all. The possibility that this low activity may have been due to trace amounts of ADP in the presence of contaminating nucleoside diphosphokinases, as shown in the reactions below, was not investigated.



Sum:



IDP differs from ADP in that the 6-amino group of the purine is replaced by a hydroxyl, and GDP, in addition to the 6-hydroxyl, has a 2-amino group. The fact that these nucleotides do not act as phosphoryl group acceptors is in accord with the postulate of Ennor and Morrison (1958) that the 6-amino group of adenine is an essential component of the nucleotide donor or acceptor.

Recently Nihei et al. (1961) investigated the activity of creatine kinase with inosine and cytidine nucleotides as substrates. These workers reported that the same maximal velocities were attained as with adenosine nucleotides, but that the K_m values were 100 to 500-fold greater than those of the adenosine nucleotides. They

concluded that the purine or pyrimidine moiety of the nucleotide influenced the affinity of the nucleotide for the enzyme, but not the phosphoryl transfer. They also interpreted their findings as being consistent with the 6-amino of the purine having a direct effect on the binding of the nucleotide to the enzyme.

It was evident (Table 16) that lombricine kinase catalysed the transfer of phosphoryl groups from ATP to guanidino-compounds possessing acidic moieties, such as lombricine, taurocyamine and 2-guanidinoethylphosphate. These findings confirmed earlier reports of Pant (1959) and Rosenberg *et al.* (1960). However the degree of phosphorylation with lombricine was much higher than with taurocyamine and 2-guanidinoethylphosphate, and other guanidino-compounds of similar carbon length to the latter compounds were not phosphorylated. Whether the faster rates found with lombricine are due to a greater affinity of this compound for the enzyme is not known, since the K_m values for taurocyamine and 2-guanidinoethylphosphate were not determined.

Thus the specificity of lombricine kinase for guanidino compounds is quite restricted, and is similar to the limited specificities exhibited by other guanidino kinases. For example, creatine kinase is

active with creatine and N-ethylguanidinoacetate; arginine kinase with D- and L-arginine, homoarginine, arginine methyl-ester, canavanine and guanidine; and taurocyamine kinase with taurocyamine and β -guanidinopropionic acid (Ennor and Morrison, 1958).

It seems reasonably certain that the phosphorylation of taurocyamine, and by analogy the phosphorylation of guanidinoethylphosphate, is carried out by the same enzyme that phosphorylates lombricine. This is based on the fact that all three of these compounds were phosphorylated by an homogenous lombricine kinase, and that the ratios of the specific activities with taurocyamine and lombricine as substrates remained constant at approximately 0.25 throughout all stages of the fractionation (Table 17).

This finding was compatible with the fact that taurocyamine was absent from earthworms, and that the earthworm enzyme preparation was unable to phosphorylate β -guanidinopropionate, which was readily phosphorylated by taurocyamine kinase of A. assimilis extracts (Griffiths, 1958).

Lombricine kinase was markedly activated by Mg^{2+} and Mn^{2+} and to a lesser degree by Co^{2+} and Ca^{2+} .

Previous workers have shown that creatine kinase is activated by Mg^{2+} , Mn^{2+} and Ca^{2+} ; arginine kinase by Mg^{2+} and Mn^{2+} ; and taurocyamine kinase by Mg^{2+} , Mn^{2+} , Ca^{2+} and Co^{2+} (Ennor and Morrison, 1958). Thus lombricine kinase resembles the other guanidino kinases in its metal requirements. The role of the divalent cations in the activation of lombricine kinase will be discussed more fully in Chapter 4.

Lombricine kinase may be regarded as a sulphhydryl enzyme since it loses catalytic activity when some or all of its -SH groups undergo chemical modification. Experimental results showed that it was extremely sensitive to inhibition by pHMB, and the observation that a large excess of cysteine failed to reverse this inhibition would suggest that pHMB caused denaturation of the enzyme. Arsenite failed to inhibit lombricine kinase, from which it may be inferred that the -SH groups essential for activity are not in sufficiently close proximity to form a stable γ -As ring (Peters, 1952). The lack of inhibition by dinitrophenol was in accord with similar findings reported for creatine kinase and arginine kinase (Ennor and Morrison, 1958).

SUMMARY

1. Lombricine kinase which catalyses the reaction
$$\text{ATP} + \text{lombricine} \rightleftharpoons \text{phospholombricine} + \text{ADP}$$
was isolated from the muscular body wall of the earthworm, Megascolides cameroni. After fractionation with $(\text{NH}_4)_2\text{SO}_4$ the earthworm extract was chromatographed on TEAE-cellulose, and further purified by adsorption on CM-cellulose. The lombricine kinase thus prepared was electrophoretically homogenous, with specific activities varying between 30 and 40.
2. Whole earthworms were found unsuitable as a starting material for lombricine kinase preparations, as intestinal peptide hydrolases destroyed lombricine kinase during the purification procedures.
3. The pH optima were 8.6 and 7.2 in the forward and reverse directions respectively.
4. Lombricine kinase was found to be activated by Mg^{2+} and Mn^{2+} ions, and to a lesser extent by Co^{2+} and Ca^{2+} ions in both forward and reverse reactions.
5. Specificity studies showed that D- and L-lombricine, taurocyamine and 2-guanidinoethylphosphate were phosphorylated in the forward reaction. The rates with the latter two compounds were much less than with lombricine, and 2-guanidinoethylphosphate was

phosphorylated more slowly than taurocyamine. Of the nucleotides studied only ADP and deoxy-ADP participated in the forward reaction, but the rate of phosphoryl group transfer with deoxy-ADP was much less than with ADP.

6. Lombricine may be classified as an -SH enzyme because of its inhibition by sulphhydryl reagents.

CHAPTER 4.

KINETIC STUDIES OF THE ACTIVATION OF LOMBRICINE

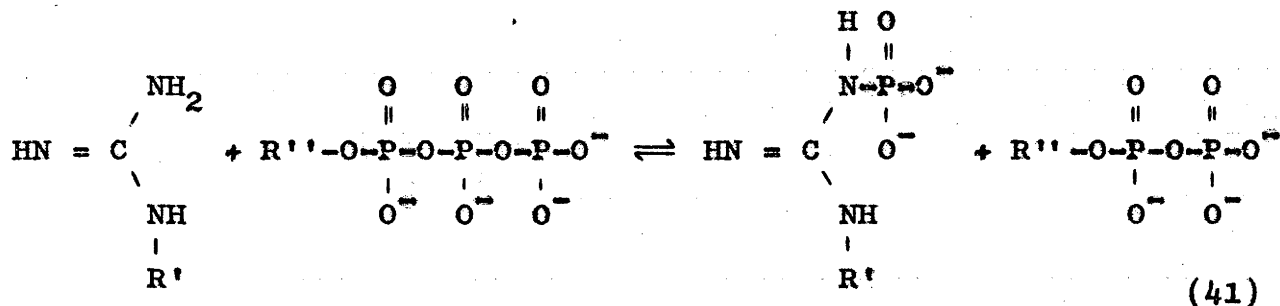
KINASE BY MAGNESIUM

CHAPTER 4

KINETIC STUDIES OF THE ACTIVATION OF
LOMBRICINE KINASE BY MAGNESIUM

INTRODUCTION

The general reaction catalysed by the guanidino kinases may be illustrated as follows:



Guanidino-compound + ATP

Phosphagen + ADP

Where R' = the remainder of the particular phosphagen base, and R'' = adenine-ribose. From this formulation it is evident that these enzymes catalyse the reversible transfer of phosphoryl groups. This has been confirmed for the creatine-kinase reaction by the application of ¹⁸O studies, which demonstrated that phosphoryl group transfer occurred without exchange of the phosphoryl oxygens with either substrate or water (Harrison, Boyer and Falcone, 1955). It follows therefore that the guanidino kinases resemble ATPase and various kinases, since these latter

enzymes also transfer phosphoryl groups, with a similar site of bond rupture, namely the terminal O-P bond of ATP (Cohn, 1956).

Although it is possible for phosphoryl group transfer to occur by a double displacement mechanism with the formation of phosphoenzyme intermediates, no evidence favouring such a mechanism has been adduced for guanidino kinase reactions. Product inhibition studies of the reaction catalysed by creatine kinase indicated that ATP and ADP competed for one common binding site on the enzyme surface, whereas an independent site was concerned with the binding of creatine and PC (Kuby et al., 1954b). Similar findings were reported by Griffiths et al. (1957) for the arginine kinase reaction. It was concluded that these independent binding sites were in juxtaposition with the area of phosphoryl group transfer common to both, thereby permitting phosphoryl group transfer to occur as a single displacement reaction. Similarly other kinase reactions are considered to proceed by direct transfer of the phosphoryl group from donor to acceptor (Koshland, 1954; Reynard, Hass, Jacobsen and Boyer, 1961).

Further evidence in support of a single displacement mechanism in guanidino kinase reactions was provided by the observation that the thermodynamic dissociation constant of the creatine kinase-MgADP²⁻ complex obtained by equilibrium dialysis was the same as that obtained

from kinetic studies (Kuby and Mahowald, 1958). Such a finding is not compatible with a double displacement mechanism involving a phosphoenzyme intermediate, but is consistent with a single displacement mechanism (Noda et al., 1960).

In addition the inability to demonstrate exchange reactions between [β - ^{32}P]ADP and ATP catalysed by creatine kinase (Ulbrecht, Ulbrecht and Wustrow, 1957; Noda et al., 1960) provided strong evidence against the participation of phosphoenzyme intermediates. In this connection it is of interest to note that many phosphatases catalyse exchange reactions. To account for this phosphoenzyme intermediates have been proposed for reactions catalysed by phosphoglyceromutase, glucose-6-phosphatase, phosphoserine phosphatase and alkaline phosphatases (Neuhaus and Byrne, 1959; Agren, 1959; Pizer, 1960; Hass and Byrne, 1960). Kennedy and Koshland (1957) isolated a phosphoenzyme by incubation of crystalline phosphoglucomutase of rabbit muscle with ^{32}P and McCoy and Najjar (1959) demonstrated through kinetic studies that a phosphoenzyme intermediate was involved in the reaction catalysed by yeast phosphoglucomutase.

All phosphoryl group transfers involving the adenine nucleotides display an absolute requirement for divalent cations (Lardy, 1951). Guanidino kinases are activated by Mg^{2+} in all instances, but the transition

element Mn^{2+} may substitute for Mg^{2+} in vitro, this ready substitution of one metal for the other constituting the general rule (Cotzias, 1961). Other metals such as Ca^{2+} or Co^{2+} also serve as activators in some cases.

The guanidino kinases may be classified as true metal-enzymes as defined by Malmstrom and Rosenberg (1959) in which the metal ion is freely dissociable from the enzyme. This contrasts with the much firmer metal-enzyme interaction observed with a true metallo-enzyme, as illustrated by carboxypeptidase, in which the Zn^{2+} ion is not readily dissociable from the enzyme (Vallee, Rupley, Coombs and Neurath, 1960).

It is generally accepted that the activating metal ion partakes in the formation of an enzyme-metal-substrate (EMS) complex, since enzymic activity reaches a limiting value both with increasing metal or increasing substrate concentrations (Malmstrom and Rosenberg, 1959). This implies that the metal ion is essential, and no enzymic action can proceed in the absence of the necessary divalent cation. However distinctions are recognised in the manner in which the EMS complex arises. This may be illustrated by the divergent theories advanced to explain the metal activation of the guanidino kinases. In the case of creatine kinase activation by Mg^{2+} , Kuby et al., (1954b) concluded from kinetic studies that there was an

initial combination of Mg^{2+} with nucleotide to form a metallo-substrate, which was the true substrate for the enzyme. On the other hand, similar kinetic studies of the Mg^{2+} activation of the reaction catalysed by arginine kinase led Griffiths et al. (1957) to postulate that there was an initial binding of Mg^{2+} to the enzyme molecule, with subsequent interaction of this complex with free nucleotide.

The fundamental weakness of the above theories lay in the assumption that only one component of the reaction system was capable of interacting with the enzyme. Having recognised this limitation Morrison et al. (1961) proceeded to study the kinetics of the Mg^{2+} activation of the reaction catalysed by creatine kinase, in which the three possible pathways for the formation of an enzyme- Mg^{2+} -nucleotide complex were taken into account. Their kinetic data were consistent with the formation of the EMS complex by reaction of the enzyme with the free forms of Mg^{2+} and nucleotide, as well as with the Mg^{2+} -nucleotide complex.

The experiments presented in this chapter are concerned with the kinetic studies of the activation of lombricine kinase by Mg^{2+} . The general approach devised by Morrison et al. (1961) was adopted, and the results obtained were qualitatively and quantitatively similar to those reported by the above workers.

THEORY

The general kinetic equations describing the metal-activation of an enzyme and one substrate have been formulated by Dixon and Webb (1958) and applied to the study of the Mg^{2+} activation of creatine kinase (Morrison et al. 1961) and lombricine kinase (see below). Two fundamental assumptions have been made in formulating these equations.

Firstly, it is assumed that the enzyme (E) may interact with either the free metal (designated by M or μ) or free substrate (designated by S or σ) or with a metallo-substrate complex (MS) to form an enzyme-metal-substrate complex (EMS), and that this complex is the only source of reaction products. Since the formation of the ternary complex directly from free components in a trimolecular reaction is improbable (Dixon and Webb, 1958) the EMS complex is envisaged as the end result of three sets of bimolecular reactions involving combinations of E, M, and S in different sequences.

Structurally four different complexes could be formed (EMS, ESM, MES, $E \begin{smallmatrix} M \\ S \end{smallmatrix}$) but for the purposes of the present discussion the same complex is considered to be formed by each pathway. However the precise nature of this complex need not be specified (Dixon and Webb, 1958; Morrison et al. 1961). In the following treatment the substrate S refers to nucleotide, for no consideration has

been given to the possibility of lombricine or PL forming part of the ternary complex. Also no assumptions have been made with regard to the effect of S on the binding of M to the enzyme, and vice versa.

Secondly, it is assumed that all reactions leading to the formation of the ternary complex are in rapid equilibrium. It follows that the concentration of EMS will be independent of the pathway of formation.

From the above considerations the following equilibria, with their corresponding constants apply:

PATHWAY I

Combination between free enzyme and free substrate



PATHWAY II

Combination between free enzyme and free metal



PATHWAY III

Combination between free enzyme and metal-substrate



From the definition of the equilibrium constants:

$$K_1 K_2 = K_3 K_4 = K_5 K_6 = \frac{[E] [M] [S]}{[EMS]} \quad (45)$$

A velocity equation in terms of free components may be obtained from any four of the above equilibria e.g.

$$v = \frac{ke}{\frac{K_1 K_2}{\sigma \mu} + \frac{K_2}{\mu} + \frac{K_4}{\sigma} + 1} \quad (46)$$

In addition to the terms already defined k is the velocity constant of the breakdown of EMS, e is the total enzyme concentration, and v is the observed initial velocity. Equation (46) can be converted into a variety of other forms in accordance with equation (45).

The reciprocal form of equation (46) may be written:

$$\frac{1}{v} = \frac{1}{ke\sigma} \left[\frac{K_1 K_2}{\mu} + K_4 \right] + \frac{1}{ke} \left[\frac{K_2}{\mu} + 1 \right] \quad (47)$$

This is an equation of a straight line. Thus when $1/v$ is plotted against $1/\sigma$ for various values of μ (designated as μ') then a series of straight lines will be obtained cutting the ordinate at

$$\frac{1}{V_{\mu'}} = \frac{1 + \frac{K_2}{\mu'}}{ke} \quad (48)$$

and the abscissa at

$$-1/K_{\mu'} = \frac{1 + \frac{K_2}{\mu'}}{\frac{K_1 K_2}{\mu'} + K_4} \quad (49)$$

In this fashion a series of values for $V_{\mu'}$ and $K_{\mu'}$ may be obtained.

When the values of $1/v_{\mu}$, are plotted against $1/\mu$, essentially as described by Florini and Vestling (1957) a straight line is obtained which cuts the base line at $-1/K_2$.

Morrison et al. (1961) rearranged equation (49)

$$\text{to the form } K_{\mu} \left[\frac{K_2}{\mu} + 1 \right] = \frac{K_1 K_2}{\mu} + K_4 \quad (50)$$

so that a plot of $K_{\mu} \left[\frac{K_2}{\mu} + 1 \right]$ against $1/\mu$ gives

K_4 and $\frac{K_4}{-K_1 K_2}$ as the ordinate and abscissa intercepts

respectively. Hence values for K_1 , K_2 and K_4 are obtained. K_5 , the equilibrium constant between metal and substrate (in this instance between Mg^{2+} and either ADP^{3-} or ATP^{4-}) can be determined independently. Thus from the relationship (equation 45) the values of K_3 and K_6 are obtained.

MATERIALS

Buffers

NEM and triethanolamine, purified as described previously (Chapter 3), were adjusted to pH 7.0 and 8.6 respectively by the addition of 5N-HCl. Neither of these buffers interacts appreciably with metal ions.

Substrates

The nucleotides (ADP and ATP) and L- and D-lombricine were as described previously (Chapter 3).

PL(NH₄⁺ salt) was synthesised as described in Appendix 2.

Magnesium Chloride

A solution of MgCl₂·6H₂O (approximately 0.1M) was standardised by adsorbing 5 ml. portions onto a column (20 cm. x 1 cm.) of Dowex-50 (mesh 200-400, H⁺ form) resin, followed by washing the resin with 30 ml. of distilled water. The effluent acid was titrated with standard NaOH.

Enzyme

Lombricine kinase of specific activity 50 units/mg. of protein was purified as described in Chapter 3.

Removal of divalent cations from reaction components

Chelex 100 (200-400 mesh, Na⁺ form) was obtained from California Corporation For Biochemical Research, Los Angeles, California, U.S.A. Before use the resin was cycled batchwise through 1N-HCl and 1N-NaOH, and washed exhaustively with water before and after each step. When the pH of the effluent of the final water wash had fallen to 7.5, the washing was discontinued. The resin was stored wet until required. All components of the reaction systems (water, ADP, ATP, lombricine, PL, buffers and glycerol used to stabilise lombricine kinase) were treated with chelating resin as follows: Chelating resin (5 gm.) was added to the respective solutions (in about 100 ml.), and stirred for 4 hr. The solution was filtered on a sintered-glass filter, and the pH of the filtrate checked.

The interaction of the resin with the solutions was accompanied by a small pH change. The pH was readjusted with 0.1N-HCl or KOH, and the molarity of the solution adjusted with metal-free water to the final required values as described previously (Chapter 3).

Determination of the rate of the reverse reaction

The initial rate of the reverse reaction was determined by measuring the release of lombricine from PL. The method employed was essentially similar to that described in Chapter 3, but in order to increase the sensitivity of the assay the final volume was reduced from 10 ml. to 3 ml., and the quantities of the reagents used were scaled down accordingly. Thus to the reaction mixture (1 ml.) was added 0.3 ml. of EDTA-NaOH solution, followed by 1 ml. of 1-naphthol diacetyl reagent and 0.7 ml. of water. After 30 min. at room temperature the extinctions were determined at 535 m μ . Under these conditions 0.01 μ mole gave an extinction of 0.045 in cell with 1 cm. light path.

Determination of the rate of the forward reaction

The initial rate of the forward reaction was determined by measuring the formation of PL (estimated as orthophosphate liberated from PL by acid hydrolysis). The method employed was essentially similar to that described in Chapter 3, but in order to increase the

sensitivity of the assay, the final volume was reduced from 10 ml. to 3.1 ml., and the quantities of the reagents used were scaled down accordingly. Thus to the reaction mixture (1 ml.) was added 1 ml. of 0.2M-TCA. After hydrolysis 0.5 ml. ammonium molybdate (5% w/v), 0.4 ml. HClO_4 (60% w/v) and 0.2 ml. of the amino naphthol sulphonic acid reagent were added. The extinction was measured at 750 μ at 10 min. and under these conditions 0.1 μ mole of orthophosphate gave an extinction of 0.100 in a cell with a 1 cm. light path.

A series of reaction mixtures, each containing varying amounts of ATP, was prepared for each kinetic experiment. It follows that the correction factor for ATP hydrolysis etc. also varied for each incubation mixture. Allowance was made for this in each series by the inclusion of a zero time control containing a specified amount of ATP. The correction factor for the ATP present in each reaction mixture of the series was then assessed on a proportional basis in accordance with their ATP concentration.

Calculation of free metal and free nucleotide concentrations in reaction mixtures

The total $[\text{Mg}^{2+}]$ and total nucleotide necessary for maintaining free metal $[\mu]$ constant while varying free nucleotide $[\sigma]$ was calculated from the relationship

$$\text{Total nucleotide} = \frac{K_5 [\text{total metal}]}{[\mu]} - K_5 + [\text{total metal}] - \mu$$

which was derived from the equilibrium

$$K_5 = \frac{[\text{free nucleotide}][\text{free metal}]}{[\text{metal-nucleotide}]} \quad \text{or} \quad \frac{[\sigma] [\mu]}{[\text{metal-nucleotide}]}$$

$$\text{where } [\text{total nucleotide}] = [\sigma] + [\text{metal-nucleotide}]$$

$$\text{and } [\text{total metal}] = [\mu] + [\text{metal-nucleotide}]$$

It is apparent that the magnitude of K_5 which is the inverse of the stability constant of MgATP^{2-} or MgADP^{1-} must be known. Since the precise values of these stability constants are uncertain, as evidenced from the great variability of those reported in the literature, values of $70,000\text{M}^{-1}$ for MgATP^{2-} (O'Sullivan and Perrin, 1962) and $2,000\text{M}^{-1}$ for MgADP^{1-} (Morrison et al. 1961) were assumed for the above calculations. These particular values were adopted since they were obtained under conditions closely approximating those used in the present kinetic experiments.

Statistical estimation of the kinetic parameters of Michaelis-Menten equations

Provisional estimates of K and V values reported herein were determined statistically by the weighted-regression procedure of Wilkinson (1961).

RESULTS

Determination of residual activity

Preliminary experiments in the reverse reaction showed that the residual activity, i.e. the enzymic activity which proceeds in the absence of added Mg^{2+} ions, was 30% of the maximal activity obtained with $2.4 \times 10^{-4} M-Mg^{2+}$. This activity completely disappeared on the addition of $1 \times 10^{-3} M-EDTA$. Every component of the reaction system was then treated with chelating resin as described in Methods, as this resin selectively removes divalent metal ions even in the presence of high concentrations of monovalent ions. After this treatment the residual activity fell to less than 2% of the maximum velocity obtained with $4 \times 10^{-4} M-Mg^{2+}$, $7 \times 10^{-4} M-ADP$ and $1 \times 10^{-2} M-PL$. Treatment of lombricine kinase with chelating resin for 4 hr. at pH 7.0 did not cause any further appreciable alteration in the residual activity. It was therefore considered that the metal introduced with $2\mu g$ of protein was negligible. Similarly the residual activity in the forward reaction was not significant.

The kinetics of the reverse reaction

The effect of the concentration of PL on the initial velocity of the reverse reaction in the presence of $0.01 M-Mg^{2+}$ is shown in Fig.14. The K_m values for PL and the enzyme were calculated to be $7.4 \times 10^{-3} M$, while

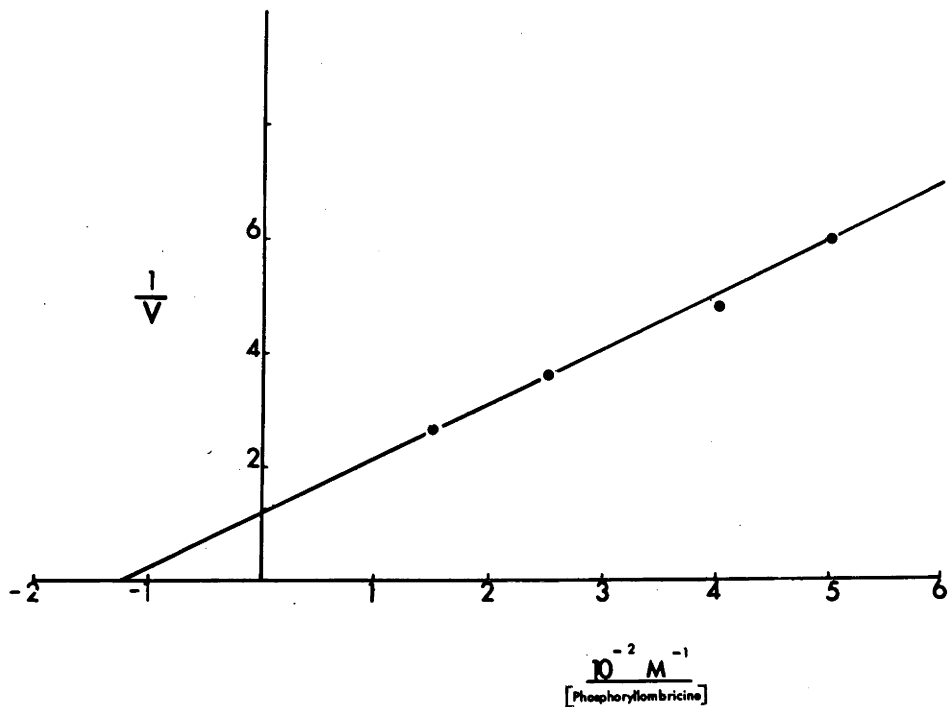


Fig.14. Effect of PL concentration on the initial velocity of the reverse reaction. The results are plotted after the manner of Lineweaver and Burk (1934). The reaction mixture (1 ml.) contained 0.1M-NEM-HCl pH 7.0, PL (NH_4^+ salt) was varied as shown, 0.01M- MgCl_2 , 0.002M-ADP, 0.5 μg of lombricine kinase. Temperature 30°. Reaction time 1 min. $v, \Delta E_{535}/\text{min.}$

the maximum velocity of the reaction corresponded to 200 μ moles of lombricine formed per mg. of protein per minute.

The activation of lombricine kinase by Mg^{2+} in relation to ADP^{3-} at pH 7.0 is shown in Fig.15 as plots of $1/v$ versus $1/\sigma$, with the concentrations of free Mg^{2+} varied from $0.8 \times 10^{-4}M$ to $4.0 \times 10^{-4}M$. A linear relationship was obtained with each plot, indicating that v and σ can be related by the Michaelis-Menten equation. The lines shown in Fig.15 were drawn using calculated values for K_{μ^+} and V_{μ^+} while the individual points shown were experimental findings. It is apparent that apart from the one plot with $\mu^+ = 0.4mM$ the lines converge to a common point on the abscissa $-\frac{1}{K_{\mu^+}}$. These results show that, although the reaction velocity is markedly dependent upon the Mg^{2+} concentration, the K_{μ^+} values can be considered to be independent of the Mg^{2+} concentration, thereby allowing an average value of $0.35mM$ to be assigned to K_{μ^+} . Clearly the binding of ADP^{3-} to the enzyme was not appreciably affected by the metal ion concentration. A summary of K_{μ^+} , and V_{μ^+} , values is presented in Table 20.

When $1/v_{\mu^+}$ values were plotted against $1/\mu^+$ a straight line was obtained (Fig.16) from which K_2 was calculated to be $0.24mM$. Plotting the calculated values

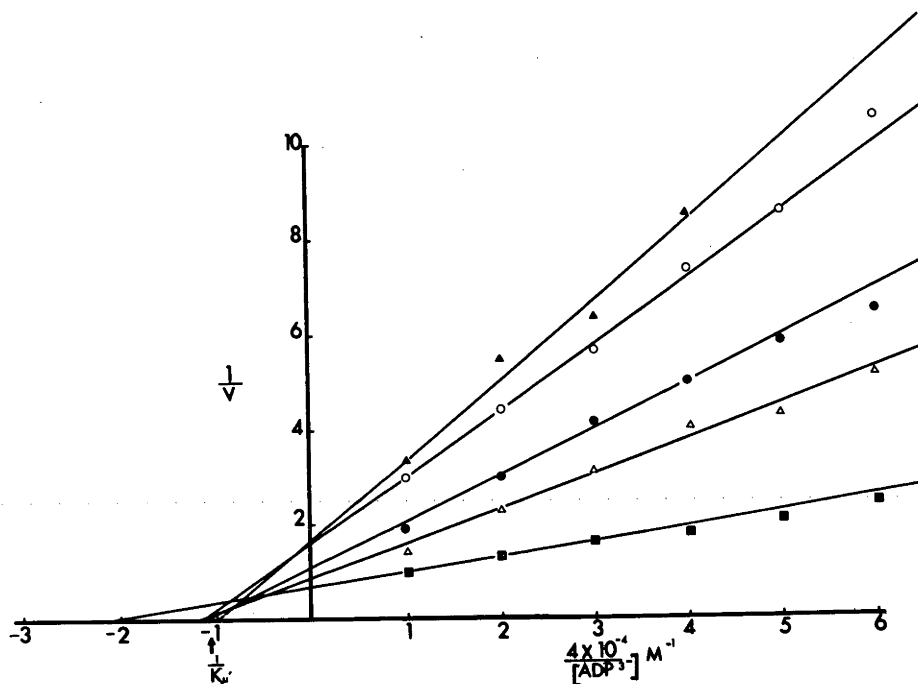


Fig.15. Double-reciprocal plots of the initial velocities in the reverse reaction at various concentrations of free $Mg^{2+}[\mu']$ against free $ADP^{3-}[\sigma]$ concentrations. The concentrations of total Mg^{2+} and total ADP^{3-} required to maintain $[\mu]$ at a fixed value $[\mu']$ while $[\sigma]$ was varied were determined as stated in the Methods. The reaction mixtures (1 ml.) contained 0.1M-NEM-HCl buffer pH 7.0, 0.01M-PL, free $Mg^{2+}[\mu']$ and free $ADP^{3-}[\sigma]$ at the concentrations shown, and $2\mu g$ lombricine kinase. Temperature 30° . Reaction time 1 min. $v, \Delta E_{535}/min.$ Values of Mg^{2+} were: \blacksquare , $4 \times 10^{-4}M$; \blacktriangle , $2 \times 10^{-4}M$; \bullet , $1.33 \times 10^{-4}M$; \circ , $1 \times 10^{-4}M$; \blacktriangle , $0.8 \times 10^{-4}M$.

TABLE 20.

Summary of $K_{\mu'}$ and $V_{\mu'}$ values for various Mg^{2+} [μ']
concentrations in the reverse reaction

Free Mg^{2+} concentration

(mM)		(mM)
μ'	$V_{\mu'}$	$K_{\mu'}$
0.4	14.97	0.179
0.2	12.32	0.322
0.133	9.13	0.347
0.1	6.12	0.341
0.08	5.97	0.402
0.066	5.0	0.326

Average value of $K_{\mu'}$ = 0.350

$V_{\mu'}$ values are expressed in arbitrary units.

The $K_{\mu'}$ and $V_{\mu'}$ values were calculated statistically by the weighted regression procedure of Wilkinson (1961).

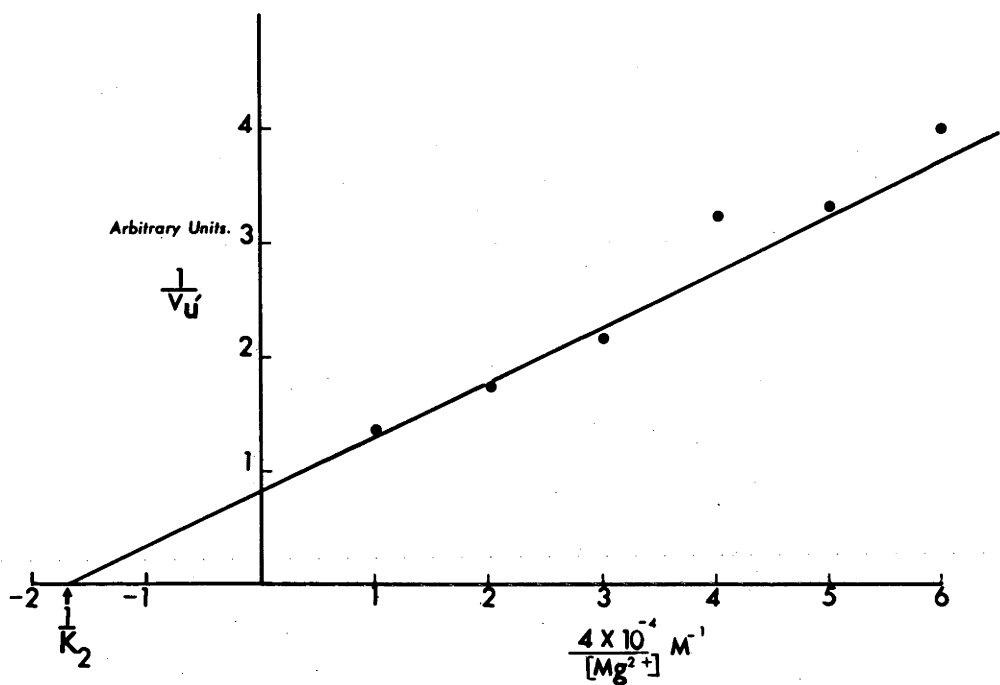


Fig. 16. Double-reciprocal plot of the maximum velocities ($v_{\mu'}$) obtained in the reverse reaction at various fixed free Mg^{2+} concentrations $[\mu']$ against the free Mg^{2+} concentration $[\mu']$. The calculated maximum velocities ($v_{\mu'}$) were presented in Table 20 expressed in arbitrary units.

$K_{\mu} \left[\frac{K_2}{\mu} + 1 \right]$ against $[\mu]$ according to equation (50)

using an average value of K_{μ} of 0.35mM gave values for K_4 and K_4/K_1K_2 as shown in Fig.17. From the foregoing plots values for K_1 , K_2 , and K_4 were obtained. As K_5 had been determined independently values were ascribed to the remaining constants K_3 and K_6 from the relationship expressed in equation (45).

Kinetics of the forward reaction

The general kinetic approach used for the reverse reaction was also employed to study the forward reaction. However the practical difficulties inherent in the assay procedure, i.e. in the determination of the amount of PL formed, reduced the sensitivity of the method, and severely restricted the range over which accurate kinetic data could be obtained.

The effect of lombricine concentration on the initial velocity of the forward reaction for both isomers of lombricine is shown in Fig.18. It is evident that although the K_m values (9mM and 7mM for D- and L-lombricine respectively) were not significantly different, the maximal velocity was greater with L-lombricine (Table 21).

In the studies of the Mg^{2+} activation of lombricine kinase in the forward reaction D-lombricine was held constant at a concentration of 0.01M. At this concentration the enzyme was not saturated with lombricine

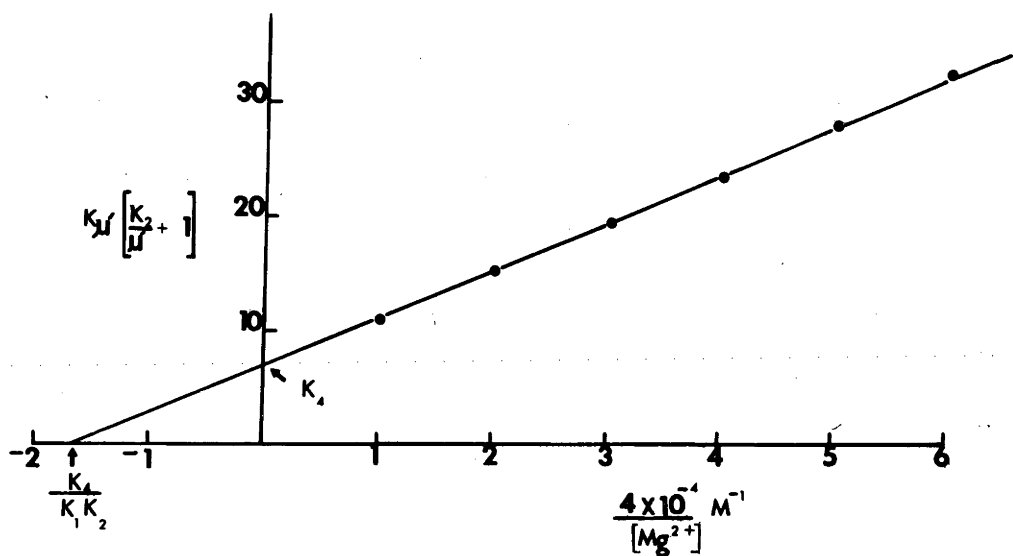


Fig.17. Plot of the calculated values $K_{\mu'} \left[\frac{K_2}{\mu'} + 1 \right]$ against the reciprocals of the concentrations of free Mg^{2+} in the reverse reaction.

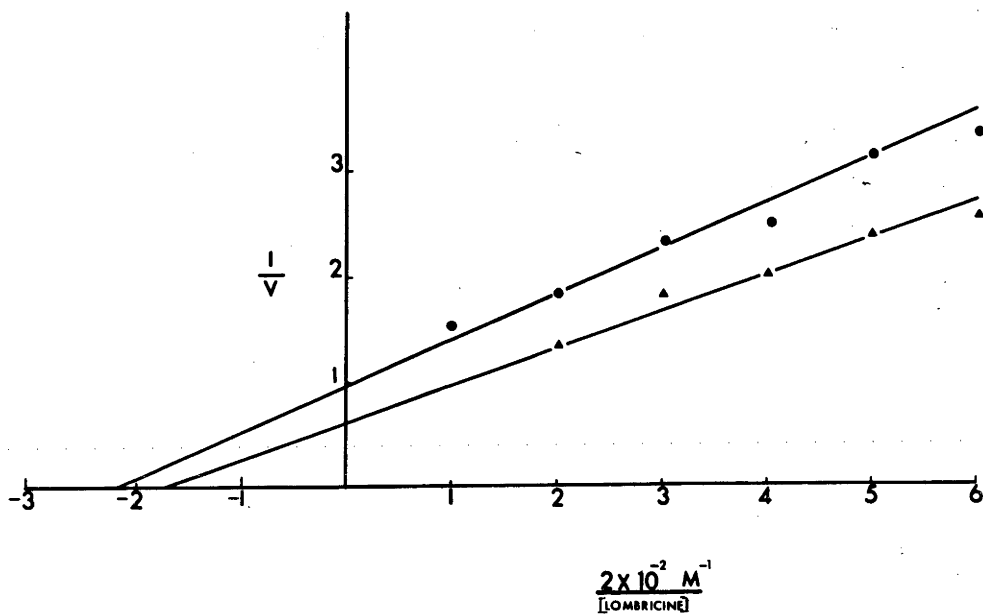


Fig.18. Effect of D- and L-lombricine concentration on the initial velocity of the forward reaction. The results are plotted after the manner of Lineweaver and Burk (1934). The reaction mixture (1 ml.) contained 0.1M-glycine-KOH buffer pH 8.6, D- or L-lombricine was varied as indicated, 0.005M-ATP, 0.005M-MgCl₂, and 10μg lombricine kinase. Temperature 30°. Reaction time 1 min. v , Δ E₇₅₀/min.

● , D-lombricine

▲ , L-lombricine

TABLE 21.

Maximum velocities and K_m values with D- and L-lombricine in the forward reaction

Substrate	K_m (mM)	V_{max} $\mu\text{moles PL formed/}$ min./mg. protein
D-lombricine	9.1	31
L-lombricine	11.7	49

but it was assumed that this would only reduce the rate by a constant factor.

Plots of $1/v$ against $1/[\text{free ATP}^{4-}]$ at various concentrations of Mg^{2+} ranging from $0.5 \times 10^{-4} \text{M}$ to $1.0 \times 10^{-4} \text{M}$ gave straight lines as predicted from equation (47). The lines shown in Fig.19 were drawn using calculated values for K_{μ} , and V_{μ} , while the individual points shown were experimental findings. The extrapolated lines converge to a point on the abscissa $-1/K_{\mu}$. It is evident that the metal affects the initial velocity in the forward reaction but the K_{μ} values may be considered in-variant of the Mg^{2+} concentration thereby allowing an average value of 0.09mM to be assigned to K_{μ} . The statistically calculated K_{μ} , and V_{μ} values are summarised in Table 22.

When $1/V_{\mu}$ values were plotted against $1/\mu$ a straight line was obtained (Fig.20) from which K_2 was calculated to be 0.1mM . For the subsequent plot (Fig.21) of the calculated value $K_{\mu} \left[\frac{K_2}{\mu} + 1 \right]$ against $1/\mu$ an average value of K_{μ} of 0.09mM was adopted and the values of K_4 and $-\frac{K_1 K_2}{K_4}$ were determined as the ordinate and abscissa intercepts respectively.

From the foregoing plots values of K_1 , K_2 and K_4 were obtained. Since K_5 was determined independently by

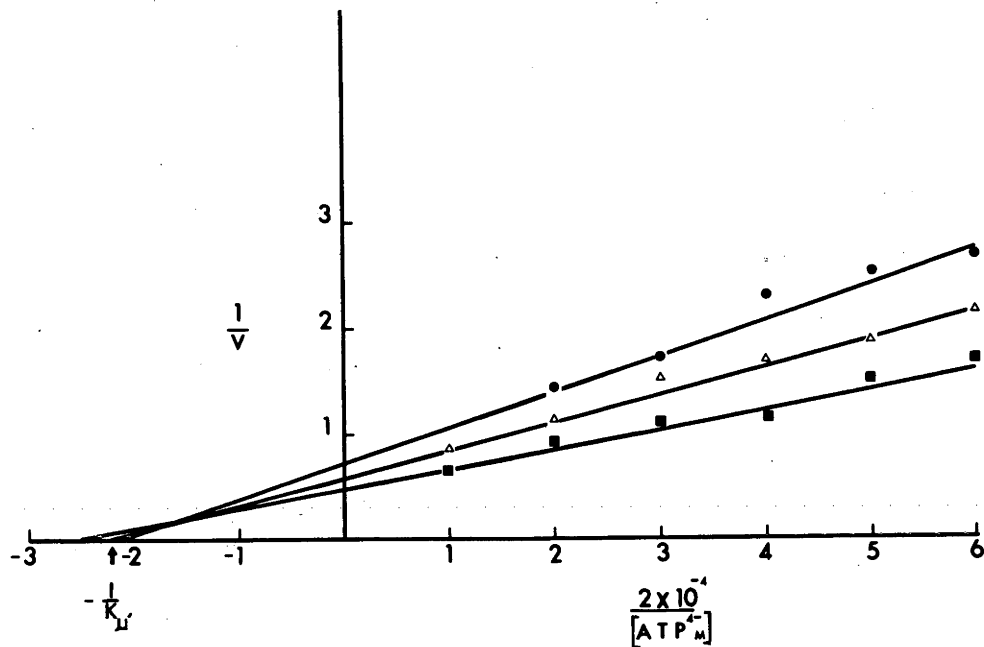


Fig.19. Double-reciprocal plots of the initial velocities in the forward reaction at various concentrations of free $Mg^{2+}[\mu']$ against free $ATP^{4-}[\sigma]$ concentrations. The concentrations of total Mg^{2+} and total ATP^{4-} required to maintain $[\mu]$ at a fixed value $[\mu']$ while $[\sigma]$ was varied were determined as stated in the Methods. The reaction mixtures (1 ml.) contained 0.1M-triethanolamine buffer pH 8.6, 0.01M-D-lombricine, free $Mg^{2+}[\mu']$ and free $ATP^{4-}[\sigma]$ as indicated, and $5\mu g$ lombricine kinase. Temperature 30° . Reaction time 1 min.

v , $\Delta E_{750}/min$. Values of Mg^{2+} were: ■, $1 \times 10^{-4}M$; Δ , $0.66 \times 10^{-4}M$; ●, $0.5 \times 10^{-4}M$.

TABLE 22.

Summary of $V_{\mu'}$ and $K_{\mu'}$ values at various fixed concentrations of $Mg^{2+}[\mu']$ in the forward reaction

Free Mg^{2+} concentration

(mM)		(mM)
μ'	$V_{\mu'}$	$K_{\mu'}$
0.1	0.51	0.078
0.066	0.42	0.09
0.05	0.34	0.095

Average value of $K_{\mu'} = 0.09$

All V values are expressed in arbitrary units.

The $K_{\mu'}$ and $V_{\mu'}$ values were determined statistically by the weighted regression method of Wilkinson (1961).

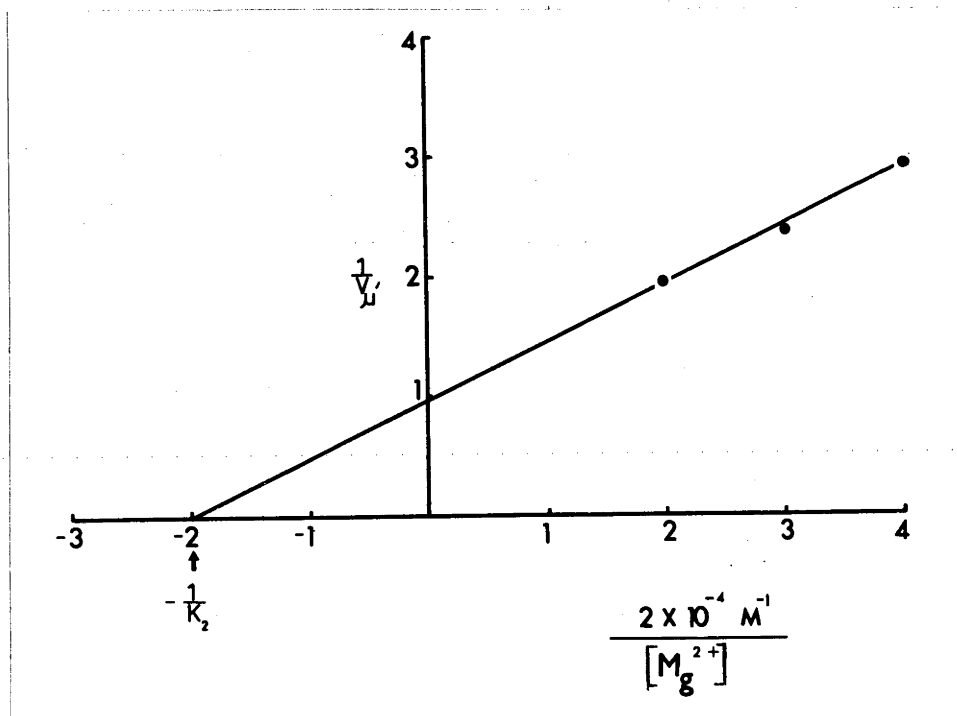


Fig.20. Double-reciprocal plot of the maximum velocities ($v_{\mu'}$) obtained in the forward reaction at various fixed free Mg^{2+} concentrations $[\mu']$ against the free Mg^{2+} concentration $[\mu']$. The calculated maximum velocities ($v_{\mu'}$) were presented in Table 22 expressed in arbitrary units.

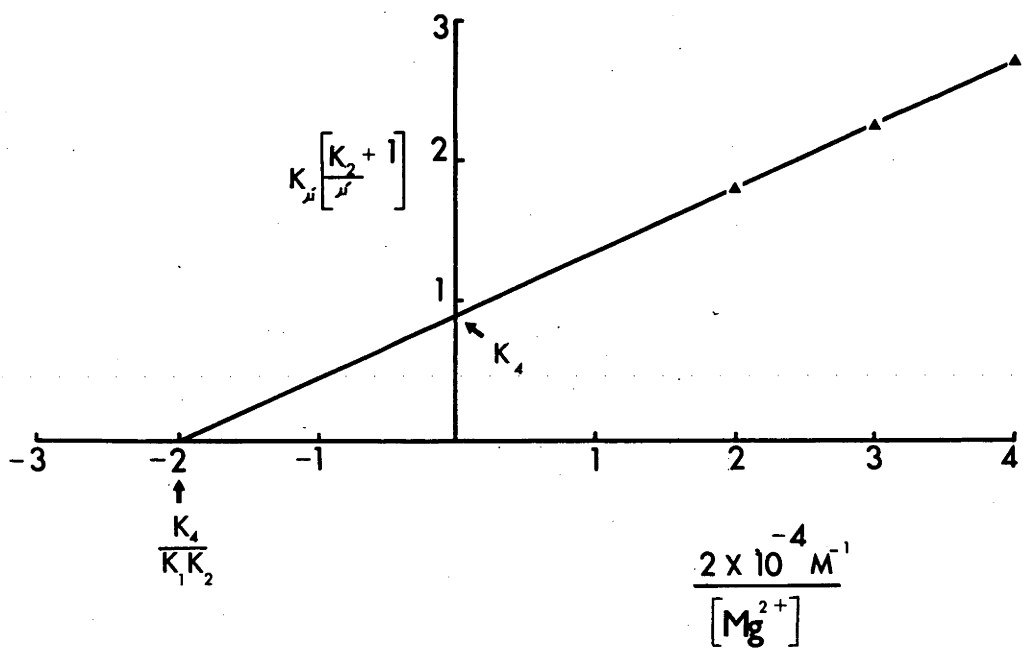


Fig.21. Plot of the calculated values $K_{\mu'} \left[\frac{K_2}{\mu'} + 1 \right]$ against the reciprocals of the concentrations of free Mg^{2+} in the forward reaction.

application of equation (45) values were ascribed to the remaining unknowns K_3 and K_6 .

The values obtained for the dissociation constants in both forward and reverse reactions are summarised in Table 23.

DISCUSSION

In the forward reaction the maximum velocity obtained with L-lombricine, the unnatural isomer, was greater than that with D-lombricine (Table 21). This finding was in accord with that of Rosenberg et al. (1960) and in addition showed that the differences in rate cannot be attributed to differences in the affinity of the enzyme for the isomers, since their K_m values were approximately the same.

In preliminary experiments a high residual activity was observed (30% maximum). In similar reaction systems Morrison et al. (1961) demonstrated that this activity was largely accounted for by Ca^{2+} and Mg^{2+} added with the substrates, as judged by direct trace element analyses. After removal of divalent cations from all the reaction components the residual activity decreased to insignificant levels (2% of maximum), which illustrates that the reaction catalysed by lombricine kinase exhibits an absolute requirement for metal ion. Furthermore, the kinetic data show that the activation which follows the

TABLE 23.

Summary of the dissociation constants of the
bimolecular reactions involved in the formation
of the ternary (EMS) complex

	<u>Forward Reaction</u>	<u>Reverse Reaction</u>
	(mM)	(mM)
K ₁	0.09	0.245
K ₂	0.1	0.24
K ₃	0.1	0.24
K ₄	0.09	0.245
K ₅	0.014	0.5
K ₆	0.64	0.12

readdition of metal ion obeys the rate laws predicted for a metal activated enzyme. On these grounds lombricine kinase may be classified as a true metal enzyme as defined by Malmstrom and Rosenberg (1959).

The possibility that the observed activation could be due to non specific effects of the metal ion, such as effects on ionic strength or electrostatic properties, or by the removal of an inhibitor as its metal complex, or by the stabilisation of the enzyme in dilute solution, can be discounted, for the reactions were carried out in mixtures of high ionic strength (0.1M) and enzyme, buffers, and substrates were highly purified. Furthermore the enzyme was quite stable for at least 4 hr. at 0° in dilute buffer at pH 7.0 when there was virtually no metal present as judged by residual activity experiments.

Because the general kinetic equation (46) describing metal ion activation is expressed in terms of the concentrations of free components, it is necessary for the concentration of one free component to remain constant while the concentration of the other free component is being varied. In this connection the approach devised by Morrison et al. (1961) has proven most useful. This approach contrasts with those previously used in which the total concentration of one component has been varied in the presence of fixed total concentration of the other.

Under such conditions the free concentrations may become quite small, particularly if the stability of the metal substrate complex is high. As a consequence the effect of the free components may not be readily apparent, especially if a restricted range of experimental conditions is employed.

This point may be illustrated by kinetic data obtained from recent studies of the activation of creatine kinase by Mg^{2+} (Noda et al. 1960; Nihei et al. 1961). It was shown that, over a limited concentration range of total metal and total nucleotide, there was good agreement between the experimental findings and predictions based on the assumption that Mg^{2+} -nucleotide was the true substrate. However, when the concentration of total nucleotide was greatly in excess of total metal the experimental findings did not fit the predicted values. Therefore it became necessary to take the effect of free nucleotide into consideration, in order to obtain agreement between experimental and theory.

In the experiments reported herein the activation of lombricine kinase by Mg^{2+} was studied primarily in relation to the concentration of free ATP^{4-} and free ADP^{3-} in the forward and reverse reactions respectively, with the concentration of the substrates D-lombricine and PL held constant at $1 \times 10^{-2} M$.

On the assumption that equilibrium kinetics are applicable, it has been shown that the experimental findings conform closely to those predicted by the general kinetic equation (46). It follows therefore, that Pathways I, II and III are possible, and values have been assigned to the dissociation constants of each of the possible reactions involved (Table 23).

Although the present results (Table 23) demonstrate that each of the three pathways leading to the formation of the ternary complex is feasible from a kinetic viewpoint, this does not imply that each of the three pathways is in fact operative. Thus, if each of the complexes of the various pathways were linked to all others by at least one route, and equilibrium conditions prevailed, then the same general form of equation (46) would be obtained. In other words, any particular pathway might proceed at zero rate overall, and, provided that both reactions of the pathway did not proceed at zero rate, the same general kinetic results would be obtained (Morrison et al. 1961).

The fact that the experimental findings are in excellent agreement with a kinetic scheme which assumes that the complexes EM, ES, and EMS are formed, demonstrates that these species, together with their corresponding dissociation constants must be taken into consideration,

even though the rates of some of the reactions of Pathway I or II may be zero.

Since the kinetic results are compatible with lombricine kinase interacting with free metal, free nucleotide, or with the metal nucleotide complex it follows that kinetic data alone are insufficient to determine which particular pathway is operative in the formation of the EMS complex. This shortcoming of the kinetic approach stems from the fact that the ternary complex is considered to be the end result of all metal activation mechanisms. Similar rate equations are obtained, therefore, for the various pathways involved in its formation, the only difference being that the kinetic constants have different physical meanings.

In order to determine if a particular pathway is favoured in the formation of the EMS complex, additional information concerning the thermodynamic equilibria between enzyme and metal or enzyme and nucleotide would be helpful. Thus, if the thermodynamic value for the binding of E to M were not the same as the kinetic value (K_3) then the suggestion that activation involves the formation of a metal-enzyme complex would not be tenable (provided that equilibrium kinetic apply).

Despite the vast amount of empirical data available no unequivocal formulation of the role of the metal ion in

the catalysis of metal ion-activated enzymes has been proposed (Malmstrom and Rosenberg, 1959). One hypothesis, originally proposed by Hellerman and Perkins (1935) for metal activation of arginase and later extended by Smith (1949) to the peptidases, envisaged that the function of the metal ion was to serve as a link between enzyme and substrate. While this concept has received support from studies with model systems (Klotz and Ming, 1954; Gurd, 1954) it has not been demonstrated unequivocally in enzyme systems (Malmstrom, 1961). In the present experiments there is little evidence that Mg^{2+} forms a link between ADP^{3-} and the enzyme in the reverse reaction, since the binding of ADP^{3-} to the enzyme is virtually the same, irrespective of whether Mg^{2+} is combined to the enzyme or not ($K_1 = K_4$). Also since $K_2 = K_3$ the binding of Mg^{2+} is not dependent upon ADP^{3-} . In the forward reaction, however, a slight drift in K_{μ} values was observed which might be construed as indicating that Mg^{2+} has a small effect on the binding of ATP^{4-} , but the number of experiments were too few to evaluate the significance of this drift. As pointed out by Morrison et al. (1961) the magnitude of the experimental error, due to the relative insensitivity of the assay procedures as well as to the low K_m of the substrates, could allow weak interactions between metal and substrate on the enzyme surface to remain undetected.

Another hypothesis proposes that the function of the metal ion might be to alter the shape of the enzyme, to mould it into its active form (Eichhorn, 1961). While there is no evidence that the metal ion per se causes this effect with guanidino kinases, there are recent reports dealing with conformational changes induced in the structure of creatine kinase on combination with all its substrates (Samuels, Nihei and Noda, 1961). Thus small but consistent increases in specific rotation were observed when creatine kinase was at equilibrium with PC, creatine, Mg^{2+} , ADP and ATP. This finding was consistent with immunoenzymological evidence (Samuels, 1961) to the effect that when creatine kinase was activated with all its substrates it was protected from combination with its specific antibody. This suggested that enzyme substrate interaction altered the configuration of a significant portion of the enzyme molecule.

It has also been proposed that the metal ion may directly partake in changing the electronic structure of the substrate molecule. This hypothesis implies that a ternary (EMS) complex is formed, an assumption which has already been made on kinetic grounds. However, further support for this assumption is derived from nuclear magnetic resonance studies (Cohn and Leigh, 1962) which have demonstrated the formation of a ternary complex between Mn^{2+} , ADP and creatine kinase; no ternary complex was formed

with PC. It was further concluded that Mn^{2+} was bonded only to the nucleotide, and that metal enzyme interaction did not take place. If this interpretation of the results is confirmed, and if the conclusions derived from the study of Mn^{2+} activation are also applicable to Mg^{2+} activation, then the assumption that Mg^{2+} -enzyme can be formed as a primary complex would be invalid. However, at this juncture it seems premature to speculate on these points.

Although at present no information is available about the structure of the ternary complex, some attempts have been made to define the nature of the Mg^{2+} -nucleotide interaction. Morrison, Ennor and Griffiths (1958) postulated that Mg^{2+} was attached to the α and β phosphoryl groups of ATP. It was envisaged that this would increase the positive charge on the terminal phosphorus atom, with subsequent weakening of the terminal O-P bond. Phosphoryl group transfer reactions catalysed by guanidino kinases could involve an electrophilic attack by the terminal phosphorus atom of ATP, on the lone pair of electrons of the guanidino NH_2 group of the phosphagen base leading to the formation of an N-P link, with rupture of the O-P bond.

In this connection studies by Cohn and Hughes (1962) on the nuclear magnetic resonance spectra of the hydrogen and phosphorus nuclei of the Mg^{2+} complexes of

ATP and ADP are of interest. These findings indicated that in solution the metal ion complexed with the O^- of the β and γ phosphate groups of ATP, and with the α and β groups of ADP; no complex with the adenine ring of ATP was detected. These authors ascribe the observed differences in the stability constants between the ADP and ATP complexes of Mg^{2+} to these differences in complexing sites. While it would be anticipated that Mg^{2+} attached to the β and γ phosphates of ATP would also have the effect of weakening the terminal O-P bond, it introduces the difficulty that the attachment of the metal to the γ -phosphate might also be expected to hinder the transfer of this group.

SUMMARY

1. The kinetics of the enzymic reaction



have been studied in both directions in relation to the activation of lombricine kinase by Mg^{2+} .

2. On the assumption that a ternary enzyme-metal-substrate complex is involved in the enzymic catalysis, and that all the reactions leading to its formation are in rapid equilibrium, the dissociation constants of all the possible intermediate steps have been determined.
3. It was concluded that the kinetic results were compatible with the view that the enzyme interacted with free nucleotide, free Mg^{2+} , or with a metal-nucleotide complex prior to the formation of the ternary complex.
4. The magnitudes of the dissociation constants indicated that Mg^{2+} had little, if any, effect on the binding of the nucleotides to the enzyme.

APPENDIX 1.

THE CHARACTERISATION OF LOMBRICINE FROM
THE EARTHWORM, MEGASCOLIDES CAMERONI

APPENDIX 1.

THE ISOLATION AND CHARACTERISATION OF LOMBRICINE FROM
THE EARTHWORM MEGASCOLIDES CAMERONI

INTRODUCTION

It has been established that D-lombricine is present in small earthworms (see Introduction). From this it was inferred in the preceding chapters that lombricine in the giant earthworm Megascolides cameroni would also be of the D-configuration. However, it was necessary to make certain of this point because extensive use has been made of this animal both in tracer studies and in the preparation of lombricine kinase.

This section deals with the isolation of D-lombricine from giant earthworms. In addition it serves to illustrate the techniques which were employed to obtain lombricine and SEP for use as substrates for enzyme reactions referred to in preceding chapters. The method employed was that described by Ennor et al. (1960) adapted to a smaller scale.

MATERIALS AND METHODS

Solvents, reference compounds, ion exchange resins, and animals were as described in Chapter 1.

Gradient Elution from Amberlite CG-120 (mesh 100-200, H⁺ form)

A gradient elution was carried out essentially as described by Hurlbert, Schmitz, Brumm and Potter (1954) using Amberlite CG-120 in a column (40 cm. x 4 cm.). An increasing gradient of HClO₄ was achieved by placing 750 ml. of water in a mixing chamber (1 l.) which was connected to a 3.1 reservoir, charged with 2 l. of 0.5N-HClO₄. After 1.5 l. of eluant had passed through the column, 1.5 l. of 0.75N-HClO₄ was added to the reservoir. Similarly at effluent volumes of 2 l., 3 l. and 4.5 l. the reservoir was replenished with 1.2 l. of 1N, 1.0 l. of 1.5N and 1 l. of 2.0N-HClO₄ respectively. A gravity feed was used to obtain a constant flow rate of 10 ml./min. through the column.

The determination of ninhydrin-reacting materials, guanidino-reacting materials, and chromatographic procedures have already been described (Chapter 1).

D-Amino acid oxidase

Sheep-kidney D-amino acid oxidase was a kind gift of Dr. H. Rosenberg. The enzyme was prepared according to the method of Negelein and Brömel (1939) and was taken to Stage III (NH₄)₂SO₄ precipitation. The precipitate containing the enzyme was stored at 0° as a suspension in 50% saturated (NH₄)₂SO₄. Portions of this suspension were centrifuged as required and the precipitate was suspended in 0.01M-pyrophosphate buffer, pH 8.1, and dialysed against

100 vol. of the same buffer, with two changes, for 24 hr. The dialysed solution was cleared by centrifugation and its volume adjusted with water so that each ml. of the solution corresponded to 1 g. of the acetone-dried kidney powder used in the preparation. The solution had been tested for the presence of catalase according to Herbert (1955) and it was found that 0.1 ml. of the solution decomposed 100 μ moles of H_2O_2 in 30 seconds. The solution was kept frozen at -10° and retained activity for at least 3 months. Assays with this enzyme were carried out in Warburg manometers at 37° in air. The vessels contained 2.0 ml. of 0.1M-sodium pyrophosphate buffer, pH 8.3, the amino acid tested and 0.3ml. of the enzyme solution, in a total volume of 3 ml.

Determination of optical rotations

Optical rotations were determined in a polarimeter measuring to 0.01° in a small bore tube 10 cm. in length, with an approximate capacity of 1 ml. Solvent and concentrations were as stated in the text.

RESULTS

Isolation of lombricine

Fresh whole earthworms (Megascolides cameroni) were disintegrated in batches of 200 g. in a Waring Blendor with 1.5 volumes of 1.5N- $HClO_4$. After 600 gm. were extracted in this manner, the combined suspension was centrifuged and the supernatant brought to pH 7 with 10 KOH. It was then

cooled to 0° , and the precipitated potassium perchlorate was removed by centrifugation and discarded. One volume of ethanol was added to the supernatant, and the precipitated glycogen was centrifuged off. The supernatant, about 3 l., was applied to a column (25 cm. x 4 cm.) of Zeo-Karb 225 (mesh 20-50, H^{+} form) at the rate of 20 ml./min. The effluent contained no guanidino-reacting materials and was discarded. The resin was washed with water until the effluent was about pH 6, and the adsorbed materials were eluted with aqueous 2.5N- NH_3 sol. at a rate of 10 ml./min. NH_3 and water were removed from the eluate under reduced pressure at 40° , with consequent reduction in volume to about 30 ml. This was applied to a column (40 cm. x 4 cm.) of Amberlite CG-120 resin (mesh 100-200, H^{+} form) and subjected to gradient elution as described in Methods. The eluate was collected in 25 ml. fractions. Fractions 96-105, 124-135, 140-156 and 180-202 contained ninhydrin-reacting materials, but only fractions 180-202 showed the presence of guanidino-reacting material. These fractions (180-202) were pooled, brought to pH 7 with 10N-KOH at 0° , and the potassium perchlorate filtered off. The filtrate (500 ml.) was applied to a column (20 cm. x 4 cm.) Zeo-Karb 225 (mesh 20-50, H^{+}) at a rate of 20 ml./min. All guanidino-reacting material was retained on the resin. The resin was washed with distilled water until the effluent

was about pH 6 and the adsorbed materials were eluted with aq. 2.5N-NH₃ sol. at the rate of 10 ml./min. NH₃ and water were removed under reduced pressure at 40° until the solution was concentrated to about 20 ml. A unidimensional paper chromatogram in Solvent system 1 of approximately 0.005 ml. of this solution showed that the bulk of the material was lombricine, with only minor contamination with other ninhydrin-reacting material. The solution was layered evenly on top of a column (40 cm. x 4 cm.) of Dowex-50 (mesh 200-400, NH₄⁺) resin and eluted with distilled water. The eluate, collected in 5 ml. fractions, was found to contain guanidino-reacting material in fractions 78-203.

Unidimensional paper chromatography of 0.005 ml. from each of these fractions in Solvent system 1 showed, in addition to lombricine, the presence of ninhydrin-reacting contaminants in fractions 113-141. Those fractions which were free of impurities were pooled, and taken to dryness under reduced pressure at 40°.

The white powder so obtained was taken up in 10 ml. of water, clarified by charcoal treatment, and lombricine crystallised by the slow addition of two volumes of ethanol. Lombricine was recrystallised from aqueous ethanol as colourless needles, and dried over P₂O₅ (yield 300 mg.). Two-dimensional paper chromatography of this crystalline material in Solvent systems 1 and 2 revealed no ninhydrin or guanidino-reacting material other than lombricine.

Lombricine from Megascolides cameroni had a $\alpha_D^{23} + 11.9^\circ$ (c 2.5 in water). Synthetic D-lombricine had a $\alpha_D^{23.5} + 16.1$ (c 0.805 in water) and lombricine from small earthworms $\alpha_D^{23.5} + 14.5$ (c 0.93 in water) (Beatty and Magrath, 1960). The lombricine isolated from giant earthworms was therefore of the D-configuration.

ISOLATION OF THE SERINE MOIETY OF LOMBRICINE

Acid hydrolysis of lombricine

50 mg. of crystalline lombricine isolated by the above procedure was hydrolysed in 1 ml. of 6N-HCl in a sealed tube for 20 hr. at 110° . After hydrolysis the HCl was removed under reduced pressure at 40° , the degradation products taken to dryness, and placed in a desiccator over KOH under reduced pressure overnight.

Isolation of the serine component of the acid hydrolysis products

The acid degradation products of lombricine are known to be serine, 2-guanidinoethylphosphate, guanidino-ethanol and orthophosphate (Robin, 1954). In order to isolate the serine component the hydrolysis products were taken up in a small volume of water and applied to a column (10 cm. x 1 cm.) of Dowex-50 resin (mesh 200-400, H^+). The acidic components of the reaction mixture were eluted from the resin with 30 ml. of water and when the effluent was approximately pH 6 the material retained on the resin

was eluted with aq. 2.5N-NH₃ sol. Water and NH₃ were removed from the eluate under reduced pressure at 40°, with concentration of the eluate to about 5 ml. Ascending paper chromatography in Solvent system 2 of 0.01 ml. of this solution showed serine to be the main component with only minor contamination with two other ninhydrin-reacting materials. Neither lombricine nor guanidinoethanol were detected with 1-naphthol-diacetyl spray, indicating that lombricine had been completely hydrolysed and that the guanidinoethanol being quite basic was retained on the column, and was not eluted by aq. 2.5N-NH₃ sol. The solution containing serine was applied evenly to a column (40 cm. x 4 cm.) of Dowex-50 (mesh 200-400, NH₄⁺), and eluted at the rate of 5 ml./min. with 500 ml. of water, the eluate being collected in 5 ml. fractions. Ascending paper chromatography of 0.01 ml. in Solvent system 2 showed the presence of serine in fractions 39-60. No ninhydrin-reacting material other than serine was detected.

Fractions 39-60 were pooled and the volume reduced to about 3 ml. under reduced pressure at 40°. Two volumes of ethanol were added to this solution which was stood at 5° overnight. Fine needle-like crystals formed, which were filtered off and dried over P₂O₅ in a desiccator (yield 5.5 mg.). Two-dimensional paper chromatography of 5 μmoles of serine in Solvent systems 1 and 2 showed the presence of a barely-detectable

ninhydrin contaminant.

The ability of the isolated crystalline serine to act as a substrate for D-amino acid oxidase was compared with that of authentic samples of L- and D-serine, and it was found (Table 24) that the O₂ uptake with the isolated sample was that expected if the entire material was D-serine.

DISCUSSION

The results demonstrate that the lombricine isolated from the giant earthworm Megascolides cameroni has the same optical rotation as the synthetic D-isomer. As expected the serine moiety isolated from the lombricine of these animals was shown to be also of the D-configuration by enzymic studies. These results, therefore, provide conclusive evidence that D-lombricine is present in the giant earthworm. By analogy with the smaller variety of earthworms, it is logical to conclude that SEP of Megascolides cameroni is also the D-isomer. Since SEP is present in only small quantities (30 mg./kg.) sufficient material could not be spared to prove this point directly.

SUMMARY

1. The lombricine of the earthworm Megascolides cameroni was isolated and characterised as the D-isomer.

TABLE 24.

Effect of sheep kidney D-amino acid oxidase on serine
obtained by hydrolysis of lombricine of the earthworm,

Megascolides cameroni

Substrate	D-serine (authentic)	Serine (isolated)	L-serine (authentic)
Amount present			
(μ moles)	9.53	19.1	9.53
Uptake of O ₂			
(μ g.atoms/2 hr.)	11.0	18.6	0

The reaction mixtures (3 ml.) contained 0.066M-sodium pyrophosphate buffer pH 8.3, serine as indicated, and 0.3 ml. of sheep-kidney D-amino acid oxidase preparation. Assays were carried out in Warburg manometers at 37° in air.

APPENDIX 2.

THE ENZYMIC PREPARATION OF PHOSPHO LOMBRICINE

APPENDIX 2.

THE ENZYMIC PREPARATION OF PHOSPHOLOMBRICINE

INTRODUCTION

Large quantities of phospholombricine (PL) were required for kinetic investigation of lombricine kinase, and to meet this need Ennor and Rosenberg (1962) developed a procedure for the isolation of analytically pure PL (Mg^{2+} salt) in 70% yield from natural sources. However the PL content of earthworms varied with different batches, depending upon the condition of the worms prior to freezing at the time of collection. Hence the absolute yields varied from 0.17 g. to 0.29 g. of PL (Mg^{2+} salt) per kg. of earthworms. Since pure lombricine (Ennor et al. 1960) and lombricine kinase (Chapter 3) were readily available an alternative procedure was developed in which PL was prepared enzymically, and subsequently purified by a modification of the techniques employed by Ennor and Rosenberg (1962).

MATERIALS AND METHODS

D-Lombricine, ATP, glycine-KOH buffer and magnesium chloride were as described in Chapter 3.

Dowex-1- HCO_3^-

Dowex-1(mesh 200-400) anion exchange resin was converted from the Cl^- form to the HCO_3^- form by passage of

1.5M-NaHCO₃ (Martónosi, 1960) through a column (30 cm. x 4 cm.) of resin until the effluent was Cl⁻ free. The column was then washed with water until the pH of the effluent was about 8.

Magnesium acetate solution

For the precipitation of PL as a Mg²⁺ salt a 20% solution of magnesium acetate (w/v) in 50% ethanol (v/v) was used. This is referred to as magnesium acetate solution in the text.

Estimation of lombricine, PL, and orthophosphate

These compounds were estimated as described in Chapter 3.

RESULTS

The reaction mixture (100 ml.) containing 0.1M-glycine-KOH buffer pH 9.5; 0.02M-D-lombricine; 0.03M-ATP; 0.004M-MgCl₂ was incubated at room temperature, and purified lombricine kinase (0.1 ml. of a 20 mg.protein/ml. solution, specific activity 20 (see Chapter 3) was added every hour for five hours. After six hours the reaction was stopped by freezing and thawing, thereby denaturing the enzyme. Samples (0.02 ml.) taken for estimation of PL showed that 80% of the 2 m moles of lombricine originally present had been phosphorylated. In order to remove nucleotides the reaction mixture was stirred with 15 gm. of acid washed charcoal (Norit A) for 3 hours after which the charcoal was removed by filtration.

The solution was free of nucleotides as judged by measurement of the extinction of a 0.1 ml. sample at 259 m μ . The charcoal was washed twice with water (20 ml.) and the washings were added to the filtrate (vol. 115 ml.). Denatured lombricine kinase was also removed with the charcoal at this step. The filtrate contained 1500 μ moles of PL and 250 μ moles of lombricine, indicating that no significant quantities of these compounds were adsorbed onto the charcoal.

In order to separate PL and lombricine the solution was passed through a column (30 cm. x 4 cm.) of Dowex-1 (mesh 200-400, HCO₃⁻ form) ion exchange resin at the rate of 3 ml./min. Some colloidal charcoal, not removed by filtration, remained on the surface of the resin. Lombricine was eluted from the column with water and was recovered for later preparations. When the effluent was lombricine-free, 300 ml. of 0.15N-KHCO₃ was passed through to remove final traces of lombricine. PL was then eluted from the resin with 1.5N-KHCO₃, the effluent being collected in 10 ml. fractions. The fractions containing PL were pooled, and cooled to 1° in an ice bath.

With the pH monitored by a glass electrode the solution was made acid by the slow addition of a suspension of Dowex-50 (mesh 200-400, H⁺) ion exchange resin. At

approximately pH 5, vigorous evolution of CO_2 commenced and the solution was aerated to assist the removal of CO_2 . Resin was added until the pH remained steady at 3, and then the pH was adjusted with 5N-NaOH until it was stable at 7.5. The ion exchange resin was removed by filtration, washed twice with water (50 ml.) and the washings combined with the filtrate. The pooled solution (500 ml.) contained 1400 μmoles of PL with no free lombricine detectable in a sample of 1 μmole of PL. Magnesium acetate solution (10 ml.) was added, followed by 3 volumes of 95% ethanol, which precipitated PL as the magnesium salt. The precipitate was collected by centrifugation, washed twice with 95% ethanol and once with acetone, and then dried under reduced pressure in a desiccator over KOH and paraffin. The dry material was suspended in water (about 20 ml.) and centrifuged. The precipitate was extracted three times with water, and the extracts after centrifugation were combined. After addition of 1 ml. of magnesium acetate solution, the PL was precipitated with ethanol, collected and dried as described above (Weight 674 mg.). Elementary analysis showed that the material was over 95% pure. The yield was 1220 μmoles , i.e., 76% overall, with a free lombricine content of $< 0.18\%$ detected in 7 μmoles of PL.

The dry material was suspended in water (15 ml.) and centrifuged, and the residue extracted twice with water as before. The solution was converted to the NH_4^+ salt by passage through a column (40 cm. x 3 cm.) of Dowex-50 (mesh 200-400, NH_4^+ form) resin. The resin was washed with water, the eluate collected in 5 ml. fractions and fractions containing PL were pooled. Some of the PL (NH_4^+ salt), about 100 μmoles , was converted back to the Mg^{2+} salt for elementary analysis by the addition of two drops of magnesium acetate solution, followed by precipitation with three volumes of ethanol. The precipitate was collected by centrifugation, washed twice with 95% ethanol, once with acetone, and finally dried in a desiccator under reduced pressure over KOH (Found: C, 15.73; H, 5.47; N, 13.8; P, 13.52; Mg, 4.25. Calc. for $\text{C}_6\text{H}_{15}\text{O}_9\text{N}_{4.5}\text{Mg}_{0.75}$: C, 16.14; H, 5.55; N, 14.1; P, 13.9; Mg, 4.07%).

DISCUSSION

The enzymic synthesis of PL provided a simple and rapid means for obtaining high purity material in adequate amounts. Its usefulness, however, was limited by the availability of lombricine and lombricine kinase, and as such, offered little advantage over the procedure of Ennor and Rosenberg (1962). The main advantage of the enzymic procedure was that the extent of the phosphorylation reach 80%, whereas the degree of phosphorylation of

lombricine in earthworms was not predictable, and varied from 10% to 30% approximately, depending on the condition of the worms prior to extraction.

It is well known that a high pH and a low Mg^{2+}/ATP ratio tends to displace the equilibrium of the forward reaction towards the right (Noda et al., 1954b; Rosenberg, 1955; Griffiths et al., 1957) and to this end the incubation was carried out at pH 9.5 with a Mg^{2+}/ATP ratio of 1:5. Under these conditions, however, reaction rates were much reduced, necessitating several hours incubation at room temperature before equilibrium was reached, with consequent denaturation of the enzyme. To compensate for enzyme denaturation fresh lombricine kinase was added to the reaction mixture throughout the incubation period at regular time intervals.

Ennor and Rosenberg (1962) have drawn attention to the fact that conventional precipitation techniques employing the differential solubility of the alkaline earth salts of phosphagens in aqueous and ethanolic solution were of no value in the purification of PL. These authors demonstrated the utility of anion exchange resin in the HCO_3^- form (Martónosi, 1960) for the purification of PL, and this technique proved particularly valuable in removing almost all the free lombricine. The latter point was of importance in kinetic studies of the reverse reaction, in

which traces of free lombricine seriously interfered with the assay procedure.

Recently the synthesis of phospho-D- and L-lombricine in 70% yield by the preferential guanidination at the terminal amino group of D- or L-SEP with phospho-O-methylurea has been reported (Beatty, Ennor and Magrath, 1960). However, sufficient quantities have not as yet become available for use as substrates in lombricine kinase experiments.

SUMMARY

1. A method for the enzymic synthesis of N-phospho lombricine is described.
2. The purification procedure employs anion exchange resin in the HCO_3^- form as described by Ennor and Rosenberg (1962).
3. The yields and advantages of this enzymic method are compared with that involving direct extraction of PL from earthworms and with chemical synthesis.

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