THE INVERTEBRATE GUANIDINE PHOSPHORYLTRANSFERASES

AND THEIR SUBSTRATES

A THESIS

submitted for the degree

of

DOCTOR OF PHILOSOPHY

in the

Australian National University

by

DAVID ELLIS GRIFFITHS

MARCH, 1958.
In compliance with the regulations of the Australian National University, and since the work described in this Thesis has been carried out in collaboration with my supervisor, Dr. J. F. Morrison, my contribution to this work may best be described by quoting from Dr. Morrison's letter of March 11th, 1958, to the Registrar:

"... The results reported in Chapters 1, 2, 3, 6 and the first part of Chapter 4, were obtained in collaboration with me. The candidate's contribution to the early work (Chapters 1 and 2) was that of a junior worker, whilst his contribution towards the remainder was that of a co-worker. The work described in the second part of Chapter 4, Chapter 5 and the Appendices was carried out independently by the candidate..."

Candidate's signature:

[Signature]
This thesis embodies work carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, from April, 1955 to February, 1958, during the tenure of a Australian National University Research Scholarship.
ACKNOWLEDGEMENTS

It is a pleasure to express my sincere thanks to my supervisor, Dr. J. F. Morrison, for his constant interest and valuable advice during the course of this work. I would also like to thank Professor A. H. Emnor for devoting much of his time in discussion of various aspects of this work and for offering many helpful suggestions.

The skilful technical assistance of Mr. D. Maguire during the first half of this work and of Miss D. Kaak during the latter half of the work is gratefully acknowledged. I would also like to thank Mr. R. F. Adams for carrying out the electrophoretic analyses and for his cheerful assistance at all times. Thanks are also due to Mr. V. Paral for his help with the photographic work. Finally, I would like to express my thanks to Janet Griffiths for her help in many ways, particularly in arranging and checking the references.
Throughout this dissertation, the term 'phosphagen' will be regarded as referring specifically to those naturally occurring N-phosphoryl guanidine derivatives which participate, with the adenine nucleotides, in phosphoryl group transfer reactions.

The phosphagens have been variously referred to by previous workers as phosphocreatine, arginine phosphate, phosphoarginine, phosphotaurocyamine etc. Such names suggest the presence of phosphate rather than phosphoryl groups and the compounds will, therefore, be referred to as N-phosphoryl creatine, N-phosphoryl arginine, N-phosphoryl taurocyamine etc. The commonly used abbreviations, PC, PA, PT, etc., will be retained (see below).

The enzymes concerned in the transfer of the phosphoryl group of phosphagens to ADP will be termed guanidine phosphoryltransferases (Morrison, Ennor & Griffiths, 1958) and, more specifically, as creatine phosphoryltransferase, arginine phosphoryltransferase etc. In referring to the reactions catalysed by guanidine phosphoryltransferases, the term
'forward reaction' is applied to the phosphoryl group transfer from the phosphagen to ADP, and the term 'reverse reaction' to the phosphoryl group transfer from ATP to the guanidine compound as indicated in the equation:

\[
\text{Phosphorylated Guanidine} + \text{ADP} \begin{array}{c} \text{forward} \\ \text{reverse} \end{array} \text{Guanidine base} + \text{ATP}
\]

The following abbreviations will be used:

- **AMP** Adenosine - 5' - phosphate (5' - adenylic acid)
- **ADP** Adenosine - 5' - diphosphate
- **ATP** Adenosine - 5' - triphosphate
- **ITP** Inosine - 5' - triphosphate
- **p-CMB** p-Chloromercuribenzoic acid (Na salt)
- **EDTA** Ethylenediaminetetraacetic acid (Na salt)
- **TCA** Trichloroacetic acid
- **PC** N-phosphoryl creatine
- **PA** N-phosphoryl arginine
- **PT** N-phosphoryl taurocyamine
- **PG** N-phosphoryl glycocysmine
- **PL** N-phosphoryl lombricine
- **CPT** Creatine phosphoryltransferase
- **APT** Arginine phosphoryltransferase
- **TPT** Glycocysmine phosphoryltransferase
- **GPT** Glycocysmine phosphoryltransferase
True initial velocities were measured in all kinetic studies of the reactions catalysed by guanidine phosphoryl-transferases.

All temperatures are expressed in °C.

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GENERAL INTRODUCTION

(a) Guanidines and N-phosphoryl guanidines

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GENERAL INTRODUCTION

GUANIDINES AND N-PHOSPHORYL GUANIDINES

Many of the nitrogenous bases present in animal and plant tissues have been shown to be derivatives of guanidine and have the general formula -

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{C} - \text{N} - \text{R}_2 \\
\text{HN} \quad \text{R}_1
\end{array}
\]

A few of these compounds have been shown also to be present as N-phosphoryl derivatives - the so called phosphagens.

This account will deal mainly with the N-phosphorylated guanidines in so far as their discovery, distribution and physiological function is concerned. However, a discussion of the early work on the discovery and distribution of creatine and arginine is included as this work bears a close relationship to the early theories of the biochemical evolution of the phosphagens. Such restrictions are considered desirable, particularly as a complete account of even those guanidines which occur as phosphagens would inevitably involve consideration of the urea cycle, transamidination reactions etc. These latter reactions and other aspects of general
guanidine biochemistry have been frequently reviewed (Guggenheim, 1951; Ratner, 1954, 1955; Thoai & Roche, 1956) and thus will not be discussed.

The isolation, structure, and early studies of the distribution of creatine and arginine

Creatine was first isolated by Chevreul (1835) from meat extracts and later from the muscles of mammals, fish and birds by Liebig (1847) who concluded that creatine was a normal constituent of the muscles of all higher animals. Liebig found it to have the empirical formula $\text{C}_4\text{H}_9\text{O}_2\text{N}_5\cdot\text{H}_2\text{O}$ and further showed that it was converted to creatinine ($\text{C}_4\text{H}_7\text{ON}_3$) on heating with mineral acid. Subsequent work by Dessaignes (1854) and Strecker (1861) indicated that creatine was related structurally to methylguanidine and the first correct formulation of creatine was made by Strecker (1867) and Erlemeyer (1868) who represented it as methylguanidine acetic acid.

$$\begin{align*}
\text{H}^2\text{N} & \quad \text{C} - \text{N} - \text{CH}_2 - \text{COCH} \\
\text{HN} & \quad \text{CH}_3
\end{align*}$$

Creatine

(Methylguanidine acetic acid or $N'$-methyl $N'$-amidino glycine)

The structure of creatine was confirmed by synthesis from sarcosine and cyanamide (Volhard, 1868).
Arginine was isolated from the seeds of *Lupinus luteus* and *Cucurbita sp.* by Schulze & Steiger (1887) and shown to have the empirical formula $C_{6}H_{14}N_{4}O_{2}$. Its structure was deduced from degradation studies by Schulze & Winterstein (1897) and confirmed by synthesis from ornithine and cyanamide (Schulze & Winterstein, 1899).

\[
\begin{align*}
\text{H}_2\text{N} & \\
\text{C-} & \\
\text{NH-CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH-COOH} & \\
\text{H}_2\text{N} & \\
& \text{NH}_2
\end{align*}
\]

Arginine

(1-amino-4-guanidino-n-valeric acid)

The earliest investigations of the distribution of arginine were carried out by Hedin (1895, 1898) who found the compound in the acid hydrolysates of all proteins examined. These results were correctly interpreted as indicating the occurrence of the amino acid as a universal constituent of all proteins. Soon after, it was demonstrated that certain basic proteins (e.g., protamines) were composed almost entirely of arginine (Kossel & Kutscher, 1900).

In subsequent years many investigations were carried out on the distribution of arginine and creatine and the results indicated that, whilst there was little or no creatine in
invertebrate or plant tissue, it was widely distributed in the vertebrates (see review by Hunter, 1928). Studies of the invertebrates resulted in the isolation of free arginine from the crustaceans, Crangon vulgaris (Ackermann & Kutscher, 1907) and Astacus fluviatilis (Kutscher, 1914), the insect, Melolontha vulgaris (Ackermann, 1921), the molluscs, Mytilus edulis (Ackermann, 1922) and Eledone moschata (Ackermann, Holtz & Kutscher, 1923), and from the echinoid, Arbacia pustulosa (Holtz & Thielmann, 1924), whilst Okuda (1919) isolated it from the crustacean, Palinurus japonicus and the mollusc, Loligo brekeri. It thus appeared that invertebrates were characterised by the presence of arginine. In a review of the biological distribution of nitrogenous bases, Kutscher & Ackermann (1926) suggested that, as creatine and arginine showed this striking division in their distribution, the anatomical terms vertebrate and invertebrate be substituted by their biochemical counterparts, creatinate and acreatinate. This suggestion, interesting though it is in retrospect, had an unfortunate and inhibitory influence on biochemical thought in this field for many years and was made in spite of the fact that there was evidence to suggest that some invertebrates contained creatine. Thus, Myers (1920) had found both creatine and creatinine in the blood of representative coelenterates, molluscs, echinoderms and crustaceans. Traces of
creatinine were believed to be present in the mollusc *Abalone* (Albrecht, 1921) and creatinine in the nephridia of the worm *Cyclostoma elegans* (Quast, 1924). These workers estimated creatinine directly by the Jaffe reaction whilst creatine was estimated as the creatinine formed after heating tissue extracts with acid.

Hunter (1928) concluded that, owing to the non-specific nature of the Jaffe reaction, the positive findings obtained by the above authors were 'imperfectly supported'. He came to the same conclusion as Kutscher & Ackermann (1926), *viz*; that the presence of creatine was peculiar to the vertebrates.

**The discovery of PC**

The discovery of PC resulted from studies of the inorganic P content of vertebrate muscle extracts. Eggleton & Eggleton (1927a, b, c) using the method of Briggs (1922) observed that the maximum colour development was delayed and concluded that this was due to the presence of an unknown compound which broke down during the estimation with the liberation of inorganic P. They named this labile P compound phosphagen and showed that it constituted 70-80% of what had formerly been regarded as inorganic P. No evidence as to the nature of phosphagen was presented but they speculated that it might be a phosphoric ester of glycogen or of a hexose.
The elucidation of the structure of phosphagen came from the work of Fiske & Subbarow (1927a, b) who found that the major portion of what had formerly been thought to be inorganic P was an unstable compound of creatine and phosphoric acid which was hydrolysed under the acid conditions used for the estimation of inorganic P. The compound was first isolated as an impure salt in which the ratio of creatine to phosphorus was 0.96. The same authors (Fiske & Subbarow, 1929) described the isolation of the pure crystalline calcium salt of PC of molecular formula $C_4H_8O_5N_3PO_4Ca\cdot4H_2O$. The following structure was assigned to PC:

$$\begin{align*}
\text{HN} &= \text{C} - \text{P} - \text{OH} \\
\text{N} &= \text{CH}_2 - \text{COOH} \\
\text{OH} &= \text{O} \\
\end{align*}$$

$N$-phosphoryl creatine

The discovery of PA

The work of Eggleton & Eggleton (1927a, b, c) and of Fiske & Subbarow (1927a, b) on vertebrate muscle extracts prompted Meyerhof & Lohmann (1928, 1928a, b) to investigate whether a similar labile P compound was present in invertebrate muscle. They found that extracts of crustacean muscle contained an acid-labile P compound which was hydrolysed more slowly than PC. Moreover, in the presence of acid molybdate the hydrolysis of this compound was
retarded whereas the hydrolysis of PC was accelerated. The compound was isolated as a barium salt and identified as N-phosphoryl arginine. PA was also regarded as a phosphagen.

\[
\begin{align*}
HN &= C - N - P - OH \\
&\quad \quad \quad \quad \quad OH \\
&\quad \quad \quad \quad \quad NH - CH_2 - CH_2 - CH_2 - CH - COOH \\
&\quad \quad \quad \quad \quad NH_2
\end{align*}
\]

N-phosphoryl arginine

The distribution of PC and PA

Early studies of the distribution of PC and PA in the animal kingdom made use of the different stabilities of these phosphagens in acid solution, especially in the presence of molybdate. Eggleton & Eggleton (1928) found that PC was present in all vertebrates, as well as the cephalochordate Amphioxus, but absent from invertebrates. Meyerhof (1928) showed that PA was widely distributed in the invertebrate phyla and confirmed that PC was present in Amphioxus. These studies suggested that PC was the characteristic phosphagen of the vertebrates whilst PA was confined to the invertebrates, and thus provided confirmatory evidence for the findings of Kutscher & Ackermann (1926) concerning the distribution of creatine and arginine.
Meyerhof (1930) considered this difference between the vertebrates and the invertebrates as being due to a simple chemical mutation at the vertebrate level of evolution and regarded PA as the 'primitive phosphagen' from which the PC of the vertebrates had been derived. This hypothesis was tested experimentally by Needham, Needham, Baldwin & Yudkin (1932) in a detailed survey of the distribution of phosphagens in the invertebrates which was designed to cover as wide a range of invertebrate phyla as possible and especially those phyla generally regarded as transitional between the invertebrates and the vertebrates. (The results of this survey as well as those of Meyerhof (1928) are summarised in Table 1, Appendix 1). Their results confirmed that, in general, PA was the typical invertebrate phosphagen, but they also found that the sea urchin Strongylocentrotus sp. and the hemichordate Balanoglossus sp. deviated from this general pattern in as much as they contained both PA and PC. These observations were regarded as important evidence in favour of the Echinoderm - Enteropneust theory of vertebrate ancestry proposed by Bateson (1886) which was based on a morphological similarity between the larvae of enteropneusts (hemichordates) and echinoderms. The results of Needham et al. also revealed a definite trend in the distribution of PC which was related to the chordate affinities of the animal examined. The one anomaly was the tunicates. It was claimed that Ascidia mentula contained PA, but later, Borsuk, Kreps & Verjbinskaya (1933) found PC only in Styela rustica.
Further investigation of the distribution of phosphagens in invertebrates (Schutze, 1932; Baldwin & Needham, 1933; Eggleton, 1934; Baldwin & Needham, 1937; Baldwin & Yudkin, 1950) confirmed not only the widespread occurrence of PA in insects, molluscs and cephalopods but also drew attention to the Echinodermata. Investigation of the sub-groups of this phylum revealed that PA only was present in crinoids, asteroids and holothurians and PC only in ophiuroids whilst certain echinoid genera were shown to contain both PC and PA (Baldwin & Needham, 1937; Baldwin & Yudkin, 1950). These latter results, together with the demonstration of the presence of PC in the hemichordates Saccoglossus kowalevski and S. horsti led Baldwin & Yudkin (1950) to the same conclusion as previously reached by Needham et al. (1932), viz: that their results provided chemical support for the theory of Bateson (1886) that the vertebrates evolved from echinoderms by way of the hemichordates. They disagreed with Meyerhof's hypothesis that the changeover from PA to PC was a simple mutation and considered that the replacement of PA by PC

"... probably took place through an intermediate condition in which both compounds were present side by side..."

The presence of both PA and PC in echinoids was quoted as supporting evidence.
Later, more detailed studies of the distribution of phosphagens in invertebrates, especially in the phylum Annelida, led not only to the discovery of new phosphagens but also to the realization that PC was more widely distributed than was previously thought.

The annelid phosphagens

Needham et al. (1932) examined the marine annelids Sabellaria alveolata, Nereis diversicolor and Spirographis brevispira and found evidence for the presence of a phosphagen which they presumed to be PA. Borsuk et al. (1933) also found evidence for the presence of PA in Arenicola marina and it was assumed that all annelids contained PA. However, Arnold & Luck (1933) found that arginine was not present in the marine annelids they examined (Eudistyla polymorpha, Glycera sp, Polynoe brevisitosa, Nereis vexillosa and Lumbrinereis sp.) and suggested that a phosphagen other than PA was present. Further evidence was obtained by Kurtz & Luck (1937-38) who, using the method of Meyerhof & Lohmann (1928b) for the isolation of PA, isolated from the body wall muscle of Nereis brandti an impure barium salt which contained phosphate and a guanidine in bound form. The free guanidine obtained after acid hydrolysis was not acted upon by arginase and they concluded that the phosphagen of Nereis brandti was not PA.
Baldwin & Yudkin (1950) examined 24 annelid species and obtained evidence for the presence of a 'PC-like' compound in eleven of these species; in five of these, it occurred together with a 'PA-like' compound. In thirteen other species, including *Nereis diversicolor* and *Arenicola marina*, the 'PA-like' compound occurred alone. Neither compound was identified and the descriptions of these phosphagens were based on their acid hydrolysis behaviour in the presence and absence of molybdate. When the hydrolysis of the phosphagen, present in crude extracts, was accelerated by molybdate, it was regarded as a 'PC-like' compound whilst retardation of hydrolysis by molybdate was taken as being indicative of the presence of a 'PA-like' compound. That this latter compound was not PA was shown by the fact that the melting point of the picrate of the guanidine base differed from that of arginine picrate.

The structure of the 'PA-like' phosphagens present in annelids was elucidated by Roche, Thoai and co-workers in a series of researches initiated in 1951. Thoai, Roche, Robin & Thiem (1953a, b), using paper and ion exchange chromatography, isolated glycocyamine and taurocyamine as well as their N-phosphoryl derivatives from muscle extracts of *Nereis diversicolor* and *Arenicola marina*, respectively. The N-phosphoryl derivatives, isolated as impure calcium salts were shown to be chromatographically identical with synthetically prepared PG and PT and the following structures were assigned to these
compounds

\[
\begin{align*}
\text{HN} & = \text{C} \\ \\
\text{H} & \quad \text{N} - \text{P} \quad \text{OH} \\ \\
\text{NH} - \text{CH}_2 - \text{COOH} & \quad \text{HN} = \text{C} \\ \\
\text{H} & \quad \text{N} - \text{P} \quad \text{OH} \\ \\
\text{NH} - \text{CH}_2 - \text{CH}_2 - \text{SO}_3 \text{H}
\end{align*}
\]

N-phosphoryl glycocyamine \qquad N-phosphoryl taurocyamine

These structures were later confirmed by isolation and synthesis of the crystalline ammonium salts (Thoai & Thiem, 1957).

The discovery of PG and PT was followed by the isolation and characterisation of lombricine (guanidino ethyl seryl phosphate) and its N-phosphoryl derivative (PL) from Lumbricus terrestris (Thoai & Robin, 1954).

\[
\begin{align*}
\text{HN} & = \text{C} \\ \\
\text{H} & \quad \text{N} - \text{P} \quad \text{OH} \\ \\
\text{NH} - \text{CH}_2 - \text{CH}_2 - \text{O} - \text{P} - \text{O} - \text{CH}_2 - \text{CH} - \text{COOH} & \quad \text{HN} = \text{C} \\ \\
\text{OH} & \quad \text{NH}_2
\end{align*}
\]

N-phosphoryl lombricine

Claims have been made that PG and PT are present in other annelids and in sipunculids (Thoai et al., 1953b; Thoai & Roche, 1957). However, these claims were based only on the identification of the guanidine base present. It was assumed that if only one guanidine
was present in the muscle tissue of these species it was also present as its N-phosphoryl derivative.

Hobson & Rees (1955) confirmed that PC and PT were present in *Nereis diversicolor* and *Arenicola marina* and identified PC in extracts of *Glycera gigantea* and *G. convoluta*. Evidence was also obtained for the presence, in six other annelid species, of a compound whose behaviour in acid molybdate was similar to that of PC. These latter results, coupled with those of Baldwin & Yudkin (1950), suggest that PC is of wide distribution among the annelids.

Thus, in recent years, members of the phylum Annelida have been shown to contain PC, PG, PT and PL and the presence of another, as yet unidentified, guanidine called hirudonine in leech muscle indicates that yet another phosphagen may be present (Roche, Thoai, Robin & Pradel, 1955). In contrast to the results of Needham et al. (1932), recent workers have failed to demonstrate the presence of PA in any annelid. This indicates that the differential hydrolysis technique used by early workers in this field is unsatisfactory as a means of identifying phosphagens. The use of this technique will be discussed in Chapter 4 in relation to the results of a study of the effect of molybdate on the hydrolysis of PA, PT and PG.

**Phosphagens and phylogeny**

The discovery of new phosphagens in members of the phylum Annelida and the diversity of the distribution of PC in the
invertebrates (Table 2 Appendix 1) has led to the abandonment of
the theory (Meyerhof, 1930; Needham et al., 1932) that PC and PA
are characteristic of the vertebrates and the invertebrates
respectively. The occurrence of PC in several invertebrate phyla
(Porifera, Annelida, Echinodermata) as well as in the Chordata
suggests also that Meyerhof was incorrect in regarding PC as
ontogenetically newer than PA. However, within the chordate line
of evolution (echinoderms — primitive chordates — vertebrates),
there still remains a correlation between phylogeny and distribution
of PC and PA. This will be discussed further in Chapter 6.

THE ROLE OF PHOSPHAGENS IN MUSCLE METABOLISM

Following the discovery of PC in mammalian muscle extracts
in 1927, attempts were made to determine the role of this compound
in muscle metabolism. Early studies, (Eggleton & Eggleton, 1927 a,
b; Fiske & Subbarow, 1927a, b, 1929; Nachmansohn, 1928) indicated
that the contraction of mammalian muscle involved the breakdown of
PC which was rapidly resynthesised under both aerobic and anaerobic
conditions during the recovery period. The experiments of Lundsgaard
(1930 a, b, c, d; 1931 a, b) with iodoacetate-poisoned frog muscles
confirmed that the energy for muscle contraction was supplied by the
breakdown of PC and further showed that contraction continued until
PC was completely broken down. Lundsgaard showed also that the
energy for the resynthesis of PC in normal muscle was supplied by the glycolytic system. Similar results were obtained (Lundsgaard, 1931 a) with muscles of the crustacean, *Maia squinado*, which contains PA, and this indicated that similar chemical reactions took place during the contraction process of invertebrate muscle.

While these early studies demonstrated that PC and PA were involved in the energetics of the contraction process, it was not until the discovery of an enzymic relationship between the phosphagens and ATP and the implication of ATP in the contractile process, that an indication was obtained of the role of phosphagens in muscular contraction.

**The guanidine phosphoryltransferases**

The breakdown of PC to creatine and inorganic P as a result of muscular contraction (Eggleton & Eggleton, 1927 a, b; Fiske & Subbarow, 1929) suggested that there was present in muscle an enzyme which was capable of hydrolysing PC. Lohmann (1934) demonstrated that PC was broken down to creatine by muscle extracts and that the hydrolysis occurred indirectly by transfer of the phosphoryl group of PC to ADP according to the reaction

$$\text{PC} + \text{ADP} \longrightarrow \text{Creatine} + \text{ATP}$$

The reversibility of this reaction was demonstrated by Lehmann (1935).
Lohmann (1934) believed that AMP could also function as a phosphoryl
group acceptor with the formation of ADP, but subsequently (Ennor &
Rosenberg, 1954a) it was shown that the reaction occurred with AMP
only by virtue of the presence of myokinase.

Lehmann (1935) demonstrated that a similar reversible
reaction between ADP and PA was catalysed by crab muscle extracts
whilst Thoai (1957) has shown that extracts of the marine annelids,
*Arenicola marina* and *Nereis diversicolor* catalyse reversible
reactions between ADP and PT or PG respectively. Each of these
reactions has been shown to be catalysed by a specific enzyme.

It is not intended here to discuss in detail the
properties of those enzymes which catalyse the transfer of a
phosphoryl group from a phosphorylated guanidine to ADP and which
have been termed guanidine phosphoryltransferases. This will be
done, in so far as APT, CPT and TPT are concerned, in Chapters I,
2, 5 and 6, where reference will also be made to the comparative
properties of the four guanidine phosphoryltransferases (CPT, APT,
TPT and GPT) which have been studied. However, the affinity of
these enzyme systems for $\text{Mg}^{2+}$ will be mentioned here because of
the relationship between this and the postulated role of $\text{Mg}^{2+}$ and
guanidine phosphoryltransferase systems in recent theories of
muscular contraction.
Recent views of the role of phosphagens and guanidine phosphoryl-transferases in muscular contraction

The discovery that actomyosin, the main contractile protein in muscle, acts as an ATP-ase (Engelhardt & Ljubimova, 1939) suggested that the energy for muscular contraction is derived from the breakdown of ATP and not from the hydrolysis of PC as previously proposed by Lundsgaard (1930 a). Confirmation of this idea was obtained from the results of experiments on muscle model systems (see review by Weber & Portzehl, 1954). PC was therefore regarded as a 'reservoir' of phosphoryl groups which, through the mediation of CPT, maintained the steady state concentration of ATP.

Over the last 10-15 years attention has been directed mainly towards the role of ATP in muscular contraction, and studies of various muscle models (Szent-Gyorgyi, 1947, 1949; Weber & Portzehl, 1952) have led to two general theories regarding the role of ATP. One theory states that the energy released by the hydrolysis of ATP to ADP provides directly the energy for contraction, (Weber & Portzehl, 1954). The other states that contraction is not linked with the hydrolysis of ATP but occurs as the result of the combination of actomyosin with ATP, (Szent-Gyorgyi, 1953; Morales, Botts, Blum & Hill, 1955; Morales, 1956).
It is not proposed to enter into a discussion of the relative merits and demerits of the above theories, but rather will the discussion be confined to recent theories of the role of CPT as a relaxing factor in muscular contraction. The concept of a relaxing factor was introduced as a result of studies with glycerol extracted muscle fibres (Bendall, 1952). Earlier (Szent-Gyorgyi, 1949), it had been found that the contraction of these fibres produced by the addition of ATP was not followed by relaxation and furthermore no relaxation took place if the ATP was subsequently washed out. Bendall (1952) found that the addition of a muscle extract containing a unknown factor caused relaxation of a contracted muscle fibre. Moreover this factor was able to reverse the contraction process at any stage of contraction. This factor was later identified as myokinase (Bendall, 1954). About the same time other substances were discovered which were capable of bringing about similar relaxation of muscle fibres and these, together with myokinase, have been termed collectively as relaxing factors. Five such factors have now been recognised; Myokinase, (Bendall, 1954); pyrophosphate, (Bozler, 1954 a); EDTA, (Bozler, 1954 b; Watanabe, 1955); the vertebrate CPT system (Goodall & Szent-Gyorgyi, 1953) and the invertebrate APT system (Tonomura, Yagi & Matsumiya, 1955) and they have certain features in common. Thus, all have a high affinity for $\text{Mg}^{2+}$ and under the experimental conditions used are
anionic. Their action, which can be abolished by $\text{Ca}^{2+}$, is dependent on the presence of both ATP and $\text{Mg}^{2+}$ and it is believed that they are tightly bound to actomyosin by means of a Mg bridge.

The mode of action of relaxing factors has been explained differently by the two schools of thought. Weber & Portzehl (1954) and Perry (1956) consider that, in the resting or relaxed phase, the breakdown of ATP is inhibited due to the available $\text{Mg}^{2+}$ being bound by the relaxing factor, i.e., the relaxing factor and the actomyosin ATP-ase both compete for the available $\text{Mg}^{2+}$. It is believed that the stimulation of muscle releases $\text{Ca}^{2+}$ ions which combine with the relaxing factor, thus releasing $\text{Mg}^{2+}$ ions which activate the myosin ATP-ase. Contraction then occurs as a result of the hydrolysis of ATP. On the other hand, Morales et al., (1955) and Morales (1956) consider that the action of relaxing factors is related to their ability to combine with actomyosin. It is proposed that, in the resting phase, ATP and relaxing factor are both bound to the Mg-containing active site of actomyosin. Stimulation then causes the release of $\text{Ca}^{2+}$ ions which combine with the relaxing factor and causes a spatial re-orientation of the latter away from the active site, thereby permitting the ATP-Mg-actomyosin complex to contract. It follows from these theories that the guanidine phosphoryltransferase systems have a function in muscular contraction other than that of a ATP-generating system.
The results of studies of the mechanism of muscular contraction reflect the importance of $\text{Mg}^{2+}$ ions in the control of biological reactions. Similar indications have come from the study of the reactions catalysed by fructokinase, (Hers, 1952), creatine phosphoryltransferase, (Kuby, Noda & Lardy, 1954); myokinase, (Bowen & Kerwin, 1956), gluconokinase, (Leder, 1957) and myofibrillar ATP-ase, (Perry, 1956). These enzymes, which catalyse the transfer of phosphoryl groups from ATP to acceptor compounds, and which are activated by $\text{Mg}^{2+}$, are inhibited when ATP or $\text{Mg}^{2+}$ are added in concentrations greater than those required for maximal activity. Such inhibitory effects may be of importance in vivo where rapid changes in both ATP and $\text{Mg}^{2+}$ concentrations may occur (see Raaflaub, 1956). It is thus apparent that studies of the interactions of the adenine nucleotides, metals and the guanidine phosphoryltransferases may prove of value in assessing the role played by the guanidine phosphoryltransferases in the process of muscular contraction in both the vertebrates and the invertebrates.
CHAPTER I.

THE PURIFICATION AND PROPERTIES OF ARGinine

PHOSPHORYLTRANSFERASE
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PHOSPHORYLTRANSFERASE

INTRODUCTION

The enzyme arginine phosphoryltransferase (APT) which catalyses the equilibrium as expressed by the reaction

\[
PA + ADP \rightleftharpoons \text{Arginine} + ATP
\]

was shown to be present in crude extracts of crab and octopus muscle by Lohmann (1935, 1936) and Lehmann (1935, 1936). No attempts were made to purify the enzyme but studies by Lehmann, using crude lobster muscle extracts, of the effect of pH on the equilibrium of the above reaction, showed that arginine was readily phosphorylated at alkaline pH and PA was dephosphorylated at neutral pH. Lehmann and Pollack (1942) showed that glycine and cysteine activated the phosphorylation of arginine by a crude crab muscle extract in the presence of ATP.

Szörenyi, Dvornikova and Degtyar (1949) isolated APT in the crystalline form by an ammonium sulphate fract-
ionation of aqueous extracts of fresh water crab muscle but only a few details of the properties of the enzyme were described.

Later, and following the completion of the experimental work to be described, Elodi and Szorenyi (1956) reported the isolation from fresh water crab muscle extracts of crystalline APT which was homogeneous as judged by ultracentrifuge and electrophoretic studies. However, only a few kinetic studies of the APT catalysed reaction were described and these were confined to the reverse reaction.

Doubtless the inadequacy of methods hitherto available for the isolation of PA and for the estimation of arginine have precluded detailed studies of the properties of APT. The isolation of PA from crayfish muscle in good yield (Ennor, Morrison and Rosenberg, 1956) and the development of a simple reliable procedure for the estimation of arginine (Rosenberg, Ennor and Morrison, 1956) have greatly facilitated studies on APT.

The experimental work described in this chapter is concerned with the purification and general properties of APT from the tail muscle of a crustacean, the sea-crayfish (Jasus verreauxi). Details of a brief study of the APT present in the tail muscle of the fresh water crayfish, (Cherix albidus Clark) are also included.
EXPERIMENTAL

Materials

Phosphoarginine.

PA was isolated from the tail muscle of the sea-crayfish (Jasus lalandii) as described by Ennor et al. (1956). Solutions of the sodium salt were prepared by the careful addition of $\text{Na}_2\text{SO}_4$ (saturated solution) to a solution of the Ba salt, and the end point was judged by testing with Na rhodizonate. The $\text{BaSO}_4$ was removed by centrifugation and washed several times with water. The washings were added to the original supernatant and the solution, after adjustment to pH 7.2 by addition of Na HCl, was made to volume. This method gave a quantitative recovery, and the resultant solution contained less than 0.2% free arginine.

Adenosine di- and triphosphate.

ADP and ATP were commercial samples (Sigma Chemical Co.) of the hydrated Na salts of over 95% purity. Paper electrophoretic examination showed that the ATP contained trace amounts of ADP. The ATP also contained 0.3% inorganic P. Solutions of ATP were initially pH 3.6, but were immediately adjusted to pH 8.5 by the addition of NaOH before making to volume. In the case of ADP no such adjustment was necessary and the solutions were used at pH 7.6. In both cases the concentrations were checked by
measurement of the absorption at 260 μμ of dilutions in 0.1N HCl, assuming a molecular extinction value for AMP of 14.9 x 10^3.

Adenylic acid.
AMP was prepared by Dr. H. Rosenberg by the action of apyrase on ATP and was purified as described by Kerr (1941). It was obtained as the crystalline free acid and was stored as such. Solutions were made up as required and adjusted to pH 7.4 by the addition of 1N NaOH.

Inosine triphosphate.
ITP was prepared by Dr. H. Rosenberg from ATP by the method of Kleinzeller (1942).

Arginine.
A commercial sample (British Drug Houses Ltd.) of L-arginine monohydrochloride was used without recrystallisation. The purity was checked by N analysis. Solutions were made up as required and adjusted to pH 8.5 by the addition of N NaOH.

Other guanidino compounds.
Creatine, glycoccyamine and citrulline were obtained from British Drug Houses Ltd. L-Canavanine sulphate was obtained from the California Foundation for Biochemical Research. Agmatine sulphate was obtained from L. Light & Co. Ltd. Negmine (N-ethyl glycoccyamine) was a gift from Dr. Armstrong of the University of
Utah, U.S.A. These compounds were used without recrystallisation. Argininc acid, α-carbamido arginine, L-homoarginine-HCl, arginine methyl ester 2HCl, taurocyamine, β-guanidino propionic acid, ε-guanidino-n-valeric acid, α-chloro-ε-guanidino-n-valeric acid·HCl and α-N-acetyl arginine were synthesised as described in Appendix II.

All compounds were recrystallised twice before use, and were pure as judged by the melting point and one dimensional chromatography in propanol - ammonia (sp.gr.0.88) - water (60 : 30 : 10).

Solutions of PA, ATP, ADP, AMP and arginine were stored at -15°.

**Buffer systems.**

N-ethyl morpholine (Eastman-Kodak) was redistilled under reduced pressure before use. 1 M solutions were adjusted to the required pH with 5 N HCl and then diluted to 0.5M ready for use. The components of other buffer systems were products of British Drug Houses Ltd. and the solutions were made up as described previously.

**Inhibitors.**

p-chloromercuribenzoic acid was a Sigma Chemical Co. product. Diphenylchloroarsine was a gift from Sir Rudolph Peters, F.R.S., o-iodosobenzoic acid and N-ethyl maleimide were obtained from
Other inhibitors were British Drug Houses Ltd. products.

Animals

Sea-crayfish were used as the source of APT because of their large muscle mass and their availability throughout the year. Live crayfish (Jasus verreauxi) were obtained from sea water storage pens and transported to the laboratory in bags packed with wet seaweed. All specimens arrived in good condition. Fresh water crayfish (Cherix albidas Clark) were collected at Canberra and were used immediately after transport to the laboratory.

Unless otherwise stated all operations concerned with enzyme fractionation were carried out in the cold room at 5°C.

Methods

Estimation of protein.

Protein was estimated colorimetrically by the biuret method of Gornall, Bardawill and David (1949), crystalline bovine serum albumin being used as the standard.

Determination of Enzyme activity.

Unless otherwise stated, the following techniques were used to determine the activity of the enzyme.

(1) Forward reaction

PA + ADP → Arginine + ATP
To 10 ml. graduated test tubes was added 0.8 ml. of a stock solution containing 0.1 ml. of 0.5M N-ethyl morpholine (pH 7.2), 0.1 ml. of 0.05 M PA, 0.1 ml. of 0.1M MgSO₄, 0.1 ml. of 0.01 M ADP and 0.4 ml. of water. The tubes were equilibrated in a water bath at 5°C and the reaction started by the addition of 0.2 ml. of APT solution. The APT solution was diluted to the required concentration in N-ethyl morpholine buffer (0.05 M, pH 7.2); this gave a final buffer concentration of 0.06M. At the end of the incubation period the reaction was stopped by the addition of 1.0 ml. of an EDTA-NaOH mixture containing 0.4 ml. of 0.2M EDTA (adjusted to pH 7.6) and 0.6 ml. of 5N NaOH. The arginine released in the reaction was estimated in the same tubes by the method of Rosenberg et al. (1956) without any further addition of NaOH. Under these conditions the reaction followed zero order kinetics.

(2) Reverse reaction

Arginine + ATP → PA + ADP

The velocity of the reverse reaction was determined by acid hydrolysis of the PA formed and estimation of the inorganic P released. To 10 ml. graduated tubes was added 0.8 ml. of a stock solution containing 0.1 ml. of 0.1M arginine·HCl (pH 8.4), 0.1 ml. of 0.05 M ATP, 0.1 ml. of 0.1M MgSO₄, 0.1 ml. of 0.5M N-ethyl morpholine (pH 8.4) and 0.4 ml. of water. The tubes were equilibrated in a water bath at 5°C and the reaction started
by the addition of 0.2 ml. of ATP solution. The enzyme was
diluted in N-ethyl morpholine (0.05M, pH 8.4) making the
final buffer concentration 0.06M. The reaction was stopped
by the addition of 1.0 ml. of 0.2N TCA, the tubes removed
from the bath and placed in boiling water for 1 min. (Under
these conditions PA is quantitatively hydrolysed to arginine
and inorganic P). They were then rapidly cooled in an ice
bath by agitation and the inorganic P estimated by a
modification of the method of King (1932). At half minute
intervals and to each tube was added 2.0 ml. of 5% (w/v)
ammonium molybdate in 15% (v/v) H₂SO₄ and 0.5 ml. of the
reducing agent. The volume was adjusted to 10 ml. by the
addition of water and the contents mixed. Colour intensities
were measured at 30 sec. intervals, 10 min. after the addition
of the above reagents. (It should be noted that concentrations
of N-ethyl morpholine greater than 0.06M interfere with the
determination of inorganic P).

When the reaction velocity is determined by the
estimation of inorganic P arising from PA as a result of
hydrolysis in TCA, allowance has to be made for the inorganic
P present in the ATP and that released from ATP as a result
of hydrolysis in 0.1 N TCA at 100° and in acid molybdate during
colour development. The correction may be determined in one
of two ways: the complete system is stopped at zero time with
TCA, the mixture heated at 100°, the colour developed and the extinction value determined at a fixed time following colour development. The value so obtained is then subtracted from the extinction values obtained after a similar treatment of the reaction system following incubation. Alternatively, the reaction is carried out for various time periods and treated as described above. The slope of the line obtained as a result of the extrapolation to zero time of a plot of the extinction values against reaction time gives a measure of the reaction velocity. The correction value is given by the point where the line cuts the ordinate. Both methods make due allowance for the formation of inorganic P from ATP as a result of hydrolysis in TCA and acid-molybdate and for the inorganic P present in the ATP and should, therefore, give the same correction value. Experimentally it has been found that the latter method gives a value which is about 10% higher than that obtained by the former and it would seem likely that this is the zero time value for when this correction is used the reaction rate is proportional to the enzyme concentration. For this reason the latter procedure has been used throughout this work although no explanation can be offered for the difference mentioned. Careful attention was paid to the development time and hydrolysis conditions.
**Arbitrary Units of APT activity.**

One unit of APT activity, in the case of the forward reaction, is defined as the amount of enzyme which releases 1 μmole of arginine from PA in 1 min. at pH 7.2 and at 5°.

**Specific activity.**

Specific activity is defined as the number of units of APT per mg. of protein.

**Electrophoretic experiments.**

These were carried out both by Dr. F. Dempster of the Commonwealth Serum Laboratories, Melbourne, employing the Tiselius technique, and by Mr. R.F. Adams of this department, using the Perkin Elmer model 38A apparatus.

**Zone electrophoresis experiments.**

Zone electrophoresis on paper was performed using a commercial apparatus (L.K.B. Sweden) with direct current supplied by dry cells. This apparatus was of the horizontal open strip type. Zone electrophoresis of proteins was carried out on Schleicher and Schull paper No. 2043B and phosphate buffer (0.04M, pH 7.4) containing 5% (v/v) ethylene glycol was used as the conducting medium. In order to detect the protein bands, the paper strips were first dried at 90° for 30 min. The dried strip was stained with a solution containing 0.05% bromphenol blue,
1.0% HgCl₂ and 2.0% acetic acid in water. The stained strips were washed free of excess dye with 0.5% aqueous acetic acid, then several times with water and once with ethanol (Rosenberg, 1955). Protein appeared as blue bands on an off-white background. In order to determine the relative amounts of protein in each protein band the strips were cut up and eluted with 0.01N NaOH. The eluates were cleared of filter paper debris by centrifugation and the dye colour was read in a Unicam spectrophotometer at 590 mμ against a blank solution made by elution of a non-stained portion of the paper strip. The values obtained are based on the assumption that the different proteins present have the same binding capacity for the dye on a weight basis (see Durrum, 1955).

When paper strips were examined for enzymic activity, a series of strips were run in parallel. One of these was used for protein development and the position of the protein bands on the other strips was deduced after comparison with the stained strip. The paper strips were then partially dried in vacuo at 0° for 2 - 3 hr. and areas corresponding to the position of the protein bands were cut out. Portions of these strips were placed in tubes containing the stock solution for the forward reaction and into tubes
containing a modified stock solution designed to detect myokinase and phosphoamidase activity. The mixture was then stirred thoroughly to ensure elution of any enzyme present. After incubating for 30 min. at room temperature, the mixture was cleared by centrifugation and any arginine released detected as usual.

Spectrographic analysis.

Spectrographic analyses were carried out by Dr. D.J. David of the Commonwealth Scientific and Research Organisation, Canberra. The results obtained represent a visual assessment of the concentration of each metal on an ash basis.

RESULTS

Attempts to isolate APT from the tail muscle of *Jasus verreauxi* by the method Szorenyi *et al.* (1949) developed for fresh water crab muscle were unsuccessful. It was found that the fractionation pattern for APT from sea-crayfish muscle differed considerably from that found by the above authors for APT from fresh water crab muscle. Therefore, attention was turned to the development of a method for the isolation of the enzyme from sea-crayfish muscle. This method is given below.
Extraction of tissue.

The tail muscles of 6 live crayfish were rapidly excised and passed twice through a mincer. A 500g. sample of the minced muscle was extracted by stirring with three volumes of distilled water for 30 min. and then centrifuged for 15 min. at 1,000g. and 0°. The product did not sediment well and it was necessary to pour off carefully the opalescent uppermost layer from the middle gelatinous layer. The two bottom layers were extracted with 1.5 volumes of water (750 ml.) by stirring for 30 min. and again centrifuged at 1,000g. for 15 min. at 0°. The opalescent supernatant was poured off from the firmly packed residue and combined with the first extract. The pH of the combined extracts was 6.6. Both of these extracts were of the same specific activity and further quantities of the enzyme, also of the same specific activity, could be obtained as a result of a third extraction with 1.5 volumes of water. This extract contained only a small amount of protein and was discarded.

1st Ammonium sulphate fractionation.

For convenience, two 500 ml. samples of the extract were taken and fractionated simultaneously. Solid \((\text{NH}_4)_2\text{SO}_4\) (31.5g./100 ml.) was added to each sample with mechanical
stirring. The precipitate was removed by centrifugation at 5000g, for 10 min. at 0° and discarded. To the clear colourless supernatant, solid (NH₄)₂SO₄ (10.5g./100 ml. of original extract) was added slowly with mechanical stirring. A grey precipitate was removed by centrifugation at 5000g, for 10 min. at 0° from a clear colourless supernatant which was discarded. The two precipitates were each dissolved in 100 ml. of water and combined. (During solution of these precipitates a sheen was seen in solutions of high protein concentration. Examination revealed that this was due to diamond shaped crystals which dissolved on dilution. These crystals were shown to possess glyceraldehyde-3-phosphate dehydrogenase activity by Dr. C. Kratzing of this department).

2nd Ammonium sulphate fractionation.

Ammonium hydroxide (17N) was added to a saturated solution of (NH₄)₂SO₄ at 5° so that on dilution (1:10) the pH was 8.2. This solution was added dropwise with mechanical stirring to the enzyme solution obtained from the previous step until 0.7 saturation was reached. The grey precipitate was removed by centrifugation at 5000g, for 10 min. at 0° and discarded. The supernatant was then brought to 0.8 saturation by the further addition of the alkaline (NH₄)₂SO₄.
solution. The suspension was centrifuged at 5000g. for 15 min. at 0°, the supernatant discarded, and the white precipitate dissolved in water to give a final volume of 50 ml.

Attempts to increase the specific activity of this enzyme preparation were unsuccessful. Low temperature fractionation with both ethanol and propanol at pH 6.6 and 8.2 in the presence and absence of Mg²⁺ ions led to marked loss of activity. The enzyme was not precipitated by magnesium sulphate.

Although APT constituted the major portion of the final product, it could not be induced to crystallise. The addition of saturated (NH₄)₂SO₄ solutions of varying pH in amounts just insufficient to cause precipitation gave rise to amorphous precipitates only.

A summary of the yields and degrees of purification of APT obtained with a typical preparation is shown in Table 1. It can be seen that the fractionation procedure gives approximately a 3-fold increase in the purity of the enzyme with an overall recovery of 21%.

Tests for the presence of other enzymic activities in the APT preparation.

Tests for the presence of phosphoamidase, ATP-ase and myokinase
### TABLE 1

**Summary of Yields and Specific Activities of Fractions obtained during the Fractionation of APT.**

Wt. of muscle - 500 g. Details are described in the text.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml.)</th>
<th>Total protein (g.)</th>
<th>Total units ($x 10^{-3}$)</th>
<th>Specific activity (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>2050</td>
<td>22.1</td>
<td>530</td>
<td>24</td>
<td>(100)</td>
</tr>
<tr>
<td>First ammonium sulphate precipitate</td>
<td>570</td>
<td>8.3</td>
<td>282</td>
<td>34</td>
<td>53</td>
</tr>
<tr>
<td>Second ammonium sulphate precipitate</td>
<td>54</td>
<td>1.7</td>
<td>110</td>
<td>67</td>
<td>21</td>
</tr>
</tbody>
</table>
activities in the APT preparation were carried out in the forward reaction system using the enzyme at a concentration 1000 times greater than that used in the standard activity determination. Phosphoamidase activity was tested for at pH 7.2 and 8.4 by determination of arginine released from PA in the absence of ADP; no such activity was detected. When ADP was replaced by ATP there was again no release of arginine from PA indicating the absence of a Mg$^{2+}$ activated ATP-ase, which conclusion was confirmed by the fact that there was no release of inorganic P when the enzyme was incubated with ATP at pH 7.2 and 8.4 in the presence of Mg$^{2+}$. The replacement of ADP by ATP and AMP led to the release of arginine. Thus an enzyme with myokinase-like activity was a contaminant of the APT preparation. It was concluded that this was a minor contaminant for when tested at the usual test level of 1 µg. of protein/ml., no trace of myokinase activity was detected. No tests for other enzymic activities were made.

Electrophoretic analysis.

The electrophoretic pattern of the final preparation of APT is illustrated in Fig. 1. All the components migrated to the anode at pH 8.6. An attempt was made to carry out a second
Fig. 1. Electrophoretic patterns of the APT preparation obtained after fractionation as described in the text. Veronal buffer, pH 8.6; ionic strength 0.1; temperature, 1°; field strength 6.6 volt/cm.; protein concentration, 1%; time, 272 min.

(a) descending boundary (b) ascending boundary
run in acetate buffer (pH 5.7), but on dialysis of the enzyme against the buffer prior to electrophoresis, there was marked precipitation of protein. It was not possible to redissolve the precipitate by dialysis against buffers with higher pH values. From a study of the effects of pH on the enzyme activity and protein content of the APT solution the enzyme would appear to have an isoelectric point in the vicinity of pH 5. Because of the rapid denaturation of the enzyme at pH values below 5.7, it was not possible to carry out an electrophoretic run at pH values where the protein would be positively charged. It was calculated that the major component constituted 67% of the total protein and had a mobility of $-2.84 \times 10^{-5} \text{cm}^2\text{volt}^{-1}\text{sec}^{-1}$.

**Zone electrophoresis on paper.**

The APT preparation was also subjected to zone electrophoresis on paper in order to determine whether or not the main component was responsible for the APT activity. Staining with bromphenol blue indicates the presence of a major component and a faster moving minor component (Fig. 2). From a second strip, run at the same time, areas corresponding to the stained sections were excised and added to the standard test system for the forward reaction as described in the
Fig. 2. Separation by paper zone electrophoresis of the APT preparation obtained as described in the text. Conditions:- Phosphate buffer (0.04M, pH 7.4) containing ethylene glycol (5% v/v); current 8mA; time, 22 hr.; temperature, 22°.

(a) APT  (b) contaminating protein.
Methods section. The results showed that the major, slower moving component possessed APT activity, whereas the faster moving component showed none. Neither component showed myokinase or phosphoamidase activity.

Elution of the stained protein bands, and subsequent determination of the optical density of the eluate, indicated that APT constituted 80% of the total protein. This compares with a value of 67% obtained from the run in the Tiselius electrophoresis apparatus. Durrum (1955) has pointed out the errors which can arise during staining of protein on filter paper strips with dye. It is therefore possible that the results obtained by zone electrophoresis are subject to greater error than those obtained by the Tiselius technique.

The fact that a preparation of high purity was obtained with only a 3-fold increase in specific activity indicates that APT forms a high proportion of the protein in the muscle extract. This is also indicated by the finding that only four components could be detected in the crude extract by zone electrophoresis on paper.

General properties of APT.
The APT preparation could be dialysed against water and N-ethyl morpholine and phosphate buffers (0.05M, pH 7.2)
without loss of enzymic activity. There was no loss of activity when the protein solution was freeze-dried following dialysis against phosphate buffer, but after dialysis against water, freeze-drying led to an almost complete loss of activity. When water was added to the water dialysed, freeze-dried protein the solution was opalescent and the pH was 4.4, which low pH presumably accounts for the loss of activity. Although the enzyme could be dialysed against phosphate buffer and freeze-dried, it was more convenient to store the solution at 5° in the presence of an equal volume of glycerol. Suitable reaction rates were obtained with high dilutions of the glycerol solution so that the concentrations of glycerol (approximately 0.01%) and \((\text{NH}_4)_2\text{SO}_4\) in the final reaction mixture were such that they had no effect on the activity of the enzyme.

The initial specific activity of all APT preparations (67-80) fell during the first three days of storage to a value of 42-46. This decrease in activity was independent of the method of storage, but after its occurrence the specific activity of the glycerol solutions of APT remained constant for at least twelve months. In the absence of glycerol, it was found that aqueous and \((\text{NH}_4)_2\text{SO}_4\) solutions of the enzyme became opalescent within a week and a
precipitate settled on the walls of the vessel. There was, however, no fall in the specific activity of the supernatant. On the other hand, glycerol solutions of the enzyme remained clear.

**Effect of temperature and dilution on APT activity.**

The initial experiments designed to determine APT activity in the forward reaction were carried out at 30° but the results were unreliable. This was at first believed to be due to the effect of temperature on the enzyme and an incubation temperature of 5° was then chosen. However, even at this temperature it was found that the observed specific activity varied with the degree to which the enzyme was diluted before the addition to the test medium. Thus a large volume of highly diluted enzyme was not as active as a small volume of a correspondingly more concentrated enzyme even though the reaction was linear with time in all cases. This effect was most marked when the concentration of protein in the diluted enzyme solution was less than 2.5 µg./ml. and absent at concentrations greater than 5 µg./ml. This inactivation of the enzyme at high dilutions was apparently not due to oxidation of -SH groups for the effect was observed even when cysteine was present. A temperature of 5° was chosen for all subsequent work as the reaction rate was slowed
sufficiently to permit the use of larger amounts of enzyme and thus to avoid inactivation of the type referred to above. The enzyme was completely inactivated when heated to 50° for 5 min. The addition of arginine afforded no protection against this inactivation.

**Effect of enzyme concentration on the reaction velocity.**

The initial velocity is proportional to the enzyme concentration in both the forward and reverse reactions over the concentration range studied (Fig. 3 & 4). It will be noted that the velocity of the forward reaction is about 5 times faster than that of the reverse reaction.

**Effect of pH on the reaction velocity.**

The relationship between pH and the maximum velocity in the forward reaction (Fig. 5) indicates that the maximum velocity is reached at pH 6.8 in phosphate, pH 7.2 in glycerophosphate, and at pH 7.0 in N-ethyl morpholine. At the pH optimum the reaction rates were the same in phosphate and glycerophosphate, but were slightly lower in N-ethyl morpholine. Although the pH optimum in N-ethyl morpholine is at pH 7.0, further experiments on the forward reaction in this buffer were carried out at pH 7.2 as better buffering capacity was obtained at this pH. Tris-(hydroxymethyl)-aminomethane was not used as a buffer as it interferes with the estimation of arginine (cf. Rosenberg et al. 1956).
Fig. 3. Effect of enzyme concentration on the reaction velocity of the forward reaction at pH 7.2. Conditions as described in the text, except that various volumes of a stock enzyme solution (5μg. of protein/ml.) were added. Reaction time, 4 min.; temperature, 5°.
Fig. 5. Effect of pH on the initial velocity of the forward reaction. The system was as described in the text. The enzyme concentration was 1 µg, of protein/ml. and reactions were run for 3 and 6 min. The pH was determined with a glass electrode in a duplicate set of tubes. Temperature, 5°C.

○, N-ethylmorpholine (0.06M);
●, phosphate (0.06M);
△, glycerophosphate (0.06M).
In the reverse reaction (Fig. 6) the pH activity curves show that the optimum pH is about 8.4 in N-ethyl morpholine, glycine and glycyglycine buffers. At this pH the reaction velocity is the same in each of the three buffers.

Activation of APT by divalent cations.
The purified APT preparation shows only a small residual activity in the absence of divalent cations (Fig. 7). The activity of the enzyme in the forward reaction is markedly increased by the addition of Mg\(^{2+}\) but Ca\(^{2+}\) is without effect. Mn\(^{2+}\) activated the enzyme to a greater degree than Mg\(^{2+}\) but as this ion interferes with the arginine estimation it was not possible to obtain a precise figure for the degree of activation. In the reverse reaction (Fig. 8) it was possible to demonstrate activation of the enzyme by both Mg\(^{2+}\) and Mn\(^{2+}\), but here again there was no activation by Ca\(^{2+}\). There was no activation of APT by Ba\(^{2+}\), Sr\(^{2+}\), Fe\(^{2+}\), Fe\(^{3+}\), Co\(^{2+}\), Cu\(^{2+}\), Cd\(^{2+}\), Zn\(^{2+}\) and Al\(^{3+}\) as tested in the reverse reaction.

The activity of the APT preparation in the absence of added ions (residual activity) was not removed by dialysis against 0.01M EDTA (pH 7.2) for 16 hr., followed by dialysis against water. However, when EDTA (0.01M) was added to the test medium there was a complete loss of residual activity.
Fig. 6. Effect of pH on the initial velocity of the reverse reaction. The system was as described in the text. The enzyme concentration was 15 μg. of protein/ml. and the reactions were run for 5, 10 and 15 min. The pH was determined with a glass electrode in a duplicate set of tubes. Temperature, 5°.

○, N-ethylmorpholine (0.06M);
●, glycine (0.06M);
▲, glycylglycine (0.06M).
Fig. 7. Activation of APT by bivalent metals as determined in the forward reaction. Conditions are as described in the text. Temperature, 5°.
Fig. 8. Activation of APT by bivalent metals as determined in the reverse reaction. Conditions as described in the text. Temperature, 5°C.
Effect of inhibitors on APT activity.

The effect of inhibitors on APT activity in the forward reaction is summarised in Table 2. The inhibition by iodoacetate, p-CMB, o-iodosobenzoate, N-ethyl maleimide and diphenylchloroarsine characterises APT as an -SH enzyme. The high affinity of the enzyme for Mg$^{2+}$ is indicated by the fact that a EDTA : Mg$^{2+}$ ratio of 2 : 1 is required for complete inhibition of the reaction at pH 7.2.

The inhibition by 2,4-dinitrophenol is unusual as this compound has not been shown to inhibit transphosphorylation at the substrate level. The nature of the inhibition was further investigated and it was found that when tested on the reverse reaction at pH 8.4 and pH 7.2 no inhibition resulted. A similar result was obtained when tested on the forward reaction at pH 8.4. It would thus seem that 2,4-dinitrophenol has no effect on the transphosphorylation; its inhibitory action on the forward reaction at pH 7.2 may therefore be due to interaction with one of the substrates.

The enzyme is very sensitive to p-CMB, 100% inhibition being obtained at $10^{-7}$M. This inhibition was only partially reversed by cysteine which was added in concentrations 10 and 20 times that of p-CMB (see Table 3).
TABLE 2.

Effect of Various Substances on APT Activity in the Forward Reaction

The enzyme (1 μg./ml.) was incubated for 5 min. in the presence of the inhibitor, N-ethyl-morpholine (0.06M, pH 7.2), PA (5mM) and Mg$^{2+}$ ions (5mM). The reaction was started by the addition of ADP (mM) and stopped after 5 min. Total volume, 1.0 ml.; temperature 5°.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (M)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodoacetate</td>
<td>$1 \times 10^{-2}$</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-3}$</td>
<td>19</td>
</tr>
<tr>
<td>p-CMB</td>
<td>$1 \times 10^{-7}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-8}$</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-8}$</td>
<td>27</td>
</tr>
<tr>
<td>o-iodosobenzoate</td>
<td>$1 \times 10^{-3}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-4}$</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-5}$</td>
<td>19</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>$1 \times 10^{-4}$</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-5}$</td>
<td>10</td>
</tr>
<tr>
<td>Diphenylchloroarsine</td>
<td>$1 \times 10^{-4}$</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-5}$</td>
<td>12</td>
</tr>
<tr>
<td>Chloroacetophenone</td>
<td>$1 \times 10^{-4}$</td>
<td>0</td>
</tr>
<tr>
<td>Arsenite</td>
<td>$1 \times 10^{-3}$</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>$2 \times 10^{-2}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-2}$</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-3}$</td>
<td>32</td>
</tr>
<tr>
<td>2:4-Dinitrophenol</td>
<td>$1 \times 10^{-5}$</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>$1 \times 10^{-4}$</td>
<td>39</td>
</tr>
</tbody>
</table>
TABLE 3.

Effect of Cysteine on p-CMB Inhibition of APT

<table>
<thead>
<tr>
<th>p-CMB concentration (M)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(a)</td>
</tr>
<tr>
<td>1 x 10^-8</td>
<td>27</td>
</tr>
<tr>
<td>5 x 10^-8</td>
<td>75</td>
</tr>
<tr>
<td>1 x 10^-7</td>
<td>100</td>
</tr>
</tbody>
</table>

(a) No cysteine

(b) Cysteine : p-CMB = 10:1

(c) Cysteine : p-CMB = 20:1

Reaction mixture: 0.1 ml. PA (0.05M), 0.1 ml. MgSO₄ (0.1M), 0.1 ml. N-ethylmorpholine (0.5M, pH 7.2), 0.1 ml. APT (10 μg./ml.), p-CMB of required concentration, 0.4 ml. water; this mixture was incubated for 10 min. at 5°C. The reaction was started by the addition of 0.1 ml. of ADP (0.01M).

Cysteine was added in series (b) and (c) 5 min. after zero time, i.e. 5 min. after preincubation with inhibitor.

In all tubes containing cysteine, 0.1 ml. of 1 x 10^-4 M p-CMB was added to the reaction mixture after the reaction had been stopped with EDTA-NaOH mixture. In this way inhibition of the colour development of arginine by cysteine was avoided.
Specificity of APT.

The ability of APT to phosphorylate other guanidine compounds was determined in the reverse reaction by estimating both the disappearance of free guanidine and the formation of acid labile P. In the case of canavanine, it was possible to determine only the formation of acid labile P as this compound does not give the guanidine colour reaction with α-naphthol and diacetyl. It can be seen (Table 4) that apart from arginine there is also phosphorylation of arginine methyl ester, L-homo-arginine and L-canavanine. However, under the conditions used, the degree to which these compounds were phosphorylated was less than in the case of arginine. On much longer incubation, the degree of phosphorylation of these compounds increased and approached that of arginine. There was no phosphorylation of agmatine, citrulline, 6-guanidino-n-valeric acid, α-carbamido arginine, β-guanidino propionic acid, creatine, glycocyamine, negmine, taurocyamine, argininic acid, α-chloro-6-guanidino-n-valeric acid and α-N-acetyl arginine.

These latter compounds as well as canavanine, homo-arginine, lysine and ornithine had no effect on the rate at which arginine was phosphorylated when present in equimolar concentrations.

Under the standard test conditions, arginine methyl
TABLE 4.

Specificity of APT; Phosphorylation of Guanidines

The reaction mixture contained substituted guanidine (2 mM), ATP (5 mM), Mg$^{2+}$ ions (mM) and N-ethylmorpholine (0.06 M, pH 8.4). The enzyme (final concentration, 46 μg of protein/ml.) was added and the tubes were incubated for 17 hr. at 5°. Total volume 1.0 ml.

<table>
<thead>
<tr>
<th>Substituted guanidine</th>
<th>(a)*</th>
<th>(b)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
<td>88</td>
<td>91</td>
</tr>
<tr>
<td>L-Arginine methyl ester</td>
<td>65</td>
<td>67</td>
</tr>
<tr>
<td>L-Canavanine</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>L-Homoarginine</td>
<td>23</td>
<td>26</td>
</tr>
</tbody>
</table>

*(a) Calculated on basis of the free guanidine remaining.

*(b) Calculated by determination of the guanidine P formed.
ester was phosphorylated at the same rate as arginine whereas
the rate of phosphorylation of homoarginine and canavanine was
negligible.

APT is specific for ADP in the forward reaction; AMP
does not act as a phosphate acceptor. In the reverse
reaction ATP could not be replaced by ITP or ADP.

Isolation of APT from the tail muscle
of Cherix albidus Clark

Specimens of fresh water crayfish (Cherix albidus Clark) were
available only in the summer months. On completion of the
work on APT isolated from sea-crayfish an attempt was made to
isolate the enzyme from the tail muscle of Cherix albidus by
the procedure described by Szorenyi et al. (1949).

The precipitate formed by the addition of (NH₄)₂SO₄
(43.2g./100 ml. extract) to the extract was removed by
filtration and the filtrate allowed to stand for 48 hr. at
5°. No crystalline material was formed, but the amorphous
precipitate which appeared after 18 hr. contained large
amounts of APT of high specific activity (90). Electrophoretic
analysis of this fraction in veronal buffer (µ = 0.1) at
pH 8.6 showed the presence of three components, the major
component being assumed to be APT. It was estimated that
APT constituted about 50% of the total protein present.
The only experiments carried out with this APT preparation indicated that both Mg\(^{2+}\) and Mn\(^{2+}\) ions markedly activated the enzyme and that Ca\(^{2+}\) ions were without effect.

**Spectrographic analyses of APT preparations from fresh water and sea crayfish.**

APT preparations from fresh water and sea crayfish were dialysed against nine changes of distilled water at 5° and freeze-dried. The dry material was ashed and examined by arc spectrography.

The results (Table 5) of visual examination of the spectrograms indicated a much higher content of Mg\(^{2+}\) ions than of Mn\(^{2+}\) ions in both preparations. It is also of interest to note that both samples contained comparatively large amounts of Ca\(^{2+}\) ions, an ion which is incapable of activating the enzyme.

**DISCUSSION**

In the present work the major portion of the protein possessing APT activity was precipitated from the crude extract of sea-crabfish muscle in the fraction obtained between the addition of 31.5 and 42g. of ammonium sulphate/100 ml.; the enzyme remaining in the supernatant was of much lower specific activity. This result differs sharply from that of Szorenyi.
TABLE 5.

Spectrographic Analysis of APT Preparations

(a) Figures represent a visual assessment of the concentration of each metal on an ash basis.

<table>
<thead>
<tr>
<th></th>
<th>10%</th>
<th>1-10%</th>
<th>0.1-1%</th>
<th>0.1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea-crayfish</td>
<td>Na, Al, Cu, Si, Ca, P</td>
<td>Mg, Fe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh-water crayfish</td>
<td>Si, Al, Fe</td>
<td>Cu, Mg, Pb</td>
<td>Cd, Mn, Na</td>
<td></td>
</tr>
</tbody>
</table>

(b) Comparison of the relative concentrations (in arbitrary units) of metals present in the ash of each preparation as determined from the spectrograms.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ash (%)</th>
<th>Na</th>
<th>Cu</th>
<th>Al</th>
<th>Si</th>
<th>Fe</th>
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</thead>
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<tr>
<td>Sea-crayfish</td>
<td>2.2</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Fresh-water crayfish</td>
<td>0.22</td>
<td>0.05</td>
<td>7</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ash (%)</th>
<th>Mg</th>
<th>Ca</th>
<th>P</th>
<th>Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea-crayfish</td>
<td>2.2</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Fresh-water crayfish</td>
<td>0.22</td>
<td>6</td>
<td>7</td>
<td>10</td>
<td>0.5</td>
</tr>
</tbody>
</table>
et al. (1949), who isolated crystals of APT from the supernatant obtained after the addition of 43.2g. of ammonium sulphate/100 ml. In one respect, however, the present results confirm those of these workers; diamond shaped protein crystals were isolated from the supernatant (43.2g. ammonium sulphate/100 ml.) after standing for 24 hr. at 5°. These were identical in appearance with those designated 'crystals B' photographed by Szorenyi et al. (1949), who did not identify them as possessing enzymic activity. Experiments carried out by Dr. C. Kratzing of this department have indicated that they possess glyceraldehyde-3-phosphate dehydrogenase activity. On examination in the Perkin Elmer electrophoresis apparatus at pH 8.6 only one component was found to be present. In a recent communication, Szorenyi, Elodi and Devenyi (1956) have described similar results obtained with extracts of the fresh water crabs, Potamobius astacus and Potamobius leptodactylus. They identified the crystalline protein as D-glyceraldehyde-3-phosphate dehydrogenase and gave a detailed account of its properties.

An examination of fresh water crayfish muscle extracts also failed to confirm the findings of Szorenyi et al. (1949), but it is of interest to note that whereas the fractionation pattern was different from that described by
these workers it was also different from that observed in the sea-crayfish muscle. Use of the fractionation procedure of Elodi and Szorenyi (1956), which is essentially similar to that of Szorenyi et al. (1949), would also have resulted in loss of enzyme as the APT is precipitated at the ammonium sulphate concentrations used by these authors. It is possible that these differences may be due to species variation, but in any event it is apparent that the fractionation procedure described by Szorenyi et al. (1949) cannot be applied to the isolation of APT in crystalline form from the tail muscle of either sea water or fresh water crayfish available for the present work.

There are major differences in the properties of the present APT preparation as compared with the crystalline preparations of Szorenyi et al. (1949) and Elodi and Szorenyi (1956). Thus the present APT preparation shows greater stability in aqueous solution and on dialysis than the crystalline preparations. However, it is well known that purification of proteins often increases their lability and the comparative stability of the present preparation may be due to the stabilising effect of the contaminating protein. The present preparation of APT is activated by Mg$^{2+}$ and Mn$^{2+}$ only and shows very little residual activity (i.e. activity
in absence of added cations). On the other hand the crystalline APT preparation of Elodi and Szorenyi shows appreciable residual activity and furthermore, is activated from 2 to 300% by Cu$^{2+}$, Ca$^{2+}$, Mn$^{2+}$ and Mg$^{2+}$. This comparatively high residual activity may be due to the presence of Mn$^{2+}$ bound to the enzyme as Szorenyi et al. (1949) found that their crystalline APT preparation contained 0.27% Mn. Szorenyi and co-workers therefore consider Mn$^{2+}$ to be the natural activator of APT. Spectrographic analysis of both the fresh water and sea-crayfish APT obtained in the present work showed only a low concentration of Mn$^{2+}$ and that Mg$^{2+}$ ions were present in higher concentration than Mn$^{2+}$ ions. It is not known which of these two ions is the natural activator of APT but the inhibition of residual activity by EDTA [Elodi and Szorenyi (1956) and present work] suggests that Mg$^{2+}$ or Mn$^{2+}$ ions bound to the enzyme are concerned in APT activity.

An ill-defined pH optimum for the reverse reaction in glycine buffer was found to lie at pH 8.4 in the present work, whereas Szorenyi et al. (1949) have claimed a well-defined pH optimum, in the same buffer, at pH 9.1. Another difference between the present results and those of the above authors lies in the isoelectric points; thus in crayfish APT the isoelectric point lies at about pH 5.0, but an unusually
A low figure of 3.5 was reported for crab muscle APT.

N-ethylmorpholine was chosen as the buffer for the general experiments carried out with APT. This was based on the assumption that the concentration of free metal ion would not be reduced by reaction with the buffer and thus complications of kinetic studies would be avoided. It is noteworthy therefore that the APT activity in the reverse reaction at the pH optimum is the same in N-ethyl morpholine as in glycine and glycylglycine, which coordinate with Mg\(^{2+}\) ions (Monk, 1951). Moreover in the forward reaction the velocity of the enzymic reaction in N-ethylmorpholine is less than in phosphate and glycerophosphate buffers, which also form complexes with Mg\(^{2+}\) ions (Tabor and Hastings, 1943).

The inhibitions of the enzyme by three classes of -SH reagents indicates that APT is dependent upon the presence of thiol groups for activity. The inhibition by diphenylchloroarsine indicates that the enzyme forms a stable monothioarsenite, and the failure of arsenite to inhibit suggests that the thiol groups are not in sufficiently close proximity to form a stable As-ring (Peters, 1952). The fact that complete reversal of p-CMB inhibition by cysteine was not obtained suggests that p-CMB causes denaturation of the enzyme.
The inhibition by 2:4-dinitrophenol is of interest in that it occurs at the substrate rather than the enzyme level. The inhibition was not further investigated and it is not known whether 2:4-dinitrophenol acted by combination with ADP or with the α-amino or guanidino group of PA. Ennor and Rosenberg (1954a) have reported an inhibiting effect by 2:4-dinitro-o-cresol on CPT; doubtless a similar explanation would account for this effect.

The experiments on the specificity of APT give some indication of the groups required for interaction of the enzyme and the substrate. The failure of the enzyme to phosphorylate argininic acid, agmatine, \( \delta \)-guanidino-\( \alpha \)-valeric acid, \( \alpha \)-chloro-\( \delta \)-guanidino-\( \alpha \)-valeric acid, \( \alpha \)-carbamido arginine and \( \alpha \)-N-acetyl arginine suggests that the \( \alpha \)-amino group of arginine is an essential requirement of the substrate. The 'free' carboxyl group is not essential as both arginine and arginine methyl ester were phosphorylated at the same rate. These results may be compared with those obtained with arginase (Greenberg, 1951), for which enzyme a free carboxyl group but not an \( \alpha \)-amino group is required. An intact guanidino group is required, as evidenced by the failure of APT to phosphorylate citrulline. The fact that homoarginine and canavanine are phosphorylated indicates that the enzyme is
not completely specific. However, the rate of phosphorylation of these compounds is slow as compared with arginine. APT is thus similar to arginase and argininosuccinase (Greenberg, 1951; Walker, 1953) in that there is little or no differentiation between guanidino and guanidoxy groups. Elodi and Szorenyi (1956) observed that D-arginine is phosphorylated at 75-90% of the rate that L-arginine is phosphorylated and that creatine, glycocyamine and arginine bound in protein (e.g., salmon protamine) are not phosphorylated.

A comparison of the present results with those obtained with CPT by Kuby et al. (1954), Ennor and Rosenberg (1954) and Rosenberg and Ennor (1955) shows that there is a similarity in the properties of the two enzymes. Both are \(-\)-SH enzymes of the monothiol type; both are activated by \(\text{Mg}^{2+}\) and \(\text{Mn}^{2+}\) ions, although CPT is also activated by \(\text{Ca}^{2+}\) ions. The pH optimum for the forward reaction in each case is about pH 7 and for the reverse reaction is from pH 8.4 to 8.8. Under optimum conditions the velocity of each forward reaction is about six times as fast as the reverse reaction. This similarity is perhaps not surprising as the CPT-PC system presumably plays the same role in the metabolism of vertebrate muscle as the APT-PA system plays in the metabolism of invertebrate muscle.
SUMMARY

1. A method is described for the isolation of a highly active preparation of APT from the tail muscle of the sea crayfish (*Jasus verreauxi*). The purity was estimated to be 67 - 80%.

2. The enzyme had a pH optimum of 6.6 - 7.0 and 8.4 - 8.5 in the forward and reverse reactions respectively, as expressed by the equation: \( PA + ADP \rightleftharpoons arginine + ATP \).

3. \( Mn^{2+} \) and \( Mg^{2+} \) ions activated the enzyme in both the forward and reverse reactions. There was no activation by \( Ca^{2+} \) ions.

4. The \(-SH\) nature of the enzyme was established as a result of the inhibition by iodoacetate, p-CMB, etc. There was also inhibition by EDTA and 2,4-dinitrophenol.

5. The enzyme phosphorylated homoarginine and canavanine. There was no phosphorylation of agmatine, argininic acid, \( S\)-guanidino-n-valeric acid, \( \alpha\)-carbamidoarginine, citrulline, creatine, negmine, glycocyamine, \( \beta\)-guanidinopropionic acid, taurocyamine, \( \alpha\)-chloro-\( S\)-guanidino-n-valeric acid or \( \alpha\)-acetyl arginine.

6. AMP could not act as a phosphate acceptor in the forward reaction. ITP or ADP could not replace ATP in the reverse reaction.
CHAPTER 2.

A STUDY OF THE KINETICS OF THE REACTION

CATALYSED BY ARGinine PHOSPHORYLTRANSFERASE
CHAPTER 2.

A STUDY OF THE KINETICS OF THE REACTION CATALYSED

BY ARGININE PHOSPHORYLTRANSFERASE

INTRODUCTION

The general properties of arginine phosphoryltransferase (APT) which catalyses the approach to equilibrium as expressed by the reaction

$$PA + ADP \rightleftharpoons \text{Arginine} + \text{ATP}$$

have been described in Chapter I. Although the properties of the enzyme have also been studied to some extent by Lohmann (1935), Lohmann (1936), Szorenyi et al. (1949) and Elodi and Szorenyi (1956), no detailed study has been made of the kinetics of the reaction. This has been undertaken in the present work which was also designed to elucidate the role of $\text{Mg}^{2+}$ in the mechanism of the reaction. It has previously been shown that $\text{Mg}^{2+}$ activates APT (Chapter I) and it is well known that $\text{Mg}^{2+}$ activates other phosphoryltransferases (Lehninger, 1950). However, little work has been directed towards an understanding of the role of $\text{Mg}^{2+}$ in phosphoryl group transfer reactions. The results obtained indicate that $\text{Mg}^{2+}$ reacts with APT to form an active $\text{Mg}$-APT complex which is responsible for catalysing
the reaction as expressed above.

EXPERIMENTAL

The materials and experimental methods together with the preparation of the enzyme used in the present investigation have been fully described in Chapter 1. The APT preparation had a purity of between 67 and 80%. The enzyme was stored at 5° in the presence of an equal volume of glycerol and diluted with 0.05 M N-ethyl morpholine of the appropriate pH before addition to the test system. Although the preparation was contaminated by myokinase, this activity was not detectable at the low enzyme concentrations and short reaction periods used for the kinetic studies. Even with the larger enzyme concentrations and longer incubation periods which were used for the equilibrium experiments, it appeared that the presence of myokinase did not influence the equilibrium values. The detailed descriptions of the experimental conditions are given in the legends to the figures and tables. All experiments have been carried out at 5° for reasons previously explained (see Chapter 1).

The $K_m$ values represent a measure of the substrate concentration required for half maximum velocity and are not necessarily true dissociation constants of the enzyme.
substrate complexes.

**RESULTS**

**Effect of divalent cations on the reaction velocity.**

The effect of Mg$^{2+}$ concentration on the reaction velocity of the reverse and forward reactions is shown in Fig. 9 and 10 respectively. In the case of the reverse reaction (Fig. 9) it is seen that there is a relationship between the initial velocity and the ATP and Mg$^{2+}$ concentrations. Thus for each concentration of ATP, a maximum velocity is reached when the Mg$^{2+}$ concentration is approximately equal to that of ATP. The maximum velocity is also dependent upon the concentration of ATP. When the Mg$^{2+}$:ATP ratio exceeded 1:1 as a result of increasing the Mg$^{2+}$ concentration, there was no decrease in the reaction velocity.

In the case of the forward reaction at pH 7.2 (Fig. 10) maximum enzymic activity at the three levels of ADP was attained when the concentration of Mg$^{2+}$ was greater than that of ADP. There was no relationship between the concentrations of ADP and Mg$^{2+}$ as was the case in the reverse reaction, for the ratio of Mg$^{2+}$:ADP at maximum enzymic activity varied from 3 to 8 according to the ADP concentration. When the Mg$^{2+}$ concentration was increased considerably above that
Fig. 9. Effect of Mg$^{2+}$ concentration on the initial velocity of the reverse reaction in the presence of various concentrations of ATP. The reaction mixture contained arginine ($1 \times 10^{-2}$ M), N-ethylmorpholine (0.06 M, pH 8.4) and APT (25 µg. of protein/ml.). Total volume, 1.0 ml.; temperature 5°.
Fig. 10. Effect of Mg$^{2+}$ concentration on the initial velocity of the forward reaction in the presence of various concentrations of ADP. The reaction mixture contained PA (5 x 10$^{-3}$ M), N-ethylnorpholine (0.06 M, pH 7.2) and APT (1 μg. of protein/ml.). Total volume, 1.0 ml.; temperature 5°C.
required for maximum velocity, there was a fall in the reaction rate which was dependent upon the ADP concentration; the fall was greater with lower concentrations of ADP.

**Dissociation constants of APT for Mg$^{2+}$ and Mn$^{2+}$.**

In order to elucidate the role of divalent metals in the action of APT, the Mg$^{2+}$ concentration was varied whilst the other components of both the forward and reverse reaction systems were held constant. When the reciprocals of the initial reaction velocities were plotted against the reciprocals of the Mg$^{2+}$ concentrations according to the method of Lineweaver and Burk (1934), straight lines were obtained. Fig. 11 shows the results obtained for the forward reaction at pH 7.2 and pH 7.9, whilst Fig. 12 shows the results obtained for the reverse reaction at pH 6.4 and pH 7.9. Similar results were obtained when the Mn$^{2+}$ concentration in the reverse reaction system was varied (Fig. 12).

The above results conform to a Michaelis-Menten relation, i.e., to the equation $v = \frac{V \cdot S}{K_S + S}$ where $v$ = initial reaction velocity, $V$ = maximum reaction velocity, $S$ = cation concentration and $K_S$ = the dissociation constant of the metal-APT complex formed according to the equation:

$\text{APT} + \text{Me}^{2+} \rightleftharpoons \text{Me} + \text{APT}$

They are, therefore, consistent with the hypothesis that one
Fig. 11. Effect of $\text{Mg}^{2+}$ concentration on the initial velocity of the forward reaction. The results are plotted according to the method of Lineweaver & Burk (1934). The conditions were the same as those described in Fig. 10.

Conc. of ADP ($1 \times 10^{-3}$ M). $v = \mu$moles/mg$_s$/min.
Fig. 12. Effect of Mg$^{2+}$ and Mn$^{2+}$ concentrations on the initial velocity of the reverse reaction. The results are plotted according to the method of Lineweaver & Burk (1934). The conditions were the same as those described in Fig. 9. Conc. of ATP ($5 \times 10^{-3}$M). $v$ = μmoles/mg/min.
Mg$^{2+}$ or Mn$^{2+}$ ion combines with each active centre of the enzyme to form the active complex.

The calculated dissociation constants and $V_{\text{max}}$ values are listed in Table 6. When the concentrations of ADP and ATP are sufficiently high to saturate the enzyme, viz. $1 \times 10^{-3}\text{M}$ and $5 \times 10^{-3}\text{M}$ respectively it will be seen that the dissociation constant of the Mg - ATP complex at pH 7.9 differs as determined in the forward and reverse reactions. Moreover, the dissociation constant of the Mg - ATP complex, as determined in the forward reaction, was increased as a result of a 5-fold increase of the ADP concentration. The dissociation constant of the metal-APT complex is the same for both Mg$^{2+}$ and Mn$^{2+}$, although the $V_{\text{max}}$ value is greater in the presence of Mn$^{2+}$. This finding is in agreement with the previous finding (Chapter 1.) that the activation of ATP by Mn$^{2+}$ is greater than by Mg$^{2+}$.

**Effect of substrate concentrations on the reaction velocity.**

(a) Forward reaction

The effect of ADP on the reaction velocity of the forward reaction at pH 7.2 was determined under two sets of experimental conditions. In the first, all the components of the reaction system, including Mg$^{2+}$ were held constant and the ADP concentration was varied. The results plotted
Dissociation Constants of Mg - APT and Mn - APT Complexes

Conditions for the forward reaction were as described in Fig. 10; those for the reverse reaction as described in Fig. 9.

<table>
<thead>
<tr>
<th>Metal</th>
<th>pH</th>
<th>[ADP] (M)</th>
<th>Dissociation Constant (M)</th>
<th>V_{max}, (\mu moles/mg./min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>7.2</td>
<td>1 x 10^{-3}</td>
<td>5.4 x 10^{-4}</td>
<td>57.0</td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>1 x 10^{-3}</td>
<td>4.3 x 10^{-4}</td>
<td>45.5</td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>5 x 10^{-3}</td>
<td>1.0 x 10^{-3}</td>
<td>-</td>
</tr>
<tr>
<td>Reverse reaction ([ATP] = 5 x 10^{-3} M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>7.9</td>
<td>-</td>
<td>8.7 x 10^{-4}</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
<td>-</td>
<td>1.5 x 10^{-3}</td>
<td>10.7</td>
</tr>
<tr>
<td>Mn^{2+}</td>
<td>8.4</td>
<td>-</td>
<td>1.5 x 10^{-3}</td>
<td>12.5</td>
</tr>
</tbody>
</table>
according to the method of Lineweaver and Burke (1934) are shown in Fig. 13. With a Mg$^{2+}$ concentration of $5 \times 10^{-3} \text{M}$, a Michaelis-Menten relation is obtained over a limited range of ADP concentrations, i.e. between $10^{-3}$ and $4 \times 10^{-4} \text{M}$.

Within this range, the $K_m$ value of APT for ADP was calculated to be $2.4 \times 10^{-4} \text{M}$. As would be expected from the results shown in Fig. 10, when the ADP concentration falls below $4 \times 10^{-4} \text{M}$ in the presence of $5 \times 10^{-3} \text{M}$ Mg$^{2+}$ the reaction velocity decreases more markedly, so that the reciprocal values are higher than those required for a straight line plot. Thus at the lower concentrations of ADP, the Michaelis-Menten relation does not hold. When the Mg$^{2+}$ concentration was varied simultaneously with the ADP concentration so as to maintain a Mg$^{2+}$ : ADP ratio of 5 : 1, a Michaelis-Menten relation was obtained over the whole range of ADP concentrations investigated (Fig. 13). Under these conditions, the $K_m$ value was calculated to be $3 \times 10^{-4} \text{M}$. This value is similar to that obtained above.

The effect of PA concentration on the reaction velocity in the presence of Mg$^{2+}$ at a concentration of $5 \times 10^{-3} \text{M}$ is also shown in Fig. 13. It was calculated that the $K_m$ value of APT for PA was $1.2 \times 10^{-3} \text{M}$. The same value was obtained when the Mg$^{2+}$ concentration was increased to $1 \times 10^{-2} \text{M}$. It
Fig. 13. Effect of PA and ADP concentrations on the initial velocity of the forward reaction. The results are plotted as described by Lineweaver & Burk (1934). The reaction mixture in all cases contained N-ethylmorpholine (0.06 M, pH 7.2) and APT (1 µg. of protein/ml.). o—o variable substrate: PA, Mg$^{2+}$ (5 x 10$^{-3}$ M), ADP (2 x 10$^{-3}$ M); △—△ variable substrate: ADP, Mg$^{2+}$ (5 x 10$^{-3}$ M), PA (1 x 10$^{-2}$ M); •—• variable substrate: ADP, (Mg$^{2+}$ also varied to give Mg : ADP = 5 : 1). Total volume 1.0 ml.; temperature 5°. v = µmoles/mg./min.
will be noted that identical maximum velocities were obtained irrespective of whether ADP or PA was the variable substrate. Thus in each case the concentrations of the non-variable substrates were sufficient to saturate the enzyme.

(b) Reverse Reaction

The relationship between the initial reaction velocity of the reverse reaction and the ATP concentration was determined under conditions where the Mg\(^{2+}\) concentration was fixed at \(1 \times 10^{-2}\) M and also where the ratio of Mg\(^{2+}\) : ATP was maintained at 1 : 1. The results plotted according to the method of Lineweaver and Burk (1934) are shown in Fig. 14. In the latter case, the reaction velocity at each level of ATP was lower than that obtained when the Mg\(^{2+}\) concentration was fixed at \(1 \times 10^{-2}\) M except for the one instance where the Mg\(^{2+}\) : ATP ratio of 1 : 1 is common to each plot.

It can be seen (Fig. 14) that when the arginine concentration was varied and the other components of the system were held constant a straight line was obtained when the results were plotted as described by Lineweaver and Burk (1934). It will be noted that the maximum velocity was greater than that obtained with ATP as the variable substrate. It would seem therefore that the enzyme was not saturated with respect to arginine under the conditions chosen for the determination.
Fig. 14. Effect of arginine and ATP concentrations on the initial velocity of the reverse reaction. The results are plotted as described by Lineweaver & Burk (1934). The reaction mixture in all cases contained N-ethylmorpholine (0.05 M, pH 6.4) and ATP (15 μg. of protein/ml.). ●—●, variable substrate: arginine, Mg$^{2+}$ (1 x 10$^{-2}$M), ATP (1 x 10$^{-2}$M); ○—○, variable substrate: ATP, Mg$^{2+}$ (1 x 10$^{-2}$M), arginine (1 x 10$^{-2}$M); △—△, variable substrate ATP, (Mg$^{2+}$ also varied to give Mg : ATP = 1 : 1). Total volume 1.0 ml., temperature 5°. v = μmoles/mg./min.
of the $K_m$ of APT for ATP. The $K_m$ of APT for arginine was calculated to be $3.2 \times 10^{-3} \text{M}$.

A summary of the $K_m$ and $V_{max}$ values obtained for the substrates of APT is given in Table 7.

Inhibition of the forward and reverse reactions by the reaction products.

It was of interest to determine whether or not the products of the forward and the reverse reactions had any effect on the velocity of the reaction. For technical reasons it was not possible to test the effect of arginine in the forward reaction or PA in the reverse reaction. However, it was possible to test the effect of ATP in the forward reaction and ADP in the reverse reaction. Table 6 shows that ATP does not inhibit the forward reaction when added at the same concentration as the ADP, and that it is only when the ATP concentration is 10 times that of the ADP that inhibition occurs. There was only a low degree of inhibition by AMP. These results suggest that the affinity of APT for ADP under the conditions of the forward reaction is very much greater than for ATP or AMP. Alternatively, the results could be explained as being due to a reduction of the $\text{Mg}^{2+}$ concentration and hence the concentration of the active $\text{Mg} - \text{APT}$ complex as a result of the formation of $\text{Mg} - \text{ATP}$ and $\text{Mg} - \text{AMP}$ complexs.

In contrast to the above results there is marked inhibition
TABLE 7.

K<sub>m</sub> Values and Maximum Velocities

Conditions as described in Figs. 13 and 14.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (M)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (μmoles/mg./min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward reaction (pH 7.2)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>1.2 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>61.0</td>
</tr>
<tr>
<td>ADP</td>
<td>2.4 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>61.0</td>
</tr>
<tr>
<td></td>
<td>3.0 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>56.0</td>
</tr>
<tr>
<td><strong>Reverse reaction (pH 8.4)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>3.2 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>25.0</td>
</tr>
<tr>
<td>ATP</td>
<td>2.5 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>3.8 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>9.8</td>
</tr>
</tbody>
</table>

Mg<sup>2+</sup> constant

Mg<sup>2+</sup> : ADP = 5 : 1

Mg<sup>2+</sup> : ATP = 1 : 1
TABLE 8.

Inhibition of the Forward Reaction by ATP and AMP

The reaction mixture contained PA (5 x 10^{-3}M), ADP (1 x 10^{-3}M), Mg^{2+} (1 x 10^{-2}M), N-ethylmorpholine (0.06 M, pH 7.2) and APT (1 μg. of protein/ml.). Total volume, 1.0 ml. Temperature 50°.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration (M)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1 x 10^{-2}</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td>AMP</td>
<td>4 x 10^{-3}</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-3}</td>
<td>16</td>
</tr>
</tbody>
</table>
of the reverse reaction by ADP. Fig. 15 shows that the inhibition is competitive, the degree of inhibition depending both on the concentration of the ADP and ATP. Calculation of the $K_i$ value for ADP gave a figure of $1.5 \times 10^{-3} M$ which is the same as that obtained for the dissociation constant of the ATP-ATP complex.

**Effect of temperature on the reaction velocity.**

The effect of temperature on the reaction velocity is shown in Fig. 16. Two Arrhenius plots are shown; one for the forward reaction at pH 7.2 and the other for the reverse reaction at pH 8.4. In order to measure true initial reaction velocities, the same enzyme concentration was used at all temperatures, but the reaction time was progressively decreased with increasing temperature. The forward reaction followed the Arrhenius equation over the range 0° - 35°. From the slope of the line, the apparent activation energy was calculated to be 9,500 cal./mole. The Arrhenius equation held also for the reverse reaction over the same temperature range, although there was a sharp break at about 10°. The apparent activation energy was 21,500 below 10° and 9,500 cal./mole above 10°.

**Equilibrium studies.**

The effect of pH and Mg²⁺ concentration on the percentage phosphorylation of arginine is shown in Fig. 17. It is
Fig. 15. Competitive inhibition of the reverse reaction by ADP with ATP as the variable substrate. The results are plotted according to the method of Lineweaver & Burk (1934). The reaction mixture contained N-ethylmorpholine (0.06 M, pH 8.4), arginine \((1 \times 10^{-2} \text{M})\), \(\text{Mg}^{2+} \,(1 \times 10^{-2} \text{M})\) and APT (15 \, \mu\text{g. of protein/ml.}).

Total volume, 1.0 ml.; temperature, 5°. ○—○, no added ADP;
•—•, ADP \((1 \times 10^{-3} \text{M})\); △—△, ADP \((2 \times 10^{-3} \text{M})\). \(v = \mu\text{moles/mg./min.}\)
Fig. 16. Effect of temperature on the initial velocity of the forward and reverse reaction. The results are graphed as Arrhenius plots. Conditions for the forward reaction were as described in Fig. 10; conditions for the reverse reaction were as described in Fig. 9. T = absolute temp; K = μmoles/mg./min. ○—○, forward reaction; Δ—Δ, reverse reaction.
Fig. 17. Effect of pH and Mg$^{2+}$ concentrations on the percentage phosphorylation of arginine. The reaction mixture contained PA (5 x 10$^{-3}$ M), ADP (1 x 10$^{-3}$ M), N-ethylmorpholine (0.06 M) and APT (45 µg. of protein/ml.); incubated for 2 hr. at 5°. The pH was checked by means of a glass electrode in a duplicate set of tubes. o—o Mg$^{2+}$ (1 x 10$^{-2}$ M); Δ—Δ Mg$^{2+}$ (1 x 10$^{-3}$ M); •—• Mg$^{2+}$ (2 x 10$^{-4}$ M).
apparent that increasing pH favours the phosphorylation of arginine at the three concentrations of Mg$^{2+}$ used and that the degree of phosphorylation is dependent on the Mg$^{2+}$ concentration at any particular pH. As the Mg$^{2+}$ concentration decreases the percentage phosphorylation of arginine increases.

**Enzymatic Activity in the absence of added Mg$^{2+}$**

A comparison was made of the $K_m$ values of APT for ADP and PA in the presence and absence of Mg$^{2+}$ and for this purpose an APT preparation before dilution with glycerol was dialysed against 0.01 M phosphate buffer (pH 7.4). In the absence of Mg$^{2+}$ the time-activity curve (using a protein concentration of 5 µg/ml.) showed a rapid fall off of the reaction rate. This occurred after 4 min. when only 0.13 µmoles of arginine had been released in the presence of 5 µmoles of PA and 1 µmole of ADP; up to 4 min. the reaction was linear. Thus for the determination of the $K_m$ values the volume of the reaction mixture was doubled and the reaction time reduced to 3 min.

It was found that the $K_m$ of APT for ADP was $1.5 \times 10^{-4}$ M as compared with $3.0 \times 10^{-4}$ M in the presence of Mg$^{2+}$, whereas the $K_m$ of APT for PA was $2.2 \times 10^{-3}$ M as compared with $1.2 \times 10^{-3}$ M in the presence of Mg$^{2+}$.
The dialysed APT preparation also catalysed the reverse reaction in the absence of Mg$^{2+}$, but the amount of protein required to obtain suitable reaction rates was so high that it interfered with the P estimation. Hence it was not possible to determine the $K_m$ values of APT for ATP and arginine.

**Inhibition of the forward reaction by Ca$^{2+}$.**

When Ca$^{2+}$ was added to the forward reaction catalysed by APT in the absence of Mg$^{2+}$, there was no inhibition up to concentrations of $1 \times 10^{-2}$M. On the other hand in the presence of $1 \times 10^{-2}$M Mg$^{2+}$, $1 \times 10^{-2}$M Ca$^{2+}$ produced an inhibition of 72%. Thus the effect of Ca$^{2+}$ is to lower the increase in activity due to Mg$^{2+}$. Further investigation of the inhibition of the reaction by Ca$^{2+}$ showed that it was competitive (Fig. 1B), the degree of inhibition depending upon the relative concentrations of Mg$^{2+}$ and Ca$^{2+}$. It was calculated that the $K_i$ value for Ca$^{2+}$ was $8.3 \times 10^{-5}$M as compared with a dissociation constant of $3.6 \times 10^{-4}$M for Mg - APT. The affinity of APT for Ca$^{2+}$ is therefore much greater than its affinity for Mg$^{2+}$.

**DISCUSSION**

The nature of the activation produced by Mg$^{2+}$ in trans-
**Fig. 18.** Competitive inhibition of the forward reaction by \( \text{Ca}^{2+} \) when \( \text{Mg}^{2+} \) concentration was varied. The reaction mixture contained PA (5 x 10^{-3} \text{M}), ADP (1 x 10^{-3} \text{M}), N-ethylmorpholine (0.06 M, pH 7.2) and APT (1.5 \mu g. of protein/ml.). Total volume, 1.0 ml., temperature 5^\circ. \circ--\circ, no added \text{Ca}^{2+}; \bullet--\bullet, \text{Ca}^{2+} (2 \times 10^{-4} \text{M}); \Delta--\Delta, \text{Ca}^{2+} (5 \times 10^{-4} \text{M}).
phosphorylation reactions is of some interest; on general grounds it is possible that a Mg - ATP complex is formed which acts as the "active" substrate. Such a conclusion has been reached by Kuby, et al. (1954), Hers (1952) and Liebecq (1953) as a result of the study of reactions catalysed by creatine phosphoryltransferase, fructokinase and hexokinase respectively (see also Lardy and Parks, 1956). They found that maximum reaction velocities were attained when the Mg2+ and ATP concentrations were approximately equal. A similar result has also been obtained in the present work but seems open to other interpretation, for Mg2+ could also act by influencing the velocity of the breakdown of the enzyme-substrate complex or by reacting with the apoenzyme to form an active Mg-enzyme complex.

In assessing the validity of the conclusion drawn by the above workers it should be remembered that it is based upon a relationship obtained when enzymic activities, at varying concentrations of ATP, are plotted against the concentration of added Mg2+ and that this is tacitly assumed to be equal to the concentration of free Mg2+. Such an assumption is, however, invalid for, as Burton and Krebs (1953) and Smith and Albery (1956) have shown, both ATP and ADP form complexes with Mg2+ so that the concentration of 'free
$\text{Mg}^{2+}$', 'free ATP' and Mg - ATP will vary according to the amount of $\text{Mg}^{2+}$ and ATP added.

The relationship between the velocity of the APT reaction (in both the forward and reverse directions) and the $\text{Mg}^{2+}$ concentration is consistent with the idea that $\text{Mg}^{2+}$ reacts with the enzyme in the ratio of one $\text{Mg}^{2+}$ ion to each active centre according to the equation:

$$\text{Mg}^{2+} + \text{APT} \rightleftharpoons \text{Mg} - \text{APT}$$

Thus the Mg - APT complex can be considered as the active enzyme complex. The dissociation constant of Mg - APT as determined from the plot of $1/v$ against $1/\text{Mg}^{2+}$ (where the concentration of $\text{Mg}^{2+}$ is taken to be the same as that added) will not be a true dissociation constant for the Mg - APT complex. The value so calculated will lie between the true dissociation constant of the Mg - APT complex and that of either Mg - ATP or Mg - ADP, and will depend on the relative dissociation constants of Mg - APT and Mg - ATP or Mg - ADP.

It would be expected that, under the same experimental conditions and provided that there are no secondary reactions between $\text{Mg}^{2+}$ and the substrates, the dissociation constant of the Mg-enzyme complex would be the same irrespective of whether it was determined as a result of study of the forward or reverse reactions. This expectation was realised experim-
ntally for the results (Table 6) show that at pH 7.9 the value is lower when calculated from the forward reaction than when calculated from the reverse. Thus less Mg\(^{2+}\) is apparently required to half-saturate the enzyme in the presence of ADP than in the presence of ATP. Such a result is, however, consistent with the interpretation that Mg\(^{2+}\) reacts with the enzyme, for the dissociation constant of Mg - ADP is greater than that of Mg - ATP and thus more Mg\(^{2+}\) would be available to interact with the enzyme. It can also be seen (Table 6) that a 5-fold increase in ADP concentration results in an increase in the dissociation constant of Mg - ATP which is consistent with the idea that ADP reduces the amount of Mg\(^{2+}\) available for reaction with the enzyme.

The differences in the values for the dissociation constant may be accounted for by the fact that the abscissae values of the plots (Fig. 11 and 12) represent the concentrations of Mg\(^{2+}\) added and thus do not allow for any reduction as a result of interaction with ADP or ATP. It is however possible to calculate the 'free Mg\(^{2+}\)' concentration from the dissociation constants of Mg - ATP and Mg - ADP (Burton and Krebs, 1953) and for the purpose of calculation it has been assumed that Mg\(^{2+}\) ions react, in the system investigated, only with ATP, ADP and APT; all other possible reactions have been
neglected. On this basis the Mg$^{2+}$ ions which are not in combination with ATP or ADP are referred to as 'free Mg$^{2+}$'. If the reciprocals of the values so obtained are plotted against $1/v$ the dissociation constants of Mg - ATP as determined under various conditions may be calculated and are shown in Table 9 (underlined). It will be seen that these values are very close to each other and show much less variation than those calculated directly from Fig. 11 and 12. They are also considerably lower than these latter which are dependent upon the concentration of ATP or ADP in the system and the dissociation constants of Mg - ATP and Mg - ADP. These results indicate that the role of Mg$^{2+}$ ions in the ATP reaction lies in a combination of the metal with the enzyme and that the formation of Mg - ATP and Mg - ADP are inevitable side reactions.

A characteristic feature of the results plotted in Fig. 9 lies in the fact that maximum enzymic activities are obtained at varying concentrations of Mg$^{2+}$ depending on the concentrations of ATP. If however, the activities are replotted (Fig. 19) against the concentrations of 'free Mg$^{2+}$' determined as before, maximum enzymic activity is obtained when the latter is $2 \times 10^{-3}$ M and this value is independent of the concentration of ATP. It is also worthy of note that no relationship between enzymic activity and
# TABLE 9

## Dissociation Constants of Mg-APT from Plots of $1/v$ against $1/\text{total Mg}^{2+}$ and $1/\text{'free Mg}^{2+}$

<table>
<thead>
<tr>
<th>Determined in the presence of</th>
<th>[ADP] or [ATP] (M)</th>
<th>Calculated dissociation constant $K_d$</th>
</tr>
</thead>
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<tr>
<td></td>
<td>pH 7.2</td>
<td>pH 7.9</td>
</tr>
<tr>
<td>ADP</td>
<td>$1 \times 10^{-3}$</td>
<td>$5.4 \times 10^{-4}$*</td>
</tr>
<tr>
<td></td>
<td>$(3.6 \times 10^{-4})^\dagger$</td>
<td>$(3.1 \times 10^{-4})^\dagger$</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-3}$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>$(3.3 \times 10^{-4})$</td>
</tr>
<tr>
<td>ATP</td>
<td>$5 \times 10^{-3}$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$(2.4 \times 10^{-4})(4.7 \times 10^{-4})$</td>
<td>$(2.4 \times 10^{-4})(4.7 \times 10^{-4})$</td>
</tr>
</tbody>
</table>

* Calculated from a plot of $1/v$ against $1/\text{total Mg}^{2+}$.

$^\dagger$ Calculated from a plot of $1/v$ against $1/\text{'free Mg}^{2+}$.'
Fig. 19. Effect of 'Free Mg$^{2+}$' concentration on the initial velocity of the reverse reaction. Activity measurements are taken from those illustrated in Fig. 9; 'Free Mg$^{2+}$' concentrations were calculated using the dissociation constant of Burton & Krebs (1953) for Mg-ATP.
Mg - ATP concentration could be demonstrated (Fig. 20). In the case of the forward reaction (Fig. 10) maximum enzymic activity is again reached at Mg$^{2+}$ concentrations which are dependent upon the ADP concentration. There is, however, no simple relationship between the concentrations of ADP and Mg$^{2+}$ when maximum activity is reached. If these results are recalculated and the enzymic activity plotted against 'free Mg$^{2+}$' concentration it is found that maximum enzymic activity is reached at each level of ADP when the concentration of 'free Mg$^{2+}$' is approximately $2.5 \times 10^{-3}$ M. This value is similar to that obtained for the reverse reaction.

It will be noted that the maximum velocities in the case of the forward reaction (Fig. 10) fall within a much narrower range of Mg$^{2+}$ concentrations and that they are much closer to the true values than is the case with the reverse reaction (Fig. 9). These findings are due to the fact that the amounts of ADP added in the forward reaction were less than the amounts of ATP added for the reverse reaction and since the dissociation constant of Mg - ADP is higher than that of Mg - ATP it follows that the values for concentrations of 'free Mg$^{2+}$' in the forward reaction must be closer to those for total Mg$^{2+}$ than is the case in the reverse reaction.
Fig. 20. Effect of Mg-ATP concentration on the initial velocity of the reverse reaction. Activity measurements are taken from those illustrated in Fig. 9. Mg-ATP concentrations were calculated using the dissociation constant of Burton & Krebs (1953) for Mg-ATP.
The decrease in reaction velocity of the forward reaction (Fig. 10) in the presence of high concentrations of Mg$^{2+}$ is doubtless due to a reduction of 'free ADP' concentrations as a result of interaction with Mg$^{2+}$. In this case the ratio of Mg$^{2+}$ : ADP was much greater than was the ratio of Mg$^{2+}$ : ATP in the reverse reaction where no fall off of reaction velocity was noted (Fig. 9).

When the $K_m$ of ATP for ADP was determined in the presence of a constant amount of Mg$^{2+}$ a Michaelis-Menten relation was obtained only over a limited range of ADP concentrations for the velocity was much less at lower levels of ADP than that required. This was due to a reduction of the ADP concentrations by Mg$^{2+}$ for a Michaelis-Menten relation was obtained over the entire range of ADP concentrations when $1/v$ was plotted against $1/{\text{'free ADP'}}$ rather than $1/{\text{total ADP}}$. The $K_m$ value obtained from this plot (Table 10) must be regarded as the true value and it will be noted that it is appreciably lower than that calculated on the basis of total ADP. When the Mg$^{2+}$ concentration was varied simultaneously with the ADP to maintain a Mg : ADP ratio of 5 : 1, a Michaelis-Menten relation was obtained over the range of ADP concentrations studied. The $K_m$ value (Table 10) was close to that obtained when the Mg$^{2+}$ concentration was kept constant, both values being
<table>
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<th>Substrate</th>
<th>Concentration of Mg$^{2+}$ (M)</th>
<th>$K_m$ Value of APT (M)</th>
<th>$K_m$ Value of APT (M)</th>
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</thead>
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<tr>
<td>ADP</td>
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<td>$2.4 \times 10^{-4}$</td>
<td>$4.3 \times 10^{-5}$</td>
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<td>$3.8 \times 10^{-3}$</td>
<td>$6.3 \times 10^{-3}$</td>
<td></td>
</tr>
</tbody>
</table>

* (a) Calculated from a plot of $1/v$ against $1/\text{total substrate}$.  

\$ (b) Calculated from a plot of $1/v$ against $1/\text{free substrate}$.  

TABLE 10.  

$K_m$ Values of APT for ADP and ATP
calculated from a plot of $1/v$ against $1/\text{total ADP}$. However, the $K_m$ value calculated from a plot of $1/v$ against $1/\text{free ADP}^1$ is considerably higher than what is considered as the true value and higher than that obtained on the basis of total ADP. This high value is probably due to the fact that as the $Mg^{2+}$ concentration was reduced concomitantly with the ADP concentration (maintaining a $Mg : ADP$ ratio of $5 : 1$) it limited the concentration of the $Mg - APT$ complex. Thus the fall off in rate was due to a reduction of both the ADP and $Mg - APT$ concentrations.

When the ATP concentration was varied in the presence of a fixed concentration of $Mg^{2+}$ and when both the ATP and $Mg^{2+}$ concentrations were varied so as to maintain a $Mg : ATP$ ratio of $1 : 1$, a Michaelis-Menten relation between the reaction velocity and the ATP concentration was found (Fig. 14). It can be seen (Table 10) that although the $K_m$ values calculated from a plot of $1/v$ against $1/\text{total ATP}$ differ slightly, they are considerably different from those calculated from a plot of $1/v$ against $1/\text{free ATP}$. The value obtained from the latter plot in the presence of a fixed amount of $Mg^{2+}$ must be considered as the true $K_m$ value of APT for ATP. The high value calculated from a similar plot of the results obtained when the $Mg^{2+}$ concentration was varied is probably due to the concent-
ration of $Mg^{2+}$ limiting the concentration of $Mg$ - APT as was the case with the forward reaction.

The influence of $Mg^{2+}$ on the reaction can also be seen from the equilibrium studies. As the concentration of $Mg^{2+}$ is increased, the equilibrium shifts in favour of arginine and ATP. This can be explained as being due to the greater affinity of $Mg^{2+}$ ions for ATP than for ADP.

There is additional supporting evidence to suggest that the function of $Mg^{2+}$ is to form a complex with the apoenzyme. In the experiments carried out with the dialysed APT preparation to which no $Mg^{2+}$ was added the enzyme showed activity in both the forward and reverse reactions. It was found that the $K_m$ of APT for ADP was lower than when $Mg^{2+}$ was present although higher than that calculated on the basis of the 'free ADP' present when $Mg^{2+}$ was added; on the other hand, the $K_m$ value of APT for PA was increased. Although no explanation can be offered for the higher value, the results do indicate that the binding of $Mg^{2+}$ by PA must be sufficiently weak so as to have no effect on the reaction. The activity of the enzyme in the absence of added $Mg^{2+}$ is probably due to $Mg^{2+}$ bound to the enzyme. Spectrographic analysis (Chapter 1) has shown that $Mg^{2+}$ is present in a dialysed APT preparation.

Further evidence for the idea that $Mg^{2+}$ react with APT
to form an active Mg - APT complex comes from the results of
the inhibition of APT by Ca\(^{2+}\). Ca\(^{2+}\) have no effect on the
forward reaction catalysed by APT in the absence of Mg\(^{2+}\),
whereas they competitively inhibit the enzyme in the presence
of Mg\(^{2+}\). Thus the interaction of Ca\(^{2+}\) and ADP to form Ca -
ADP does not appreciably reduce the concentration of free ADP.
This can be explained by the fact that the K\(_m\) value of APT for
ADP (4.3 x 10\(^{-5}\)M) is much lower than the dissociation constant
of Ca - ADP [1.6 x 10\(^{-4}\)M; (Distefano and Neuman, 1953); 1.55 x
10\(^{-3}\); (Smith and Alberthy, 1956)]. The inhibition of the
reaction when Mg\(^{2+}\) are present must therefore be due to the
competition by Ca\(^{2+}\) and Mg\(^{2+}\) for the same site on the enzyme
surface. It is noteworthy that the affinity of APT for Ca\(^{2+}\)
is greater than for Mg\(^{2+}\), although there is no activation by
Ca\(^{2+}\). These results also imply that in APT as isolated, the
linkage of Mg\(^{2+}\) to the enzyme is different from and stronger
than that resulting from the addition of Mg\(^{2+}\) to the enzyme
system.

The competitive inhibition of the reverse reaction by
ADP and the inhibition of the forward reaction by ATP suggest
that ATP and ADP are bound to APT at the same site on the
enzyme surface. It is possible that this linkage is through
Mg\(^{2+}\), as this metal complexes predominantly in O-type linkages
(Williams, 1953). The fact that ATP cannot be replaced by inosine triphosphate in the reaction (Chapter 1) indicates that the adenine moiety of ATP is also involved in the linkage of ATP or ADP to the enzyme.

It is believed that the present results indicate that Mg$^{2+}$ react with the apoenzyme to form an active Mg - ATP complex, and that reaction of free ADP or ATP with the active enzyme involves linkage with Mg$^{2+}$. It is possible that PA and arginine are attached at another site on the enzyme surface in sufficiently close proximity to the ADP - ATP site so that P can be directly transferred without liberation as inorganic P. This hypothesis, inasmuch as it concerns the reaction investigated, may be schematically represented as in Fig. 21, and will be further elaborated in Chapter 3.

Although this scheme is based on the results of experiments in which Mg$^{2+}$ was used to activate the enzyme, it should be borne in mind that ATP is also activated by Mn$^{2+}$. It is therefore probable that the mechanism of the activation of ATP by Mn$^{2+}$ is similar to that postulated for Mg$^{2+}$.

It should be pointed out that recalculation of the results of Kuby et al. (1954), Hers (1952) and Liebecq (1953) might well indicate that a similar central reaction, i.e. formation of a metal-enzyme complex, is concerned in the
Fig. 21. Schematic representation of the mechanism of the transphosphorylation reaction catalysed by APT. All reactions are reversible. The abbreviations AP and A represent N-phosphoryl arginine and arginine respectively.
reactions catalysed by CPT, fructokinase and hexokinase, and that this type of reaction may be one generally applicable to phosphoryl group-transfer reactions.

SUMMARY

1. The kinetics of the reaction, PA + ADP $\rightleftharpoons$ arginine + ATP, catalysed by APT, have been studied.

2. It has been shown that when enzymic activity is plotted against the total Mg$^{2+}$ concentration, maximum activity is reached at a concentration of Mg$^{2+}$ which is dependent on the concentration of ADP or ATP. When enzymic activity is plotted against the concentration of 'free Mg$^{2+}$', maximum activity is reached at a Mg$^{2+}$ concentration which is independent of the concentration of ADP or ATP.

3. A Michaelis-Menten relation between enzymic activity and the concentration of Mg$^{2+}$ and Mn$^{2+}$ was demonstrated. The dissociation constants of the Mg - APT complex, determined under various conditions, were calculated from plots of $1/v$ against $1/\text{total Mg}^{2+}$ and $1/v$ against $1/'\text{free Mg}^{2+}'$.

4. The $K_m$ values of the four substrates of the reaction were determined. Those for ADP and ATP were calculated from plots of $1/v$ against $1/\text{total substrate}$ and $1/v$ against $1/'\text{free substrate}'$.
5. The forward reaction was inhibited by ATP and AMP; the reverse reaction was inhibited competitively by ADP. It was concluded that ADP and ATP are bound to the enzyme at the same site.

6. There was competitive inhibition of the forward reaction by Ca$^{2+}$.

7. The effect of temperature on the velocity of the forward and reverse reactions was studied and the apparent activation energies were calculated.

8. The equilibrium of the reaction was influenced by pH and the concentration of Mg$^{2+}$.

9. The conclusion was reached that Mg$^{2+}$ reacts with APT to form an active Mg – APT complex which in turn reacts with the free forms of ATP and ADP.
CHAPTER 3.

THE ACTIVATION AND INHIBITION OF ARGinine

PHOSPHORYLTRANSFERASE BY DIVALENT METALS
CHAPTER 3.

THE ACTIVATION AND INHIBITION OF ARGININE PHOSPHORYLTRANSFERASE BY DIVALENT METALS

INTRODUCTION

Enzymes which catalyse phosphoryl group transfer reactions involving ATP have, with few exceptions, an absolute requirement for divalent cations (Lardy, 1951). In most instances, this requirement is fulfilled by Mg$^{2+}$ or Mn$^{2+}$ and, more rarely, by Ca$^{2+}$ and Co$^{2+}$. However, there is as yet no direct experimental evidence to indicate the role which metal ions play in the activation of phosphoryl group transfer or of the mechanism by which this transfer takes place, but recently various suggestions have been made.

Koshland (1954) has considered that the general mechanisms of group transfer, elucidated from studies of organic chemical reactions, can be applied to enzymic phosphoryl group transfer reactions. He has regarded enzymic phosphoryl group transfer in terms of substitution reactions, as expressed by the equation

\[ B - X + Y \rightarrow B - Y + X, \]

and suggested that transfer may take place by one or other of the following general mechanisms:
(i) by single displacement, where the role of the enzyme is to achieve juxtaposition of the substrates and also to polarise the electrons of the substrate molecules so as to increase their tendency to react. The acceptor substrate Y then makes a direct nucleophilic attack on the donor B-X with the result that the Y-B bond begins to form while the B-X bond is breaking. 

(ii) by double displacement, which involves the formation of an intermediate enzyme-B complex. A nucleophilic group on the enzyme first attacks and cleaves the B-X bond thus creating an enzyme-B complex which is then attacked in a second step by Y to give the final product Y-B. The attack by Y may take place from solution or from a specific site on the enzyme molecule to which Y is adsorbed.

Koshland favours the former mechanism for phosphoryl group transfer reactions as it is directly analogous to the displacements observed in the chemical reactions of phosphate. He also regards the CPT reaction as supporting evidence for this mechanism as the formation of a N-P bond is unequivocal evidence for a nucleophilic displacement on the terminal P atom of ATP. Koshland has proposed a general mechanism for phosphoryl group transfer which, when applied to the reactions catalysed by the guanidine phosphoryltransferases, can be illustrated as shown below -
According to this mechanism, the activated complex would have the structure:

\[
\begin{align*}
\text{RN} & \quad \text{P} \\
\text{H} & \quad \text{Ad-PO}_3\text{PO}_3\text{H-PO}_3^- \\
\end{align*}
\]

(where the dotted lines indicate the bonds being formed and broken). Here the lone pair of electrons of the nitrogen atom makes a nucleophilic attack on the terminal P atom of ATP.

The above mechanism for enzymic phosphoryl group transfer does not ascribe a role to activating cations such as \(\text{Mg}^{2+}\) and \(\text{Mn}^{2+}\), but many theories to account for this activation have been proposed. These may conveniently be divided into three groups which propose: (i) that the primary effect of the metal is on the enzyme protein. The binding of metal ions may change the net charge on the enzyme so as to favour the binding of substrate or coenzyme, or alternatively, may stabilise the active configuration of the enzyme molecule. (ii) that the metal may act as a bridge between the enzyme and the substrate (Hellerman, 1937; Smith, 1949, Lehninger, 1950), thereby forming a ternary chelate complex with the enzyme and the substrate (Calvin, 1954). The metal ions
may serve, not only to increase the binding of the substrate to the enzyme, but also to bring about its activation. A similar conclusion has been reached by Szent-Györgyi (1957) who has proposed that the activation of myosin ATP-ase by Mg$^{2+}$ is due to the fact that Mg forms with the enzyme and ATP an enzyme-Mg-ATP chelate, which facilitates electron shifts from the terminal phosphoryl group and causes labilisation of the P-O-P bond.

On the other hand Klotz (1954) and Klotz & Loh Ming (1954) have pointed out that Mg$^{2+}$ and Mn$^{2+}$ do not enter into chelate complexes as readily as do ions such as Ni$^{2+}$, Cu$^{2+}$ and Pb$^{2+}$, which do not activate phosphoryl transferases. They argue that if chelating ability were important, the metals which form stronger complexes with ATP than Mg$^{2+}$ or Mn$^{2+}$ should also function as activators. They have postulated that as a result of forming co-ordination bonds with enzyme and substrate the metal ion stabilises the activated state of the enzyme-substrate complex. (iii) Other authors (Najjar, 1951; Boyer & Harrison, 1954) have considered that metal activations involve the formation of reactive metallosubstrates (e.g. Mg-ATP complexes). The combination of ATP with Mg$^{2+}$ would favour an increase in the residual charge on the terminal P atom of ATP, thus leading to increased ease of attack by nucleophilic reactants. It has been claimed (Kuby et al., 1954; Hers, 1952; Lardy & Parks, 1956) that kinetic studies
indicate that Mg-ATP is the active substrate in the phosphoryltransferase reactions studied, but it has been shown that the premises on which these claims are based are open to other interpretations (Chapter 2).

It has been shown (Chapter 1) that APT is similar to other phosphoryltransferase enzymes in that it is activated by Mg$_{2+}$ and Mn$_{2+}$. Kinetic studies (Chapter 2) have indicated that the activation by Mg$_{2+}$ is due to the formation of an active Mg-APT complex which reacts with the free forms of the nucleotides and a general scheme was proposed for the reversible phosphoryl group transfer catalysed by APT (Fig. 21). This hypothesis will be elaborated further in this chapter and a possible mechanism for the phosphoryl group transfer catalysed by APT will be presented. Some results of the inhibition of APT by the metals of the first transition series and the alkaline earth metals, other than Mg$_{2+}$, will also be presented.

RESULTS AND DISCUSSION

(a) The mechanism of the APT catalysed reaction

The following hypothesis on the mechanism of the phosphoryl group transfer catalysed by APT is based on experimental evidence derived from a study of this enzyme (Chapters 1 and 2), and takes into consideration the mechanisms
of phosphoryl group transfer proposed by Koshland, (1954) and Klotz (1954).

The juxtaposition of the phosphoryl group donor and acceptor on the enzyme surface, together with the role of the metal activator, is represented diagrammatically in Fig. 22. It is envisaged that the nucleotide is attached to the enzyme surface through the $-\text{NH}_2$ group (ITP cannot serve as a phosphoryl donor) and, as Mg has a coordination number of either 4 or 6, through the Mg attached to the enzyme. At pH 8.4 all the $-\text{OH}$ groups attached to the P atoms are completely ionised so that there exist 3 positions at which magnesium could be chelated to form either a 4 or 6 membered ring. Reaction of magnesium with the ATP terminal phosphoryl group which is transferred is unlikely for this would stabilise the ground state of the ES complex and increase the activation energy. Furthermore, as ADP competes with ATP for the same site on the enzyme surface it would seem that the same groups are involved in the attachment of these two nucleotides, i.e., the $\alpha$ and $\beta$ phosphoryl groups. If this is the case it would be anticipated that ADP could not serve as a phosphoryl group donor and that AMP could not function as an acceptor. Such predictions are in accord with the experimental findings.

At pH 8.4 arginine would have the structure indicated (Fig. 22) and since a free guanidine $-\text{NH}_2$ and an $\alpha$-amino group
Fig. 22. Hypothetical picture of the transfer of the phosphoryl group from ATP to arginine in the APT catalysed reaction.
are essential it would seem that both these groups are involved in the attachment of the guanidine to the enzyme surface.

On the basis of the modes of attachment of the phosphoryl donor and acceptor to the enzyme surface as envisaged (Fig. 22), it becomes possible to suggest a sequence of events which culminate in phosphoryl group transfer. Normally O is more electro-negative than P and by virtue of the attachment through magnesium the excess positive charge on the terminal P would be increased with a resultant weakening of the -O-P bond. This phosphoryl group could now make an electrophilic attack on the lone pair of electrons of the arginine -NH₂ which is considered to be in sufficiently close proximity for such an attack to occur. The transition state can then be pictured as one in which there is bonding between the two substrates on the enzyme surface. When the reactants are PA and ADP a similar situation is, of course, reached as a result of the phosphoryl group of PA making an electrophilic attack on the negatively charged O of the terminal phosphoryl group of ADP. Rupture of the bonds will then occur and at a point which will be dependent on the concentration of the substrates present.

The role of Mg²⁺ in this mechanism is, therefore, to bind enzyme and nucleotide substrate in a ternary complex which facilitates electron shifts from the terminal phosphoryl group of
ATP and causes labilisation of the P-O-P bond (cf. Szent-Györgyi, 1957). This scheme gains support from the work of Lowenstein (1957) who showed that Mn\(^{2+}\) catalysed the non-enzymic transfer of the phosphoryl group from ATP to inorganic P to form pyrophosphate according to the equation -

\[
\text{ATP} + 3\overline{P} \xrightarrow{\text{Mn}^{2+}} \text{ADP} + 3\overline{PP}
\]

The catalysis of this reaction can be explained as being due to an interaction of Mn\(^{2+}\) and ATP which results in labilisation of the terminal phosphoryl group.

It is also possible that an imidazole group on the enzyme surface may function as an intermediary for the transfer of a phosphoryl group. Rathlev & Rosenberg (1956) have shown that aminophosphate can react non-enzymically with imidazole to form both mono- and di-phosphoryl imidazole, which in turn are capable of directly phosphorylating creatine. There is however, no information as to whether or not imidazole groups are concerned with guanidine phosphoryltransferase activity.
(b) The inhibition of APT by divalent cations

The reason for the marked activation of phosphoryltransferases by \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) is not immediately apparent. These metals have different chemical properties, one being a member of the alkaline earths and the other of the first transition series, and in an attempt to correlate the properties of members of these two groups with their influence on enzyme activity some experiments have been carried out with APT.

**Results**

The effect of divalent cations on the \( \text{Mg}^{2+} \)-activated APT reaction is summarised in Table 11 which shows that inhibition is caused by \( \text{Be}^{2+}, \text{Sr}^{2+}, \text{Ca}^{2+}, \text{Ba}^{2+}, \text{Ni}^{2+}, \text{Co}^{2+}, \text{Zn}^{2+} \) and \( \text{Cd}^{2+} \). Further investigation of the inhibition of the reaction by \( \text{Sr}^{2+} \) and \( \text{Ba}^{2+} \) showed that it was competitive (Fig. 23), the degree of inhibition depending on the relative concentrations of \( \text{Mg}^{2+} \) and \( \text{Sr}^{2+} \) or \( \text{Ba}^{2+} \). Thus, the effect of these metals is similar to that caused by \( \text{Ca}^{2+} \) (Fig. 18). The \( K_I \) values of these cations (calculated from the equation

\[
K_I = \frac{[I]}{K_m} \cdot \frac{K_m}{K_{app} - K_m}
\]

of \( \text{Mg}^{2+} \), and \( K_{app} \), the apparent Michaelis constant in the presence of \( \text{Ca}^{2+}, \text{Sr}^{2+} \) and \( \text{Ba}^{2+} \), were: \( \text{Ca}^{2+}, 8.3 \times 10^{-5} \text{M} \) (Chapter 2);
The Effect of Divalent Metals on the Activity of Mg-Activated APT.

(The reaction mixture contained 0.1 ml. of 0.5 M N-ethylmorpholine (pH 7.2), 0.1 ml. of 0.05 M PA, 0.1 ml. of 0.1 M MgSO₄, 0.1 ml. of 0.01 M ADP, 0.2 ml. APT (6 µg. protein/ml.) and 0.4 ml. of water. All reactions were for 5 min. at 5°C).

<table>
<thead>
<tr>
<th>Metal</th>
<th>Metal Conc.</th>
<th>Ionic Radius</th>
<th>Inhibition %</th>
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<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
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<td>84</td>
</tr>
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</tr>
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<td>Co</td>
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<td>0.72</td>
<td>29</td>
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</table>

* Inhibition occurs at lower Mg²⁺ concentration. Thus 10⁻³ M Ba produces inhibition of 7 and 12% in the presence of 2 x 10⁻³ M and 6.7 x 10⁻⁴ M Mg²⁺ respectively.
Fig. 23. Competitive Inhibition of the forward reaction by Sr$^{2+}$ and Ba$^{2+}$ when Mg$^{2+}$ concentration was varied. The reaction mixture contained PA (5 x 10$^{-3}$M), ADP (1 x 10$^{-3}$M), N-ethylmorpholine (0.06 M, pH 7.2) and ATP (6 µg. of protein/ml.). Total volume, 1.0 ml. Temperature 5°. ———, no added cations; o--o, Ba$^{2+}$ (1 x 10$^{-3}$M); ▲▲, Sr$^{2+}$ (5 x 10$^{-4}$M).

$\nu$ = initial velocity (arbitrary units).
Sr\(^{2+}\), 2.5 \times 10^{-4} \text{M}; \quad \text{Ba}^{2+}, 3.6 \times 10^{-3} \text{M}.

The inhibition caused by Be\(^{2+}\), Ni\(^{2+}\) and Co\(^{2+}\) was shown to be non-competitive.

**Discussion**

It is difficult to explain why Mg\(^{2+}\) and Mn\(^{2+}\) alone of the alkaline earths and transition series metals, respectively, should activate APT, while the other metals of these groups inhibit. However, some insight into the problem may be gained from a consideration of the types of co-ordination complexes formed by these metals. Williams (1953) has shown that Mg\(^{2+}\) and Mn\(^{2+}\) form co-ordination complexes predominantly with O-containing ligands and as kinetic studies (Chapter 2) have indicated that Mg\(^{2+}\) and Mn\(^{2+}\) react by combining with APT, it is possible that co-ordination complexes with O-containing ligands are formed at the active site. The competitive inhibition of Mg-activated APT by Ca\(^{2+}\), Sr\(^{2+}\) and Ba\(^{2+}\) (Figs. 18, 23), therefore, is probably due to the fact that these metals form similar co-ordination complexes with O-containing ligands on the enzyme and thus compete for the active site. This inhibition decreases in the order Ca\(^{2+}\), Sr\(^{2+}\), Ba\(^{2+}\) which is also the order of increasing ionic radii (Table 11) and this suggests that the magnitude of the ionic radius is important. Thus while the alkaline earth metals whose ions have radii greater than Mg\(^{2+}\) inhibit by
displacement of the $Mg^{2+}$, they are apparently unable to induce the spatial arrangement required for the transfer of the phosphoryl group. If this theory is tenable, $Mn^{2+}$ presumably activates by virtue of the fact that its ionic size, whilst greater than that of $Mg^{2+}$ is less than that of $Ca^{2+}$. The non-competitive inhibition by $Be^{2+}$, the other member of the alkaline earths, is probably due to the fact that it has a high affinity for O-containing ligands with which it forms stable covalent complexes, and thus has a direct effect on the enzyme either by changing the total charge or by altering the active configuration.

The activating effect of $Mn^{2+}$ is probably due to the fact that it alone of the transition series metals forms complexes predominantly with O-containing ligands. The other metals (e.g. $Ni^{2+}$ and $Co^{2+}$) complex predominantly with N-containing ligands and their inhibitory effect is probably due to a direct effect on the enzyme protein. A similar explanation probably holds for the inhibition caused by $Zn^{2+}$ and $Cd^{2+}$ which may also react with -SH groups which are essential for activity (Chapter 1).

SUMMARY

1. A possible mechanism for the phosphoryl group transfer catalysed by APT has been presented.

2. The inhibition of the Mg-activated APT reaction by divalent metal cations of the alkaline earths and first transition series has been studied.
3. There was competitive inhibition of the Mg-activated AFT reaction by Sr$^{2+}$ and Ba$^{2+}$ and non-competitive inhibition by Be$^{2+}$, Ni$^{2+}$ and Co$^{2+}$. 
CHAPTER 4.

STUDIES OF PHOSPHORYLATED GUANIDINES
CHAPTER 4

STUDIES OF PHOSPHORYLATED GUANIDINES

A. THE SYNTHESIS OF N-PHOSPHORYL TAUCROCYAMINE

PT was first isolated from the annelid, Arenicola marina by Thoai et al. (1953a, b). These authors also reported the synthesis and isolation of PT as an impure calcium salt following the phosphorylation of taurocyamine by POCl₃ in an alkaline medium, but no method has been described for the synthesis of the pure compound.

In connexion with studies of the enzyme taurocyamine phosphoryltransferase (TPT), which catalyses the reaction

\[ \text{PT} + \text{ADP} \rightleftharpoons \text{Taurocyamine} + \text{ATP} \]

a method was developed for the synthesis and isolation of the pure barium salt of PT.

After completion of this work, the synthesis and isolation of PT as a crystalline ammonium salt was reported by Thoai & Thiem (1957). This latter method involves the handling of large volumes and is thus less convenient than the method to be described below.
EXPERIMENTAL

Materials

Taurocyamine

This compound was prepared as described in Appendix 2. The purity of the material was checked by N analysis and paper chromatography.

Taurocyamine Phosphoryltransferase

TPT was purified from aqueous extracts of *Arenicola assimilis* as described in Chapter 5 and stored in 50% (v/v) glycerol at -10°; the solution contained 1.86 mg. of protein/ml.

Myosin

This was prepared by Dr. H. Rosenberg of this Department from rabbit skeletal muscle as described by Bailey (1942). It was stored in 50% (v/v) glycerol at -10° and contained 2.5 mg. of protein/ml.

Alkaline phosphatase

The sample of purified intestinal alkaline phosphatase used was a gift from Professor R.K. Morton, University of Adelaide.

Methods

Estimation of Taurocyamine

Taurocyamine was estimated by the method of Rosenberg *et al.* (1956) for the estimation of arginine. Total taurocyamine
was estimated by the same method after hydrolysis of the N-P bond in N HCl at 100° for 10 min. and the difference in taurocyamine concentration before and after hydrolysis was taken as 'bound' taurocyamine.

**Estimation of inorganic P**

Inorganic P was estimated by the method of Ennor & Stocken (1950).

All hydrolyses and enzyme experiments were carried out with the Na salts of PT and Fraction II and these were prepared by passage of solutions of the Ba salts through columns of Zeo-Karb 225 (Na form).

**RESULTS**

**Initial experiments**

Taurocyamine was reacted with POCl₃ in an alkaline medium and the Ba salt of the product isolated from the mixture. This salt was assumed to be the mono-N-phosphoryl derivative, i.e. C₃H₈O₆N₃PSEα but analysis of the material dried over P₂O₅ in vacuo at 100° showed Ba = 40.8% (Theory = 35.8%) and a P : taurocyamine ratio of 1.10. Two samples prepared subsequently gave a P : taurocyamine ratio of 1.33 and 1.44, indicating contamination by a P containing compound. Some evidence as to the nature of this compound was obtained by determination of P and taurocyamine released during hydrolysis in N HCl at 37°. The
results indicated a progressive decrease with time of the P : taurocyamine ratio from a maximum value of 2.2 (Table 12) and this was interpreted as indicative of the presence of mono- and di-N-phosphoryl taurocyamine. As a consequence some of the material was placed on an anion-exchange resin (IRB -400, Cl form) from which two fractions were subsequently eluted. One fraction was eluted with 0.1M NaCl and had a P : taurocyamine ratio of 1 : 1; the other, which was eluted with 2M NaCl had a ratio of 2 : 1 (Fraction II). The details of the synthesis and separation of the two phosphorylated derivatives are given below.

Preparation of crude PT

Taurocyamine (20g., 0.12 moles) was added to a three-necked flask (2 litre) containing 20 ml. of water and 50 ml. of 10 N NaOH. The mixture was rapidly stirred and 38 ml. (0.43 moles) of POCl₃ and 170 ml. of 10 N NaOH added over a period of 1 hr. and at such a rate that the mixture remained strongly alkaline. The contents of the flask were maintained between -5⁰ and 0⁰ by periodic immersion in a solid CO₂ - ethanol mixture. When the additions were completed the reaction mixture was stirred for a further 30 min. at -5⁰. The precipitate of NaCl and sodium phosphate was removed by filtration on a Buchner funnel (Whatman No. 531 paper) and the solid residue extracted with 200 ml. of ice-cold water in a Waring blendor. The mixture was filtered and
**TABLE 12.**

**Hydrolysis of Crude PT.**

Crude PT (1.1 x 10^{-3} M on basis of taurocyamine) was hydrolysed in N HCl at 37°.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>µMoles/ml. P</th>
<th>µMoles/ml. Taurocyamine</th>
<th>Molar Ratio P/Taurocyamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0.047</td>
<td>0.0215</td>
<td>2.2</td>
</tr>
<tr>
<td>5</td>
<td>0.075</td>
<td>0.038</td>
<td>1.98</td>
</tr>
<tr>
<td>10</td>
<td>0.127</td>
<td>0.07</td>
<td>1.81</td>
</tr>
<tr>
<td>40</td>
<td>0.406</td>
<td>0.250</td>
<td>1.63</td>
</tr>
<tr>
<td>50</td>
<td>0.458</td>
<td>0.302</td>
<td>1.52</td>
</tr>
<tr>
<td>120</td>
<td>0.845</td>
<td>0.588</td>
<td>1.44</td>
</tr>
<tr>
<td>160</td>
<td>1.04</td>
<td>0.755</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>1.47*</td>
<td>1.09</td>
<td></td>
</tr>
</tbody>
</table>

* These results were obtained from a sample hydrolysed in N HCl at 100° for 15 min.
the residue re-extracted twice with 100 ml. of cold water. The residue was then discarded, the filtrates combined and allowed to stand at 5° for 24 hr. The crystalline precipitate which formed was removed by filtration and discarded. The filtrate was then adjusted to pH 7.6 - 8.0 by the addition of 5N HCl. On allowing to stand for a further 1 - 2 hr. in the cold a second crystalline precipitate was obtained which was filtered off and discarded. Ethanol (4 vol.) was then added to the filtrate and the oil which separated was allowed to settle for 24 hr. at 5°. The supernatant liquid was discarded after removal by decantation and the semi-solid oil dissolved in 500 ml. of water. This solution was clarified by filtration and the Na salts again precipitated as an oil by the addition of ethanol (4 vol.). This mixture was allowed to stand for 24 hr. at 5°, the supernatant discarded and the oil dissolved in 500 ml. of water and filtered. These two precipitations removed the free taurocyamine and a large proportion of the inorganic P (Table 13).

**Isolation of PT (Mono-N-phosphoryl taurocyamine)**

The aqueous solution of the twice precipitated oil was passed through a column of Deacidite FF (Cl form, 66 x 3 cm.) at approximately 5 ml./min. and the effluent tested at intervals for the presence of bound taurocyamine. In all preparations it was found that some bound guanidine (Fraction 0) passed through the
TABLE 13.

Analytical Data on Fractions Obtained during the Isolation of the Ba salts of Mono-N-phosphoryl taurocyamine and Fraction II.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml.)</th>
<th>Taurocyamine (g.)</th>
<th>Ba salt (g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Free</td>
<td>Total</td>
</tr>
<tr>
<td>Initial filtrate</td>
<td>1220</td>
<td>6.2</td>
<td>15.3</td>
</tr>
<tr>
<td>Solution of oil (1st ppt.)</td>
<td>500</td>
<td>0.3</td>
<td>6.3</td>
</tr>
<tr>
<td>2nd ppt.)</td>
<td>500</td>
<td>0.03</td>
<td>7.6</td>
</tr>
<tr>
<td>Fraction 0</td>
<td>960</td>
<td>0.03</td>
<td>0.7</td>
</tr>
<tr>
<td>Mono-N-phosphoryl taurocyamine</td>
<td>2980</td>
<td>0.01</td>
<td>4.7</td>
</tr>
<tr>
<td>Fraction II</td>
<td>540</td>
<td>0.003</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* These figures represent the final yields after two further precipitations in the absence of added Ba acetate. This procedure results in appreciable losses, but is necessary if serious contamination of the product with Ba acetate is to be avoided.
column to the extent of 1-10% of the total bound guanidine present in the original solution.

Chromatographic examination of this material after hydrolysis and development on paper in methanol - acetic acid - water, (80 : 10 : 20); n-butanol - 6N HCl, (70 : 30) and phenol - water (80 : 20 (w/v)) saturated with SO₂, indicated the presence of two guanidines. One of these was identical in behaviour with taurocyamine but the other, which had a higher Rₜ in all solvents, was not identified. This material was discarded.

The column was then washed with water until the effluent was free of bound taurocyamine. Elution was then commenced with 0.2M NaCl (5 ml./min.) and the eluate collected in 200 ml. fractions. Qualitative tests for bound taurocyamine were carried out on each fraction and fractions 1-15 were combined. Elution with additional 0.2M NaCl removed only trace amounts of bound taurocyamine. Ba acetate (75 ml. of M solution) was then added to the combined eluates and the pH adjusted to pH 8.2 by the addition of N NaOH. The precipitate was removed by filtration and the filtrate adjusted to pH 4-5 by the addition of 5N HCl. The solution was then placed in a flask which was evacuated on a water pump to remove CO₂ and the solution brought to pH 6-6.5 by the addition of a freshly prepared solution of NaOH. Ethanol, (3 vol.) was added and the amorphous precipitate collected by centrifugation, washed with ethanol and ether and dried in vacuo over CaCl₂. The dry material
was dissolved in 250 ml. of water, adjusted to pH 6 and precipitated by the addition of ethanol (2 vol.) in the absence of added Ba acetate. The precipitate was collected as before, dried and again similarly reprecipitated. Yield, 7.0g. (Table 13).

The material on analysis gave C, 8.8%; S, 7.1%; Taurocyamine, 39.0%; H, 2.6%; P, 7.3%; Ash (BaP₂O₇), 52.5%; N, 9.6%; Ba, 31.6%; H₂O, 6.7%. Calculated for C₃H₈O₆N₃PSBa₂H₂O : C, 8.6%; S, 7.6%; Taurocyamine, 40.0%; H, 2.9%; P, 7.4%; Ash (BaP₂O₇), 53.8%; N, 10.0%; Ba, 32.7%; H₂O, 8.6%.

The compound is believed to possess the structure:

```
                  H
                  /
                  /
N—P—Ba
```

Attempts to isolate the compound as the crystalline Na, K and Li salts were unsuccessful because of their markedly hygroscopic nature, but the crystalline ammonium salt was prepared.

Preparation of crystalline ammonium salt

The Ba salt (400mg.) was dissolved in 20 ml. of water and passed through a column of Zeo-Karb 225 (NH₄ form). The column was washed with water and the washings (30 ml.) combined
with the original effluent. Ethanol (6 vol.) and acetone (6 vol.) were then added and the finely dispersed oil which separated, crystallised in fine needles on standing at 5° overnight. Yield 172 mg.

The material on analysis gave: taurocyamine, 58.1%; P, 10.9%; calculated for C₅H₃O₆N₃SP (NH₄)₂, taurocyamine, 59.2%; P, 11.0%.

Isolation of Fraction II

Following elution of mono-N-phosphoryl taurocyamine the column was treated with 2M NaCl to elute Fraction II which was collected in 540 ml. of eluate. Ba acetate (50 ml. of M solution) was added and the solution became slightly opalescent, but cleared when adjusted to pH 4 - 5 by the addition of 5 N HCl. Dissolved CO₂ was removed as before and the pH brought to 7. (At higher pH a precipitate appears and this increases with increasing alkalinity). Ethanol (2 vol.) was then added and the pH re-adjusted to 7 by the addition of 5N NaOH. The precipitated Ba salt was recovered by centrifugation, washed with ethanol and ether and dried in vacuo over CaCl₂. The dry material was dissolved in water and precipitated in the absence of additional Ba acetate with 2 volumes of ethanol. The precipitate after drying was redissolved, similarly reprecipitated and again dried. Yield 55g. (Table 13).
Fraction II gave on preliminary analysis a Ba : P : taurocyamine ratio of 2 : 2 : 1 and it was anticipated that the compound would be di-N-phosphoryl taurocyamine and that its structural formula could be expressed thus:

\[
\begin{array}{c}
\text{Ba} \\
\text{P} \\
\text{N} \\
\text{C} \\
\text{N} \\
\text{P} \\
\text{Ba}
\end{array}
\]

Full elementary analysis, (Table 14), revealed the presence of an excess of C, H, and S over that demanded by theory on the basis of the above formulation. It seemed likely that the excess of these elements might be due to the presence of a contaminant eluted from the column by 2M NaCl. To test this supposition some of the material was dissolved, and the solution passed through another column of Deacidite FF (Cl form). The column was washed with water and eluted with 0.1 M NaCl until appreciable amounts of bound guanidine appeared. As the bound guanidine which did appear was due to very slow displacement of Fraction II by 0.1M NaCl the concentration of the eluant was increased. On elution with 0.4M NaCl, Fraction II was eluted as a single homogeneous peak. Increase of the concentration of the eluant from 0.4M to
<table>
<thead>
<tr>
<th>Element or Group</th>
<th>%</th>
<th>Moles (N = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>7.17</td>
<td>4.0</td>
</tr>
<tr>
<td>H</td>
<td>3.22</td>
<td>21.6</td>
</tr>
<tr>
<td>N</td>
<td>6.24</td>
<td>3.0</td>
</tr>
<tr>
<td>S</td>
<td>5.43</td>
<td>1.1</td>
</tr>
<tr>
<td>P</td>
<td>9.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Ba</td>
<td>36.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Taurocyamine</td>
<td>23.6</td>
<td>1.0</td>
</tr>
<tr>
<td>H₂O</td>
<td>9.0</td>
<td>3.4</td>
</tr>
</tbody>
</table>
to 1M and 2M did not result in displacement of any more bound taurocyamine. The bound taurocyamine eluted by 0.4M NaCl was isolated as a Ba salt in the manner described above. On analysis the material gave figures similar to those in Table 14. Attempts made to crystallise the compound as an ammonium, K or Na salt were unsuccessful.

In view of these results the question arose as to whether Fraction II was a di-N-phosphoryl-guanidine and whether taurocyamine was in fact the guanidine released under mild conditions of hydrolysis. This latter point was checked by paper chromatography of the guanidine released by hydrolysis in 0.1N HCl at 65°. The material was chromatographed (Whatman 3MM paper) in a variety of solvent systems and in all cases behaved similarly to authentic taurocyamine. The solvent systems used were: acetone: NH₃ solution (2N): water (60:30:10); propanol: NH₃ solution (sp.gr. 0.88): water (60:30:10), Hanes & Isherwood (1949); and pyridine: isopentanol: water (80:40:70), Roche, Thoai & Hatt (1954).

Stability in acid

Some experiments were carried out to determine the relative stabilities of PT and Fraction II in acid solution; hydrolysces were carried out in N HCl at 37°. In the case of PT, taurocyamine and P were released in equimolar proportions
throughout the course of the hydrolysis (Fig. 24). The compound was 50% hydrolysed in 72 min.

The behaviour of Fraction II under similar conditions presented a marked contrast. Thus, owing to a difference in stabilities of the phosphoryl linkages, the P : taurocyamine ratio in the early stages of the hydrolysis was about 4 : 1 and fell as hydrolysis proceeded. Fraction II was 50% hydrolysed in about 130 min. at which time the P : taurocyamine ratio was about 2.3 : 1.

**Stability in alkali**

No taurocyamine was released from either PT or Fraction II when heated in N NaOH for 60 min. at 37°.

**Action of phosphatase**

In an endeavour to obtain some idea of the nature of the phosphoryl linkages in Fraction II and to compare its behaviour with that of PT, both compounds were incubated with alkaline phosphatase.

The results indicated that P and taurocyamine were released from PT in equimolar proportions throughout the course of the reaction and that the compound is completely hydrolysed by the enzyme. With Fraction II (Fig. 25) on the other hand the rate of P liberation was much greater than that of taurocyamine. Thus, at the end of 5 min. the molar ratio P : taurocyamine was 18 and after 20 min. had fallen to 5. After 75 min., both P and
Fig. 24. Hydrolysis of PT; µmoles of taurocyamine and P released on incubation in N HCl at 37°. Initial concentration of PT = 1.04 µmoles/ml.
Fig. 25. Action of alkaline phosphatase on Fraction II.
The reaction mixtures contained ethanolamine (0.05M, pH 9.2), alkaline phosphatase (18μg. of protein/ml.), fraction II (0.98mM with respect to taurocyamine), temperature 37°. o—o, taurocyamine released; Δ—Δ, P released.
taurocyamine were being released at a slow constant rate with a P : taurocyamine ratio of approximately 2.1 - 2.2 : 1. Fraction II was hydrolysed only to the extent of 55% after 240 min.

**TPT experiments**

The ability of Fraction II to function as a substrate for TPT was tested in the forward reaction in the presence and absence of myosin. This latter enzyme was added to ensure that the maximum amount of taurocyamine would be released. As controls, similar experiments carried out with PT as substrate indicated that at equilibrium 55% of the taurocyamine was released (Table 15). In the presence of myosin all the bound taurocyamine was released. Such a result would be expected as PT is the natural substrate for the enzyme. The results with Fraction II indicate that only a small proportion of the bound taurocyamine is released even in the presence of myosin.

**DISCUSSION**

The reaction between POCl₃ and glycocyamine or creatine to yield N-phosphorylated derivatives was initially described by Zeile & Fawaz (1938), Fawaz & Seraidarian (1946) and subsequently by Ennor & Stocken (1948), who isolated PC as the Na salt. These communications did not refer to any side reactions and only one product was isolated. It was, therefore anticipated that the
TABLE 15.

PT and Fraction II as substrates for TPT

Reaction mixture: ADP, \(10^{-3}\text{M}\); Mg\(^{2+}\), \(2.5 \times 10^{-3}\text{M}\); Ca\(^{2+}\), \(5 \times 10^{-3}\text{M}\); N-ethylmorpholine, \(0.05 \text{ M, pH 7.2}\); TPT, \((50 \mu\text{g protein/ml})\); PT, \(2.67 \times 10^{-3}\text{M}\); or Fraction II, \((1.33 \times 10^{-3}\text{M}, \text{calculated on bound taurocyamine content})\).

Total volume, 1.5 ml. Reaction time, 8 hr. at 37\(^\circ\).

<table>
<thead>
<tr>
<th>PT</th>
<th>Fraction II</th>
</tr>
</thead>
<tbody>
<tr>
<td>No myosin</td>
<td>No myosin</td>
</tr>
<tr>
<td>Myosin (800 \mu\text{g protein/ml})</td>
<td>Myosin (800 \mu\text{g protein/ml})</td>
</tr>
</tbody>
</table>

Taurocyamine

| µmoles/ml. | 1.48 | 2.67 | 0.16 | 0.28 |

Taurocyamine

| % released | 55 | 100 | 12 | 21 |

Taurocyamine in absence of myosin

| 0.55 |

Taurocyamine in presence of myosin

| 0.57 |
phosphorylation of taurocyamine with POCl$_3$ under similar conditions would yield but one product i.e. a mono-N-phosphoryl derivative. Such, however, was not the case and the separation of this latter compound from a mixture could be accomplished only by the use of an anion exchange resin from which it could be eluted with 0.2M NaCl. Although PT could be crystallised as the ammonium salt it was isolated as a pure amorphous Ba salt.

PT is completely hydrolysed by alkaline phosphatase which is believed to attack only N - P and ester phosphate linkages (Morton, 1955). In the dry state as the Ba salt it is unstable and slowly breaks down over a period of 6 months at room temperature to yield the free base and inorganic P. It has, however, been found that PT is stable in solution as the Na salt if stored at -15°; such solutions have been kept for 6 months without decomposition.

The other product of the phosphorylation is readily separated from PT but is of unknown constitution except insofar as it yields, on acid hydrolysis, 2 moles of P to 1 of taurocyamine. There is however, no evidence as to the manner in which the P is bound although it is clear from the phosphatase experiments that a part of the P is bound in an N - P linkage. If it be assumed that mono-N-phosphoryl taurocyamine (PT) is the natural substrate for TPT and that the enzyme has a high degree of specificity for
this substrate, additional evidence as to the nature of Fraction II can be obtained from the action of this enzyme upon PT and Fraction II. The fact that the ratio of the taurocyamine released in the absence and in the presence of myosin is about the same with PT and Fraction II as substrates is indicative of a contamination of the latter with PT. Thus it may be calculated that Fraction II is contaminated to the extent of 20% with PT. (The sample used for this experiment was about 1 month old, so it is possible that it was contaminated with PT). Contamination could arise as a result of the breakdown of the original 'compound' as first isolated from the ion exchange resin. This is perhaps likely particularly as Fraction II when isolated as the Ba salt breaks down in such a way that 50% of the bound taurocyamine appears as the free guanidine even when stored in the dry state at room temperature (24°C) over 4 months.

**SUMMARY**

1. A method is described for the preparation of the Ba salt of PT.
2. A phosphorylated product in addition to the above is obtained. This can be readily separated from the reaction mixture, but is of unknown constitution.
3. Some of the properties of PT are described.
B. STUDIES OF THE ACID HYDROLYSIS OF PHOSPHORYLATED GUANIDINES

INTRODUCTION

PC and PA are completely hydrolysed by heating for 1 min. in N/10 acid at 100° (Meyerhof and Lohmann, 1928a; Lohmann, 1936) as also are the recently discovered phosphagens PG, PT and PL (Thoai et al., 1953a; Thoai and Robin, 1954). The N-P bond is therefore markedly unstable as compared with the P-O-P bond of the purine and pyrimidine nucleotides and the C-C-P bonds of the hexose phosphates.

In a study of the effect of pH on the hydrolysis of PC, Fiske and Subbarow (1929) found that the maximum rate of hydrolysis was proportional to the concentration of the monoanion and these results indicate that the monoanion is the unstable species. Little is known of the behaviour of PA, PG and PT in acid solution except that the rate of hydrolysis of PA is greater in 0.01 N HCl than in 1N HCl (Lohmann, 1936).

The different acid labilities of PC and PA in the presence of molybdate ion was noted by Lohmann (1928) and Meyerhof and Lohmann (1928a) who found that whereas the hydrolysis of PC was catalysed by molybdate, that of PA was retarded. Following this discovery the retarding effect of molybdate was used as a
criterion for the presence of PA in extracts of animal tissues (Needham et al., 1932; Baldwin, 1933). It was believed that, irrespective of the conditions of hydrolysis or the concentration of molybdate, a precise value would be obtained for the retardation factor which was defined as the rate of hydrolysis in the absence of molybdate / rate of hydrolysis in the presence of molybdate. However, it soon became apparent that this was not the case and under different experimental conditions values for the retardation factor ranging from 1.5 to 30 were obtained with PA. This, together with the discovery of other naturally occurring phosphagens whose hydrolysis behaviour in acid molybdate was similar to that of PA (Baldwin and Yudkin, 1950), indicated that the use of the numerical value of the retardation factor was no longer a valid criterion for the identification of PA.

Investigations of the effect of molybdate on the acid hydrolysis of phosphagens have been confined mainly to experiments with PC. Barker, Ennor and Harcourt (1950) showed that molybdate catalysed the direct formation of creatinine from PC and it was suggested that the reaction mechanism involved a splitting out of phosphomolybdate from a creatine phosphomolybdate complex, followed by ring closure. Weil-Malherbe and Green (1951) found that in the presence of molybdate the maximum rate of hydrolysis of PC takes place at pH 2.5, the rate in the presence of molybdate being 135 times that in its
absence. No investigations of the effect of acid molybdate on PG, PT and PL have been made other than that of Fawaz and Seraidarian (1946) who found that, under the conditions of the Fiske and Subbarow P estimation, PG is 15% hydrolysed in 30 min. at 28° whereas PC is completely hydrolysed.

The results of studies of the acid hydrolysis of PA, PG and PT in the presence of molybdate show that there is not only retardation of the hydrolysis of PA but also of PG and PT; this retardation is dependent on both the phosphagen : molybdate ratio and the acid concentration.

EXPERIMENTAL

Materials

N-phosphorylarginine.

BaPA was isolated by the method of Ennor et al. (1956) from the tail muscle of the sea-crayfish Jasus verreauxi. Solutions of the Ba salt were made up as required and the pH adjusted to 7 - 7.2 with N HCl.

N-phosphoryltaurocyamine.

The crystalline ammonium salt was prepared as described in Section A of this chapter.

N-phosphorylglycocamine.

The crystalline ammonium salt was prepared as described in Appendix 2.
Ammonium Molybdate

This was a B.D.H. product, A.R. Grade of molecular formula $\text{(NH}_4\text{)}_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$.

Buffer solutions

Sodium acetate-HCl buffers were used over the pH range 1 - 5.5. For higher pH values N-ethyl morpholine buffers were used. The final concentration of buffer in all cases was 0.1M.

Methods

All experiments were carried out in a water bath at 37° and solutions of the reagents were preincubated for 10 - 15 min. prior to the start of the reaction.

Hydrolysis of phosphagens

At zero time, 10.0 ml. of acid or acid-molybdate solution of the required concentration were mixed rapidly with 10.0 ml. of a 2 x $10^{-3}$M solution of the phosphagen. Samples were removed at various time intervals and pipetted into 10 ml. graduated tubes containing 1 ml. of NaOH-EDTA mixture (0.6 ml. of 5N NaOH, 0.4 ml. of 0.2M EDTA) and the required amount of N NaOH to neutralise the acid in the sample removed. The guanidine liberated was estimated by the method of Rosenberg et al. (1956). The final concentrations in the reaction mixture were:

- Phosphagen, 0.001 M; ammonium molybdate, 0.002 M (except where otherwise stated); HCl, 1.0N, 0.5N or 0.1N.
The effect of pH on the rate of hydrolysis of phosphagens was studied as follows: At zero time, the reaction was started by pipetting the required amount of phosphagen solution (0.005 M) into various buffer solutions (containing molybdate if required), and mixing rapidly. The final concentrations in the reaction mixture were: phosphagen, 0.001 M; ammonium molybdate, 0.002 M; buffer solution, 0.1 M. Samples were removed for guanidine analysis after incubation at 37° for 10 and 20 min. and the guanidine content estimated as described above. pH measurements were made with the glass electrode on samples removed approximately 5 min. after the start of the reaction.

RESULTS

The hydrolysis curves obtained with PA, PT and PG at various acid concentrations indicated that in each case the reaction conformed to first-order kinetics (see Fig. 24 for hydrolysis of PT in N HCl). A comparison of the times required to effect 50% hydrolysis of these phosphagens (Table 16) shows that their acid lability decreases in the order PA > PT > PG, irrespective of the acid concentration. However, a decrease of the acid concentration causes an increase of the hydrolysis rate of each phosphagen so that the rate in 0.1N acid is 30 - 50% greater than that in 1.0N acid. The results with PA are in agreement with those of Lohmann (1936) who, using a crude
TABLE 16.

Acid Hydrolysis of PA, PG and PT

Conditions have been described in the text. Temperature 37°.

<table>
<thead>
<tr>
<th>Phosphagen</th>
<th>Acid</th>
<th>Rate (μmoles of guanidine released/10 min.)</th>
<th>Time for 50% Hydrolysis (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A*</td>
<td>B</td>
</tr>
<tr>
<td>PT</td>
<td>1.0</td>
<td>0.084</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.097</td>
<td>0.064</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.118</td>
<td>0.00</td>
</tr>
<tr>
<td>PG</td>
<td>1.0</td>
<td>0.064</td>
<td>0.055</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.079</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.091</td>
<td>0.022</td>
</tr>
<tr>
<td>PA</td>
<td>1.0</td>
<td>0.129</td>
<td>0.121</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.136</td>
<td>0.053</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.194</td>
<td>0.00</td>
</tr>
<tr>
<td>PCV</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* No molybdate present.

\[ \phi \] 2 x 10^{-3} M ammonium molybdate present.

\[ V \] Results of Barker, Ennor & Harcourt (1950).

** 22% hydrolysis in 3 hr.
sample of PA, found a 45% increase in its hydrolysis rate on reducing the acid strength from 1.0N to 0.1N. Further investigations of the relationship between acid strength and the rate of hydrolysis (Figs. 26, 27, 28) revealed that there is a maximum rate within the pH range 1.0 to 3.0 for each of the three phosphagens.

At a particular acid concentration, the addition of molybdate caused a decreased rate of hydrolysis of PA, PG and PT (Table 16), but the effect became much more marked as the acid concentration was decreased from 1N to 0.1N. The rate of hydrolysis in the presence of $2 \times 10^{-3}$ M molybdate and 0.1N acid was such that after 3 hr. only trace amounts of guanidine were released from PA and PT whilst PG was hydrolysed to the extent of 22%. A study of the influence of pH on the effect of molybdate showed that the rates of hydrolysis of PA, PT and PG are minimal within the same pH range as that in which maximum rates are obtained in the absence of molybdate. At lower pH values the effect of molybdate is decreased and this is also the case at higher pH values. At pH 4.5 - 5.5, the rate of hydrolysis in the presence of molybdate is approximately the same as in its absence.

Further investigations of the effect of molybdate (Figs. 29, 30, 31) showed that the degree of hydrolysis is dependent on the phosphagen : molybdate ratio as well as on the acid
Fig. 26. Effect of pH on the hydrolysis of PA. PA, 0.001M; temperature, 37°; reaction period 10 min. o—o, No molybdate; •—•, Molybdate (0.002M) present. pH measurements were made as described in the text. The points at pH 0, 0.65, and 1.0 represent the % hydrolysis in 1N, 0.5N and 0.1N HCl, respectively.
Fig. 27. Effect of pH on the hydrolysis of PG. PG, 0.001M; temperature, 37°C; reaction period 10 min. o—o, No molybdate; •——•, Molybdate (0.002M) present. pH measurements were made as described in the text. The points at pH 0, 0.65 and 1.0 represent the % hydrolysis in 1N, 0.5N and 0.1N HCl, respectively.
Fig. 28. Effect of pH on the hydrolysis of PT. PT, 0.001M; temperature 37°; Reaction period 10 min. o—o, No molybdate; •—•, Molybdate (0.002M) present. pH measurements were made as described in the text. The points at pH 0, 0.65 and 1.0 represent the % hydrolysis in 1N, 0.5N and 0.1N HCl, respectively.
Fig. 29. Hydrolysis of PA: The effect of the molybdate: PA ratio on the rate of hydrolysis. PA, 0.001M; temperature 37°; reaction period, 20 min. •—•, 1.0N HCl; o—o, 0.5N HCl; △—△, 0.1N HCl.
Fig. 30. Hydrolysis of PG: The effect of the molybdate : PG ratio on the rate of hydrolysis. PG, 0.01M; temperature 37°; reaction period, 20 min. •--•, 1.0N HCl; o—o, 0.5N HCl; △—△, 0.1N HCl.
Fig. 31. Hydrolysis of PT: The effect of the molybdate: PT ratio on the rate of hydrolysis. PT, 0.001M; temperature 37°; reaction period, 20 min. •—•, 1.0N HCl; o—o 0.5N HCl; △—△, 0.1N HCl.
As the concentration of molybdate increased, the degree of inhibition of hydrolysis also increased, the effect being more marked at lower acid concentrations.

Because of the structural similarity of PC and PG, it might have been expected that PG would also give rise to a cyclic amidine as a result of acid hydrolysis, especially in the presence of molybdate. However, glycocyamidine could not be detected in reaction mixtures by means of the Jaffe reaction and almost quantitative recoveries of glycocyamine were obtained following complete hydrolysis of PG in both acid and acid-molybdate solution.

**DISCUSSION**

The fact that the maximum rate of acid hydrolysis of PA, PG and PT occurs within the range between pH 1.0 and 3.5 suggests that the monoanions are more unstable species than either the dianions or uncharged molecules. Similar conclusions have been reached by Fiske and Subbarow (1929) as far as PC is concerned and it would thus appear that N-phosphorylated guanidines are similar, in this respect, to a number of ester-P compounds (see review by Barnard, Bunton, Llewellyn, Oldham, Silver and Vernon, 1955). An explanation for the enhanced hydrolysis of the monoanion has been proposed recently by Herr and Koshland (1957) as a result of studies of the acid hydrolysis of butyl thiophosphate.
They concluded that hydrolysis of the monoanion takes place by means of a concerted acid and base catalysis and the mechanism proposed by these authors takes into account the decreased rate below pH 2 and above pH 3.5 - 4. Later work with PC (Koshland and Herr, 1957) has indicated that the acid hydrolysis of this compound can be considered as a phosphoryl group transfer reaction with water as the acceptor molecule. The qualitative similarity between the present results (Figs. 26, 27, 28) and those obtained by Herr and Koshland suggests that the mechanism they propose may well be applicable to the hydrolysis of the N-P bond of phosphagens.

The demonstration that the retardation of the hydrolysis of PA is dependent on the molybdate : phosphagen ratio, as well as on the acid concentration (Fig. 29), explains the wide variation in the values obtained for the retardation factor for PA (Meyerhof and Lohmann, 1928a; Baldwin and Needham, 1933; Lohmann, 1936; Baldwin and Yudkin, 1950).

Many of these values were obtained as a result of the determination of the rate of hydrolysis of PA in crude tissue extracts following the addition of various amounts of acid or acid-molybdate (0.25% - 2% molybdate; 0.01N - 1.0N acid). Even if PA were the only invertebrate phosphagen and if the concentrations of molybdate and acid were standardised, it is clear that the value obtained would be dependent on the concentration
of PA. However, because of the presence in crude extracts of other P compounds which react with molybdate, and of compounds which function as buffers, the final concentration of molybdate and acid is not necessarily the same as that added. These considerations, together with the possible presence of PG and PT in invertebrate muscle extracts, indicate that the numerical value for the retardation factor is no longer valid as a criterion for the identification of PA.

The effect of molybdate on the hydrolysis of PA, PG and PT can be explained by postulating the formation of dissociable molybdate-phosphagen complexes in which the N-P bond is more stable than in the phosphagens. Indeed there is evidence that such complexes are formed for it is possible to precipitate a molybdate-PA complex from acid solution by the addition of ethanol (Ennor, Morrison and Rosenberg, 1956; unpublished observations). The fact that minimal hydrolysis in the presence of molybdate, occurs at pH values where the acid hydrolysis is maximal suggests that it is the phosphagen monoanion which complexes with the molybdate. It follows then that the stability of the complex will depend not only on the stability constant, but also on the pH (Figs. 26, 27, 28). This argument would explain the increased hydrolysis which is observed at pH values below pH 1.0 and above pH 3.0. The concentration of the complex will, of course, depend on that of the molybdate ion
and as this increases an increased inhibition of hydrolysis is observed (Figs. 29, 30, 31). The order of stability of the molybdate-phosphagen complexes would seem to be $PA > PT > PG$ (cf. Figs. 26, 27, 28) which is the reverse of the order of the acid stabilities of these phosphagens (Table 16).

Further study is necessary to elucidate the mechanism of hydrolysis in the presence of molybdate and in this connection the observation that a yellow coloured complex is formed at pH values from 1.0 to 2.5 but not in 1.0N HCl suggests that the breakdown of the molybdate-phosphagen complexes may be followed by spectrophotometric methods.
SUMMARY

1. The acid lability of the phosphagens studied decreases in the order PA > PT > PG.

2. In all cases maximum hydrolysis occurred within the pH range 1.0 - 3.0, and the conclusion was reached that the phosphagen monoanion was the unstable species.

3. Molybdate retarded the acid hydrolysis of PG and PT as well as that of PA; the degree of retardation being dependent on both the molybdate : phosphagen ratio and the acid concentration.

4. The conclusion was reached that molybdate forms a stable complex with the monoanions of PA, PG and PT.
CHAPTER 5.

THE PURIFICATION AND PROPERTIES OF TAUCRYAMINE

PHOSPHORYLTRANSFERASE
CHAPTER 5.

THE PURIFICATION AND PROPERTIES OF TAUCRYAMINE PHOSPHORYLTRANSFERASE

INTRODUCTION

The enzyme taurocyamine phosphoryltransferase (TPT) which catalyses the equilibrium as expressed by the reaction:

\[ \text{PT} + \text{ADP} \rightleftharpoons \text{Taurocyamine} + \text{ATP} \]

was first shown to be present in extracts of the annelid, *Arenicola marina* by Hobson (1955). Later, Thoai (1957) obtained partially purified preparations of the enzyme as a result of fractionation of extracts of *A. marina* with ethanol. He reported that the pH optima of the forward and reverse reactions were pH 7.1 and 8.9, respectively, and that the enzyme was specific for taurocyamine. However, because of the marked lability of the preparation and the presence of a very active ATP-ase, no satisfactory kinetic studies could be made.

The experimental work to be described in this chapter is concerned with the isolation of purified TPT from *Arenicola assimilis*, which is more stable than that reported by Thoai (1957) and which is suitable for kinetic studies. A preliminary
investigation of the general properties of TPT is also described.

EXPERIMENTAL

Materials

N-ethyl morpholine and imidazole were products of Eastman-Kodak Ltd. and sodium cacodylate and sodium acetate were obtained from British Drug Houses Ltd. The organic solvents used (95% ethanol and acetone) were purified by distillation. N-amidino acetamide, p-guanidino benzoic acid, and N-amidino sulphanilic acid were gifts from I.C.I. Ltd. Ethylguanidine-HBr and 2-guanidino ethanol-HBr were prepared by Dr. H. Rosenberg of this Department and γ-guanidino-n-butyric acid was a gift from Mr. L. S. Walters of the South Australian Brewing Co. Ltd., Adelaide. Other guanidine compounds, nucleotide substrates and inhibitors were the same as described in Chapter 1 and Appendix 2.

N-phosphoryl taurocyamine

BaPT was prepared as described in Chapter 4. Solutions of NaPT were prepared by passing solutions of the Ba salt through a column of Zeo-Karb 225 (Na form). The column was washed with water, the filtrate and washings combined and made to volume. The molarity of the solution was checked by estimation of the taurocyamine released after hydrolysis in N HCl for 1 min. at 100° (Rosenberg et al., 1956).
Buffer solutions

N-ethyl morpholine and imidazole (1M) were adjusted to the required pH with 5N HCl or 5N NaOH and then diluted to a final concentration of 0.5M. Sodium acetate-acetic acid buffers were prepared by the addition of 0.5N acetic acid to 0.5M solutions of sodium acetate until the required pH was obtained, and then diluted to 0.25M. In all cases the final pH of the buffer solutions was checked with the glass electrode.

Calcium phosphate gel

This was prepared by the method of Keilin & Hartree (1938) and used at a concentration of 25mg./ml.

Enzyme Source

Specimens of Arenicola assimilis were collected at Shoreham, Victoria, cleaned of sand and mucus and frozen by dropping into liquid air; they were then removed, placed in polythene bags and stored in solid CO₂ for transport to the laboratory.

Methods

Protein was estimated by the biuret method of Gornall, Bardawill & David (1949), crystalline bovine serum albumin being used as the standard.
Unless otherwise stated, enzymic activity was determined as described in Chapter 1, except that PA and arginine were replaced by PT and taurocyamine respectively.

**Arbitrary units of TPT activity**

One unit of TPT activity is defined as the amount of enzyme which releases 1 μmole of taurocyamine from PT in 1 min. at pH 7.2 and at 5°.

**Specific activity**

This is defined as the number of units of TPT per mg. of protein.

**Electrophoretic experiments**

These were carried out in the Perkin-Elmer (model 38A) apparatus.

**RESULTS**

Initial experiments indicated that poor mechanical disruption of *Arenicola marina* was obtained when specimens were extracted in a Waring blender with either acetone or aqueous solutions. As a consequence the total amount of enzyme obtained was low. It was subsequently found that increased amounts could be obtained by aqueous extraction of powdered preparations of *A. marina* prepared by freeze-drying and grinding. Such preparations which were stable for 2 - 3 weeks when stored in vacuo at 5°,
were therefore used as the starting material. Many difficulties were experienced in obtaining a satisfactory fractionation procedure for the purification of TPT. The enzyme itself is labile, even in purified form, and markedly more so in crude extracts of the whole animal. The increased lability in crude extracts was undoubtedly due to the presence of proteolytic enzymes extracted from the gut of the animal but it was impracticable to dissect out the body wall muscle as was done by Thoai (1957) for the larger species A. marina. However, the lability of TPT in crude extracts could be reduced, but not abolished, by the addition of chloramphenicol, EDTA and KCN to the extracting medium. Each compound alone was effective in reducing the loss in enzymic activity, but the combination of all three was more effective. Because of the inability to stabilize the enzyme, it was necessary to develop a rapid fractionation procedure and after repeated trials the following one was adopted:

Preparation of initial extract

Unless otherwise stated, all operations concerned with the fractionation of TPT were carried out in the cold room at 2°C. 100g. of dry worm powder was extracted by stirring with 10 vol. of a chloramphenicol-EDTA-KCN solution (chloramphenicol, 500 μg./ml.; EDTA, 10⁻³ M; KCN, 10⁻³ M; pH 6.9) for 10 min. The
mixture was centrifuged at 0° for 5 min. at 5000g. and the supernatant filtered through fluted papers (Whatman No. 531). The centrifugate was re-extracted for 10 min. with 5 vol. of the same solution and the mixture centrifuged at 0° for 10 min. at 5000g. The supernatant was filtered and the two extracts combined to give a dark red opaque solution.

**Calcium phosphate gel treatment**

Calcium phosphate gel (0.25 vol., 25mg./ml.) was added to the extract, the mixture stirred for 1 - 2 min. and then centrifuged at 0° for 10 min. at 1000g. The supernatant was a clear light red coloured solution (pH 6.1 - 6.3).

**Acetone fractionation at alkaline pH**

The pH of the supernatant was adjusted to 7.7 - 7.8 by the addition of one-ninth volume of potassium phosphate buffer (1M, pH 8.0) and the solution then cooled to 0° in a dry ice - ethanol bath. Acetone (-20°) was added with mechanical stirring at a rate of approximately 100 ml./min. to a concentration of 40% (v/v). During this addition the temperature of the solution was gradually lowered to -5°. After stirring for 5 min. the precipitate was removed by centrifugation at -5° for 10 min. at 1000g. and discarded. The temperature of the slightly cloudy supernatant was brought to -5° and acetone (-20°)
was added (approximately 100 ml./min.) with mechanical stirring to a final concentration of 60% (v/v). After stirring for 5 min. at -5° the mixture was centrifuged at -5° for 10 min. at 1000g.

The precipitate was dissolved in 250 ml. of potassium phosphate buffer (0.1M, pH 6.3) and clarified by centrifugation at 0° for 5 min. at 5000g. The protein concentration of the solution was then adjusted to 5 mg./ml. by dilution with the same phosphate buffer. If the pH of the resultant solution was higher than 6.5 - 6.6, it was adjusted to pH 6.5 with 0.5N acetic acid.

**Acetone fractionation at acid pH**

The solution was cooled to 0° in a dry ice-ethanol bath and 0.25 vol of acetone (-20°) added (approximately 100 ml./min.) with mechanical stirring. At the same time the temperature was gradually lowered to -5°. The mixture was stirred at this temperature for 5 min. and further acetone added (20-30 ml./min.) to a concentration of 37% (v/v). The mixture was stirred for 10 min. at -5° and then centrifuged at -2° for 10 min. at 1000g. (If the temperature falls below -5° at this step, the enzyme is partially precipitated). The supernatant was cooled to -5°, and acetone (-10°) added (20-30 ml./min) with stirring to a concentration of 50% (v/v). After stirring for 10 min. at -5° the mixture was centrifuged as before. The precipitate was taken up in 60 ml. of phosphate buffer (0.1M, pH 7.3; containing 10^{-3}M
EDTA) and the protein concentration of the solution adjusted to 5 mg. of protein/ml. by dilution with the same buffer.

**Ammonium sulphate fractionation**

The solution was brought to 52% saturation by the addition of an ammonium sulphate solution, (saturated at 2°, and containing $10^{-3}$ M EDTA), and the precipitate removed by centrifugation at 0° for 10 min. at 5000g. The supernatant was retained, and brought to 90% saturation by the addition of solid ammonium sulphate. The precipitate was collected by centrifugation at 0° for 40 min. at 5000g. and dissolved in 20 ml. of phosphate buffer (0.1M, pH 6.3 containing $10^{-3}$ M KCN). The solution was dialysed for 1 hr. against three changes of phosphate buffer (0.01M, pH 6.3; containing $10^{-3}$ M KCN) in order to reduce the ammonium sulphate concentration prior to fractionation with ethanol.

**Ethanol fractionation**

The dialysed solution was cooled to 0° and maintained at this temperature throughout the ethanol fractionation. Ethanol (-5°) was added at a slow rate (5 ml./min.) with mechanical stirring to a concentration of 52% (v/v) and the resultant precipitate removed by centrifugation at 0° for 5 min. at 5000g. The ethanol concentration of the supernatant was brought to 63% (v/v) in similar fashion and the precipitate
collected by centrifugation at 0° for 5 min. at 5000 g. The precipitate was dissolved in 8 – 10 ml. of 10^{-3} M KCN, and clarified by centrifugation at 0° for 5 min. at 5000 g. It was then diluted with an equal volume of glycerol and stored at -10°.

The fractionation was carried out without interruption to avoid loss of enzymic activity which otherwise occurred at stages prior to the ethanol fractionation.

The yields and degrees of purification of a typical TPT preparation obtained by this fractionation procedure (Table 17) show that a 40-fold increase in the purity of the enzyme with an overall recovery of 10% was obtained. Occasionally products were obtained following fractionation with acetone at pH 6.5 which had specific activities as high as 14 – 18, and when these were further fractionated they yielded products with specific activities ranging from 45 - 60. No explanation for this variation can be offered.

Tests for the presence of other enzymes

Tests for the presence of phosphoamidase, ATP-ase and myokinase activities in the enzyme preparation were carried out as described in Chapter 1 except that PA was replaced by PT in the test system. The enzyme was used at a concentration 200 times greater than that used in the standard activity determination. Traces only of myokinase and phosphoamidase
TABLE 17.

Summary of Yields and Specific Activities of Fractions obtained during the Fractionation of TPT

Wt. of worm powder, 100g. Details are described in the text.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml.)</th>
<th>Total protein (g*)</th>
<th>Total Units</th>
<th>Specific Activity</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>1300</td>
<td>20.9</td>
<td>16,500</td>
<td>0.79</td>
<td>(100)</td>
</tr>
<tr>
<td>Gel supernatant</td>
<td>1410</td>
<td>7.9</td>
<td>10,400</td>
<td>1.32</td>
<td>63</td>
</tr>
<tr>
<td>40-60% Acetone ppt.</td>
<td>243</td>
<td>2.0</td>
<td>6,750</td>
<td>4.3</td>
<td>53</td>
</tr>
<tr>
<td>37-50% Acetone ppt.</td>
<td>77</td>
<td>0.54</td>
<td>5,250</td>
<td>9.6</td>
<td>32</td>
</tr>
<tr>
<td>52-90% (NH₄)₂SO₄ ppt.</td>
<td>24</td>
<td>0.25</td>
<td>4,600</td>
<td>18.5</td>
<td>28</td>
</tr>
<tr>
<td>Dialysed enzyme</td>
<td>29</td>
<td>0.20</td>
<td>2,800</td>
<td>14.0</td>
<td>17</td>
</tr>
<tr>
<td>52-63% ethanol ppt.</td>
<td>8.3</td>
<td>0.055</td>
<td>1,700</td>
<td>31</td>
<td>10</td>
</tr>
</tbody>
</table>
activities were detected but a $\text{Mg}^{2+}$ and $\text{Ca}^{2+}$-activated ATP-ase was present. However its activity was not great enough to interfere with the kinetic studies of the reverse reaction. The purified preparation also showed traces of arginase activity in contrast to the highly active arginase activity shown by crude extracts.

**Stability of the enzyme preparation.**

The initial activity of all preparations stored in glycerol fell by about 25% during the first 24 hr. of storage, but thereafter the decrease was very slow. 80% of the initial activity was lost after six months storage. On the other hand, TPT preparations dissolved in $10^{-3}$ M KCN lost 50% of their activity in 3 days.

**Electrophoretic analysis.**

The electrophoretic pattern of a typical TPT preparation (Fig. 32) indicates the presence of one major and three minor components. The TPT activity was shown to be associated with the major component which constituted 50% of the total protein.

**Effect of enzyme concentration on reaction velocity.**

The initial velocity is proportional to the enzyme concentration in both the forward (Fig. 33) and reverse reactions.
Fig. 32.  Electrophoretic patterns of a TPT preparation obtained as described in the text. Phosphate buffer, pH 7.2, $\mu = 0.2$; temperature $4^\circ$; protein concentration 0.7%; time 60 min.
Fig. 33. Effect of enzyme concentration on the reaction velocity of the forward reaction at pH 7.2. Conditions as described in the text except that various volumes of a stock enzyme solution (50 μg. of protein/ml.) were added. Reaction time, 5 min.; temp. 5^\circ C.
Effect of pH on the reaction velocity.

The relationship between pH and reaction velocity in the forward reaction (Fig. 34) indicates that maximum velocity is reached in the range between pH 6 - 6.6 in all buffers (imidazole-HCl; cacodylate-HCl; sodium acetate-acetic acid). At the pH optimum, the rates were the same in imidazole and cacodylate buffers but were slightly higher in sodium acetate-acetic acid buffer. In the reverse reaction the pH-activity curve shows a broad maximum in the range pH 8.2 - 8.8 in N-ethyl morpholine buffer.

Effect of divalent cations on TPT activity.

The purified TPT preparation showed only a small residual activity in the absence of divalent cations. The activity of the enzyme in the forward reaction was markedly increased by Mn$^{2+}$, Mg$^{2+}$ and Co$^{2+}$, and to a lesser degree by Ca$^{2+}$ (Fig. 35). There was no activation by Ba$^{2+}$, Sr$^{2+}$, Fe$^{2+}$ and Fe$^{3+}$, and inhibition was produced by Ni$^{2+}$, Zn$^{2+}$ and Cu$^{2+}$. In the reverse reaction (Fig. 36) it was possible to demonstrate activation of the enzyme by Mn$^{2+}$, Mg$^{2+}$, Co$^{2+}$ and Ca$^{2+}$. The activation by Ca$^{2+}$ was more marked than was the case in the forward reaction.

Effect of inhibitors on TPT activity.

The effect of inhibitors on TPT activity in the forward reaction is summarised in Table 18. The inhibition by
Fig. 34. Effect of pH on the initial reaction velocity of the forward reaction. The system was as described in the text. The enzyme concentration was 20 µg. of protein/ml. and the reactions were run for 3 and 6 min. The pH was determined with a glass electrode in a duplicate set of tubes.

- , N-ethyl morpholine (0.06M); ■, acetic acid-sodium acetate (0.05M); ○, imidazole-HCl (0.06M);
△, cacodylate-HCl (0.06M).
Fig. 35. Activation of TPT by bivalent metals as determined in the forward reaction. Conditions as described in the text. Enzyme, 20 μg. of protein/ml.; reaction period 5 min.; temp. 5°C.
Fig. 36. Activation of TPT by bivalent metals as determined in the reverse reaction. Conditions as described in the text. Enzyme, 100 µg. of protein/ml.; reaction period 5 min.; temp. 50°.
TABLE 16.

Effect of various Substances on TPT Activity in the Forward Reaction

The enzyme (20 μg. of protein/ml.) was incubated for 5 min. in the presence of the inhibitor, N-ethylmorpholine (0.06M, pH 7.2), PT (5 x 10^{-3}M) and Mg^{2+} (1 x 10^{-3}M). The reaction was started by the addition of ADP (1 x 10^{-2}M, 0.1 ml.) and stopped after 5 min. by the addition of 1.0 ml. EDTA - NaOH mixture. Total volume, 1.0 ml.; temperature, 5°.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (M)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodoacetate</td>
<td>4 x 10^{-3}</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 x 10^{-2}</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5 x 10^{-3}</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-3}</td>
<td>34</td>
</tr>
<tr>
<td>p-CMB</td>
<td>1 x 10^{-6}</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-7}</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-8}</td>
<td>0</td>
</tr>
<tr>
<td>2 : 4 Dinitrophenol</td>
<td>4 x 10^{-4}</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-4}</td>
<td>8</td>
</tr>
<tr>
<td>N-ethyl maleimide</td>
<td>4 x 10^{-4}</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-4}</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>1 x 10^{-5}</td>
<td>12</td>
</tr>
</tbody>
</table>
iodoacetate, p-CMB and N-ethyl maleimide characterizes TPT as an -SH enzyme. The enzyme is very sensitive to p-CMB, 100% inhibition being obtained at $10^{-6}$M. TPT was inhibited by high concentrations of EDTA which presumably reacts by chelation with $\text{Mg}^{2+}$.

**Specificity of TPT.**

The ability of TPT to phosphorylate other guanidine compounds was determined in the reverse reaction by estimating the disappearance of free guanidine. In those cases where disappearance of free guanidine occurred, confirmatory tests for the formation of acid labile P were also carried out. This check was necessary as disappearance of free guanidine may occur by means other than phosphorylation. This was found to be the case on incubation with arginine where disappearance of guanidine was noted but no labile P was formed. This was later found to be due to contamination of the TPT preparation with trace amounts of arginase.

It can be seen (Table 19) that apart from phosphorylation of taurocyamine there is also phosphorylation of $\beta$-guanidino-propionic acid and $\gamma$-guanidino-n-butyric acid. Glycocyamine, creatine and arginine are not phosphorylated as was found by Thoai (1957). There was also no phosphorylation of 2-guanidino-ethanol, ethylguanidine, $\delta$-guanidino-n-valeric...
TABLE 19.

Specificity of TPT: Phosphorylation of Guanidines

The reaction mixture contained substituted guanidine \((2 \times 10^{-3} \text{m})\), \(\text{Mg}^{2+} (1 \times 10^{-3} \text{M})\), ATP \((5 \times 10^{-3} \text{M})\) and N-ethylmorpholine \((0.06 \text{M}, \text{pH } 8.4)\). The enzyme \((350 \mu\text{g. of protein/ml.})\) was added and the tubes were incubated for 14 hr. at 5°C. Total volume 1.0 ml.

<table>
<thead>
<tr>
<th>Guanidine derivative</th>
<th>Phosphorylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurocyamine</td>
<td>74</td>
</tr>
<tr>
<td>(\gamma)-guanidino-n-butyric acid</td>
<td>17</td>
</tr>
<tr>
<td>(\beta)-guanidino propionic acid</td>
<td>35</td>
</tr>
</tbody>
</table>

* Calculated on basis of free guanidine remaining.
acid, N-amidino acetamide, p-guanidino benzoic acid or N-amidino sulphanilic acid. The latter two compounds were tested as the spatial distance between the guanidino group and the sulphonic or carboxyl groups is similar to that found in taurocyamine and \( \beta \)-guanidino-propionic acid, respectively.

Studies of the specificity of the nucleotide substrates required for TPT activity showed that AMP cannot replace ADP in the forward reaction nor can ADP replace ATP in the reverse reaction. The 6-amino group of the adenine nucleotides appears to be necessary for activity as ATP could not be replaced by ITP in the reverse reaction.

**DISCUSSION**

The TPT preparation obtained by the procedure described above is superior to that obtained by Thoai (1957) both with respect to activity and stability. Thoai's preparation was calculated to have a specific activity of 1.44 (forward reaction 37\(^\circ\)) whilst in the present work TPT preparations with specific activities (at 37\(^\circ\)) varying from 110 to 200 were obtained. Thoai also reported that his preparation lost 50\% of its activity in 16 hr. at -10\(^\circ\), whereas TPT prepared by the present method lost only 25\% of its activity in one week on storage in 50\% (w/v) glycerol at -10\(^\circ\).
In common with other guanidine phosphoryltransferases, TPT is activated by $\text{Mg}^{2+}$ and $\text{Mn}^{2+}$ and in its activation by $\text{Ca}^{2+}$ is similar to mammalian and invertebrate CPT. However, it differs from all other known guanidine phosphoryltransferases in that it is activated by $\text{Co}^{2+}$. The activation by $\text{Mn}^{2+}$ is greater than that by $\text{Mg}^{2+}$ and in this respect is similar to APT (Chapter 1) and mammalian CPT (Kuby et al., 1954).

Ill-defined pH optima for the forward and reverse reactions at pH 6 - 6.6 and 8.2 - 8.8 respectively, were found in the present work whereas Thoai (1957) reported the pH optima to lie at pH 7.1 and 8.9 respectively. The pH optima are thus similar to those found for APT (Chapter 1) and CPT (Kuby et al., 1954).

The inhibition of enzymic activity by iodoacetate, p-CMB and N-ethyl maleimide indicates that TPT is dependent upon the presence of thiol groups for activity. The inhibition by 2,4-dinitrophenol was not further investigated but may well be similar to that described in Chapter 1 for APT.

The experiments on the specificity of TPT give some indication of the groups required for interaction of the enzyme with the substrate. The non-phosphorylation of 2-guanidinoethanol and ethylguanidine indicates that an acidic group on carbon atom 1 is essential for activity. Moreover the phosphorylation of $\beta$-guanidino-propionic acid (Table 19) indicates
that the sulphonylic group is not essential and can be replaced by a carboxyl group. However, the rate of phosphorylation of taurocyamine was much greater than that of β-guanidino-propionic acid so that presumably the sulphonylic group interacts better with the enzyme than does the carboxyl group. The phosphorylation of γ-guanidino-n-butyric acid and the non-phosphorylation of glycocyamine suggests that TPT would react with the higher but not the lower homologue of taurocyamine but these were not available for test.

A comparison of the present results with those obtained for APT (Chapter 1) and CPT (Kuby et al., 1954) shows points of similarity in their properties. All are dependent upon intact -SH groups for their activity and are activated by Mg$^{2+}$ and Mn$^{2+}$. The pH optima for the forward reaction in each case is about 6.5 - 7 and for the reverse reaction is about pH 8.5.

SUMMARY

1. A method is described for the purification of TPT from Arenicola assimilis.

2. The enzyme had a pH optimum of 6.0 - 6.6 and 8.2 - 8.8 in the forward and reverse reactions respectively, as expressed by the equation

\[ \text{PT} + \text{ADP} \xrightarrow{\text{Taurocyamine} + \text{ATP}} \]

3. Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$ and Co$^{2+}$ activated the enzyme in both the
forward and reverse reactions.

4. The enzyme phosphorylated β-guanidino-propionic acid and Y-guanidino-n-butyric acid. There was no phosphorylation of glycocyanine, creatine, arginine, 2-guanidino-ethanol, ethyl-guanidine.

5. There was inhibition of enzyme activity by EDTA and 2:4-dinitrophenol. The -SH nature of the enzyme was established as a result of the inhibition by iodoacetate, p-CMB and N-ethyl maleimide.

6. AMP could not act as an acceptor in the forward reaction. ITP or ADP could not replace ATP in the reverse reaction.
CHAPTER 6.

A STUDY OF THE DISTRIBUTION OF GUANIDINE PHOSPHORYLTRANSFERASES AND THEIR SUBSTRATES

IN ECHINOIDS AND TUNICATES
CHAPTER 6

A STUDY OF THE DISTRIBUTION OF GUANIDINE
PHOSPHORYLTRANSFERASES AND THEIR SUBSTRATES IN
ECHINODIDS AND TUNICATES.

INTRODUCTION

The phylum Echinodermata and the sub-phyla Urochordata (Tunicata) and Hemichordata (Enteropneusta), assumed prominence in the field of comparative biochemistry as a result of the work of Needham et al. (1932) who found that the echinoid Strongylocentrotus lividus and the hemichordate Balanoglossus salmoneus contained both PC and PA. Earlier, Meyerhof (1930) had advanced the hypothesis that PA was the characteristic phosphagen of invertebrate muscle, whilst PC was characteristic of the vertebrates. The occurrence of both phosphagens in echinoids and hemichordates thus lent biochemical support (Needham et al., 1932) to the morphological data upon which Bateson (1886) based his theory that the vertebrates evolved from an echinoderm stock by way of the hemichordates. The distribution of phosphagens thus appeared to follow the general pattern proposed by Meyerhof (1930), namely, that PC was a typical chordate characteristic (see Table 1, Appendix 1). The one exception was the tunicates which were stated to
contain PA only (Needham et al., 1932). This claim was based on equivocal chemical evidence but has been generally accepted (Baldwin, 1948; Florkin, 1949; Wald, 1952) in spite of the claim by Borsuk et al. (1933) that tunicates contain PC.

Later work has shown that the premises on which Meyerhof's hypothesis were based are not correct. It has been shown that PC is not confined to vertebrates and those animals intermediate between vertebrates and invertebrates, but that it is also present in sponges (Roche and Robin, 1954) and annelids (Hobson and Rees, 1955). Moreover, other phosphorylated guanidines, e.g. PT, PG and PL, also occur in invertebrate muscle (Thoai et al., 1953 a, b; Thoai and Robin, 1954).

Much of the earlier work on echinoids and tunicates was concerned with the study of the distribution of guanidine compounds. Arnold and Luck (1933) showed that arginine was present in the jaw muscles of Strongylocentrotus franciscanus, whilst Holtz and Thielmann (1924) isolated arginine from Arbacia pustulosa. The isolation of arginine from the tunicates Ciona intestinalis and Phallusia mammilata was claimed by Flossner (1932). The first positive identification of creatine in echinoids was made by Greenwald (1946), who isolated potassium creatinine picrate from the testes of Strongylocentrotus sp. Greenwald also identified creatine in the tunicates, Boltenia sp. and Microcosmus sp.
In all cases the identification of PC and PA in echinoids, tunicates and hemichordates has been based on the differential acid hydrolysis of these compounds in the presence of molybdate (Needham et al., 1932; Borsuk et al., 1933; Baldwin and Needham, 1937; Baldwin and Yudkin, 1950). This method is unsatisfactory as a means of identification of phosphagens (Chapter 4) and it is apparent that new specific methods for the detection and identification of creatine, arginine, PC and PA are required. Such methods, utilising colorimetric guanidine analysis, paper chromatography and specific enzyme assays, have been developed and will be described in the experimental section which follows.

Little work has been done on the echinoid and tunicate enzymes which catalyse the phosphoryl group transfer reactions between ATP and arginine or creatine. Baldwin and Needham (1937) showed that extracts of the jaw muscles of Paracentrotus lividus and Sphaerechinus granularis were capable of phosphorylating creatine and arginine. Yudkin (1954) showed that extracts of the jaw muscles of the echinoids Diadema setosum, Lytechinus variegatus, Tripneustes esculentus, and Echinometra lacunter were able to phosphorylate both arginine and creatine when incubated with ATP at pH 7.8. Jaw muscle extracts of the echinoid Arbacia punctulata were able to phosphorylate arginine only. There have been no reports of the presence of CPT or APT in tunicates.
Two echinoid species, *Centrostephanus rodgersii* and *Heliocidaris erythrogramma* Valenciennes; and two tunicate species *Pyura stolonifera* Heller and *Pyura subculata* have been investigated for guanidine and phosphagen content. An investigation of the guanidine-phosphoryltransferases involved in the metabolism of these compounds has also been carried out. A hemichordate, *Balanoglossus* sp. was similarly investigated.

**EXPERIMENTAL**

**Materials**

**Animals.**

Specimens of *Centrostephanus rodgersii* and *Heliocidaris erythrogramma* were collected in the Sydney area during the summer months. The gonads and jaw apparatus (Aristotle's lantern) were excised and frozen immediately by dropping into liquid $N_2$. When the tissues had reached the temperature of liquid $N_2$, they were removed, placed in polythene bags and stored in solid $CO_2$ for transport to the laboratory. No distinction was made between male and female gonads.

*Balanoglossus* sp. was collected in the Sydney area and frozen whole by the procedure described above.

The tunicate species examined, *Pyura stolonifera* Heller and *Pyura subculata* were collected on the south coast of New South Wales. The sphincter muscles of the mouth and atrial
region were excised immediately after collection, cleaned of fat and portions of the gonads and alimentary canal and were then frozen by the procedure described above.

**Phosphagens and guanidine compounds.**

The compounds used have been described previously in Chapter 1.

**Arginase.**

Arginase was prepared from ox liver by the method of Greenberg (1951). The preparation was taken to stage F and freeze-dried. The enzyme was dissolved in 0.05M N-ethyl morpholine buffer (pH 8.4) before use. Such solutions contained sufficient Mn^{2+} to activate the enzyme but insufficient to interfere with the colorimetric method for the estimation of guanidines.

**Creatine phosphorlytransferase.**

CPT was prepared as described by Ennor and Rosenberg (1954a) by Dr. H. Rosenberg of this department. The freeze-dried enzyme was dissolved in 0.05M N-ethylmorpholine buffer of the appropriate pH before use.

**Arginine phosphorlytransferase.**

APT was prepared as described in Chapter 1 and stored as a 2.3% (w/v) solution in 50% (v/v) glycerol. Dilutions of the enzyme were made with 0.05M N-ethyl morpholine buffer of the appropriate pH.
Methods

Estimation and detection of guanidines.

The guanidine content of tissue extracts was estimated by the colorimetric method of Rosenberg et al. (1956). Guanidines were demonstrated on paper chromatograms by spraying with a 1:1 mixture of 5 N NaOH and the α-naphthol-diacetyl reagent of Rosenberg et al. (1956). Monosubstituted guanidines were demonstrated also by the method of Acher and Crooker (1952).

Estimation of protein.

Protein was estimated colorimetrically by the biuret method of Gornall, Bardawill and David (1949), crystalline bovine serum albumin being used as the standard.

Study of guanidines

Extraction of guanidines.

The method used for the extraction of guanidines from tissues was essentially that of Thoai et al. (1953b). The frozen material was comminuted in a Waring blender with 2 vol. of water and the mixture then adjusted to pH 3 by the addition of 10N H₂SO₄. (Large amounts of CO₂ were evolved on the addition of acid to extracts of the jaw apparatus of echinoids). It was then boiled for 2 min., cooled and the precipitate removed by centrifuging. Samples were withdrawn for the determination of the guanidine content and the remainder treated with a saturated
solution of basic lead acetate until there was no further precipitation, and the excess of lead was removed with $\text{H}_2\text{S}\cdot$

All the extracts obtained were clear, but were sometimes coloured and in these cases the colour was removed by treatment with a small amount of active charcoal. The extract was then freeze-dried and the residue dissolved in a small volume of water; insoluble material was removed by centrifuging and the supernatant examined enzymically and by paper chromatography.

**Paper chromatography.**

Paper chromatographic examination of the tissue extracts was carried out by the ascending method at room temperature ($20 - 22^\circ$). Sheets of Whatman no. 3MM paper (12 in. x 12 in.) were used as a better resolution of guanidines was obtained than with Whatman no. 1 paper.

Many solvent systems were investigated, including those recommended by Block and Zweig (1955) and Roche, Thoai and Hatt (1954). Development of tissue extracts in alkaline solvents led to excessive 'tailing' of the guanidine spots, and there was not sufficient resolution of guanidines in the acidic solvents used by these authors to enable positive identification. As will be seen below, extracts of the tissue of *H. erythrogramma*, *Balanoglossus sp.*, *P. stolonifera* and *P. subculata*...
contain arginine and creatine, and excellent separation of these guanidines was obtained with n-butanol-6N HCl (70:30, v/v; Makisumi, 1952) and phenol - water (60:20, w/v) saturated with SO₂ (Crawford, 1951). The following solvents were used in conjunction with n-butanol - 6N HCl and phenol - water - SO₂ even though the separation of guanidines was not as good; n-propanol-NH₃ solution (sp. gr. 0.88) - water (60:30:10, Hanes and Isherwood, 1949); pyridine - isopentanol - water (60:40:70, Roche et al., 1954); acetone - n-propanol - NH₃ solution (2N) - water (50:10:30:10); acetone - NH₃ solution (2N) - water (60:30:10), and n-propanol - dioxan - NH₃ solution (2N) (40:10:50).

The guanidines present in tissue extracts were further characterised by enzymic means as the presence of impurities made the preparation of crystalline derivatives difficult. Moreover, the enzymes used (arginase, CPT and APT) show such a high degree of specificity that they provide an excellent means for the characterisation of arginine and creatine.

Enzymic characterisation and estimation of arginine.

Tissue extracts, adjusted to pH 8.5 with N-NaOH, were incubated with arginase in a system which contained 0.1 ml. of extract, 0.1 ml. of N-ethyl morpholine buffer (0.5M, pH 8.5), 0.1 ml. of arginase (containing 0.1 - 1.0mg. of protein) and 0.7 ml. of water. The mixture was incubated at 37° for 1 - 2
hr., after which 1.0 ml. of a EDTA-NaOH mixture containing 0.4 ml. of 0.2 M EDTA (adjusted to pH 7.6) and 0.6 ml. of 5N NaOH was added. The presence of arginine was detected by estimation of the total guanidine before and after incubation with arginase. By this means it was also possible to estimate the amount of arginine in the extract as it is almost completely destroyed by arginase. Appropriate control experiments were always carried out to ensure that the extracts contained no substances capable of inhibiting arginase activity and thus producing misleading results.

Enzymic characterisation of arginine and creatine, using CPT and APT.

Arginine and creatine were also characterised by incubation with APT and CPT in a system which contained 0.1 ml. of extract (pH 8.5), 0.1 ml. of ATP (5 x 10^{-2}M), 0.1 ml. of MgSO_4(10^{-2}M), 0.1 ml. of N-ethyl morpholine buffer (0.5M, pH 8.5), 0.2 ml. of APT or CPT containing 1 mg. of protein and 0.4 ml. of water. The reaction mixture was incubated at 5°C for 1 - 2 hr. and the guanidine disappearance measured after the addition of 1.0 ml. of the EDTA-NaOH mixture described above.

When chromatographic analysis of the guanidines present in tissue extracts indicated the presence of both creatine and arginine, enzymic identification of the guanidines was carried out by colorimetric estimation after each of the
following treatments:
(1) incubation of samples of the extract with APT and CPT;
(2) incubation of a sample of the extract with CPT after destruction of the arginine with arginase;
(3) Incubation of the separated guanidine with the appropriate guanidine phosphoryltransferase.

For this purpose arginine and creatine were separated by passing the extract through a column of Zeo-Karb 216 (Na form) when creatine passed through. The column was washed with water and the adsorbed arginine eluted with 2N NH₃. The eluate was freeze-dried after removal of most of the ammonia by aeration. This method was used only for the extracts from the jaw muscles of H. erythrogramma which contained an unknown inhibitor of arginase.

**Study of the phosphagens**

**Isolation of phosphagens.**

The jaw apparatus of echinoids and tunicate muscle were extracted with 2 vol. of ice-cold 9% (w/v) TCA by treatment in a Waring blender for 2 min. The mixture was filtered on a Buchner funnel (Whatman no. 531 paper) and the filter cake extracted with 1 vol. of ice-cold 5% (w/v) TCA. After filtration the filtrates were combined, adjusted to pH 9 by the addition of 10 N NaOH and the resultant precipitate was removed by centrifugation. These operations were carried out
in the cold room at 5°. The pH of the extract was then adjusted to 8.2 by the addition of 5 N HCl, excess of barium acetate (M) was added and the pH readjusted to 8.2. The insoluble barium salts were removed by centrifugation. To the supernatant was added 4 vol. of ethanol, the pH was adjusted to 8.2 and the precipitate allowed to settle overnight. The precipitate was removed by centrifugation, washed with ethanol and ether and dried in vacuo.

Echinoid and tunicate gonads were extracted as described above, but 1 vol. of ethanol was added to the TCA extract before neutralisation in order to precipitate the glycogen. Further fractionation was carried out as described by Le Page (1951).

The water soluble, ethanol insoluble barium salts containing the phosphagen were suspended in a small volume of water and sufficient 5 N HCl to effect solution was added. The solution was immediately adjusted to pH 9 by the addition of 5N NaOH. The inorganic P so precipitated was removed by centrifugation and discarded; 4 vol. of ethanol was added to the supernatant and the barium salt of the phosphagen collected by centrifugation, washed with ethanol and ether and dried in vacuo.

**Preparation of the sodium salts of the phosphagens.**

Conversion of the barium salts into sodium salts by the addition of Na₂SO₄ led to a marked loss of phosphagen when PC was present,
so that an ion-exchange column was used from which 85% recoveries were obtained. Solutions of the barium salts were passed through a column of Zeo-Karb 225 (Na form). The effluent was freeze-dried and the residue dissolved in a small volume of water. In no case did the material so prepared contain guanidine other than that bound to P.

**Paper chromatography.**

Paper chromatography of the tissue phosphagens in solvent systems used by other authors, e.g. propanol - NH$_3$ - water (Hobson and Rees, 1955), pyridine - isopentanol - water (Roche et al., 1954), proved unsatisfactory owing to the marked 'tailing' of the compounds. Moreover, there was such poor separation of the marker phosphagens, PA, PC, PT and PG, that these solvents could not be relied upon for use in positive identification of unknown phosphagens. Attempts were made to find solvents which would give good separation of the various phosphagens, but they were unsuccessful. The phosphagens were therefore identified by chromatography of the free bases after acid hydrolysis. The barium salts of the phosphagens were dissolved in N HCl and allowed to hydrolyse by standing at room temperature for 24 hr. Alternatively, the solutions of the sodium salts of the phosphagens were spotted on the paper and hydrolysed by allowing the paper to remain for 1 hr. in a jar filled with HCl vapour before development with solvent. Both
procedures were effective in hydrolysing the phosphagens of echinoid and tunicate tissues. The conditions for chromatography were the same as those described previously.

**Enzymic characterisation of the phosphagens.**

Solutions of the sodium salts of the phosphagens were incubated separately with APT and CPT (1mg. of protein/ml.) for 1 hr. at 5°C under the conditions described for the determination of APT activity in the forward reaction (see Chapter 1). The release of arginine or creatine by the respective phosphoryltransferases (which contained no phosphatase or phosphoamidase activity) in the presence of ADP was taken as evidence for the presence of the relevant phosphagen.

**Study of the guanidine phosphoryltransferases**

The jaw apparatus of both echinoids and the muscle of both tunicates were examined for the presence of guanidine phosphoryltransferases. The frozen tissue was passed twice through a mincer and extracted with 2 vol. of water by stirring mechanically for 30 min. The mixture was centrifuged for 15 min., the residue re-extracted with 1 vol. of water for 30 min., centrifuged, and the supernatants were combined. The extract was then fractionated with solid (NH₄)₂SO₄, which was added with mechanical stirring. After centrifuging at 5000g. for 5 min., the supernatants were carefully poured off and the
protein precipitates obtained were dissolved in the minimum amount of water. Enzyme fractions from *Pyura stolonifera* were dialysed overnight against 0.01 M phosphate buffer, pH 7.0, before testing for CPT and APT activities. All operations were carried out at 5°C. APT and CPT activities were determined under the same conditions as described in Chapter 1 for the determination of APT activity. In each case, the appropriate controls for phosphoamidase activity were set up.

RESULTS

(a) Guanidines.

Paper chromatography showed that both creatine and arginine were present in *H. erythrogramma*, *P. stolonifera* and *P. subculata*, but only trace amounts of arginine were present in both species of *Pyura*. In extracts of *C. rodgersii* only arginine was detected whilst in *Balanoglossus* sp. both creatine and arginine were identified. Extracts of *Balanoglossus* also contained a third unidentified guanidine which was present in approximately the same concentration as arginine. The results of the investigation of the guanidine content of the above animals are summarised in Table 20.

(b) Phosphagens.

A study of the phosphagens present (Table 21) shows that PA only is present in the jaw muscles and gonads of


**TABLE 20.**

Distribution of guanidines in *C.rodgersii*, *H.erythrogramma*,
*P.stolonifera*, *P.subculata* and *Balanoglossus sp.*

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tissue</th>
<th>Guanidine content (mg./100g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Creatine</td>
</tr>
<tr>
<td><em>C.rodgersii</em></td>
<td>Jaw muscles</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Gonads</td>
<td>0</td>
</tr>
<tr>
<td><em>H.erythrogramma</em></td>
<td>Jaw muscles</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Gonads</td>
<td>77</td>
</tr>
<tr>
<td><em>P.stolonifera</em></td>
<td>Sphincter muscle</td>
<td>73</td>
</tr>
<tr>
<td><em>P.subculata</em></td>
<td>Sphincter muscle</td>
<td>78</td>
</tr>
<tr>
<td><em>Balanoglossus sp.</em></td>
<td>Whole animal</td>
<td>24*</td>
</tr>
</tbody>
</table>

* In these cases arginine was detectable by paper chromatography, but was less than 1% of the total guanidine present.

* Only an approximate value for the creatine content can be given as an unidentified guanidine of approximately the same concentration as arginine was present.
TABLE 21.

Distribution of phosphagens in *C. rodgersii*, *H. erythrogramma*, *P. stolonifera* and *P. subculata*.

Methods for the identification of phosphagens have been described in the text.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tissue</th>
<th>Phosphagen</th>
<th>Guanidine liberated by</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. rodgersii</em></td>
<td>Jaw muscles</td>
<td>PA</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Gonads</td>
<td>PA</td>
<td>+</td>
</tr>
<tr>
<td><em>H. erythrogramma</em></td>
<td>Jaw muscles</td>
<td>PA &amp; PC</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Gonads</td>
<td>PA &amp; PC</td>
<td>+</td>
</tr>
<tr>
<td><em>P. stolonifera</em></td>
<td>Sphincter muscle</td>
<td>PC</td>
<td>o</td>
</tr>
<tr>
<td><em>P. subculata</em></td>
<td>Sphincter muscle</td>
<td>PC</td>
<td>o</td>
</tr>
</tbody>
</table>
C. Rodgersii, whereas these tissues in H. erythrogramma contain both PA and PC. No detailed investigations were carried out to determine the relative amounts of each of the phosphorylated guanidines in the tissues of H. erythrogramma, but qualitative analyses indicated that the ratio of PA : PC was about the same as that of the free guanidines.

In P. stolonifera and P. subculata PC only was identified. Repeated attempts to demonstrate the presence of PA were unsuccessful, even when kilogram quantities of muscle were used. It was thus concluded that PA was not present or was present in concentrations less than 1% of that of PC.

No phosphagens were detected on examination of extracts of Balanoglossus sp. The material used appeared to have deteriorated on storage and this, coupled with the fact that only small quantities were available, is a possible reason for non-detection of phosphagens.

(c) Guanidine phosphoryltransferases.

Examination of aqueous extracts of the jaw muscles of C. Rodgersii showed that only APT was present. Fractionation of the extract (Table 22) showed that the major portion of the enzymic activity was precipitated between the addition of 42 and 56 g of (NH$_4$)$_2$SO$_4$/100 ml. No CPT activity was detected in the original extract or in any of the fractions obtained as a result of (NH$_4$)$_2$SO$_4$ fractionation. The APT isolated (purified
 TABLE 22

APT and CPT Activity of Fractions isolated by Ammonium Sulphate Fractionation of Extracts (100 ml.) of the Jaw Apparatus of *C. rodgersii* and *H. erythrogramma*.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>C. rodgersii</th>
<th>H. erythrogramma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APT</td>
<td>APT</td>
</tr>
<tr>
<td></td>
<td>Total Units</td>
<td>Total Units</td>
</tr>
<tr>
<td>Original</td>
<td>230</td>
<td>12</td>
</tr>
<tr>
<td>1</td>
<td>68</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>244</td>
<td>10.4</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>74.0</td>
</tr>
<tr>
<td>6</td>
<td>625</td>
<td>81.0</td>
</tr>
</tbody>
</table>

A unit of enzyme activity is defined as the amount of enzyme which releases 1 µmole of creatine or arginine from PC or PA in 1 min. at pH 7.2 and 5°.

Specific activity (S.A.) is defined as the number of units of enzyme activity per mg. of protein.

Fraction 1 was obtained by the addition of 21g. of (NH₄)₂SO₄ to the extract. Fractions 2-5 inclusive were obtained by the stepwise addition of 7g. of (NH₄)₂SO₄ to the supernatant from the preceding fraction. Fraction 6 was obtained by the addition of 14g. of (NH₄)₂SO₄ to fraction 5 (or fraction 4 in the case of *C. rodgersii*).
3.6 fold) did not phosphorylate creatine, glycocyamine or taurocyamine when tested under the standard conditions for the reverse reaction (see Table 4, Chapter 1). The enzyme is similar to that isolated from sea-crayfish muscle (see Chapter 1) in that it is activated by $\text{Mg}^{2+}$ and $\text{Mn}^{2+}$ but not by $\text{Ca}^{2+}$ ions (Fig. 37).

Aqueous extracts of the jaw muscles of *H. erythrogramma* contained both APT and CPT, which could be separated by $(\text{NH}_4)_2\text{SO}_4$ fractionation. Table 22 shows that CPT is precipitated between the addition of 28 and 35g. of $(\text{NH}_4)_2\text{SO}_4/100$ ml., whereas APT is precipitated between the addition of 42 and 63g. of $(\text{NH}_4)_2\text{SO}_4/100$ ml. It is also clear that the total units of activity of APT and CPT are approximately equal. The APT isolated was activated by $\text{Mg}^{2+}$ and $\text{Mn}^{2+}$ ions but not by $\text{Ca}^{2+}$ ions, and in this respect is similar to APT isolated from crayfish muscle and from *G. rodgersii*. The CPT, on the other hand, was activated by all three cations and is thus similar to CPT isolated from rabbit muscle (Rosenberg and Ennor, 1955; Kuby et al., 1954). A comparison of the activation of the two enzymes by $\text{Mg}^{2+}$, $\text{Mn}^{2+}$ and $\text{Ca}^{2+}$ ions is shown in Fig. 38.

Aqueous extracts of the sphincter muscles of *P. stolonifera* and *P. subculata* contained both CPT and APT. The APT activity was extremely low, being barely detectable after a 12-15 hr. incubation. The CPT and APT activities of *P. stolonifera* were
Fig. 37. Activation by bivalent metals of APT from the jaw muscles of *C. rodgersii*. Fractions were isolated and activity determinations carried out in the forward reaction as described in the text. APT, 12μg./ml. incubated for 3 min. at $5^\circ$. 
Fig. 38. Activation by bivalent metals of APT and CPT fractions from the jaw muscles of *Heliocidaris erythrogramma*. Fractions were isolated and activity determinations carried out in the forward reaction as described in the text.

☐ CPT fraction; ☐, APT fraction.
only partially separable by ammonium sulphate fractionation.
The major portion of the CPT activity was precipitated between
the addition of 35 and 42g. of \((\text{NH}_4)_2\text{SO}_4/100\) ml. of extract,
whilst the major portion of the APT activity was precipitated
between the addition of 42 and 49g. \((\text{NH}_4)_2\text{SO}_4/100\) ml. of extract,
(see Table 23). These results suggest that two different enzymes
are responsible for the CPT and APT activities and evidence in
support of this conclusion was obtained by studies of the metal
activation of the CPT and APT fractions. The CPT activity was
increased in the presence of \(\text{Mg}^{2+}, \text{Mn}^{2+}\) and \(\text{Ca}^{2+}\) ions, whilst
the APT activity was increased in the presence of \(\text{Mg}^{2+}\) and \(\text{Mn}^{2+}\)
ions only (Fig. 39, 40).

Table 24 shows the effect of inhibitors on CPT isolated
from \textit{P. stolonifera}. The inhibition by iodoacetate, p-CMB, and
\(\alpha\)-iodosobenzoate characterises the CPT of \textit{P. stolonifera} as a
\(-\text{SH} \) enzyme.

**DISCUSSION**

The present work has clearly established that the echinoid
\textit{Centrostephanus rodgersii} contains only one phosphagen (PA) and
thus provides the second example of a mono-phosphagen type in
this group. The first example was described by Baldwin and Yudkin
(1950), who found PA only in \textit{Arbacia punctulata}. The presence
of PG in the two tunicate species examined has also been clearly
TABLE 23.

APT and CPT Activity of Fractions isolated by Ammonium Sulphate Fractionation of Extracts (100 ml.) of the Sphincter Muscles of P. stolonifera.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>10^3 Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APT</td>
</tr>
<tr>
<td>Original</td>
<td>(0)</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.86</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>5</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Activities were determined under the conditions of the forward reaction as previously described.

Fraction 1 was obtained by the addition of 28g. of ammonium sulphate to the extract. Fractions 2-5 inclusive were obtained by the stepwise addition of 7g. of ammonium sulphate to the supernatant from the previous fraction.

* This fraction was used for all experiments on CPT activity.

# This fraction was used for all experiments on APT activity.
Fig. 39. Activation by bivalent metals of CPT isolated from *P. stolonifera*. Fraction 3 was isolated as described in Table 23; activity determinations were carried out as described in the text. Temperature 5°C.
Fig. 40. Activation by bivalent metals of APT isolated from *P. stolonifera*. Fraction 4 was isolated as described in Table 23. Activity determinations were carried out in the forward reaction as described in the text. Temp. 5°.
TABLE 24.

Effect of inhibitors on activity of CPT isolated from P. stolonifera.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodoacetate</td>
<td>10.0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>29</td>
</tr>
<tr>
<td>p-CMB</td>
<td>0.01</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>0</td>
</tr>
<tr>
<td>o-iodosobenzoate</td>
<td>0.01</td>
<td>100</td>
</tr>
<tr>
<td>N-ethylmaleimide</td>
<td>0.1</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>18</td>
</tr>
<tr>
<td>Diphenylchloroarsine</td>
<td>0.1</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>12</td>
</tr>
<tr>
<td>Arsenite</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>2:4-Dinitrophenol</td>
<td>1.0</td>
<td>0</td>
</tr>
</tbody>
</table>

The enzyme (200μg of protein) was incubated for 5 min. at 5° in the presence of the inhibitor, N-ethyrmorpholine buffer (0.05M, pH 7.0), PC (5 x 10⁻³M), and MgSO₄ (5 x 10⁻³M). The reaction was started by the addition of ADP (0.1 ml. of 10⁻²M solution), and stopped with 1 ml. of EDTA-NaOH mixture after 5 min. Temperature 5°.
demonstrated. The general assumption (cf. Baldwin and Needham, 1937; Florkin, 1949; Wald, 1952) that both PA and PC are found in echinoids, and PA only in tunicates, is thus disproved and the results emphasise the necessity for examining a number of genera within a class before concluding that a particular phosphagen(s) is characteristic of that class.

Arginine and creatine were the only guanidines detected in echinoids and tunicates by the paper chromatographic techniques used. Arginine, creatine and a third, unidentified, guanidine were detected in extracts of Balanoglossus sp. It is also possible that other guanidines were present at concentrations less than 1% of the total guanidine. Thus it might have been expected that H. erythrogramma and P. stolonifera and P. subculata, which contain PC, would also contain trace amounts of glycocyamine in view of the results of Thoai and Robin (1951) and Roche, Thoai, Robin, Garcia and Hatt (1952), which indicated the presence of glycocyamine and the enzyme methylating glycocyamine in Paracentrotus lividus and Sphaerechinus granularis.

Although Baldwin and Needham (1937) and Yudkin (1954) showed that extracts of echinoid jaw muscles were able to phosphorylate both creatine and arginine, their results did not indicate whether this phosphorylation was carried out by one or two enzymes. The present work clearly establishes the presence of two enzymes of approximately equal activities in H. erythro-
Moreover, the properties of the APT and the CPT present are similar to those of APT isolated from crustacean muscle (see Chapter 1), and CPT isolated from rabbit muscle (Kuby et al., 1954). The jaw apparatus of echinoids contains seven different groups of muscles (Hyman, 1955) and it has been shown by Needham et al. (1932) that both the protractor and retractor muscles of *Strongylocentrotus lividus* contain PA and PC. It would seem, therefore, that in this species and in *H. erythrogramma* both phosphagens are present in the same muscles and it follows that the free guanidines and the guanidine phosphoryltransferases are also present.

CPT and APT were present in both the tunicate species examined. The two enzymes were partially separated by ammonium sulphate fractionation and were shown to have the same general properties as crustacean APT and mammalian CPT. All the phosphoryltransferases examined, echinoid and tunicate, exhibit the same behaviour towards $Mg^{2+}$, $Mn^{2+}$ and $Ca^{2+}$, and enzyme inhibitors as the respective phosphoryltransferases from the muscles of crustaceans and mammals. The specific activities of the aqueous extracts of tunicate muscle were very low as compared with those obtained with echinoid extracts and the APT activity was only 5 - 6% of the CPT activity.

The low phosphoryltransferase activity in tunicates as compared with echinoids may be correlated with the speed of
action and function of the muscle masses concerned. Thus, tunicate muscle exhibits very slow cycles of contraction and relaxation which are concerned with intake and expulsion of sea-water from the atrial cavity. Echinoid muscles, on the other hand, exhibit a much higher degree of activity and load-bearing capacity. A similar correlation is indicated from the work of Tonomura et al. (1955) who have shown that homogenates of the fast adductor muscle of Pecten possess much higher APT activity than do those of the slow adductor muscle, and further investigation of such a correlation would be of interest in the light of recent theories of the role of the guanidine phosphoryltransferase systems in muscular contraction (see Morales and Botts, 1956).

The occurrence of PA and PC and their respective phosphoryltransferases in some echinoids and of APT and CPT in tunicates presents a peculiar problem in muscle metabolism. It is difficult to picture the functional interrelationship which might exist between the two phosphagens and the respective enzymes, for although the role of PA in invertebrate muscle appears to be similar to that of PC in vertebrate muscle, it is hard to conceive of the two phosphagens both serving as 'reservoirs' for the adenylic system in the same muscle. However, in the absence of evidence to the contrary, it must be assumed that such is the role of the two phosphagens, although the
possibility (Ennor and Rosenberg, 1952) that one or other of these phosphagens "...may contribute to endergonic reactions directly and without the mediation of the latter (adenylic) system..." must be borne in mind. (In this connection, the recent demonstration of the synthesis of PC by a direct reaction between creatine and phosphoglycerate catalysed by mammalian muscle extracts is of interest; Cori, Abarca, Frankel and Traverso-Cori, 1956). It is not clear how the function of each phosphagen may be determined. Ennor and Rosenberg (1954b) have shown that the incorporation of $^{32}$P into PC in rabbit muscle is extremely rapid, and it is likely that this would also occur with PA and PC in echinoid muscle. It would thus be difficult to detect any differential incorporation of $^{32}$P into the two phosphagens, so that the application of $^{32}$P to the problem would seem of little value.

**Phylogenetic considerations.**

The important position that phosphagen and guanidine phosphor-yltransferase distribution holds in theories of biochemical evolution has been well documented (Meyerhof, 1941; Baldwin, 1948; Florkin, 1949 and Wald, 1952). The discovery of new phosphagens, coupled with the discovery of a wider distribution of PC in invertebrates has disproved the hypothesis that PC is a typically chordate characteristic and raises the question whether a correlation between phosphagen distribution and
phylogeny is strictly valid. The subject was last reviewed in 1950 by Baldwin and Yudkin (1950) and a reassessment in view of recent results would seem to be appropriate (see Table 2, Appendix 1).

Present evolutionary theories based on phylogenetic considerations are conveniently summarised in a 'phylogenetic tree' (see Fig. 41). The Metazoa, above the helminth level of organisation, are broadly separable into two main groups. One, comprising phyla Mollusca, Arthropoda and Annelida, differs fundamentally from the other, which includes the phyla Echinodermata and Chordata. The Molluscan and Arthropod lines of evolution which culminate in the highly organised and complex groups, the Cephalopods and Insects, all contain PA and the associated phosphoryltransferase. The Echinoderm-Chordate line shows a gradual change in phosphagen content from PA to PC culminating in the vertebrates which contain PC only (Table 25). There is thus evidence of a relationship between echinoderms, hemichordates and vertebrates such as was postulated on morphological and embryological grounds by Bateson (1886). The demonstration of PC and CPT in tunicates described above, is consistent with and provides biochemical support for, the accepted phylogenetic classification of the tunicates. These results remove an anomaly based on the results of Needham et al. (1932) who claimed that PA was present in tunicates. The
Fig. 41. Phylogenetic tree (modified from Prosser et al., 1950).
TABLE 25.

Distribution of Phosphagens and Guanidine Phosphoryl-transferases in Echinodermata and Chordata.

<table>
<thead>
<tr>
<th></th>
<th>PA</th>
<th>PC</th>
<th>APT</th>
<th>GPT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Echinodermata</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crinoidea</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asteroidea</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holothuroidea</td>
<td>+</td>
<td>-</td>
<td>*</td>
<td>+</td>
</tr>
<tr>
<td>Echinoidea</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>or</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ophiuroidea</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chordata</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemichordata</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urochorda</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cephalochorda</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vertebrata</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

This table has been compiled from the results of Baldwin & Needham (1937); Baldwin & Yudkin (1950) and the present work.

* Verbinskaya, Borsuk & Kreps (1935) claim both PA and PC to be present in *Cucumaria frondosa*; Greenwald (1946) has isolated creatine from the testes of *Holothuria tubulosa* and *Cucumaria frondosa*.

Φ Recent work of Baldwin & Yudkin (1950) indicates that PC only is present in Hemichordates. The present work indicates that the main guanidine present in *Balanoglossus sp.* is creatine.
demonstration of PC in tunicates is of especial interest in the light of the recent theory of vertebrate evolution proposed by Berrill (1955). This author has advanced a theory of vertebrate evolution from tunicate ancestors based on an intensive study of the embryological development of these groups.

It can be seen from Table 25 and Fig. 41 that a correlation between phosphagen distribution and phylogeny does exist within the Echinoderm-Chordate line of evolution. The dangers of drawing premature conclusions are obvious in the light of previous theories of the comparative biochemistry of phosphagens (Meyerhof, 1930; Needham et al., 1932). Present views are aptly described in the words of Baldwin and Yudkin (1950) - '...the nature of the phosphagen is merely one character and cannot, any more than any other single character such, for example, as metamericism, be regarded as the sole basis of, but only as supporting evidence for, phylogenetic relationships...'.

Examination of the phosphagen content of a large number of genera from many phyla must be made before definite conclusions can be drawn as to the phylogenetic implications of phosphagen distribution.
SUMMARY

1. Two echinoids, Centrostephanus rodgersii and Heliocidaris erythrogramma Valenciennes, and two tunicates Pyura Stolonifera Heller and Pyura subculata, have been examined for the presence of free guanidines, phosphagens and their respective guanidine phosphoryltransferases.

2. *C. rodgersii* has been shown to contain arginine, PA and APT. *H. erythrogramma*, on the other hand, contained both creatine and arginine together with PC, PA, CPT and APT.

3. The CPT and APT in *H. erythrogramma* are readily separable and are similar in properties to those enzymes isolated from vertebrate and invertebrate sources.

4. *P. stolonifera* and *P. subculata* have been shown to contain creatine and arginine (trace amounts), PC, CPT and APT.

5. The CPT and APT in *P. stolonifera* are partially separable by ammonium sulphate fractionation and are similar in properties to those enzymes isolated from vertebrate and invertebrate sources.

6. Methods for the chromatographic and enzymic characterization of creatine and arginine are described.
APPENDIX 1

THE DISTRIBUTION OF PHOSPHAGENS IN INVERTEBRATES
APPENDIX 1

THE DISTRIBUTION OF PHOSPHAGENS IN INVERTEBRATES

The distribution of phosphagens in the invertebrates has been summarized in the following two tables. Table 1, which summarizes the distribution of phosphagens as known in 1932, has been included as it is of value in assessing the evolutionary theories based on the distribution of phosphagens proposed by Meyerhof (1930) and Needham et al. (1932). Results of recent investigations have been incorporated into Table 2, which summarizes the current knowledge of the distribution of phosphagens.
Table 1.

The Distribution of phosphagens in Invertebrates.

The results presented in this Table are those of Meyerhof (1928) and Needham et al. (1932).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Phosphagen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COELENTERATA</strong></td>
<td></td>
</tr>
<tr>
<td>Pleurobrachia pileus</td>
<td>PA</td>
</tr>
<tr>
<td><strong>PLATYHELMINTHES</strong></td>
<td></td>
</tr>
<tr>
<td>Planaria vitta</td>
<td>PA</td>
</tr>
<tr>
<td><strong>NEMERTINEA</strong></td>
<td></td>
</tr>
<tr>
<td>Lineus longissimus</td>
<td>PA</td>
</tr>
<tr>
<td><strong>SIPUNCULOIDEA</strong></td>
<td></td>
</tr>
<tr>
<td>Sipunculus nudus</td>
<td>PA</td>
</tr>
<tr>
<td><strong>ANNELIDA</strong></td>
<td></td>
</tr>
<tr>
<td>Sabellaria alveolata</td>
<td>PA</td>
</tr>
<tr>
<td>Spirographis brevispira</td>
<td>PA</td>
</tr>
<tr>
<td>Nereis diversicolor</td>
<td>PA</td>
</tr>
<tr>
<td><strong>ARTHROPODA</strong></td>
<td></td>
</tr>
<tr>
<td>Astacus fluviatilus</td>
<td>PA</td>
</tr>
<tr>
<td><strong>MOLLUSCA</strong></td>
<td></td>
</tr>
<tr>
<td>Pecten sp.</td>
<td>PA</td>
</tr>
<tr>
<td>Sepia officinalis</td>
<td>PA</td>
</tr>
<tr>
<td>Octopus vulgaris</td>
<td>PA</td>
</tr>
<tr>
<td>Phylum</td>
<td>Phosphagen</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>ECHINODERMATA</strong></td>
<td></td>
</tr>
<tr>
<td>Holothuroidea</td>
<td></td>
</tr>
<tr>
<td>Synapta inhaerens</td>
<td>PA</td>
</tr>
<tr>
<td>Asteroidea</td>
<td></td>
</tr>
<tr>
<td>Asterias glacialis</td>
<td>PA</td>
</tr>
<tr>
<td>Echinoidea</td>
<td></td>
</tr>
<tr>
<td>Strongylocentrotus lividus</td>
<td>PA &amp; PC</td>
</tr>
<tr>
<td><strong>CHORDATA</strong></td>
<td></td>
</tr>
<tr>
<td>Urochordata (Tunicata)</td>
<td></td>
</tr>
<tr>
<td>Ascidia mentula</td>
<td>PA</td>
</tr>
<tr>
<td>Hemichordata (Enteropneusta)</td>
<td></td>
</tr>
<tr>
<td>Balanoglossus salmoneus</td>
<td>PA &amp; PC</td>
</tr>
<tr>
<td>Vertebrata</td>
<td></td>
</tr>
<tr>
<td>All classes</td>
<td>PC</td>
</tr>
</tbody>
</table>
Table 2.

**Biological Distribution of the Phosphagens**

This Table refers only to those reports of the occurrence of the phosphagens and the guanidine phosphoryltransferases for which reasonable evidence has been adduced. It does not include references to reports which are inferential in the sense that they are based on the isolation or detection of a guanidine base. Reference to published work is shown thus ( ).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Phosphagen</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PROTOZOA</strong></td>
<td>unidentified</td>
<td>(22)</td>
</tr>
<tr>
<td><strong>PORIFERA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hymeniacidon caruncula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bowerbank</td>
<td>PA (21)</td>
<td></td>
</tr>
<tr>
<td>Thetia lyncurium</td>
<td>PC (21)</td>
<td></td>
</tr>
<tr>
<td><strong>COELENTERATA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PA (19)</td>
<td></td>
</tr>
<tr>
<td><strong>PLATYHEMINTHES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PA (19)</td>
<td></td>
</tr>
<tr>
<td><strong>SIPUNCULOIDEA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sipunculus nudus</td>
<td>PA (15)</td>
<td></td>
</tr>
<tr>
<td>Phylum</td>
<td>Phosphagen</td>
<td>Enzyme</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------</td>
<td>-------------------</td>
</tr>
<tr>
<td><strong>ANNELIDA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Glycera convoluta</td>
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<td>Aphrodite aculeata</td>
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<td><strong>ARTHROPODA</strong></td>
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<tr>
<td>Crustacea (all genera)</td>
<td>PA (16, 18, 23)</td>
<td>APT (8, 12, 15, 18, 20)</td>
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<td>Insecta (all genera)</td>
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<tr>
<td>MOLLUSCA (all genera)</td>
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<td>APT (13, 27)</td>
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<td>Echinoidea</td>
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<td>Strongylocentrotus drobachiensis</td>
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Table 2 continued

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<td>Sphaerechinus granularis</td>
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<td>Diadema setosum</td>
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<td>Arbacia punctulata</td>
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<td>Lytechinus variegatus</td>
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<td>Tripneustes esculentus</td>
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**Crinoidea**

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**Asteroidea**

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**Ophiuroidea**

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<td>Ophiothrix fragilis</td>
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<td>Synapta inhaerens</td>
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<td>Thyone briareus</td>
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<td>Cucumaria frondosa</td>
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<td>Holothuria tubulosa</td>
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**CHORDATA**

**Urochordata (Tunicata)**

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<td>Ascidia mentula</td>
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<td>Pyura stolonifera</td>
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<td>Pyura subculata</td>
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**Hemichordata (Enteropneuta)**

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<td>Balanoglossus clavigerus</td>
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<td>Saccoglossus kowalevski</td>
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**Cephalochordata**

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**Vertebrata**

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TABLE OF REFERENCES FOR TABLE 2, APPENDIX 1.

APPENDIX 2

THE PREPARATION OF SUBSTITUTED GUANIDINES

AND N-PHOSPHORYL GUANIDINES
APPENDIX 2

THE PREPARATION OF SUBSTITUTED GUANIDINES

AND N-PHOSPHORYL GUANIDINES

INTRODUCTION

In the experiments involving the specificity of APT and TPT a number of guanidino compounds other than arginine or taurocyamine were employed as test substrates. The synthesis of these compounds and of N-phosphoryl glycocyamine is described below.
EXPERIMENTAL AND RESULTS

Materials

All materials employed for synthesis other than O-methyl isourea-HCl and δ-amino-n-valeric acid were commercial samples of varying degrees of purity and were recrystallised from suitable solvents before use.

Preparation of O-methyl isourea-HCl.

This compound was prepared from calcium cyanamide by the method of Kurzer and Lawson (1954) or from monosodium cyanamide by the following method.

20g. of monosodium cyanamide was suspended in 200 ml. of dry methanol and cooled in an ice bath. Dry HCl was then passed through for 1 hour. The precipitate of NaCl was then filtered off and the filtrate concentrated in vacuo to a small volume. O-methyl isourea-HCl crystallises out on cooling. The crystals were collected by filtration and recrystallised from a small volume of hot methanol. The crystals were collected and dried in vacuo over CaCl₂-KOH. Yield 12g. M.pt. 117 - 119⁰. Litt. m.pt. 118 - 119⁰.
Preparation of $\delta$-amino-$n$-valeric acid.

$\delta$-amino-$n$-valeric acid was prepared from cyclopentanone by the following reaction sequence -

\[
\begin{align*}
\text{Cyclopentanone (1)} & \longrightarrow \text{Cyclopentanone oxime (2)} \\
\alpha\text{-piperidone (3)} & \longrightarrow \delta\text{-amino-$n$-valeric acid-HCl.}
\end{align*}
\]

Reaction (1) was carried out by the procedure described by Bosquet (1943) for the synthesis of heptaldoxime. Reactions (2) and (3) were carried out as described by Schniepp & Marvel (1935). m.pt. 92°. Litt. m.pt. 92 - 94°.

Methods

All melting points were determined in a copper block and are uncorrected. N analysis was carried out by the micro-Kjeldahl-technique.

Preparation of Homoarginine ($\alpha$-amino-$\epsilon$-guanidino-$n$-caproic acid). This compound was prepared from lysine by the method of Stevens and Bush (1950). The method used was essentially the same as procedure B of the above authors, except that O-methylisourea-HCl was used instead of the free base.

Repeated attempts to crystallise the final oily product by the method of Stevens and Bush were always unsuccessful. Homoarginine hydrochloride was finally crystallised by dissolving the oil in the minimum volume of hot 95% ethanol and then adding absolute ethanol until a turbidity appeared. Homoarginine
crystallised on slow cooling. Yield 5.4g.; m.pt. 202 - 203°.

Homoarginine hydrochloride was readily recrystallised by dissolving in an equal volume of water and adding 5 vols. of 95% ethanol. Crystallisation took place after prolonged cooling. Yield 4.1g.; m.pt. 206 - 208°. Stevens and Bush state m.pt. is 207 - 209°, %N = 24.8%; theory = 24.9%.

Preparation of Arginine methyl ester dihydrochloride.

This compound was prepared by the method of Fischer and Suzuki (1905). 3g. arginine hydrochloride was suspended in 100 ml. of dry methanol and dry HCl passed through for 1 hour. The arginine hydrochloride dissolved within 10 min. The methanol was removed by distillation in vacuo. The residue (4g.) was dissolved in 95% ethanol and ether added to turbidity. Crystals formed in 2 min. Recrystallised 3 times. Yield 2g. m.pt. 196°; Litt. value 195°. % N = 21.4%. Theory = 21.5% N.

Preparation of α-carbamido-arginine.

This compound was prepared by the method of Boon and Robson (1935). 2g. of arginine hydrochloride and 2g. of potassium cyanate were dissolved by shaking in 16 ml. of water and heating on a water bath for 1 hr. and allowing to stand overnight. A heavy crystalline precipitate formed which was washed with cold water and recrystallised from a small volume
of hot water and dried in vacuo. Yield 1.8g. m.pt. 174°; Litt. value 172°. % N = 31.9. Theory = 32.2% N.

Preparation of 6-guanidino-n-valeric acid.

This compound was prepared by action of O-methylisourea hydrochloride on 6-amino-n-valeric acid hydrochloride, according to the general guanylation procedure of Schutte (1943). The preparation of 6-amino-n-valeric acid is described above.

3.8g. 6-amino-n-valeric acid hydrochloride dissolved in 10 ml. concentrated ammonia solution (sp. g. 0.88) was mixed with a solution of 3.5g. O-methyl isourea hydrochloride and left for 24 hours. The precipitate which forms was filtered off and recrystallised from hot water. Yield 1.2g. m.pt. 265 - 266°, 273° on rapid heating. Hellerman and Stock (1938) state the m.pt. of the crude acid is 273° but after grinding with alcohol and recrystallising from hot water the m.pt. is 283 - 284°. On carrying out this procedure the melting point was found to be 273°. After a further recrystallisation the m.pt. was 273 - 274°. The compound gave only one guanidine spot after paper chromatography in propanol-ammonia solvent system (Propanol-ammonia sp. g. 0.88 - water 60 : 30 : 10). % N = 26.4; theory = 26.43 % N.

Preparation of Argininic acid (α-oxy-6-guanidino-n-valeric acid).
This compound was prepared in poor yield by the method of Felix and Schneider (1938). m.pt. 226°; Litt. m.pt. 228°. \% N = 23.8. Theory = 24.0 \% N.

Preparation of α-chloro-6-guanidino-n-valeric acid monohydrochloride.
This compound was prepared by the method of Hamilton and Ortiz (1955). m.pt 147-49°; Litt. m.pt. 150°. \% N = 18.2. Theory = 18.25 \% N.

Preparation of α-N-acetyl arginine.
This compound was prepared by the method of Birnbaum, Winitz and Greenstein (1956). \% N = 22.1. Calc. for C_{8}H_{16}O_{3}N_{4} + 2H_{2}O; N = 22.2 \%.

Preparation of β-guanidino-propionic acid.
This was prepared from β-alanine and S-methylisothiourea by the method described for the preparation of taurocyamine. The product was recrystallised several times from hot water. 32.3 \% N; Theory 32.5 \% N.

Preparation of taurocyamine (2-guanidino-ethane-sulphonic acid).
This compound was prepared by the guanylation of taurine with S-methylisothiourea sulphate in ammoniacal solution, according to the general method of Schutte (1943).

30g. Taurine were dissolved in 100 ml. of concentrated ammonia solution by warming to 55 - 60°. 50g. S-methylisothiourea
sulphate was then added in small portions. A vigorous reaction took place at each addition due to the evolution of methyl mercaptan. The evolution of methyl mercaptan was assisted by the addition of a few pieces of porous pot. Taurocyamine began to precipitate after 20 min. and the reaction was left to proceed overnight. The reaction mixture was then vigorously aerated for 30 min. to remove dissolved methyl mercaptan and the precipitate of taurocyamine was filtered off and washed with a small volume of water. A further crop of taurocyamine was obtained by adding 1 volume of ethanol to the filtrate, and this was combined with the previous crop. It was recrystallised by dissolving in the minimum amount of hot water, boiled with a small amount of animal charcoal, and filtered. The filtrate crystallised immediately on cooling and the crystals were collected by filtration, washed with absolute alcohol and dried. Yield = 26g.; 65% of theory; m.pt. 258°. It was recrystallised in similar fashion. Yield = 21g.; 51% of theory. A higher yield may be obtained by treating the filtrates with ethanol, m.pt. = 262-264°. Thoai and Robin (1954) state the m.pt. is 228°, while a value of 260° has been reported by Engel (1875). N = 25.2; theory = 25.2 % N.

Preparation of N-phosphoryl glycocyamine

PG was prepared by the phosphorylation of glycocyamine with POCl₃ as described by Rosenberg (1955), and isolated as an impure
crystalline sodium salt. This was dissolved in water and adsorbed onto a Deacidite FF (Cl form) column and then PG was eluted off with 0.2M NaCl as described in Chapter 4 for the isolation of PT. No evidence was obtained for the presence of a di-N-phosphoryl derivative. PG was isolated as an impure Ba salt which was then dissolved in water and passed through a column of Zeo-Karb 225 (NH₄ form). Ethanol (6 vol.) and acetone (6 vol.) were then added to the filtrate and the crystalline ammonium salt separated on cooling. This was recrystallised by dissolving in the minimum amount of water and adding ethanol (6 vol.). The crystals were collected by centrifugation and dried in vacuo. % glycocyamine 49.8%; % P = 13.3. Theory for C₁₃H₁₄⁰₅N₆P, 50.7% glycocyamine and 13.4% P.
SUMMARY

The synthesis of the following compounds has been described:

O-methyl isourea-HCl.
δ-amino-n-valeric acid.
Homoarginine (α-amino-δ-guanidino-n-caproic acid).
Arginine methyl ester dihydrochloride.
α-carbamido-arginine.
δ-guanidino-n-valeric acid.
Arginiminic acid (α-oxy-δ-guanidino-n-valeric acid).
α-chloro-δ-guanidino-n-valeric acid.
α-N-acetyl arginine
β-guanidino-propionic acid.
Taurocyamine (2-guanidino-ethane-sulphonic acid).
N-phosphoryl glycoxyamine (PG).
REFERENCES


Minneapolis: Burgess Publ. Co.


108. " " (1930c). " " 220, 8.
111. " " (1931b). " " 233, 322.


