STUDIES OF ENZYMIC PHOSPHORYL GROUP TRANSFER:
Transphosphorylation Reactions Involving
N-Phosphorylcreatine and Adenine Nucleotides

A THESIS
submitted for the degree
of
DOCTOR OF PHILOSOPHY
in the
Australian National University
by
MARY DELMA DOHERTY

JANUARY, 1961.
STATEMENT

The regulations of the Australian National University require that a statement be made describing which parts of the work in this thesis have been carried out by myself. This may best be done by quoting from a letter written by my supervisor, Dr. J. F. Morrison, on the 12th January, 1961, to the Registrar of the University:

"...The results reported in Chapter 2 were obtained in collaboration with me. The work reported in Chapters 1, 3 and 4 was carried out independently by the candidate...."

Candidate's signature:

[Signature]

[Institution Stamp]
This thesis embodies work carried out in the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, from October, 1957, to August, 1960, during the tenure of an Australian National University Research Scholarship.
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I am indebted to my many colleagues in the Department for helpful advice and discussion. I should like to mention particularly Dr. R.L. Blakely and Dr. W.H. Elliott.

The skilful technical assistance of Miss D. Kaak during the early part of this work and of Mrs. M. Labutis during the latter part is gratefully acknowledged. I would also like to thank Mr. R.F. Adams for carrying out electrophoretic analyses, and Mr. V. Paral and his staff for help with photographic work.

I have been given valuable assistance by the staff of the Australian National University Library and I should like to thank in particular Mrs. D. McLeod.

Finally, I am very grateful to the Australian National University for the award of a Research Scholarship.
The following abbreviations have been used:

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<tr>
<td>ADP</td>
<td>adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>ADP ribose</td>
<td>adenosine diphosphate ribose</td>
</tr>
<tr>
<td>Ammediol</td>
<td>2-amino-2-methyl-1,3-propanediol</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine-5'-phosphate (adenylic acid)</td>
</tr>
<tr>
<td>APT</td>
<td>arginine phosphoryltransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>CDP</td>
<td>cytosine-5'-diphosphate</td>
</tr>
<tr>
<td>CMP</td>
<td>cytosine-5'-phosphate</td>
</tr>
<tr>
<td>CPT</td>
<td>creatine phosphoryltransferase</td>
</tr>
<tr>
<td>CTP</td>
<td>cytosine-5'-triphosphate</td>
</tr>
<tr>
<td>1:3-DPGA</td>
<td>1:3-diphosphoglyceric acid</td>
</tr>
<tr>
<td>DPN (Coenzyme 1)</td>
<td>diphosphopyridine nucleotide</td>
</tr>
<tr>
<td>DPNase</td>
<td>diphosphopyridine nucleotidase</td>
</tr>
<tr>
<td>DPNH₂</td>
<td>reduced diphosphopyridine nucleotide</td>
</tr>
<tr>
<td>FDP</td>
<td>fructose-1:6-diphosphate</td>
</tr>
<tr>
<td>3-GAP</td>
<td>D-glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine-5'-diphosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>GMP</td>
<td>guanosine-5'-phosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>IDP</td>
<td>inosine-5'-diphosphate</td>
</tr>
<tr>
<td>IMP</td>
<td>inosine-5'-phosphate</td>
</tr>
<tr>
<td>ITP</td>
<td>inosine-5'-triphosphate</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethyl morpholine</td>
</tr>
<tr>
<td>NMN</td>
<td>nicotinamide mononucleotide</td>
</tr>
<tr>
<td>P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>orthophosphoric acid</td>
</tr>
<tr>
<td>PA</td>
<td>N-phosphorylarginine</td>
</tr>
<tr>
<td>PC</td>
<td>N-phosphorylcreatine</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>3-PGA</td>
<td>3-phosphoglyceric acid</td>
</tr>
<tr>
<td>PP</td>
<td>inorganic pyrophosphate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloracetic acid</td>
</tr>
<tr>
<td>TPN (Coenzyme I1)</td>
<td>triphosphopyridine nucleotide</td>
</tr>
<tr>
<td>TPNH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>reduced triphosphopyridine nucleotide</td>
</tr>
<tr>
<td>Versene (EDTA)</td>
<td>ethylenediaminetetraacetic acid (Na salt)</td>
</tr>
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All temperatures are expressed in °C.

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GENERAL INTRODUCTION

Historical

Harden and Young (1906a, b; 1908) were the first to recognise that phosphate played an important role in metabolism. They found that during the fermentation of glucose by cell-free yeast extracts, inorganic phosphate disappeared with the concomitant formation of hexose diphosphate. This demonstration of a relationship between carbohydrate and phosphate metabolism aroused a general interest in organic phosphate compounds and led not only to the discovery of a wide variety of these compounds, but ultimately to the recognition of the significance of phosphorylation in the transfer of energy in biochemical reactions.

The early work on phosphate metabolism was severely hampered by the lack of a method which would permit the estimation of inorganic phosphate in the presence of acid-labile organic phosphate compounds. Inorganic phosphate was assayed by gravimetric methods which were based on the ability of orthophosphate to form an insoluble complex with molybdate. The difference in the values of the inorganic phosphate estimated before and after ashing a sample of test material was taken to represent the organic phosphate present. As a consequence of the prolonged exposure of the test material to acid conditions during precipitation of the molybdate complex considerable hydrolysis of organic phosphate compounds occurred and thus the estimates of inorganic phosphate were too high.
Bloor (1918a, b) recognised the existence of naturally occurring organic phosphate compounds which are unstable under acid conditions. He assayed inorganic phosphate by a nephelometric method based on the formation of a strychnine-phosphomolybdate complex which allowed the rapid estimation of inorganic phosphate (Pouget & Chouchak, 1909) and this reduced the time of exposure of the labile compounds to acidic conditions and thus minimized the degree of acid hydrolysis. The values that he obtained for inorganic phosphate in red blood corpuscles were very much lower than those obtained by the gravimetric method and he demonstrated that as much as 80% of the total phosphate of the corpuscles was derived from acid-labile organic phosphate compounds.

Despite these reports by Bloor, it was not until more systematic studies of the phosphate content of tissues were carried out using rapid, sensitive colorimetric methods for the estimation of inorganic phosphate (Bell & Doisy, 1920; Briggs, 1922, 1924; Fiske & Subbarow, 1925) that it became generally recognised that acid-labile organic phosphate compounds occurred in tissues.

Lohmann and Jendrassik (1926) noted that when a colorimetric method was used to assay fresh muscle extracts for inorganic phosphate there was a delay in the development of maximum colour. The true significance of this observation was not at first recognised as it was assumed that the retardation of colour development was due to
the presence of interfering substances. Eggleton & Eggleton (1927a, b, c) recognised, however, that this delay in colour development was due to the decomposition of an unstable compound during the estimation. Further studies ultimately led to the discovery of the group of compounds which play a role in phosphoryl group transfer reactions. The discovery, properties and metabolic relationship of these and related compounds are discussed in the following sections.

N-PHOSPHORYLCREATINE

Discovery

Although Eggleton and Eggleton (1927a, b, c) recognised the presence of labile organic phosphate compounds in muscle extracts they failed to identify this material which they referred to as 'phosphagen'. They originally believed it to be a phosphoric ester of glycogen or of a hexose. It remained for Fiske and Subbarow (1927a, b) to identify the acid-labile compound as N-phosphorylcreatine (PC). This compound was originally isolated from extracts of skeletal muscle in a crude form as a calcium salt and was shown to have a ratio of creatine to phosphate of 0.96. The isolated material was hydrolysed by acid to inorganic phosphate and free creatine and the rate of the hydrolysis was markedly increased in the presence of molybdate. These properties of PC therefore explained the failure of the early workers to detect this compound.
Creatine was already known to be a constituent of muscle and, prior to the discovery of PC, considerable interest had been taken in the metabolic significance of creatine and the related anhydride, creatinine (Hunter, 1928). Creatine was originally isolated from meat extracts in 1835 (Chevruel, 1835) and subsequently was shown to be methyl-guanidino-acetic acid (Strecker, 1867). The synthesis of creatine from sarcosine and cyanamide (Volhard, 1868) is consistent with the structure shown below:

![Creatine Structure](image)

PC was later isolated in pure form both as barium and calcium salts (Fiske & Subbarow, 1928; 1929a) and the molecular formula of the crystalline calcium salt was shown to be $C_4H_8N_5O_5PCa\cdot4H_2O$. On the basis of the chemical properties of the isolated material and its relationship to creatine, PC was assigned the following structure:

![N-Phosphorylcreatine Structure](image)
The term phosphagen which was used by Eggleton and Eggleton (1927a, b, c) to describe their unidentified material was also applied to the phosphorylated derivative of arginine which was isolated from crayfish muscle (Meyerhof & Lohmann, 1928a) and later to the other N-phosphorylated substituted guanidines found in nature. This led Ennor and Stocken (1958) to suggest that the term phosphagen be regarded as a generic name embracing all (and restricted to) those naturally occurring phosphorylated guanidine compounds which function as stores of phosphate-bond energy from which phosphoryl groups may be transferred to ADP to form ATP as a result of enzymatic catalysis.

Chemical Synthesis of N-Phosphorylcreatine

The first chemical synthesis of PC was achieved by the phosphorylation of creatine with POCl₃ in an alkaline solution (Zeile & Fawaz, 1938) and the material was isolated as the calcium salt. Modifications of this original method have been used for the preparation of the crystalline sodium salt of PC (Ennor & Stocken, 1948; Pradel, Thiem, Pin & Thoai, 1959). PC has also been obtained by reaction of a phosphorylcyanamide derivative with an ester of sarcosine to give the corresponding phosphagen ester which was then hydrolysed (Cramer & Vollmar, 1958). An interesting reaction which also led to PC formation involved the reaction of diphosphorylimidazole with creatine at pH 13 - 14.
(Rathlev & Rosenberg, 1956), but as yet this method has not been used for any large scale synthesis of PC.

**Discovery of the Adenine Nucleotides**

**Adenylic Acid**

Crystalline preparations of adenylic acid were first isolated independently by Jones and Kennedy (1918, 1919) and Thannhauser (1919) from alkaline hydrolysates of yeast nucleic acids. Later, crystalline compounds with the same chemical composition as the yeast adenylic acid were isolated from blood (Hoffman, 1925) and from muscle (Embden & Zimmermann, 1927) by methods which did not involve alkaline hydrolysis. It was assumed at the time that the compounds isolated from these different sources were identical. However, while the adenylic acid prepared from muscle was deaminated by adenylic deaminase (Schmidt, 1928) to give ammonia and inosinic acid that from yeast was not attacked. It was thus clear that the nucleotides isolated from these two sources were not, in fact, identical. A study of the chemical and physical properties of these compounds led Embden and Schmidt (1929) to suggest that isomers of adenylic acid exist which differed in the position of attachment of the phosphate on the ribose group. Attempts were then made to establish the structure of these compounds.

The structure of the adenylic acid isolated from muscle (AMP) was the first to be determined. Enzymic deamination of
this compound gave rise to inosinic acid, a nucleotide whose structure was already established (Levene & Jacobs, 1911). This evidence, together with a study of the ability of AMP to complex with either copper or boric acid led Klimek and Parnas (1932) to assign the following structure to AMP:

![Adenosine-5'-phosphate (AMP)](image)

When this structure was originally formulated no evidence was available as to whether the ribose is attached at the N-7 or the N-9 position of the adenine moiety but later spectrophotometric results were obtained which were consistent with attachment at the N-9 position (Gulland & Holiday, 1936; Gulland & Story, 1938). Synthesis of AMP (Baddiley & Todd, 1947) verified the structure as shown above and confirmed that the glycosidic linkage has the β-configuration.

Yeast adenylic acid was originally assumed to be a single compound although the position of attachment of the phosphate on the ribose moiety was in doubt. It is now recognised, however, that two isomers of adenylic acid are produced on alkaline hydrolysis of yeast ribonucleic acid. These were separated by column
chromatography (Cohn, 1950) and named adenylic acid \( a \) and adenylic acid \( b \) to denote the sequence of their emergence from the column. Adenylic acid \( a \) and adenylic acid \( b \) were identified as adenosine-2'\(^{-}\)-phosphate and 3'\(^{-}\)-phosphate respectively (Brown, Fasman, Magrath & Todd, 1954; Khym & Cohn, 1954). The occurrence of the two isomers in the yeast adenylic acid preparation is a consequence of the intermediate formation of cyclic adenosine-2'\(^{-}\)-3'\(^{-}\)-phosphate during the hydrolysis of the phosphate bond linking the 3' position of one nucleotide to the 5' position of another in the ribonucleic acid (Brown & Todd, 1953; Markham & Smith, 1952).

Strong alkali readily hydrolyses the cyclic structure to give adenosine-2'\(^{-}\) and adenosine-3'\(^{-}\)-phosphate. The two nucleotides do not occur naturally in yeast and may be regarded as artifacts arising as a result of alkaline hydrolysis. It has, however, been reported that adenosine-3'\(^{-}\)-phosphate occurs in some snake venoms (Doery, 1956) but the metabolic significance of the compound is not known.

**Adenosine Triphosphate**

ATP was isolated independently by Fiske and Subbarow (1929b) and Lohmann (1929) and the crystalline silver salt was shown to have the composition \( C_{10}H_{13}O_{13}N_{5}P_{3}Ag_{5} \). Evidence for a structural relationship to AMP was provided by the fact that in the presence of barium and alkali the isolated material was hydrolysed to AMP. In addition, muscle extracts hydrolysed ATP to give two moles of orthophosphate and one mole each of ammonia and inosinic acid.
Doubts were, however, raised with regard to the structure of the isolated nucleotide which Lohmann had designated adenosine-5'-triphosphate. Barrenscheen and Filz (1932) found that the isolated material was not deaminated by adenylic deaminase unless it was first treated with a phosphatase which Jacobsen (1931) had isolated from liver. Moreover, they found that the rate of deamination of the isolated material by nitrous acid was slow compared with that of AMP and suggested that these results indicated that the two labile phosphate groups were attached as pyrophosphate to the amino group of adenine. However, the preparation of inosine triphosphate from the isolated material by treatment with nitrous acid (Lohmann, 1932) supported Lohmann's original suggestion that the compound isolated was adenosine-5'-triphosphate. Subsequently it was recognised that adenylic deaminase shows an absolute specificity for AMP and therefore would not attack ATP until that nucleotide had been hydrolysed by an ATPase. Complete synthesis of ATP (Baddiley, Michelson & Todd, 1949) verified the structure shown below:

![Adenosine-5'-triphosphate (ATP)](attachment:image)
In view of the differences in the structures of the adenylic acids isolated from yeast nucleic acid and from muscle, special interest was taken in the structure of the adenosine triphosphate isolated from different sources. Wagner-Jauregg (1936) demonstrated that the adenosine triphosphate obtained from yeast was identical with the product obtained from rabbit muscle. Although ATP has been isolated from a number of species of bacteria, it has been claimed that the nucleotide isolated from the autotrophic bacterium *Thiobacillus thioxidans* is adenosine-3'-triphosphate (Le Page & Umbreit, 1943). Barker and Kornberg (1954) were unable to confirm the reported isolation of adenosine-3'-triphosphate but they did, however, isolate a small amount of ATP from the autotroph.

**Adenosine Diphosphate**

The barium salt of adenosine diphosphate (ADP) was first isolated from a reaction mixture in which ATP had been hydrolysed by a dialysed crab muscle extract (Lohmann, 1935). The isolated material was shown to be a diphosphate of adenosine and in view of its relationship to ATP was assigned the structure of adenosine-5'-diphosphate and this has been confirmed by synthesis (Baddiley & Todd, 1947).

**Pyridine Nucleotides**

A discussion of the isolation, structure and properties of the pyridine nucleotides has been included as these compounds...
may undergo non-enzymic and enzymic hydrolysis to give adenine nucleotides (Chapter 2).

The history of the pyridine nucleotides dates from the demonstration by Harden and Young (1906a) that cell-free yeast extracts lose the ability to ferment glucose when the extract is dialysed and that the activity can be restored by the addition of a heat stable, dialysable material which they termed 'cozymase'. Later it was shown that this coenzyme occurred not only in yeast but also in animal tissues and Meyerhof (1918) obtained an active preparation of cozymase from frog muscle. In 1923, von Euler and Myrbäck (1923) began a study of the chemical nature of cozymase, which they prepared from yeast, but by 1931 the only definite evidence regarding the structure of the coenzyme was that it was related to adenylic acid (von Euler & Myrbäck, 1931). However, it was shown that neither AMP nor ATP had cozymase activity.

The elucidation of the structure of cozymase was aided to a large extent by the study of a closely related compound discovered by Warburg and Christian (1931). They found that aerobic oxidation of glucose-6-phosphate to 6-phosphogluconic acid required in addition to the enzyme preparation a heat stable, dialysable cofactor which is present in mammalian erythrocytes. This cofactor was partially purified from erythrocytes by Warburg, Christian and Griese (1935) and was shown to contain nicotinamide, ribose, phosphorus and adenine. Subsequently the presence of
nicotinamide in cozymase was demonstrated (von Euler, Albers & Schlenk, 1935) and it was recognised that the two cofactors had a similar chemical composition and differed only in the fact that the cofactor from erythrocytes contained an additional phosphate moiety. Later, von Euler and Adler (1938) demonstrated that the erythrocyte cofactor was converted to cozymase by enzymic dephosphorylation.

In 1936 both cozymase (Coenzyme I) and the erythrocyte cofactor (Coenzyme II) were obtained in highly purified form and this opened the way to the final elucidation of the structure of the two coenzymes (von Euler, Albers & Schlenk, 1936; Warburg & Christian, 1936). As a result of the efforts of numerous investigators the structure of Coenzyme I was well established by 1942 (Schlenk, 1942; Singer & Kearney, 1954). The position of the third phosphate of Coenzyme II however, remained in doubt and it was not until 1950 that Kornberg and Pricer (1950a) showed that the additional phosphate was in the position which corresponded to adenylic acid \( a \); i.e. Coenzyme II contains the adenosine-2'-5'-diphosphate moiety. This formulation is also supported by results obtained with the specific 3'-nucleosidase of barley which does not attack Coenzyme II (Wang, Shuster & Kaplan, 1954). Complete synthesis of Coenzyme I has been achieved (Hughes, Kenner & Todd, 1957) and this confirmed that the coenzymes have the structures shown below:-
DPN (Coenzyme I) $R = H$

or

TPN (Coenzyme II) $R = \text{–PO(OH)}_2$

(Oxidized Forms)

Following the chemical identification of the two coenzymes they became known as diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN). However, this terminology for the nicotinamide nucleotide coenzymes is incorrect and also confusing since these names not only fail to describe accurately the structure of the coenzymes but are, in fact, the chemical names for other compounds (Dixon, 1960). The nomenclature suggested recently by the Enzyme Commission of the International Union of Biochemistry to overcome these difficulties is the use of the following:

DPN - Nicotinamide-adenine dinucleotide (NAD)

and

TPN - Nicotinamide-adenine dinucleotide phosphate (NADP)

However, throughout this work the former nomenclature will be retained.
Isomers of DPN

When DPN was originally purified it was assumed to consist of a single component. It has now been shown, however, that DPN occurs in two isomeric forms which differ only in the configuration of the nicotinamide ribose linkage. Only one of these isomers is active as the coenzyme (Kaplan, Ciotti, Stolzenbach & Bachur, 1955) and it is generally accepted that the linkage of the active isomer of DPN has the β-configuration, although, this has not been definitely proven (Hughes, Kenner & Todd, 1957).

The inactive isomer of DPN, or α-DPN as it is usually called, was first isolated from a commercial preparation of DPN which contained from 10-15% of material resistant to hydrolysis by DPNase (Kaplan et al., 1955). Although this material had the same chemical composition as DPN it did not function as an electron carrier and did not support the growth of Hemophilus parainfluenzae which is the bioassay method used for the detection of the coenzyme. Although the nicotinamide ribose linkage of α-DPN appears to be resistant to enzymic hydrolysis the pyrophosphate bond is cleaved by either snake venom pyrophosphatase or the liver DPNH₂ pyrophosphatase.

No role has been assigned to α-DPN but, as it has been reported to occur in crude tissue extracts (Kaplan et al., 1955), it would seem that it is not just an artifact caused by inversion during the isolation procedure. The level of the isomer
in commercial preparations of DPN varies from 1.9% - 15% (Kaplan et al., 1955; Siegel, Montgomery & Bock, 1959). This may be due to the introduction of chromatographic isolation techniques for Hughes et al. (1957) found that during the elution of their synthetic DPN from an anion-exchange column there was some resolution of the two isomers, with β-DPN being the more easily eluted.

**Cleavage of the Pyridine Nucleotides**

This discussion will be restricted to those enzymic and non-enzymic reactions which result in the formation of adenine-containing nucleotides by cleavage of either the nicotinamide ribose linkage or the pyrophosphate bond of the pyridine nucleotides. **DPNases.** Both DPN and TPN are hydrolysed by DPNases to give nicotinamide and an adenine-containing nucleotide. The nucleotide arising as a result of cleavage of DPN with Neurospora DPNase was isolated by Kaplan, Colowick and Nason (1951) and shown to be ADP ribose. Hydrolysis of TPN by DPNase has been used to prepare 2′-phosphoadenosine diphosphoribose (Neufeld, Kaplan & Colowick, 1955). However, the DPNases do not attack the reduced pyridine nucleotides, nicotinamide ribose, nicotinamide mononucleotide and the unnatural isomer of DPN (α-DPN).

The DPNases are widely distributed and occur in animal tissues, moulds and bacteria. However, the enzymes prepared from these sources differ markedly in their sensitivity to nicotinamide, the DPNases from animal sources being far more sensitive to
inhibition by this compound than the enzyme prepared from
Neurospora (Zatman, Kaplan & Colowick, 1953). They also differ
in the type of inhibition which occurs. Thus, DPNase prepared
from spleen is inhibited non-competitively and 50% inhibition
occurs at $1.5 \times 10^{-3}$ M nicotinamide, whereas the enzyme from
Neurospora, although insensitive to nicotinamide at that concen-
tration, is competitively inhibited at higher concentrations.
The DPNases from animal sources can catalyse an exchange between
labelled nicotinamide and DPN and also between DPN and analogues
of nicotinamide to give the corresponding analogue of the coenzyme
but the Neurospora DPNase could not be shown to catalyse any
exchange. It has also been found that species specificity occurs
among the animal DPNases with regard to inhibition by iso-nicotinic
acid hydrazide (Zatman, Kaplan, Colowick & Ciotti, 1954).

Pyrophosphatases. Both the oxidized and the reduced forms of the
pyridine nucleotides are hydrolysed by pyrophosphatases from
kidney particles, snake venom and potatoes (Kornberg & Lindberg,
1948; Kornberg & Pricer, 1950b) forming either oxidized or reduced
nicotinamide mononucleotide and an adenine-containing nucleotide.
In the case of DPN and DPNH$_2$ the adenine-containing nucleotide is
AMP while the corresponding nucleotides from TPN or TPNH$_2$ is
adenosine-$2'\text{-}5'$-diphosphate. In addition to the non-specific
pyrophosphatases it has been shown that liver contains an enzyme
which is specific for the reduced coenzymes (Jacobson & Kaplan, 1957)
but which will also attack the oxidized and the reduced forms of α-DPN.

**DPN Pyrophosphorylase.** The enzyme, DPN pyrophosphorylase, which catalyses the cleavage of DPN in the presence of inorganic pyrophosphate according to the reaction:

\[ \text{DPN} + \text{PP} \rightarrow \text{NAD} + \text{ATP} \]

has been partially purified from both liver and yeast (Kornberg, 1950). Kornberg found that this enzyme would also attack DPNH₂ but he demonstrated that TPN was not a substrate. More recently, it has been shown (Preiss & Handler, 1958a; Atkinson & Morton, 1960) that DPN pyrophosphorylase preparations also catalyse the synthesis of desamido-DPN from ATP and nicotinic acid mononucleotide. In the presence of glutamine, ATP and the enzyme DPN synthetase desamido-DPN is converted to DPN and it would appear that this is the pathway operative for the synthesis of this coenzyme in a number of species (Preiss & Handler, 1958b).

**Non-enzymic Hydrolysis.** There has been no report of a systematic investigation of the chemical stability of the pyridine nucleotides. However, it has been shown that these compounds are destroyed by heat and that the oxidized and the reduced forms of the coenzymes differ markedly in their sensitivity to acid and alkali.

Colowick, Kaplan and Ciotti (1951) studied the rate of destruction of DPN at 100° and found that hydrolysis occurs over a wide range of pH. The rate of hydrolysis, however, increases
markedly when the pH is raised above a certain critical value which varies with the particular buffer used. They reported that at pH values close to neutrality the hydrolysis of DPN involves only the nicotinamide ribose bond. This bond appears to be the most labile in the molecule and there is some evidence that the nicotinamide ribose bond undergoes hydrolysis during storage of the pyridine nucleotides at low temperatures (Chapter 2). Treatment of DPN with dilute alkali (0.1N NaOH) at 25° causes cleavage of both the nicotinamide ribose and the pyrophosphate bonds with the resultant formation of nicotinamide, ribose-5'-phosphate and AMP (Kaplan, Colowick & Barnes, 1951). The earlier report that ADP was also a product of the alkaline hydrolysis of the coenzyme (Vestin, Schlenk & von Euler, 1937) could not be confirmed. The lability of TPN to heat or alkali is similar to that of DPN.

In contrast to the relative stability of the oxidized coenzymes in acid, the reduced compounds are immediately destroyed in 0.1N acid even at room temperature (Warburg & Christian, 1934). As it has been found that DPNH₂ is slowly hydrolysed to ADP ribose at pH 4.7 at room temperature it would seem that the nicotinamide ribose bond is also the most unstable bond in the reduced pyridine nucleotides (Burton & Kaplan, 1954). The reduced pyridine nucleotides are reported to be stable at alkaline pH.
The Enzymic Relationship of N-Phosphorylcreatine and the Adenine Nucleotides

Lehnartz (1928) seems to be the first to have suggested an interrelationship between PC and an adenine nucleotide. He found that when AMP was added to rabbit muscle extracts, which were capable of carrying out the glycolytic reactions, there was initially a rapid hydrolysis of PC but as incubation proceeded the phosphagen was resynthesized. Although Lehnartz recognised that PC was not synthesized by transfer of phosphate from AMP he was unable to explain his results. Subsequent investigations have shown that these results were presumably due to the fact that the addition of AMP led to ATP formation with the concomitant hydrolysis of PC and that later glycolysis provided the energy for the resynthesis of the phosphagen.

Meyerhof and Lohmann (1931, 1932) recognised that the synthesis of PC from creatine was dependent upon the production of ATP. They found that there was no synthesis of PC using muscle extracts which were incapable of catalysing the glycolytic reactions, either as a result of ageing or dialysis, unless ATP was added. Subsequently it was recognised that the adenine nucleotides were also involved in the hydrolysis of PC. Lohmann (1934) found that aged muscle extracts were unable to hydrolyse PC but that the addition of ATP or AMP to the system brought about rapid release of creatine. He represented the reactions involved
as follows:-

\[
\begin{align*}
\text{ATP} & \rightarrow \text{AMP} + 2 \text{H}_2\text{O} \\
\text{AMP} + 2 \text{PC} & \rightarrow \text{ATP} + 2 \text{Creatine}
\end{align*}
\]

Lehmann (1935, 1936) demonstrated the reversibility of the reaction and as a result of kinetic studies concluded that the phosphorylation of AMP by PC occurs in two steps. This view was supported by Banga (1943) who claimed that crude extracts of rabbit muscle could be resolved into two components. The one which catalysed the reaction

\[
\text{PC} + \text{ADP} \rightleftharpoons \text{Creatine} + \text{ATP}
\]

was called ATP-creatine phosphopherase, while the other which she claimed catalysed the reaction

\[
\text{PC} + \text{AMP} \rightleftharpoons \text{Creatine} + \text{ADP}
\]

was named ADP-creatine phosphopherase.

Banga achieved partial purification of the enzyme which she called ATP-creatine phosphopherase. This enzyme was further purified by Askonas (1951) and was later obtained in a crystalline form and called ATP-creatine transphosphorylase (Kuby, Noda & Lardy, 1954). Ennor and Morrison (1958) have suggested that this enzyme could be referred to as creatine phosphoryltransferase (CPT) and this terminology will be used here.

Some doubts have been cast on the existence of the enzyme ADP-creatine phosphopherase. Banga did not in fact demonstrate the formation of ADP and it has been subsequently shown that AMP
may be phosphorylated by PC in the presence of the enzymes myokinase and CPT (Chappell & Perry, 1954). This reaction will be discussed in further detail in Chapter 3.

Role of N-Phosphorylcreatine in Muscular Contraction

Both Fiske and Subbarow (1927a, b) and Eggleton and Eggleton (1927a, b, c) recognised that PC played a role in muscular contraction and were able to show that the concentration of this compound was lowered as a result of muscular fatigue and that it was absent from extracts of muscle in heat rigor. Subsequently it was found that the hydrolysis of PC liberated a large amount of heat (Meyerhof & Suranyi, 1927; Meyerhof & Lohmann, 1927, 1928b) and the suggestion was made that PC may supply the energy for muscular contraction. This concept was supported by the observation that frog muscle poisoned with iodoacetate, and thus unable to resynthesis PC, ceased to contract when the phosphagen had been completely hydrolysed (Lundsgaard, 1930a, b, c, d; 1931a, b). Lundsgaard recognised that the energy for the resynthesis of PC in normal muscle was supplied by the glycolytic system but he was unaware of the pathway involved. It was not until after the discovery of the enzymic relationship of PC and ADP that the precise role of PC in muscular contraction could be appreciated.

The discovery that the main contractile protein of muscle, actomyosin, acts as an ATPase (Engelhardt & Ljubimowa, 1939) suggested that the energy for muscular contraction is derived from
the breakdown of ATP and not directly from PC as had been suggested by Lundsgaard. This relationship of the two energy sources was confirmed by the demonstration that ATP but not PC could induce contraction of actomyosin threads (Szent-Gyorgyi, 1942) or single muscle threads (Weber, 1951). There has been a great deal of discussion (Weber & Portzehl, 1954; Perry, 1956; Szent-Gyorgyi, 1958) as to whether the hydrolysis of ATP by myosin ATPase is the immediate source of the chemical energy for muscle contraction but as yet no definite answer is available. However, the role of PC seems to be clearly that of a 'reservoir' of phosphoryl groups, which through the mediation of CPT, maintain a steady state concentration of ATP.

**The Role of Adenine Nucleotides in Phosphoryl Group Transfer Reactions**

Following the isolation of ATP in 1931, investigations were undertaken to establish the role played by this compound in phosphate metabolism. It was found that ATP acted as a phosphoryl group donor in many metabolic reactions and that ADP could accept a phosphoryl group from a wide variety of phosphorylated compounds. By 1944 it was generally accepted that these nucleotides were essential for the intermolecular transfer of the phosphoryl group (Green & Colowick, 1944) although it was recognised that intramolecular phosphate transfer reactions did not require these nucleotides. There are, however, some cases where the transfer of a phosphoryl group from one molecule to another can occur
without involving nucleotides. Axelrod (1948) was the first to demonstrate a transfer of this type when he found that citrus juice could catalyse a direct phosphoryl group transfer from aryl phosphates to aliphatic alcohols. Later it was shown that both acid and alkaline phosphatases can catalyse the transfer of a phosphoryl group directly from PC, PEP or glucose-1-phosphate to glycerol, glucose or fructose (Meyerhof & Green, 1950; Morton, 1953). However, it seems doubtful whether these reactions have biological significance as the concentration of both the phosphoryl group donor and the alcohol acceptor must be high for transfer to occur. In a general sense the phosphatases may be considered as phosphoryl-transferring enzymes which may transfer this group either to water or to an alcohol acceptor. Cori, Abarca, Frenkel and Traverso-Cori (1956) claim to have demonstrated a direct transfer of a phosphoryl group from 1,3-diphosphoglyceric acid to creatine and this will be discussed in detail later.

**Transphosphorylation Reactions Between the Nucleotides**

Adenine nucleotides are involved in the majority of the phosphoryl group transfer reactions which occur between nucleotides. The first reaction of this type to be reported was that catalysed by myokinase (adenylate kinase) (Colowick & Kalckar, 1943) namely:

\[ 2 \text{ADP} \rightleftharpoons \text{AMP} + \text{ATP} \]

Following the discovery that mono-, di- and triphosphates of uridine, guanosine and cytosine (in addition to adenosine) occur
in tissues (Hurlbert, Schmitz, Brumm & Potter, 1954) investigations were made to detect transphosphorylation reactions between nucleotides containing different bases. A number of such transfer reactions have been observed and for convenience they have been divided into three main groups.

(a) **Phosphoryl Group Transfer from ATP to Nucleoside Monophosphates.**

The general equation;

$$ATP + NMP \rightleftharpoons ADP + NDP$$

(where N = uridine, guanosine, cytosine or adenosine) represents the type of reaction catalysed by a group of enzymes known as ATP-nucleoside monophosphate kinases (Lieberman, Kornberg & Simms, 1955; Strominger, Heppel & Maxwell, 1959) which have been partially purified from yeast and liver. None of the enzymes has been obtained in pure form, but the fractionation studies of Strominger et al. (1959) seem to indicate that there are distinct enzymes specific for UMP, CMP, GMP and AMP. It has not been possible to demonstrate an ATP-IMP kinase in either liver extracts or preparations from pea seeds (Kirkland & Turner, 1959) but it has been reported (Keleti & Telegdi, 1960a, b) that crystalline triose phosphate dehydrogenase can catalyse a transphosphorylation between these two nucleotides. However, it does not appear that this enzyme functions simply as an ATP-nucleoside monophosphate kinase as the transfer is coupled with the reduction of DPN.
(b) Phosphoryl Group Transfer from AMP to Nucleoside Triphosphates.

The reactions catalysed by the class of enzymes known as nucleoside triphosphate-AMP kinases may be illustrated as follows:

\[ \text{NTP} + \text{AMP} \rightleftharpoons \text{NDP} + \text{ADP} \]

where N = uridine, guanosine, inosine, cytosine or adenosine.

Heppel, Strominger and Maxwell (1959) have demonstrated that the above transfer reactions occurred on the addition of UTP, GTP, ITP and CTP, as well as AMP, to a preparation from liver acetone powder which was free of ATP-nucleoside monophosphate kinase activity. However, it remains to be shown whether the reactions observed are catalysed by a single enzyme with low specificity or whether specific enzymes are involved for each of the nucleoside triphosphates.

The reversal of the above reaction results in the phosphorylation of a nucleoside diphosphate by ADP and as the former compound may arise as a result of the transfer of a phosphoryl group from ATP to the nucleoside monophosphate, it is possible to transfer two phosphoryl groups from ATP to a nucleoside monophosphate. An example of this type of transfer is the phosphorylation of CMP by ATP which is catalysed by liver preparations (Herbert & Potter, 1956) and presumably occurs as follows:

\[ \text{ATP} + \text{CMP} \rightleftharpoons \text{ADP} + \text{CDP} \rightleftharpoons \text{AMP} + \text{CTP} \]

(a) ATP-CMP kinase

(b) AMP-CTP kinase
(c) Phosphoryl Group Transfer from ATP to Nucleoside Diphosphates.

The reaction:

$$\text{ATP} + \text{NDP} \xrightleftharpoons{} \text{ADP} + \text{NTP}$$

(where $N$ = uridine, inosine, guanosine or cytosine) has been reported to be catalysed by preparations from rabbit muscle, yeast and firefly lanterns (Krebs & Hems, 1953; Berg & Joklik, 1954; Balfour & Samson, 1959). No attempt has been made to isolate specific enzymes from any of these preparations and Strominger (1960) points out that the overall reaction represents the sum of the reactions catalysed by a nucleoside triphosphate-AMP kinase and myokinase. As these two enzymes occur in extracts of yeast and muscle and as no rigorous precautions were taken to exclude AMP from the reaction mixtures in which the transfer reactions were studied, doubt must be cast on the existence of ATP-nucleoside diphosphate kinases in these tissues. However, the same criticism cannot be applied to the results obtained with firefly lantern extracts. These preparations emit light when ATP is added but do not show this activity when ATP is replaced by any of the other nucleoside triphosphates or by ADP. However, there is emission of light when the preparations are incubated with UTP, ITP, CTP or GTP together with ADP. AMP cannot replace ADP in this reaction. Thus, the preparations must catalyse the transfer of a phosphoryl group from the nucleoside triphosphates to ADP. No attempt has been made to fractionate the preparation and it remains to be shown whether the preparation contains a single ATP-nucleoside diphosphate kinase of
Nucleoside Transphosphorylations not Involving Adenine Nucleotides

Although the transphosphorylation reactions so far discussed establish that adenine nucleotides are involved in the majority of the reactions leading to the synthesis of di- and triphosphates of cytosine, guanosine and uridine there have been two reports that nucleotide transphosphorylations can occur without involving an adenine nucleotide.

It has been shown (Lieberman, Kornberg & Simms, 1955; Joklik, 1955) that yeast extracts catalyse the reaction:

\[ \text{UTP} + \text{UMP} \rightleftharpoons 2 \text{UDP} \]

However, no precautions were taken by Lieberman and co-workers (1955) to exclude ADP and ATP from their preparation and the transfer reaction observed may in fact represent the sum of a number of reactions in which these adenine nucleotides act catalytically. Joklik (1955) attempted to remove nucleotides from his yeast preparation but he offers no proof of the absence of ATP or ADP and in view of the difficulty of completely eliminating these compounds (see Chapter 3) it would appear that the only satisfactory method of demonstrating direct transfer would be the isolation of the purified enzyme.

The above requirement has been satisfied in the case of crystalline myokinase (Noda, 1958) which has been shown to catalyse the following transfer:

\[ 2 \text{CDP} \rightleftharpoons \text{CMP} + \text{CTP} \]
Thus, this enzyme does not show an absolute specificity for ADP but it does appear that an amino group suitably located on the purine or pyrimidine moiety is essential since there was no reaction when myokinase was incubated with the 5'-diphosphates of inosine, guanosine or uridine.

**Role of the Nucleotides of Cytosine, Guanosine and Uridine**

Originally it was assumed that the only role of the triphosphates of cytosine, guanosine and uridine was in the synthesis of nucleic acids. However, it has now become evident that these nucleotides are involved in many of the synthetic reactions of the cell but, unlike ATP, they appear to play only a limited role in phosphoryl group transfer reactions. In general, they react by transferring a nucleotidyl group to a phosphorylated acceptor with the resultant formation of a substituted nucleoside pyrophosphate and the elimination of a molecule of pyrophosphate. Over the last few years it has become increasingly clear that these substituted nucleotides of cytosine, guanosine and uridine play specialised roles in metabolic reactions. These latter compounds are synthesized by pyrophosphorylases which catalyse reactions of the following type:–

\[
\text{UTP} + \text{sugar-1-phosphate} \rightleftharpoons \text{UDP-sugar} + \text{PP}
\]

A number of substituted uridine diphosphates containing glucose, galactose, glucuronic acid, acetylglucoseamine and acetylgalactosamine have been isolated from natural sources (Leloir &
Cardini, 1960). They are involved in isomerization reactions such as those of glucose to galactose, acetylglucosamine to acetylmannosamine and glucuronic acid to galacturonic acid and they are also active as glycosyl group donors for the synthesis of such substances as sucrose, lactose, glucuronides, cellulose, chitin, hyaluronic acid, glycogen and polymers containing acetyl-neuraminic acid. In addition, substituted uridine diphosphates appear to be involved in the synthesis of cell wall material of several bacteria (Strominger, 1960).

The first demonstration of a specific function for a cytidine nucleotide as an enzymic co-factor was provided by Kennedy and Weiss (1955, 1956) who found that CDP-choline and CDP-ethanolamine are essential intermediates in the synthesis of phospholipids. Two other substituted cytidine dinucleotides, CDP-ribitol and CDP-glycerol, have been isolated from Lactobacillus arabinosus and it has been suggested that they may act as donors of ribitol phosphate and glycerophosphate for the formation of a polymer which contains phosphate, glycerol and ribitol and which is found in the same organism (Strominger, 1960).

The role of guanosine nucleotides is somewhat less well understood than that of either the cytosine or uridine compounds (Utter, 1960; Strominger, 1960). It has been shown that GTP plays a role in protein synthesis but the mechanism of this reaction is still obscure. GDP-mannose has been isolated from hen oviduct and
from micro-organisms and it has been speculated that this compound may be involved in the formation of mannose containing compounds such as the glycoproteins, ovomucin, or the cell wall mannins. It has been demonstrated, however, that GDP-mannose is enzymically converted to GDP-fucose in a multistep reaction involving TFNH$_2$ (Ginsburg, 1960). This reaction is of interest as L-fucose occurs not only in bacterial polysaccharides but also in animals and is a component of the blood group specific polysaccharides.

Parenthetically, it might be mentioned that while it has been emphasised that CTP, GTP and UTP are involved in the formation of substituted diphosphate derivatives, it should not be overlooked that ATP may also take part in reactions of this type. DPN, desamido-DPN, FAD and Dephospho-Coenzyme A are substituted diphosphates of adenosine which are synthesized in the presence of the appropriate pyrophosphorylases from ATP and the corresponding phosphorylated derivative. The reactions involved may be illustrated by considering the synthesis of DPN in the presence of DPN pyrophosphorylase (Kornberg, 1950):

$$\text{ATP} + \text{NMP} \rightleftharpoons \text{DPN} + \text{PP}$$

ATP can also take part in reactions which lead to the formation of anhydrides of AMP with the elimination of a molecule of pyrophosphate. An example of this type of reaction is the formation of enzyme bound amino acid adenylates which appear to be intermediates in protein synthesis. The tryptophan activating enzyme has been extensively
studied (Kingdon, Webster & Davie, 1958) and may be cited as an example:

\[
\text{Enzyme} + \text{ATP} + \text{Tryptophan} \rightarrow \text{Enzyme-Adenyl-Tryptophan} + \text{PP},
\]

**Phosphoryl Group Transfer Reactions Involving Nucleoside Triphosphates Other Than Adenosine Triphosphate**

As yet the nucleotide specificity has been studied for only a limited number of the ATP-kinases and where this has been done using relatively crude enzyme preparations it is necessary to distinguish between a direct phosphoryl group transfer from a nucleoside triphosphate other than ATP and a reaction in which ADP or ATP may act catalytically by transfer reactions involving the nucleoside triphosphate-AMP kinases. There are, however, reports of reactions for which nucleoside triphosphates other than ATP may act as phosphoryl group donors and for which ATP is inactive.

Ling and Lardy (1954) found that the enzyme phosphofructokinase could utilise both ITP and UTP, in addition to ATP, for the phosphorylation of fructose-6-phosphate. The \( K_M \) values of the enzyme for these nucleotides were similar and ranged from 3 to \( 7 \times 10^{-5} \)M. As the maximum velocity was only slightly greater with ATP than with the other nucleotides and as the rate of phosphorylation in the presence of UTP was not enhanced by the addition of ADP, it would appear that UTP was involved directly in the phosphorylation reaction. Later it was reported that CTP could also serve as a phosphoryl group donor in this reaction.
It has also been claimed that the enzyme glycerokinase, which catalyses the phosphorylation of glycerol to form L-α-glycerophosphate can utilize either ATP or UTP but that ITP is not effective as the phosphoryl group donor (Bublitz & Kennedy, 1954). Another enzyme which may show a low specificity for nucleotides is mevalonic kinase (Tchen, 1958). Studies with a partially purified preparation from yeast have shown that mevalonic acid is phosphorylated on the addition of GTP, CTP, UTP or ATP, but no rigorous proof of a direct phosphorylation from nucleoside triphosphates other than ATP was presented. These results contrast with those of Levy and Popjak (1960) who found that mevalonic kinase prepared from liver was active with only ATP or ITP.

Strominger (1955) has shown that in the presence of pyruvic kinase PEP was able to phosphorylate ADP, UDP, CDP and GDP. Similar results were obtained by Tietz and Ochoa (1958) who also demonstrated a transfer from PEP to IDP. These latter workers found that the crystalline pyruvic kinase prepared from rabbit muscle also catalysed a CO₂ dependent phosphorylation of fluoride and were able to show that the nucleotide specificity for this reaction was similar to that found for pyruvic kinase. However, Davidson (1959) has claimed that although GDP and IDP function as substrates for pyruvic kinase in the direct phosphoryl group transfer from PEP, UDP and CDP are not phosphorylated unless either ADP or ATP is also present.
The dephosphorylation of nucleotide triphosphates by myosin has been shown to be relatively non-specific (Kielley, Kalckar & Bradley, 1956) but the activity towards the different nucleotides varies markedly with the ionic composition of the medium. Thus in the presence of Ca$$^{++}$$ GTP, UTP and ITP were rapidly hydrolysed whereas CTP and ATP were only slowly attacked, but in the presence of K$$^+$$ and EDTA the only nucleotides which were hydrolysed at appreciable rates were ATP and CTP.

The enzyme oxaloacetic carboxylase may be cited as an example of an enzyme which catalyses the transfer of a phosphoryl group from a nucleoside triphosphate other than ATP (Kurahashi, Pennington & Utter, 1957). Either ITP or GTP may act as the phosphoryl group donors for this transfer but ATP, CTP, UTP and xanthosine triphosphate have been shown to be inactive. It has also been reported that pig kidney preparations catalyse an oxidation of $$\alpha$$-ketoglutarate which is coupled to the uptake of inorganic phosphate and requires GDP as the specific phosphoryl group acceptor (Sanadi, Gibson, Ayengar & Jacob, 1956).

**Mechanism of Phosphoryl Group Transfer Reactions**

Originally no attempt was made to distinguish between the transfer of a phosphoryl ($$\text{-} \overset{\text{P}}{\text{O}} _{\text{OH}} \overset{\text{OH}}{\text{O}}$$) group and a phosphate ($$\text{-} \overset{\text{P}}{\text{O}} _{\text{OH}} \overset{\text{OH}}{\text{O}}$$) group. In the case of the transfer from PC to ADP no ambiguity arises as the only group available for transfer is a phosphoryl group. However, transfer reactions involving ATP and other organic phosphates could theoretically occur by transfer of
either the phosphoryl or the phosphate grouping. The elucidation of the type of cleavage involved has been based on studies carried out using $^{18}O$. Cohn (1949) first used this method to determine the type of bond cleavage which occurs on hydrolysis of organic phosphates. She carried out the hydrolytic reactions in the presence of water labelled with $^{18}O$ and found that although acid hydrolysis of glucose-1-phosphate brought about cleavage of the C-O bond, both acid and alkaline phosphatases catalysed the cleavage of the O-P bond. Subsequently, this method was applied to the study of the transfer reactions catalysed by pyruvic kinase, CPT, phosphoglucomutase, phosphoglycerate kinase (Harrison, Boyer & Falcone, 1955) hexokinase, Mg$^{++}$ activated ATPase and myokinase (Cohn, 1956) and it was found that these enzymes, which all require Mg$^{++}$ for activity, catalyse the transfer of a phosphoryl and not a phosphate group. From the evidence now available it would seem that divalent ions have an activating effect in all the reactions involving the transfer of a phosphoryl group, i.e. reactions in which the O-P bond is cleaved. On the other hand, there is no requirement for metal ions for reactions which involve hydrolysis of the C-O bond of ester phosphates with elimination of phosphate and transfer of a glycosyl group. Examples of this type of transfer are the reactions catalysed by phosphorylase and sucrose phosphorylase. The metal ions which are usually involved in the activation of the phosphoryl group transfer reactions are Mg$^{++}$;
Mn$^{++}$ and Ca$^{++}$ and in some cases Co$^{++}$. However, particular enzymes may be activated by a number of different metal ions or they may show an absolute specificity for one of these divalent ions.

The mechanism by which divalent metal ions activate phosphoryl group transfer reactions is not known with certainty, although some studies of the mechanism of activation have been attempted using the guanidine phosphoryl transferases. Kuby, Noda and Lardy (1954a) studied the kinetics of the reaction catalysed by CPT and concluded that the metal activation involved the formation of metal-nucleotide complexes which are the active substrates for the enzyme. However, in these studies the effect of the binding of metal ion by the nucleotide present as substrate was not considered and the velocities have been plotted against the concentration of total Mg$^{++}$ added. Griffiths, Morrison and Ennor (1957) have taken this effect into consideration and calculated the concentration of free Mg$^{++}$ present in the reaction mixture using quoted stability constants. They have concluded, as a result of their studies of the kinetics of the reaction catalysed by APT, that the role of the metal ion is in the formation of an active metal-enzyme complex although they suggest that the metal also assists in the binding of the nucleotide to the enzyme surface.

Some studies have also been made of the effect of divalent metal ions on the non-enzymic transfer of a phosphoryl group from ATP. Nanninga (1957) studied the effect of metal ions
on the non-enzymic hydrolysis of ATP and found that while Ca\(^{++}\) accelerated the non-enzymic hydrolysis of ATP, Mg\(^{++}\) was inhibitory. These results raise the possibility that the type of chelate present in solution may not be the same as that involved in the enzymic reaction. Another reaction which has been studied is the non-enzymic transphosphorylation which involves the formation of inorganic pyrophosphate from ATP and orthophosphate and which is catalysed by Mn\(^{++}\), Ca\(^{++}\), and Cd\(^{++}\) (Lowenstein, 1957; 1958). Although Mg\(^{++}\) was found to be inactive when tested alone this ion did act synergistically when added together with low concentrations of the active metal ions (Lowenstein, 1960a). It was also found that the transphosphorylation catalysed by divalent metal ions was further stimulated by alkali metal ions (Lowenstein, 1960b). Potassium ions were found to show the greatest degree of stimulation and this is of some interest as a number of enzymes involved in phosphoryl group transfer from ATP have been shown to have an absolute requirement for, or be stimulated by alkali metal ions (Parks, Ben-Gershom & Lardy, 1957; Kachmar & Boyer, 1953; Melnick & Buchanan, 1957).

Reactions of N-Phosphorylcreatine not Involving Nucleotides

Hydrolysis of PC by Phosphoamidases and Phosphatases. PC may undergo hydrolytic reactions which do not involve the adenine nucleotides. Thus, the N-P bond of PC may be hydrolysed by a phosphoamidase or by the relatively non-specific phosphatases
to give inorganic phosphate and creatine. Although there have been several claims for the presence of specific phosphoamidases in tissues (Ichihara, 1933; Gomori, 1948; Holter & Li, 1950; Meyer & Weinmann, 1957) the assay methods used to detect these enzymes would not distinguish between phosphoamidases and acid and alkaline phosphatases which would also split the N–P bond (Morton, 1955). However, there has been a report of the partial purification of a phosphoamidase from beef spleen (Singer & Fruton, 1957) which attacks PC but the rate of hydrolysis of this compound is much slower than the rate of hydrolysis of the synthetic substrates tested. Although this preparation is not completely specific for the hydrolysis of the N–P bond and it does hydrolyse phenylphosphates, it does not attack aliphatic phosphate esters.

The phosphatases show a fairly broad spectrum of specificity and both acid and alkaline phosphatases hydrolyse ester phosphates and PC (Morton, 1955). However, Perlmann (1954) has reported that the intestinal phosphatase may be made specific for the N–P bond by variation of the pH of the reaction mixture. This enzyme will split both N–P and ester–P bonds at alkaline pH but only N–P bonds are cleaved at pH 6.

The ability of the acid and alkaline phosphatases to catalyse the transfer of a phosphoryl group directly from PC to glycerol, glucose or fructose (Meyerhof & Green, 1950; Morton, 1953)
has already been discussed and although this reaction is of interest as it represents an intermolecular phosphoryl group transfer which does not involve the adenine nucleotides it is probably of little biological significance.

Formation of Creatinine. The formation of creatinine has been attributed to the non-enzymic breakdown of PC (Borsook & Dubnoff, 1947) and although there is a striking relationship between the amount of creatinine excreted per day by man and the amount which could theoretically be formed non-enzymically from PC, it is possible that creatinine may be formed enzymically. In fact, a claim has been made that creatinine is formed enzymically by transfer of the phosphoryl group from PC to hexose monophosphate with the resultant formation of hexose diphosphate (Caputto, 1954; Caputto & Carpenter, 1955). Following the publication of full experimental details (Carpenter, McCay & Caputto, 1958) the problem was reinvestigated by Van Pilsum and Hiller (1959) using more specific analytical procedures for the assay of creatine, creatinine and PC (Van Pilsum, Martin, Kito & Hess, 1956). They found that rabbit muscle extracts did catalyse the transfer of a phosphoryl group from PC to hexose monophosphate, but that there was no significant formation of creatinine although creatine could be detected. The release of creatine obtained was due presumably to contamination of the muscle extract by either ATP or ADP which could act catalytically in phosphoryl group transfer reactions involving CPT. It is worthy of note that the original claim for
an apparent net production of creatinine, assayed by the non-specific alkaline picric acid method (Jaffe Reaction) could be confirmed. The material responsible for the positive reaction was not identified, but clearly it was not creatinine. Because of the lack of evidence to the contrary, it must be assumed that creatinine arises non-enzymically from PC.

Synthesis of N-Phosphorylcreatine not Involving Adenosine Triphosphate. There have been a number of suggestions that PC may be synthesized by a route which does not involve transfer of a phosphoryl group from ATP and although one of the postulated pathways is discussed in detail in Chapter 2 a general survey is given here to complete this discussion.

The results of isotopic experiments have been interpreted as indicating that PC may be synthesized by a pathway which does not involve the adenine nucleotides (Sacks & Altshuler, 1942; Harvey, 1955). These claims are based on the finding that the rate of incorporation of $^{32}$P into PC was faster than into ATP. Conflicting results were obtained by Ennor and Rosenberg (1954a) using rabbit skeletal muscle. They were unable to demonstrate any significant difference in the specific activity of PC and the terminal group of ATP but Harvey (1955) claims that their results are due to the fact that the animals were anaesthetized with Nembutal which compound was shown to affect phosphoryl group transfer reactions. However, the interpretation of the results
obtained with $^{32}$P is difficult, if not impossible, as the actual turnover of the phosphorus compounds under study was not measured. Thus, these isotopic experiments cannot be regarded as providing unequivocal proof of an alternative pathway for PC synthesis.

It was pointed out by Ennor and Morrison (1958) that in the light of the knowledge that CPT is sensitive to inhibition by iodoacetate, the results obtained by Lundsgaard (1930a, b, c) could be interpreted as indicating a pathway for the synthesis and breakdown of PC which does not involve that enzyme. Lundsgaard found that frog muscle poisoned with iodoacetate, although unable to carry out glycolysis, could continue to contract until the phosphagen had been completely hydrolysed. Inherent in his explanation of his results is the assumption that, although glycolysis is inhibited (presumably due to the sensitivity of TPD to iodoacetate), hydrolysis of PC can still occur. However, it has been shown that CPT is also sensitive to inhibition by iodoacetate (Ennor & Rosenberg, 1954b) and both enzymes would therefore be expected to be inhibited in the experiments carried out by Lundsgaard. This problem was investigated by Padieu and Mommaerts (1960) who found that although both TPD and CTP are inhibited by iodoacetate in vitro the latter enzyme was not inhibited in vivo under conditions which completely inhibit TPD. Furthermore, it was found that PC, ATP and a number of other phosphorus compounds could protect CPT against the action of
iodoacetate and it is likely that the protection of the enzyme in vivo occurs by virtue of the presence of its substrates. Watts, Rabin and Crook (1960) have also observed protection of CPT against inhibition by iodoacetate when Mg++, ATP and creatine are present. Thus, no pathway other than the 'Lohmann reaction' need be postulated to explain PC hydrolysis in iodoacetate poisoned frog muscle.

There have been two reports by independent groups (Cori, Abarca, Frenkel & Traverso-Cori, 1956; Bresler, Rubina & Vinokurov, 1957) that the synthesis of PC can occur by transphosphorylation reactions which do not involve ATP. In 1955 Bresler and Rubina (1955) reported that incubation of RNA and ATP with myokinase resulted in the formation of a phosphorylated derivative of RNA which could be isolated and which contained 50% excess phosphate. Subsequently it was claimed (Bresler et al., 1957) that this derivative of RNA could act as a phosphoryl group donor for the synthesis of PC in the presence of CPT and creatine. In view of the interesting implications of the reported transphosphorylation, Rosenberg (1958) undertook a study of this reaction but was unable to demonstrate phosphorylation of RNA under conditions described by the Russian workers. Seraydarian and Williams (1960) assayed the acid-insoluble components of muscle for a phosphorylated derivative of either RNA or DNA but failed to detect any such compound.
The report by Cori, Abarca, Frenkel and Traverso-Cori (1956) represents the only claim for the synthesis of PC by a phosphoryl group transfer reaction which does not involve either ATP or CPT. They reported that there was a direct transfer of a phosphoryl group from 1:3-diphosphoglyceric acid to creatine and have suggested (Cori, Traverso-Cori, Lagarrigue & Marcus, 1956) that a single enzyme is responsible for the reversible transfer shown below:

\[
\text{Creatine} \quad + \quad \text{1:3-DPGA} \quad \xleftrightarrow{\text{PC} \quad + \quad \text{3-PGA}}
\]

It is of interest to note that while this direct phosphoryl group transfer would explain the results obtained in the \(^{32}\text{P}\) incorporation studies (Harvey, 1955) the reaction represents an anomalous type of phosphoryl group transfer. Thus, in addition to not involving ADP, the reaction represents an exception to the generalization that ester phosphoryl groups are transferred to form \(\text{O} - \text{P}\) bonds (Cohn, 1959) and the enzyme reported to catalyse the transfer does not fit the classification proposed by Bock (1960) for the phosphoryl transfer enzymes. Probably the most interesting aspect of the reported reaction, however, is the fact that it represents a claim for a new pathway leading to the synthesis of a phosphagen. In view of this and the unusual type of phosphoryl group transfer involved a study of the reaction reported by Cori et al. (1956) was undertaken and forms a major section of the work reported here.
CHAPTER 1.

A METHOD FOR THE DETECTION OF LOW LEVELS OF

ADENOSINE DIPHOSPHATE AND ADENOSINE TRIPHOSPHATE
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ADENOSINE DIPHOSPHATE AND ADENOSINE TRIPHOSPHATE

INTRODUCTION

The adenine nucleotides may act catalytically in the transfer of a phosphoryl group from a nucleoside triphosphate to a nucleoside monophosphate (Storminger, 1960) and any claim that a direct transfer can occur between nucleotides other than those of adenine (Joklik, 1955; Lieberman, Kornberg & Simms, 1955) must be supported by evidence that the adenine nucleotides are not involved. There have also been a number of reports that phosphoryl group transfer reactions to and from acyl phosphates can occur without the mediation of ADP or ATP (Ling & Lardy, 1954; Strominger, 1955; Tietz & Ochoa, 1958; Tchen, 1958; Cori, Abarca, Frenkel & Traverso-Cori, 1956) but only some of these claims have been supported by a demonstration that the adenine nucleotides are not acting catalytically in the observed reactions. Evidence for the non-participation of the adenine nucleotides is more convincing if purified enzymes are used, but there is still the difficulty that there may be present trace amounts of an enzyme which can catalyse phosphoryl group transfers to and from low levels of adenine nucleotides. Thus the only convincing
evidence that a direct transfer is occurring would be the demonstration of a complete absence of ADP or ATP from the incubation mixtures. For this purpose a particularly sensitive method for the detection of these compounds is required.

Assay procedures for the determination of ADP and ATP have been described by Kalckar (1947) and Strehler and Totter (1954) but because these are based on stoichiometric reactions they would not be sufficiently sensitive to detect levels of ATP or ADP which may act catalytically in phosphoryl group transfer reactions. One possible exception is the firefly luminescence method which can detect less than 0.1 μg of ATP in a reaction mixture of 0.8 ml. However, to achieve this level of sensitivity elaborate apparatus is necessary. Furthermore, since the firefly enzyme is specific for ATP it is not possible to detect ADP directly.

A more satisfactory means for the detection of low levels of either ADP or ATP is to utilise a system in which these nucleotides act catalytically rather than stoichiometrically. An assay method of this type has been described by Grisolia, Mokrasch and Hospelhorn (1958) who followed the release of pyruvate from PEP in the presence of pyruvic kinase, hexokinase, glucose and catalytic levels of adenine nucleotides. They were able to detect 0.05 μmoles of ATP (approx. 30 μg.) in a 1 ml. reaction mixture.
A more sensitive system has been used by Cori, Traverso-Cori, Lagarrigue and Marcus (1958) to assay incubation mixtures for contamination by ADP or ATP. They followed the increase in the extinction at 340 m\(\mu\) due to DPNH\(_2\) produced in the presence of glucose-6-phosphate dehydrogenase, hexokinase, CPT, glucose, DPN, PC and the test material and were able to detect a minimum level of 0.005 \(\mu\)moles of ADP or ATP in a test volume of 3 ml.

The most sensitive method of this type which has been used for the detection of adenine nucleotides is that suggested by Chappell and Perry (1954). As a result of incubating PC, glucose and nucleotide with hexokinase and CPT, they found that creatine was released from PC by addition of either ADP or ATP down to a concentration of 0.5 \(\mu\)g. in a 2 ml. reaction mixture. The advantage of any method by which the adenine nucleotides can be related to the amount of guanidine released from their phosphorylated derivatives is the ease of estimation of the latter. Because of the possibility of further increasing the sensitivity of this type of method by utilising other enzymes involved in the breakdown of ATP, an investigation of a number of such systems was undertaken. A method capable of detecting 0.1 \(\mu\)g./ml. of ADP or ATP will be described.

EXPERIMENTAL

Chemicals. Nucleotides were obtained from Sigma Chemical Co., St. Louis, Mo. AMP was purified by chromatography on Dowex-1 (formate). The nucleotide was eluted with 0.25 N formic acid,
adsorbed on acid washed charcoal (see below) and recovered by stirring three times with a 10:90 (v/v) mixture of isoamyl alcohol and water. The aqueous layer from the combined extracts was taken to dryness under reduced pressure and the product crystallised twice from hot water. Before use, Nuchar C and Norit A were boiled for 15 min. with 1 N HCl, washed free of chloride and dried at 100°. PC was prepared as the sodium salt (Ennor & Stocken, 1957) and PA was isolated from cray fish muscle (Ennor, Morrison & Rosenberg, 1956). NEM was obtained from Eastman Organic Chemicals, Rochester, N.Y. It was redistilled under reduced pressure before use and was adjusted to the required buffer pH with HCl. Crystalline bovine albumin was Fraction 5 obtained from Armour Laboratories, London.

Enzymes. Unless otherwise stated, frozen rabbit muscle was used as the source of the enzymes. Myokinase was prepared according to the method of Colowick and Kalckar (1943) but only the 0.5 - 0.8 saturated ammonium sulphate fraction was collected. CPT, prepared by method B of Kuby, Noda and Lardy (1954b), was stored at 0° after freeze-drying. Before use, solutions of CPT (10 mg. of protein/ml.) were stirred with acid washed Norit A (1 g./10 ml.) for 15 min. at 0°, filtered and then stirred overnight with Norit A. Both treatments were then repeated.
Fresh rabbit muscle was used for the preparation of myosin (Perry, 1955) and Mg-activated muscle ATPase (Kielly, 1955). APT was a preparation obtained from cray fish muscle (Morrison, Griffiths & Ennor, 1957).

METHODS

Arginine. This was estimated by the method of Rosenberg, Ennor and Morrison (1956) after stopping the enzymic reaction by addition of an alkali-ethylenediamine tetraacetic acid mixture (Morrison, et al., 1957).

Creatine. Creatine was estimated either by the method used for arginine or by a slight adaptation which was designed to increase the sensitivity of the method. In the latter case the enzymic reaction was stopped by addition of 0.3 ml. of a mixture containing 3 N NaOH and 0.03 N ethylenediamine tetraacetic acid. Creatine was estimated by adding 1 ml. of the α-naphthol diacetyl reagent and diluting to 3 ml. The colour was allowed to develop for 15 min. and was read at 535 μ using a Shimadzu spectrophotometer.

Protein. Protein was estimated either by the biuret method of Gornall, Bardawill and David (1949) or with the Folin-Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951) using crystalline bovine albumin as the standard for both methods.

Inorganic phosphorus. The method of King (1932) was used for the estimation of \( P_i \).
RESULTS

A number of systems in which guanidine phosphoryltransferases are linked to an ATP hydrolysing enzyme have been studied for their suitability in the detection of catalytic levels of ADP and ATP. The type of reactions investigated are represented in the following scheme:

\[ \text{ADP + Phosphagen} \quad \text{Guanidine Phosphoryltransferase} \quad \text{ATP + Guanidine} \]

Some preliminary experiments were carried out to determine whether the linked enzyme system, APT and myokinase, when incubated with AMP and PA would constitute a satisfactory method for the detection of low levels of ADP or ATP. It was found, however, that the addition of AMP and PA led to a rapid release of arginine without addition of the test nucleotides. Because it was likely that the reaction occurred by virtue of the presence of trace amounts of either ADP or ATP, the enzymes and AMP were subjected to further purification.
The enzymes were treated with charcoal and Dowex-1 (Cl\textsuperscript{−}) and the AMP was purified by chromatography on Dowex-1 (formate). However, this did not prevent the release of arginine. When PA and APT were replaced by PC and CPT similar results were obtained. A study of this reaction has been carried out (Chapter 3) and the results indicate that nucleotide, firmly bound to the CPT, is involved in the transfer.

As the above methods proved unsatisfactory for the detection of low levels of ADP or ATP, the linked enzyme systems involving the guanidine phosphoryltransferases and the ATPases were investigated. APT is activated by Mg\textsuperscript{2+} and inhibited by Ca\textsuperscript{2+} while the reverse is true of myosin thus, these two enzymes cannot be coupled in a catalytic system for the detection of ADP or ATP. However, the Mg-activated ATPase from muscle plus myosin may be used and when these two enzymes were added to a reaction mixture containing PA, Mg\textsuperscript{2+} and buffer there was a release of arginine which was dependent upon the addition of ADP or ATP (Table 1.1). The method was found to be capable of detecting ATP at a final concentration of 1 x 10\textsuperscript{−6} M; i.e. 0.6 μg. of ADP or ATP in a 1 ml. reaction mixture.

When ATP and PA were replaced by CPT and PC a more sensitive detection method was obtained. A creatine release was obtained in the presence of 5 x 10\textsuperscript{−7} M ATP but this was not
TABLE 1.1

Release of Arginine from N-Phosphorylarginine in the presence of low levels of Adenosine Triphosphate

The incubation mixtures contained NEM-HCl buffer (pH 7.2), 75 µmoles; PA, 5 µmoles; Mg$^{2+}$, 10 µmoles; Mg-activated muscle ATPase, 0.6 mg.; APT, 0.1 mg. and ATP in a volume of 1 ml. Tubes were incubated for 20 min. at 38° and the arginine release determined.

<table>
<thead>
<tr>
<th>ATP Concentration (M)</th>
<th>Arginine Released (µmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10^{-4}</td>
<td>5</td>
</tr>
<tr>
<td>1 x 10^{-5}</td>
<td>1.0</td>
</tr>
<tr>
<td>1 x 10^{-6}</td>
<td>0.05</td>
</tr>
<tr>
<td>5 x 10^{-7}</td>
<td>0.0</td>
</tr>
</tbody>
</table>
linear over the period studied (Fig. 1.1). The reason for the non-linearity of the creatine release is not known, but is possibly due to denaturation of one or both of the enzymes by incubation at the low substrate level. This effect appears to be even more marked at a lower ATP level \(2 \times 10^{-7} \text{M}\).

The linked systems involving either CPT or APT and the Mg-activated muscle ATPase have been successfully applied to the detection of low levels of contaminating ADP and/or ATP in samples of pyridine nucleotides and ADP ribose. However, when these methods were used to determine if samples of AMP were contaminated with ADP or ATP difficulties were encountered as a slow release of guanidine occurred which was not dependent on the addition of ADP or ATP. This release was presumed to be due to contamination of the Mg-activated ATPase by myokinase. As the former enzyme is not a soluble preparation the complete removal of contaminating myokinase was not always achieved. However, myosin can be conveniently prepared free of myokinase contamination by repeated precipitation by dilution in distilled water and as CPT is activated by Ca\(^{++}\), these two enzymes were used to check if AMP samples were contaminated by ADP or ATP. In addition, it was found that this method was more sensitive than either of the detection procedures discussed above and it was therefore investigated in greater detail. For this purpose ATP at a concentration of \(1 \times 10^{-6} \text{M}\) was added to the reaction mixtures and studies
Fig. 1.1. Release of Creatine in the presence of low levels of ATP. Reaction mixture contained NEM-HCl buffer (pH 7.3), 100 μmoles; PC, 2.5 μmoles; Mg$^{2+}$, 2.5 μmoles; CPT, 0.3 mg.; Mg-activated muscle ATPase, 0.17 mg. and ATP in a total volume of 1 ml. Temp. 38°. X—X, No additions; •—•, 2 x 10$^{-7}$M ATP; ○—○, 5 x 10$^{-7}$M ATP.
were carried out to determine the conditions under which maximum release of creatine is obtained. The effect of independently varying the concentration of myosin and of CPT on the rate of creatine release is shown in Fig.1.2. The rate of creatine release was 5.2 μmoles/min, in the presence of 0.15 mg. of CPT and 0.375 mg. of myosin and this was not further increased by doubling the CPT level. On the other hand with the higher CPT level, a four fold increase in the level of myosin did increase the rate by 40%. There is a limit, however, to the possible increase in sensitivity which may be achieved by increasing the level of enzyme. High protein increases the value of the zero time control and this is apparently due to the addition of guanidine compounds with the protein.

The release of creatine was not affected over a range of pH from 7.0 to 7.8. The method has not been tested, however, at higher pH values.

Because myosin is insoluble at low ionic strength the initial studies were carried out in the presence of 0.1 M KCl, but subsequently it has been found that this component can be omitted from the incubation mixture without affecting the creatine release. These results thus indicate that this method of detection of adenine nucleotides, unlike that suggested by Grisolia et al. (1958), is relatively insensitive to changes in the ionic strength of the reaction mixture.

Standard Procedure for the Detection of Low Levels of ADP or ATP. The method adopted routinely for the detection of low
Fig. 1.2. Effect of independently varying the concentration of myosin and CPT on the rate of creatine release. Reaction mixture contained NEM-HCl buffer (pH 7.4), 50 μmoles; CaCl₂, 10 μmoles; KCl, 100 μmoles; PC, 2.5 μmoles; ATP, 1 μmole; CPT and myosin in a 1 ml. volume. Temp. 31°C. ▲, CPT, 0.15 mg.; myosin, 0.375 mg.; □, CPT, 0.3 mg.; myosin, 0.375 mg.; ●–●, CPT, 0.3 mg.; myosin, 1.5 mg.; ○–○, CPT, 0.3 mg.; myosin, 1.5 mg. without ATP.
levels of these nucleotides was as follows:

10 μmoles of NEM-HCl buffer (pH 7.0); 5 μmoles of CaCl₂;
2.5 μmoles of PC and the test material were incubated with
0.3 mg. of CPT and myosin (0.3 - 0.5 mg.) in a total volume
of 1.0 ml. for 20 min. at 38° and the creatine release estimated.

There was some variation in the activity of different myosin
preparations as judged by the rate of creatine release. The
activity of each preparation was checked before use and the
level adjusted to give a release of 0.08 - 0.1 μmoles of
creatine/ml. after 20 min. incubation in the presence of
1 x 10⁻⁶ M ATP.

**Sensitivity of the Method.**

A significant release of creatine was obtained
in the presence of ADP or ATP at a final concentration of
1 x 10⁻⁷ M (0.06 μg./ml.) (Fig. 1.3) and although a release of
creatine has been obtained at lower nucleotide concentrations
the results were somewhat variable. The sensitivity of the
method could not be increased by prolonging the incubation.
Thus, there was no detectable increase over the value of the
blank when 1 x 10⁻⁸ M ATP was incubated in the standard
incubation mixture for 140 min. The reaction can, however,
be carried out in a reduced volume and the creatine release
estimated in a final volume of 3 ml. As the test material
can comprise as much as 3/5 of the total reaction mixture, it
is possible to use the method for the detection of ADP or ATP
Fig. 1.3. Sensitivity of the method for the detection of ATP. Conditions were as for the standard procedure described in the text with varying ATP concentrations. Myosin, 0.5 mg, in 1 ml. volume.
as a contaminant of an incubation mixture or of a substrate if present at a concentration of $1.7 \times 10^{-7}$ M or above.

**Application of the Method**

As this method was developed primarily to demonstrate that the substrates and reaction mixtures, used to study the reported direct phosphorylation of creatine by 1:3-DPGA (Cori et al., 1956), were free of adenine nucleotides, a study was made to determine whether this method could be applied in the presence of the components of the reaction mixtures used by Cori and his coworkers. The results (Table 1.2) indicate that AMP, DPN, ADP ribose, nicotinamide and Mg$^{++}$ cause some inhibition of the release of creatine. More extensive investigations of the inhibition by AMP and DPN have shown that the level of inhibition by these compounds is markedly dependent on the concentration of the inhibitory material present. However, such inhibitory effects do not preclude the use of this method to detect the presence of ATP and ADP in these compounds but the level of detection is reduced.

Studies have been carried out to determine whether the inhibition noted in the presence of AMP, DPN and ADP ribose is due to inhibition of one or both of the enzymes of the assay system. It has been found (Table 1.3) that both DPN and ADP ribose inhibit myosin ATPase at a concentration of 1.0 mM whereas AMP was not inhibitory at this concentration. ADP ribose was found to inhibit CPT activity more markedly than
TABLE 1.2

Inhibition of the Creatine Release in the Standard Detection Procedure

The release of creatine in the presence and absence of the test material was determined as described in the text. The level of ATP was $5 \times 10^{-7}$ M.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Inhibition of Creatine Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>25</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>DPN</td>
<td>2.5</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>ADP ribose</td>
<td>1.0</td>
<td>40</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.005</td>
<td>14</td>
</tr>
<tr>
<td>Magnesium Acetate</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>30</td>
</tr>
</tbody>
</table>
TABLE 1.3

Inhibition of Myosin ATPase Activity and Creatine Phosphoryltransferase Activity by DPN and ADP ribose

The determination of Myosin activity was carried out in the presence and the absence of the test compound in the following reaction mixture: NEM-HCl buffer (pH 7.0), 50 µmoles; CaCl₂, 10 µmoles; KCl, 100 µmoles; ATP, 1 µmole, and myosin, 37.5 µg. in a total volume of 1 ml. The reaction was stopped after incubation for 3 min. at 30° by adding 1 ml. of cold 20% (w/v) TCA and the protein removed by centrifugation. The estimation of inorganic phosphate was carried out on a 1 ml. sample of the supernatant solution.

The determination of CPT activity was carried out in the presence and the absence of the test compound in the following reaction mixture: NEM-HCl buffer (pH 6.0), 100 µmoles; PC, 10 µmoles; ADP, 3 µmoles; Mg²⁺, 4 µmoles and CPT, 1.0 µg. in a total volume of 1 ml. Tubes were incubated for 6 min. at 30° and the creatine release estimated.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Test Compound</th>
<th>Concentration (mM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>ADP ribose</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>DPN</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AMP</td>
<td>1.0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>CPT</td>
<td>ADP ribose</td>
<td>2.5</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>DPN</td>
<td>2.5</td>
<td>71</td>
</tr>
</tbody>
</table>
DPN at the same concentration. It has been previously reported that AMP and DPN inhibit CPT (Kuby, Noda & Lardy, 1954a).

DISCUSSION

The procedure which has been developed provides a sensitive and rapid method for the detection of low levels of ADP and/or ATP for which no special apparatus is required. Moreover, the method may be used for the detection of nucleotides in an enzymic incubation mixture without prior removal of protein. In the reaction the nucleotides act catalytically so that in 20 min. the amount of creatine released is more than 100 times the concentration of the added nucleotide.

Relatively high levels of protein are used in the assay system to achieve maximum creatine release and it is essential that the enzymes themselves are free of nucleotide contamination. For this reason the CPT used throughout these experiments was treated with charcoal. This method of purification could not be applied to myosin as there was not only severe loss of protein due to adsorption on the charcoal but also some inactivation of the enzyme. However, the release of creatine in the assay system, without added ATP, remained unchanged when there was a four-fold increase in the concentration of the myosin present so this enzyme cannot be contaminated by a measurable level of ADP or ATP.

The inhibition of the release of creatine by some compounds does limit the level of ATP or ADP which may be
detected in the presence of such compounds. However, provided internal standards are used, the method is not invalidated by the presence of these inhibitory compounds. Thus, it would be possible to detect 1 part of ADP or ATP in 2,500 parts of AMP or DPN.

The method described is not claimed to give an accurate assay of the amount of ADP or ATP present in a test sample, nor would it distinguish between these two compounds. However, the method will estimate the order of the level at which ADP plus ATP are present in a given system.
SUMMARY

1. A number of linked enzyme systems in which ADP and ATP act catalytically have been investigated as possible methods for the detection of trace levels of these compounds.

2. It has been found that myosin and CPT catalyse a release of creatine from PC which is dependent on the addition of either ADP or ATP and will detect these compounds if present at a final concentration of $1 \times 10^{-7}$M.

3. Although some substances inhibit the release of creatine in the presence of low levels of ADP or ATP the method of detection may be used in the presence of these compounds provided internal standards are used.
CHAPTER 2

THE BIOSYNTHESIS OF N-PHOSPHOPRYLCREATINE:
AN INVESTIGATION INTO THE POSTULATED
ALTERNATIVE PATHWAY
CHAPTER 2.

THE BIOSYNTHESIS OF N-PHOSPHORYLCREATINE: AN INVESTIGATION
INTO THE POSTULATED ALTERNATIVE PATHWAY

INTRODUCTION

Before 1956, it was generally accepted that the sole pathway for the synthesis of PC was the reaction catalysed by CPT:

\[ \text{Creatine} + \text{ATP} \rightarrow \text{PC} + \text{ADP} \] (1).

Although the results of isotopic experiments (Sacks & Altshuler, 1942; Harvey, 1955) had been interpreted as indicating the possible presence in muscle of a synthetic pathway for the formation of PC not involving the adenine nucleotides, there was no direct evidence for such a reaction until the report by Cori, Abarca, Frenkel and Traverso-Cori (1956). These authors claimed that extracts of skeletal muscle catalyse the direct transfer of a phosphoryl group from 1:3-DPGA to creatine to form PC according to the equation:

\[ \text{Creatine} + 1:3-\text{DPGA} \rightarrow \text{PC} + 3\text{-PGA} \] (2).

In a more detailed report (Cori, Traverso-Cori, Lagarrigue & Marcus, 1958) it was suggested that only a single enzyme was involved. Furthermore, as supporting evidence for the non-participation of ATP, it was stated that there was no inhibition of PC synthesis in the presence of either ATPase or glucose plus hexokinase and that no adenine nucleotides could be detected in the reaction mixtures at the end of the incubation.
Ennor and Morrison (1958) have pointed out that because of the ubiquitous distribution of the adenine nucleotides and CPT any demonstration of an alternative pathway of PC synthesis must unequivocally exclude CPT. Whilst Cori et al. (1958) took rigorous precautions to exclude adenine nucleotides from the reaction mixtures, they were unable to obtain a preparation free from CPT. However, the new reaction has received some acceptance. Thus Cohn (1959) has cited it as being an exception to the general rule that ester phosphoryl groups are transferred to form O-P bonds and Bock (1960) has pointed out that this reaction does not fit into his classification of phosphate transfer enzymes.

Reference has been made to the reaction by Joyce and Grisolia (1959) and by Padieu and Mommaerts (1960).

In the present work the reaction has been re-examined and an attempt has been made to ascertain whether in fact CPT is involved. No specific inhibitor of this enzyme is known and the first approach was an endeavour to separate CPT from the enzyme responsible for catalysing reaction (2). It has been found that relatively crude preparations from rat and rabbit skeletal muscle will catalyse the overall reaction described by Cori et al. (1958) under conditions similar to those described by these authors. Further fractionation, however, caused a loss of activity and since this could be recovered in greater than additive amounts by recombination of fractions, it seemed that more than one enzyme was involved. Furthermore, it has been found that DPN or DPNH₂ which was added by the above authors as part of the reaction system, not
only functions as part of the system, but also gives rise to AMP which is converted to ATP under the experimental conditions. Evidence will be presented that a direct phosphoryl group transfer is improbable and that the reaction described probably involves the participation of adenosine di- and tri-phosphate as well as CPT.

**EXPERIMENTAL**

**Chemicals.** Unless otherwise stated nucleotides and substrates were obtained from Sigma Chemical Co., St. Louis, Mo. Commercial samples of DPN (purity, 95%) and enzymically prepared DPNH₂ (purity, 87-90%) were tested before use, as described in Chapter 1, for contamination by ATP or ADP. Some samples of DPN were purified by chromatography on Dowex-1 (formate) (Kornberg, 1957) and such samples were used to prepare DPNH₂ by chemical reduction (Beisenherz, Bücher & Garbade, 1955). ADP ribose was obtained from Pabst Laboratories, Milwaukee, Wis. The preparation of acid washed Norit A and Nuchar C and the purification of AMP have been described in Chapter 1.

The barium salts of FDP and 3-PGA and the silver-barium salt of PEP were converted to sodium salts by adding them as slurry to the top of a column of Zeo-Karb 225 (Na⁺) and washing through with water. The effluent was adjusted to pH 7.4 before making to the required volume and then treated with acid-washed charcoal. PC was prepared as the sodium salt (Ennor & Stocken, 1957).
Sodium pyruvate was prepared from freshly distilled pyruvic acid which was diluted with an equal volume of water, cooled and adjusted to pH 5 with 2N NaOH. The sodium salt was then precipitated with acetone. Ammediol was obtained from Eastman Organic Chemicals, Rochester, N.Y. and neutralised with HCl. Hydroxylamine hydrochloride (British Drug Houses Ltd.) was neutralised before use with KOH. Calcium phosphate gel was prepared according to the method of Keilin and Hartree (1938).

**Enzymes.** Unless otherwise stated, frozen rabbit muscle was used as the source of the enzymes. Adenylic acid deaminase was prepared by the method of Lee (1957) to the stage of elution from calcium phosphate gel. Aldolase (Taylor, Green & Cori, 1948) and D-glyceraldehyde-3-phosphate dehydrogenase (Cori, Slein & Cori, 1948) were prepared in crystalline form. Both enzymes were twice recrystallised and stored as suspensions in 0.50 and 0.66 saturated solutions of ammonium sulphate, respectively. The preparation of myokinase, CPT and myosin have been described in Chapter 1.

Apyrase was prepared from potatoes using the method described by Thoai, Roche and An (1954) and was found not to attack AMP. DPNase was prepared from mycelia of *Neurospora crassa* (Kaplan, 1955) and had an activity of 2,300 units/mg. of protein. Alcohol dehydrogenase and hexokinase were commercial preparations obtained from Sigma Chemical Co. and lactic dehydrogenase was a sample from L. Light & Co.
Rat and rabbit muscle preparations used in the early experiments were prepared as described by Cori et al. (1958) with the exception that ammediol buffer was used. Subsequently the following procedure was adopted. All operations were carried out at 2° and centrifugations were carried out 15 min. after the addition of ammonium sulphate. The muscle was treated for 2 min. in a Waring Blender with 3 vol. of 0.01 M ammediol buffer (pH 8) and the mixture was centrifuged at 1,700 g. for 20 min. The extract was filtered through cotton wool to remove fat. Ammonium sulphate (330 g./l.) was then added slowly to the filtrate with mechanical stirring. The precipitate was removed by centrifugation and ammonium sulphate (155 g./l) was added slowly with mechanical stirring to the supernatant. The precipitate was collected by centrifugation, dissolved in 0.01 M ammediol buffer, pH 8 (0.12 volume of original extract) and dialysed against the same buffer for 16 hr. (Fraction I). The dialysed preparation was stirred for 15 min. with Dowex-1 (Cl⁻) (1 g./10 ml.) and then diluted to a protein concentration of 25 mg./ml. Ammonia solution (17 N) was added to a saturated solution of ammonium sulphate at 0° so that (on testing a dilution of 1 in 10) the pH was 7.4. This solution (1.5 vol.) was added dropwise with mechanical stirring to the enzyme solution obtained from the previous step and the precipitate removed by centrifugation. The supernatant was brought then to 0.9 saturation by the slow addition of solid ammonium sulphate (220 g./l.).
The precipitate was collected by centrifugation, dissolved in ammediol buffer (0.4 vol. of Fraction I). This preparation (Fraction II) was stable for at least 2 months at 1-2°C, but its stability was reduced after treatment with charcoal. Fraction I has also been used to obtain some of the results reported in this chapter and unless otherwise stated, was stirred before use with Norit A (1 g./10 ml.) for 15 min. at 2°C and filtered. Enzyme preparations have also been treated with Dowex-1 (100 mg./ml.) in the chloride form as the loss of protein is less than that obtained as a result of treatment with charcoal.

**METHODS**

Methods for the estimation of creatine and protein have been described in Chapter 1.

**N-Phosphorylcreatine.** FC formation was demonstrated as creatine disappearance during incubation, and verified by recovery of creatine after hydrolysis for 9 min. at 65°C at pH 1-2.

**Inorganic phosphorus.** P_i was determined either by the method of King (1932) or, if labile organic phosphorus were present, by the method of Ennor and Stocken (1950).

**3-Phosphoglyceric acid.** 3-PGA was estimated by the method of Bartlett (1959).

**Ribose.** This was estimated using the orcinol reaction as described by Hurlbert, Schmitz, Brumm and Potter (1954).

**Diphosphopyridine nucleotide.** DPN was estimated either chemically
by the formation of the cyanide complex (Colowick, Kaplan & Ciotti, 1951) or enzymically with alcohol dehydrogenase using the procedure described by Bonnichsen and Brink (1955).

Reduced diphosphopyridine nucleotide. DPNH₂ oxidation was followed by studying the rate of decrease of absorption at 340 nm using the Beckman Model DK-2 ratio-recording spectrophotometer.

Nicotinamide. Nicotinamide was determined by measurement of the absorption at 262 nm (molecular extinction, 3.1 x 10⁶) and distinguished from nucleosides or nucleotides containing nicotinamide by the increased absorption in 0.01 N HCl (molecular extinction, 5.28 x 10⁶).

Hydroxamic acids. These were estimated by the method of Lipmann and Tuttle (1945).

Detection of Low Levels of Adenine Nucleotides. A method for the detection of low levels of adenine nucleotides in the nucleotide substrates used has been described in Chapter 1. No ATP or ADP could be detected in the purified samples of DPN and AMP, or in the commercial DPN used in this work. However, the commercial DPNH₂ and ADP ribose were contaminated by ATP or ADP to the extent of 0.1 - 0.2% and 0.05 - 0.1% respectively.

Assay of Adenine Nucleotides. The determination of AMP and ATP plus ADP was carried out using adenylic acid deaminase and potato apyrase (Kalckar, 1947) and the changes in optical density were followed in a Beckman DK-2 ratio-recording spectrophotometer in the presence of 0.1 M sodium succinate buffer (pH 6.5).
Chromatography and Electrophoresis. Nucleotides and nicotinamide were chromatographed on Whatman 3 MM paper by ascending chromatography either in (a) isobutyric acid: $NH_3$ (0.88 s.gr.): water (66:1:33; by vol.) or (b) ethanol: 1.0 M ammonium acetate pH 7.5 (7:3; v/v) and were detected by visual inspection in ultraviolet light. Nicotinamide and its derivatives were also detected by the methods described by Kodicek and Reddi (1951). Nicotinamide and DPN could be rapidly separated (2 - 3 hr.) by electrophoresis either in 0.033 M borate buffer, pH 8.9 or 0.033 M phosphate buffer, pH 7.0 (Sundaram, Rajagopalan & Sarma, 1959). Dinitrophenylhydrazones were chromatographed by ascending chromatography in (c) tert-amyl alcohol: ethanol: water (50:10:40; by vol.) (Ichihara & Greenberg, 1957) or by descending chromatography in (d) butanol: 1 N NaHCO$_3$ (1:2; v/v) using bicarbonate washed papers (Seligson & Shapiro, 1952). Free acids were separated by descending chromatography in (e) 1-pentanol: 5 M formic acid (1:1; v/v) (Buch, Montgomery & Porter, 1952) and detected by spraying with bromophenol blue. Hydroxamic acids were chromatographed by descending chromatography in (f) sec-butanol: formic acid: water (75:15:10; by vol.) (Hoagland, Keller & Zamecnick, 1956), (g) ethyl acetate: acetone: water (1:2:2.75; by vol.) (Michael & Albers, 1956) and (h) butanol: acetic acid: water (4:1:5; by vol.) (Thompson, 1951) and were detected by the method of Lipmann and Tuttle (1945).
RESULTS

Because creatine release from PC can be more readily determined than can the formation of PC from creatine, the initial studies were concerned with the reaction between PC and 3-PGA to form creatine and 1:3-DPGA. It was pointed out by Cori et al. (1958) that this approach suffers from the disadvantage that the equilibrium of the reaction in this direction is unfavourable but that the disadvantage could be overcome by the addition of triose phosphate dehydrogenase, DPNH$_2$ and arsenate. This results in arsenolysis of the 1:3-DPGA formed and thus increases the release of creatine according to the following reactions:

\[
\begin{align*}
\text{PC} + 3\text{-PGA} & \xrightleftharpoons{\text{triose phosphate dehydrogenase}} 1:3\text{-DPGA} + \text{Creatine} \\
1:3\text{-DPGA} + \text{H}_2\text{O} & \rightarrow 3\text{-PGA} + \text{P}_i \\
\text{DPNH}_2 & \text{arsenate}
\end{align*}
\]

With the additions referred to above, the results obtained were similar to those reported by Cori et al. (1958). Thus charcoal-treated enzyme preparations from both rat and rabbit skeletal muscle catalysed the release of creatine from PC; the release of creatine was dependent upon the presence of 3-PGA and DPNH$_2$ and there was activation by the addition of arsenate and Mg$^{2+}$ (Table 2.1). In the present experiments, the reaction rate was linear over the first 30 min. and was unaffected by the addition
TABLE 2.1

Requirements for the release of creatine from PC

in the presence of 3-PGA

The complete reaction mixture contained ammediol buffer (pH 7.4), 100 μmoles; 3-PGA, 4 μmoles; PC, 2.5 μmoles; Mg$^{2+}$, 5 μmoles; DPNH$_2$, 0.25 μmoles; arsenate, 1 μmole; enzyme, 2 mg. of a 0.64 - 0.75 sat. ammonium sulphate fraction of rabbit muscle extract prepared as described by Cori et al. (1958). Volume, 1.0 ml.; temp. 38°; reaction time, 15 min. The enzyme preparation was stirred at 2° for 15 min. with Norit A (1 g./10 ml.) and filtered. The filtrate was stirred overnight with a similar amount of charcoal and again filtered.

<table>
<thead>
<tr>
<th>Components</th>
<th>Creatine Release (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0.315</td>
</tr>
<tr>
<td>Without arsenate</td>
<td>0.125</td>
</tr>
<tr>
<td>Without DPNH$_2$</td>
<td>0</td>
</tr>
<tr>
<td>Without 3-PGA</td>
<td>0</td>
</tr>
<tr>
<td>Without Mg$^{2+}$</td>
<td>0.045</td>
</tr>
</tbody>
</table>
of triose phosphate dehydrogenase which, as was subsequently shown, was present in all muscle preparations. The rate of creatine release from PC was dependent upon the concentration of DPNH₂ and purified DPN was far less effective than DPNH₂ (Table 2.2). Since triose phosphate dehydrogenase catalyses equally well the arsenolysis of 1:3-DPGA in the presence of either DPN or DPNH₂ (Racker & Krimsky, 1952), this result suggests that if 1:3-DPGA is formed, then its formation is dependent on the presence of DPNH₂ and therefore probably does not occur by direct phosphoryl group transfer.

In the absence of arsenate, DPNH₂ was oxidised when incubated with the enzyme preparation, 3-PGA and PC. The rate of this reaction was linear, as also was the release of creatine, but the amount of DPNH₂ oxidised at any time was greater than the amount of creatine released (Table 2.3). According to the reaction postulated by Cori et al. (1958) the creatine release should be equivalent to the 1:3-DPGA formed and if the oxidation of DPNH₂ were due to the reaction

\[
1:3-\text{DPGA} + \text{DPNH}_2 \rightarrow 3-\text{GAP} + P_i + \text{DPN}
\]

the amount of DPNH₂ oxidised could not exceed the amount of creatine released.

**Evidence for the Requirement of More than One Enzyme for the Reaction 3-PGA + PC → 1:3-DPGA + creatine**

When the enzyme preparation (Fraction I) was subjected
**TABLE 2.2**

**Effect of DPN and DPNH\textsubscript{2} on the release of creatine from PC in the presence of 3-PGA**

Reaction mixtures contained ammediol buffer (pH 7.4), 100 μmoles; 3-PGA, 4 μmoles; PC, 2 μmoles; Mg\textsuperscript{2+}, 5 μmoles; arsenate, 1 μmole; enzyme, 4.2 mg. of (A) and 5.6 mg. of (B) where A and B represent different preparations of Fraction I treated with charcoal as described in Table 1. Total volume, 1.0 ml. Tubes were incubated for 15 min. at 38°.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Concentration (mM)</th>
<th>Creatine Release (μmoles/ml.)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>DPN (Purified)</td>
<td>0.25</td>
<td>-</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>-</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>DPNH\textsubscript{2}</td>
<td>0.25</td>
<td>0.323</td>
<td>0.360</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.144</td>
<td>0.160</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.062</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>0.043</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.018</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DPNH\textsubscript{2} (Prepared chemically from purified DPN)</td>
<td>0.25</td>
<td>-</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>-</td>
<td>0.19</td>
<td></td>
</tr>
</tbody>
</table>
The relationship between DPNH₂ oxidation and the release of creatine from PC in the presence of 3-PGA

The reaction mixture contained ammoniicol buffer (pH 7.4), 0.75 mmoles; PC, 7.5 µmoles; Mg²⁺, 15 µmoles; cysteine, 60 µmoles; 3-PGA, 12 µmoles; DPNH₂, 0.3 µmoles and enzyme, 1.2 mg. of a 0.6 - 0.9 sat. ammonium sulphate fraction of muscle extract treated with charcoal as described in Table 1. The total volume was 3 ml. and the reaction mixture was incubated in spectrophotometer cells at 18°.

<table>
<thead>
<tr>
<th>Incubation Time (min.)</th>
<th>Creatine Released (µmoles/ml.)</th>
<th>DPN Formed (µmoles/ml.)</th>
<th>DPNH₂ Oxidised (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.029</td>
<td>0.05</td>
<td>52</td>
</tr>
<tr>
<td>20</td>
<td>0.058</td>
<td>0.096</td>
<td>100</td>
</tr>
</tbody>
</table>
to further fractionation with calcium phosphate gel, acetone or ethanol, it was found that the specific activity of each fraction obtained was less than that of the original solution. (Enzymic activity was determined by measuring the rate of creatine release from PC in the presence of 3-PGA, DPN, triose phosphate dehydrogenase and arsenate). As activity could be recovered in more than additive amounts by recombination of two or more fractions, it seemed that more than one enzyme was involved and that the mechanism proposed by Cori et al. (1958) was probably incorrect.

If a direct phosphoryl group transfer were operative, it would be expected that when PC, 3-PGA and hydroxylamine were incubated with the enzyme preparation (Fraction I) a hydroxamic acid derivative of 1:3-DPGA would be formed. However, under these conditions, no such derivative could be detected. The failure to obtain a hydroxamate was not due to the high KCl concentration associated with the hydroxylamine, for an equivalent amount of KCl did not inhibit the creatine release with added DPNH₂. Whether or not creatine was released in the presence of hydroxylamine could not be determined as this latter compound interfered with the colorimetric reaction employed for the determination of creatine.

The addition of DPNH₂ (2.5 x 10⁻⁴ M) to the reaction mixture caused the formation of a hydroxamate which was also obtained in the absence of PC. The reaction could not be attributed to the ADP or ATP content (0.1 - 0.2%) of the DPNH₂ used, for these adenine
nucleotides were inactive at a level of $10^{-6} M$, although activity was obtained at higher levels. When a twice-charcoaled enzyme preparation was used, DPNH$_2$ could not be replaced by DPN, although the latter was active after chemical reduction (cf. Table 2.2).

Parenthetically, it should be mentioned that DPN addition did bring about hydroxamate formation with a once-charcoaled preparation. The reason for this difference is not known, but may be related to the further removal of protein by a second charcoal treatment.

Reactions of 3-PGA catalysed by the Muscle Preparation in the absence of PC

In view of the above results, investigations were directed towards the identification of the hydroxamic acid derivative, formed from 3-PGA in the presence of either DPN or DPNH$_2$, and the elucidation of the reactions involved in its formation.

The hydroxamic acid derivative formed in the presence of 3-PGA and either DPN or DPNH$_2$, in the absence of phosphoryl group donors, was obtained under the conditions described in Table 2.4. As the enzyme preparation also contained 3-PGA-kinase, the hydroxamic acid derivative of 1:3-DPGA was prepared under the same conditions with ATP ($2 \times 10^{-2} M$) replacing the DPNH$_2$ or DPN. At the end of the incubation period (2 hr.) an equal volume of ethanol was added and the mixtures were boiled. Cations were removed from the supernatant by addition of Zeo-Karb 225 ($H^+$ form). The hydroxamates
TABLE 2.4

The effect of fluoride on the hydroxamate formation from 3-PGA in the presence of DPN and DPNH₂

Reaction mixtures contained nucleotide (non-purified), 1 μmole; 3-PGA, 8 μmoles; Mg²⁺, 5 μmoles; hydroxylamine, 0.2 mmoles; enzyme, 3.5 mg. of Fraction I and either phosphate buffer (pH 7.4), 50 μmoles or ammediol buffer (pH 7.4), 100 μmoles, in a total volume of 1 ml. After 1 hr. at 38°, the apparent hydroxamate formation was determined, by the method of Lipmann and Tuttle (1945). The results were calculated from the molecular extinction value for the hydroxamate of 1:3-DPGA as quoted by Krimsky (1959).

The inhibition of the colour formation by components of the reaction mixtures and added fluoride ions was studied using succinic anhydride as a standard.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Nucleotide</th>
<th>Fluoride added (μmoles)</th>
<th>Apparent Hydroxamate formation (μmoles/ml.)</th>
<th>Inhibition of the colour formation (%)</th>
<th>Corrected Hydroxamate formation (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>DPN</td>
<td>-</td>
<td>1.62</td>
<td>31.5</td>
<td>2.37</td>
</tr>
<tr>
<td></td>
<td>DPNH₂</td>
<td>-</td>
<td>1.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DPN</td>
<td>5</td>
<td>0.303</td>
<td>33.0</td>
<td>0.452</td>
</tr>
<tr>
<td></td>
<td>DPNH₂</td>
<td>5</td>
<td>0.176</td>
<td></td>
<td>0.262</td>
</tr>
<tr>
<td></td>
<td>DPN</td>
<td>10</td>
<td>0.016</td>
<td>34.5</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>DPNH₂</td>
<td>10</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Ammediol</td>
<td>DPNH₂</td>
<td>-</td>
<td>2.08</td>
<td>17.0</td>
<td>2.51</td>
</tr>
<tr>
<td></td>
<td>DPNH₂</td>
<td>5</td>
<td>2.25</td>
<td>18.3</td>
<td>2.79</td>
</tr>
</tbody>
</table>
formed from 3-PGA in the presence of DPN, DPNH$_2$ or ATP respectively showed identical behaviour when chromatographed in solvents (f), (g) and (h), and the presence of phosphate in each was demonstrated after chromatography in solvent (f). In this solvent there was a clear separation of the hydroxamate ($R_F = 0.1$) from $P_1$ ($R_F = 0.5$). It was thus concluded that the muscle preparations give rise to the formation of 1:3-DPGA in the presence of 3-PGA and either DPNH$_2$ or DPN.

The formation of 1:3-DPGA from 3-PGA in the presence of either DPN or DPNH$_2$ could be explained by the conversion of some 3-PGA to PEP which would then act as a phosphoryl group donor. That such an explanation is likely is indicated by the finding that hydroxamate formation with both DPN and DPNH$_2$ was inhibited by fluoride in the presence, but not in the absence, of phosphate (Table 2.4). The probable involvement of PEP was also shown by the disappearance of 3-PGA in the absence of pyridine nucleotides (cf. Table 2.6) and the formation of an iodine-labile phosphorus compound (Schmidt, 1957).

The transfer of a phosphoryl group from PEP to 3-PGA would result in the formation of pyruvate in addition to 1:3-DPGA. That such a reaction did occur was confirmed by the isolation of pyruvate from those reaction mixtures which
contained DPN or DPNH₂. The identity of the isolated material as pyruvate was confirmed by the similarity in chromatographic behaviour of the free acid and its 2:4-dinitrophenylhydrazone with that of an authentic sample of pyruvate and its hydrazone (see Methods). In addition, the melting point (214 - 215°) of the 2:4-dinitrophenylhydrazone derivative was identical with that of the authentic compound and was unchanged by admixture with the latter.

In the presence of pyridine nucleotides 3-PGA gave rise to P₁ (Fig. 2.1). Although some phosphatase activity is apparent, the addition of DPN or DPNH₂ has a marked effect on the release of P₁. The kinetics of P₁ release show that the initial rate is higher with DPNH₂ than with DPN and are consistent with P₁ being derived from 1:3-DPGA. This compound decomposes spontaneously to P₁ and 3-PGA at pH 7.4 and 38° but in the presence of triose phosphate dehydrogenase and arsenate a much more rapid breakdown occurs.

The conversion of 3-PGA to 1:3-DPGA, involving PEP, which occurs in the presence of DPN (or DPNH₂) and the muscle preparation would also be catalysed by the enzymes of the glycolytic cycle in the presence of ADP or ATP. That the appropriate glycolytic enzymes were present in the muscle preparation was demonstrated by the fact that there was a release of P₁ from 3-PGA when DPNH₂ was replaced by either ADP or ATP (Table 2.5). The greater effectiveness
Fig. 2.1. Rate of release of $P_i$ from 3-PGA on the addition of DPN and DPNH$_2$, in the presence and absence of arsenate. Reaction mixtures contained 3-PGA, 5.5 $\mu$moles; Mg$^{2+}$, 5 $\mu$moles; ammediol buffer (pH 7.4), 200 $\mu$moles and enzyme, 4 mg. of Fraction I in a total volume of 0.66 ml. Temp., 38°. The additions were $\bigcirc$, DPNH$_2$ (1 $\mu$mole) and arsenate (1 $\mu$mole); $\Box$, DPN (commercial), (1 $\mu$mole) and arsenate (1 $\mu$mole); $\blacksquare$, DPNH$_2$ (1 $\mu$mole); $\bullet$, DPN (commercial), (1 $\mu$mole).
TABLE 2.5

The effect of DPNH₂ and adenine nucleotides on the release of P₁ from 3-PGA

Reaction mixtures contained 3-PGA, 8 μmoles; Mg²⁺, 5 μmoles; ammediol buffer (pH 7.4), 100 μmoles; arsenate (pH 7.4), 1 μmole; nucleotide, 1 μmole and enzyme, 4 mg. of Fraction I in a volume of 0.56 ml. Tubes were incubated at 38°.

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>Phosphate release (μmoles/tube)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min.</td>
</tr>
<tr>
<td>None</td>
<td>0.05</td>
</tr>
<tr>
<td>DPNH₂</td>
<td>0.272</td>
</tr>
<tr>
<td>ADP</td>
<td>0.128</td>
</tr>
<tr>
<td>ATP</td>
<td>0.127</td>
</tr>
</tbody>
</table>
of DPNH₂ can be explained by the requirement of triose phosphate dehydrogenase for either DPN or DPNH₂ in the arsenolytic reaction.

Reactions Between PC and 3-PGA in the Presence of DPN and DPNH₂

Hydrolysis of PC. A comparison of the amounts of Pᵢ formed from 3-PGA and from 3-PGA + PC (Table 2.6) shows that the increased amount of Pᵢ formed in the presence of PC is equivalent to the amount of creatine released, indicating overall hydrolysis of PC. It may also be noted that in both cases the Pᵢ release is markedly increased upon the addition of pyridine nucleotides and that the hydrolysis of 3-PGA is complete. In the absence of pyridine nucleotides, there is a loss of 3-PGA which was due to the formation of PEP and presumably 2-PGA.

When 3-PGA was replaced by pyruvate there was a release of creatine from PC, but only in the presence of DPN or DPNH₂ (Table 2.7). The amount of creatine released in 20 min. was greater in the presence of DPNH₂ than in the presence of DPN. The addition of arsenate increased the rate of both reactions and this finding suggests that 1,3-DPGA is formed.

The results described above can be interpreted in one of two ways -

1) DPNH₂ and DPN may in some way be functioning as a source of ADP or ATP, or

2) the pyridine nucleotides may be acting in a novel way as phosphoryl group carriers. It is
TABLE 2.6

The release of $P_i$ from 3-PGA in the presence and absence of PC

Reaction mixtures contained 3-PGA, 4 μmoles; $Mg^{2+}$, 2.5 μmoles; ammediol buffer (pH 7.4), 100 μmoles; arsenate (pH 7.4), 0.5 μmole and enzyme, 2 mg. of Fraction I. The additions were DPN (non-purified), 0.5 μmole; DPNH$_2$, 0.5 μmole; and PC, 2.5 μmoles. Volume 0.4 ml.; temp., 38°; incubation time, 3 hr.

| Additions | PC Absent | | PC Present | | | |
|-----------|-----------|-------------|-------------|-------------|-------------|
|           | $P_i$ (μmoles) | $P_i$ (μmoles) | 3-PGA (μmoles) | $P_i$ (μmoles) | $P_i$ (μmoles) | Creatine (μmoles) | 3-PGA (μmoles) |
| None      | 0.33       | -            | 1.28         | 0.76         | -            | 0             | 1.28          |
| DPNH$_2$  | 3.63       | 3.3          | 0            | 5.28         | 4.5          | 1.17          | 0             |
| DPN       | 3.75       | 3.4          | 0            | 5.68         | 4.9          | 1.47          | 0             |
TABLE 2.7

The effect of arsenate on the release of creatine from PC in the presence of pyruvate

Reaction mixtures contained PC, 2.5 μmoles; Mg^{2+}, 5 μmoles; ammonium buffer (pH 7.4), 100 μmoles; sodium pyruvate, 10 μmoles and enzyme, 4.5 mg of Fraction I treated with Dowex-1 Cl^− (1 g./10 ml.). The additions were arsenate (pH 7.4), 1 μmole; DPN (non-purified), 0.5 μmoles and DPNH₂, 0.5 μmoles. Total volume 1 ml. Creatine release was determined after 20 min. at 38°.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Creatine release (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>DPN</td>
<td>0.055</td>
</tr>
<tr>
<td>DPN + arsenate</td>
<td>0.171</td>
</tr>
<tr>
<td>DPNH₂</td>
<td>0.176</td>
</tr>
<tr>
<td>DPNH₂ + arsenate</td>
<td>0.600</td>
</tr>
</tbody>
</table>
unlikely, on energetic grounds, that the pyridine nucleotide phospharyl carrier would be TPN. Moreover, whilst the addition of TPN resulted in a release of creatine in the presence of 3-PGA, the reaction rate was slower than with an equivalent amount of DPN.

Tests were carried out to determine if DPN and DPNH$_2$ underwent enzymic hydrolysis at pH 7.4. The results were negative, as judged by assay with both the cyanide and alcohol dehydrogenase procedures, although the methods could not be relied upon to detect less than 3% hydrolysis.

**Release of Creatine from PC in the Presence of DPN**

If the pyridine nucleotides were acting as phosphoryl group carriers, a release of creatine (or pyruvate) should be obtained when PC (or PEP) is incubated in the presence of the muscle preparation with substrate amounts of DPN. Creatine was released under these conditions (Fig. 2.2) with no detectable release of P$_i$ or enzymic destruction of DPN. (DPN, rather than DPNH$_2$, was used for these experiments as it was known to be free of contaminating adenine di- and triphosphates). The addition of apyrase, either in the presence or absence of PC, did not bring about any detectable destruction of DPN (Table 2.8). It would be expected that if ATP or ADP were formed as a result of a reaction between PC and DPN, the addition of apyrase would give rise to
Fig. 2.2. The rate of release of creatine from PC in the presence of DPN. The reaction mixture contained ammonium buffer (pH 8.5), 100 μmoles; Mg$^{2+}$, 5 μmoles; PC, 5 μmoles; DPN (commercial), 2.5 μmoles and enzyme, 8.3 mg. of Fraction II. Volume, 1 ml., temp., 38°.
TABLE 2.8

Stability of DPN under conditions leading to the DPN-dependent release of creatine from PC

Reaction mixtures contained ammediol buffer (pH 8.5), 100 μmoles; Mg²⁺, 5 μmoles and DPN (non-purified), 2.5 μmoles. Additions were PC, 5 μmoles; apyrase, 33 μg. and enzyme, 7 mg. of Fraction II (Experiment 1) or 8 mg. of Fraction I (Experiment 2). Volume, 1 ml., temp. 38°, incubation time, 4 hr. (Experiment 1) or 2 hr. (Experiment 2). For the DPN assays 0.15 ml. samples were taken and the final volume in each case was 3.6 ml.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Creatine release (μmoles/ml.)</th>
<th>Cyanide Method (E₃₂₅ m⁻¹)</th>
<th>Enzymic Method (E₃₄₀ m⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With Enzyme</td>
<td>Without Enzyme</td>
<td>With Enzyme</td>
</tr>
<tr>
<td>Experiment 1.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>0.578</td>
<td>0.583</td>
</tr>
<tr>
<td>PC</td>
<td>0.60</td>
<td>0.583</td>
<td>0.583</td>
</tr>
<tr>
<td>Experiment 2.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>0.560</td>
<td>-</td>
</tr>
<tr>
<td>PC</td>
<td>0.48</td>
<td>0.554</td>
<td>-</td>
</tr>
<tr>
<td>Apyrase</td>
<td>-</td>
<td>0.554</td>
<td>-</td>
</tr>
<tr>
<td>Apyrase + PC</td>
<td>5.0</td>
<td>0.560</td>
<td>-</td>
</tr>
</tbody>
</table>
increased hydrolysis of DPN. However, when apyrase was present, there was a quantitative release of creatine and P\textsubscript{i} from PC which was dependent upon DPN. The amount of creatine released and hence the amount of product formed was affected by pH and DPN concentration (Table 2:9), but was unaffected by the Mg concentration.

These results are consistent with the formation of a phosphorylated derivative of DPN, which could be hydrolysed by apyrase and efforts were directed towards the isolation of the compound.

The fractionation on Dowex-1 (formate) of the reaction mixture after DPN and PC were incubated with the muscle preparation at pH 8.5, was carried out as described in Table 2:10. The effluent from the Dowex-1 column was free of ribose and was identified spectrophotometrically and chromatographically as containing nicotinamide. Fraction 1 was identified chromatographically as containing DPN and treatment of this fraction with apyrase did not give rise to P\textsubscript{i}. Fraction 2 was free of acid-labile phosphate and was shown to contain adenine: ribose: phosphate in the ratio 1: 2.02: 1.8, indicating that the compound was ADP ribose. A comparison of the paper chromatographic behaviour of this compound in solvent systems (a) and (b) with that of an authentic sample of ADP ribose showed that the two compounds were identical. The presence of ATP in fraction 3 was shown by paper
Factors affecting the release of creatine from PC in the presence of DPN

The reaction mixtures (Table A) contained $\text{Mg}^{2+}$, 5 μmoles; PC, 5 μmoles; DPN (non-purified), 2.5 μmoles; enzyme, 14 mg. of Fraction II and ammediol buffer (required pH), 100 μmoles. Volume, 1 ml.; temp., 38°. Creatine was determined at the end of a 3 hr. incubation period and the pH of duplicate tubes checked after the same period. Results reported in Table B were obtained under the same conditions as described in Table A with the exception that ammediol buffer (pH 8.5) was used and the DPN concentration was varied.

<table>
<thead>
<tr>
<th>pH</th>
<th>Creatine release (μmoles/ml.)</th>
<th>DPN (μmoles/ml.)</th>
<th>Creatine release (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>0.45</td>
<td>3.5</td>
<td>0.74</td>
</tr>
<tr>
<td>7.8</td>
<td>0.49</td>
<td>2.5</td>
<td>0.60</td>
</tr>
<tr>
<td>8.4</td>
<td>0.60</td>
<td>2.0</td>
<td>0.49</td>
</tr>
<tr>
<td>8.7</td>
<td>0.68</td>
<td>1.5</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25</td>
<td>0.10</td>
</tr>
</tbody>
</table>
Analysis of the products formed as a result of the incubation of DPN with PC

The incubation mixture contained DPN (non-purified), 136 μmoles; PC, 250 μmoles; ammediol buffer (pH 8.5), 4 mmoles; Mg²⁺, 200 μmoles and enzyme, 166 mg. of Fraction II in a volume of 40 ml. The mixture was incubated for 2 hr. at 38° and the release of creatine was 0.31 μmoles/ml. The reaction mixture was passed through a column of Nuchar C (3 g.) which was washed consecutively with 100 ml. of water, 60 ml. of 0.01 N ethylenediaminetetraacetic acid (pH 7.0), and 100 ml. of water. Nucleotides were eluted with 250 ml. of a mixture of isoamyl alcohol:water:ethanol (10:40:50). The organic solvent was removed under reduced pressure and the solution freeze-dried. The solids were redissolved in 50 ml. of water, adjusted to pH 8.5 by addition of 1 N NaOH and fractionated on Dowex-1 (formate) at 2°. The fractions were recovered from the eluting solution by adsorption on and elution from Nuchar C as described above.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Eluting Solution</th>
<th>Product Identified</th>
<th>Recovery (% of total E&lt;sub&gt;260 mμ&lt;/sub&gt; material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effluent</td>
<td>Water</td>
<td>Nicotinamide</td>
<td>0.6</td>
</tr>
<tr>
<td>1.</td>
<td>Formic acid (0.1 N)</td>
<td>DPN</td>
<td>81.0</td>
</tr>
<tr>
<td>2.</td>
<td>Formic acid (4 N)</td>
<td>ADP ribose</td>
<td>10.5</td>
</tr>
<tr>
<td>3.</td>
<td>Formic acid (4 N)</td>
<td>ATP</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>(4 N) - Ammonium formate (0.4 N)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
chromatography and enzymic analysis. Thus the amount of $P_i$
released by myosin was half that released by apyrase and by
myosin plus myokinase. The product formed by the action of
apyrase was AMP as judged by analysis with adenylic acid deaminase.
Fraction 3 also contained, apart from ATP, some material which
showed absorption at 260 nm, but no attempt was made to isolate
or identify this material.

The ATP could have arisen either as a result of
(1) hydrolysis, during the isolation, of a phosphorylated derivative
of DPN, (2) contamination of the commercial DPN by some compounds
which can give rise to ATP under the experimental conditions,
(3) non-enzymic hydrolysis of DPN to AMP with subsequent phosphoryl-
ation of the latter by PC.

To determine if ATP arose as a result of the breakdown
of a phosphorylated derivative of DPN, incubation mixtures were
analysed directly for ATP after incubation of DPN and PC with the
enzyme preparation for 2 hr. under the conditions described in
Table 2,10. The high concentration of DPN precluded accurate
analysis with the myosin-CPT system, but accurate analysis could
be obtained using adenylic acid deaminase and apyrase, as these
enzymes are unaffected by the presence of DPN. The results showed
that ATP or ADP was formed and corresponded to about 10% of the
added DPN. This assay also showed that AMP was formed in the
absence of PC and that the amount was equivalent to the ADP or ATP
formed when PC was present. If the muscle enzyme preparation were omitted, there was no AMP formation, so the latter must have arisen enzymically during the incubation. The formation of AMP in the absence of PC and of ATP in the presence of PC was confirmed by chromatographic and enzymic analysis of the products isolated according to the scheme shown in Fig. 2.3.

Since no enzymic hydrolysis of DPN could be detected in the reaction mixture it thus seemed that the AMP must have arisen from a contaminant of the DPN sample. Such a contaminant could be ADP ribose. The DPN used in these experiments had not been purified for it had been shown not to be contaminated with ATP, ADP or AMP as judged by analysis with CPT plus myosin and with adenylic acid deaminase. However, such tests would not give any indication of whether or not ADP ribose was present. When this compound was added the results were similar to those obtained in the presence of DPN (Table 2.11). The addition of DPNH2 and TPN also gave essentially the same results. The yield of AMP in the absence of PC and the yield of ATP and creatine in the presence of PC were approximately the same on the addition of DPN, DPNH2 and TPN, but were very much greater with an equivalent amount of ADP ribose. Under the same conditions, but with the amount of ADP ribose reduced to 0.3 μmole, it was shown by chromatography that this compound completely disappeared. At the same time, 0.6 μmoles of creatine was released from PC.
Fig. 2.3. Isolation of the adenine nucleotides formed as a result of the incubation of DPN with PC. The reaction mixture contained emediol buffer (pH 8.5), 2 mmoles; Mg$^{2+}$, 100 μmoles; DPN (commercial), 50 μmoles; enzyme, 144 mg of Fraction II and PC, 100 μmoles. In a second incubation, PC was omitted. Volume, 20 ml., temp, 38°C, incubation time, 4 hr.

Note: Boiling the reaction mixture for 1.5 min. did not increase the total adenine nucleotides as assayed by the adenylic acid deaminase-apyrase system.
TABLE 2.11

The formation of adenine nucleotides from pyridine nucleotides and ADP ribose

Reaction mixtures contained ammediol buffer (pH 8.5), 100 μmoles; Mg²⁺, 5 μmoles; nucleotide, 2.5 μmoles; enzyme, 7.2 mg. of Fraction II; and where indicated PC, 5 μmoles. Volume, 1 ml.; temp., 38°; incubation time, 4 hr.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Additions</th>
<th>AMP (μmoles/ml.)</th>
<th>ADP or ATP (μmoles/ml.)</th>
<th>Creatine (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN (non-purified)</td>
<td>None</td>
<td>0.30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DPNH₂</td>
<td>None</td>
<td>0.31</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>-</td>
<td>0.28</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>(Trace)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPN</td>
<td>None</td>
<td>0.348</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>-</td>
<td>0.33</td>
<td>0.68</td>
</tr>
<tr>
<td>ADP ribose</td>
<td>None</td>
<td>0.89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>-</td>
<td>0.91</td>
<td>1.28</td>
</tr>
</tbody>
</table>
(Because of the specificity of adenylic acid deaminase, the formation of AMP from TPN suggests that the phosphoryl group on the 3' position of the ribose moiety has been removed by hydrolysis).

The above results with ADP ribose could be accounted for by the reactions

\[
\text{ADP ribose} \rightarrow \text{ribose-5' phosphate + AMP}
\]

\[
\text{AMP + 2PC} \rightarrow \text{ATP + 2 creatine}.
\]

Release of Creatine from PC with purified DPN

The DPN sample used in the following experiments was purified by chromatography on Dowex-1 (formate). Analysis of the effluent indicated that it contained nicotinamide in an amount equivalent to 6% of the DPN. If it is assumed that nicotinamide arises as a result of the breakdown of DPN to ADP ribose, it may be concluded that the commercial DPN is contaminated by 6% of ADP ribose. The purified DPN was also capable of bringing about the release of creatine from PC at pH 7.4 but the amount of creatine released was less than that from an equivalent amount of commercial DPN (Table 2.12); the amount by which it was less was equivalent to twice the ADP ribose content of the commercial sample. It is clear then that some of the creatine released from PC by the addition of commercial DPN, is due to the presence of ADP ribose in the latter.

The activity of ADP ribose in bringing about the release of creatine from PC suggested that the activity obtained with
TABLE 2.12

Release of creatine from PC in the presence of purified and non-purified DPN

Reaction mixtures contained ammediol buffer (pH 8.5), 100 μmoles; Mg^{2+}, 5 μmoles; PC, 5 μmoles; enzyme, 7 mg. of Fraction II and 2.5 μmoles of DPN. Total volume, 1.0 ml. Creatine release was determined after 4 hr. at 38°.

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>Creatine released (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-purified DPN*</td>
<td>0.74</td>
</tr>
<tr>
<td>Purified DPN</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*2.5 μmoles of this sample were shown to contain 0.15 μmoles of ADP ribose.
purified DPN may be due to the non-enzymic breakdown of the latter. Such a breakdown would not have been detected in previous assays, as comparisons were made with a standard DPN solution which had been incubated under the same conditions but in the absence of enzyme (cf. Table 2.8). Analysis of DPN at zero time and after incubation for 4 hr. (Table 2.13) showed that at pH 7.4 non-enzymic hydrolysis of DPN could not be detected. However, at pH 8.5 there was approximately 4 - 5% hydrolysis. It would appear that the increased formation of AMP, ATP and creatine at pH 8.5 over that at pH 7.4 is due to the non-enzymic hydrolysis of DPN to ADP ribose. The lack of stoichiometry at pH 7.4 between the amount of creatine released and the amount of ATP formed is doubtlessly due to the limitations of the methods of assay.

The formation of AMP at pH 7.4 in the presence of the enzyme preparation, without detectable breakdown of DPN could be accounted for by the relative insensitivity of the DPN assay, especially as paper electrophoretic analysis of the reaction mixture after 4 hr. at pH 7.4 showed that nicotinamide (and therefore presumably ADP ribose) was formed from DPN. However, it is also possible that AMP may arise by enzymic hydrolysis at pH 7.4 of the α-isomer of DPN which hydrolysis would not be detected by means of the cyanide and alcohol dehydrogenase reactions. A check with purified *Neurospora* DPNase showed that the purified sample of DPN did contain 2.3% of the α-isomer and this could therefore make a
The hydrolysis of DPN to AMP and the conversion of AMP to ATP in the presence of PC

Reaction mixtures contained ammediol buffer, 100 μmoles; Mg²⁺, 5 μmoles; DPN (purified), 2.5 μmoles; enzyme, 7.4 mg. of Fraction II which had been stirred at 2° with Nuchar C (50 mg./ml.) and where indicated PC, 5 μmoles. Volume, 1.0 ml. Incubation time, 4 hr.; temp., 38°. For DPN assay, 0.15 ml. samples were taken and the final volume was 3.6 ml.

<table>
<thead>
<tr>
<th>Additions</th>
<th>DPN Assay Enzymic Method (E₃₄₀ mil)</th>
<th>Apparent DPN hydrolysis (μmoles/ml.)</th>
<th>Creatine release (μmoles/ml.)</th>
<th>AMP (μmoles/ml.)</th>
<th>ATP (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero 4 hr.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>pH 8.5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.710 0.680</td>
<td>0.106</td>
<td>0</td>
<td>0.246</td>
<td>0</td>
</tr>
<tr>
<td>PC</td>
<td>0.720 0.685</td>
<td>0.124</td>
<td>0.5</td>
<td>0</td>
<td>0.246</td>
</tr>
<tr>
<td>Boiled Enzyme + PC</td>
<td>0.720 0.692</td>
<td>0.099</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>pH 7.4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.729 0.726</td>
<td>&lt;0.02</td>
<td>0</td>
<td>0.068</td>
<td>0</td>
</tr>
<tr>
<td>PC</td>
<td>0.708 0.704</td>
<td>&lt;0.02</td>
<td>0.247</td>
<td>0</td>
<td>0.076</td>
</tr>
<tr>
<td>Boiled Enzyme + PC</td>
<td>0.710 0.713</td>
<td>&lt;0.02</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
contribution towards the amount of AMP formed. A third possibility is that even the purified DPN contains small amounts of ADP ribose. It has been found that whereas no ADP ribose can be detected in freshly purified preparations of DPN by paper chromatography, this compound can be detected after storage of the DPN either in solution or as a solid at 0° or -10°.

No detailed studies have been carried out with DPNH₂, but because this compound is more unstable than DPN, it might be expected that commercial samples would contain relatively larger amounts of ADP ribose. It is on this basis that the greater activity of DPNH₂ over that of DPN in releasing creatine from PC (Table 2.7) and P₁ from 1:3-DPGA (Fig. 2.1) could be explained. Chemical reduction of DPN may also give rise to ADP ribose as well as DPNH₂. If this were true, it would offer an explanation for the finding (Table 2.2) that purified DPN is active only after chemical reduction.

Release of Creatine from PC and formation of the hydroxamic acid derivative of 1:3-DPGA in the presence of ADP ribose and AMP

If the activity of the pyridine nucleotides is due to their non-enzymic conversion to, or contamination by, ADP ribose which is then enzymically converted to AMP, it follows that similar results should be obtained when the pyridine nucleotides are replaced by AMP or ADP ribose. Indeed, this was found to be so. In the presence of 3-PGA and the muscle enzyme, the addition of either of these compounds resulted in the formation of hydroxamic
acids and moreover, were more effective than equivalent concentrations of DPN (Table 2.14). It should also be noted that higher concentrations of AMP inhibit the hydroxamic acid formation. The enzyme preparation was also capable of catalysing the overall reaction

$$\text{AMP} + 2\text{PC} \rightarrow \text{ATP} + 2\text{creatine}.$$  

The equilibrium of the reaction was completely to the right as the amount of creatine released was twice that of the added AMP.

**Synthesis of PC from 1:3-DPGA and 3-PGA**

*in the presence of DPN, DPNH₂ and AMP*

Enzyme preparations from rat and rabbit skeletal muscle were shown to synthesize PC under the conditions described by Cori et al. (1958) (Table 2.15). It was also possible to confirm Cori's observation that the synthesis of PC was not reduced by the addition of an ATP utilizing system in the form of the glucose-hexokinase system. It should be noted, however, that the reaction had ceased within 30 min. Kinetic studies were not attempted in view of the complexity of the system.

No tests have been carried out with 1:3-DPGA but synthesis of PC was achieved by the addition of either AMP or DPN to a system containing 3-PGA and the muscle enzyme (Fig.2.4).

**DISCUSSION**

The results obtained in this work clearly indicate that the reaction

$$3\text{-PGA} + \text{PC} \leftrightarrow 1:3\text{-DPGA} + \text{creatine}$$
TABLE 2.14

Hydroxamate formation from 3-PGA in the presence of DPN, ADP ribose or AMP

Reaction mixtures contained 3-PGA, 8 μmoles; Mg\(^{2+}\), 5 μmoles; ammediol buffer (pH 7.4), 100 μmoles; hydroxylamine (pH 7.0), 0.2 mM; enzyme, 7.4 mg. of Fraction II, stirred for 15 min. at 2° with Nuchar C (50 mg./ml.), and test nucleotide in a volume of 1 ml. Tubes were incubated at 38° for 20 min. and hydroxamate formation determined as in Table 4. The values have been corrected for 17% inhibition of the colour formation by the reaction mixture.

<table>
<thead>
<tr>
<th>Nucleotide added</th>
<th>Concentration (mM)</th>
<th>Hydroxamate Formation (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN (purified)</td>
<td>2.5</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.246</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP Ribose</td>
<td>2.5</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0.264</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>2.5</td>
<td>1.73</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>3.32</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>2.93</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>2.57</td>
</tr>
</tbody>
</table>
**TABLE 2.15**

**Synthesis of N-Phosphorylcreatine**

Reaction mixtures contained phosphate buffer (pH 7.4), 25 μmoles; Mg$^{2+}$, 2.5 μmoles; cysteine (pH 7.4), 0.25 μmoles; pyruvate, 2.5 μmoles; fructose-1,6-diphosphate, 1.25 μmoles; creatine, 5 μmoles; lactic dehydrogenase, 0.125 mg.; aldolase, 0.25 mg.; D-glyceraldehyde 3-phosphate dehydrogenase, 0.125 mg. and rabbit muscle enzyme, 3.75 mg. of Fraction II. Additions were DPN, 0.5 μmoles; glucose, 5 μmoles; and hexokinase, 1 mg. All enzymes were stirred for 15 min. before use with Nuchar C (50 mg./ml.) and filtered. Volume, 0.55 ml.; temp., 38°.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Incubation Time (min.)</th>
<th>PC Synthesis (μmoles/tube)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN (Sigma)</td>
<td>90</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>0.50</td>
</tr>
<tr>
<td>DPN (purified)</td>
<td>90</td>
<td>0.43</td>
</tr>
<tr>
<td>DPN (purified) + glucose</td>
<td>30</td>
<td>0.41</td>
</tr>
<tr>
<td>DPN (purified) + glucose + hexokinase</td>
<td>30</td>
<td>0.43</td>
</tr>
</tbody>
</table>
Fig. 2.4. Synthesis of PC from creatine and 3-PGA in the presence of AMP or DPN. Reaction mixtures contained phosphate buffer (pH 7.4), 50 μmoles; Mg^{2+}, 5 μmoles; creatine, 5 μmoles; 3-PGA, 20 μmoles; AMP or DPN and enzyme, 7.4 mg. of Fraction II which had been stirred for 15 min. at 2° with Nuchar C (50 mg./ml.) and filtered. △ - AMP, 0.25 μmole; ○ - AMP, 0.1 μmole; ■ - DPN, 1.0 μmole; ● - DPN, 0.25 μmole; ▲ - DPN, 0.1 μmole.

Volume, 1 ml.; temp., 38°.
described by Cori et al. (1958) and catalysed by extracts of rabbit and rat skeletal muscle cannot be regarded as a direct phosphoryl group transfer, catalysed by a single enzyme. The evidence for this is twofold. Fractionation of the extracts results in a loss of activity which is restored by recombination of two or more fractions and no hydroxamic acid derivative of 1:3-DPGA is formed on incubation of 3-PGA and PC with hydroxylamine and the enzyme. The overall reaction does occur in both directions when either DPN or DPNH₂ is present.

Presumably it is because the pyridine nucleotides were added as an essential part of the test systems, that Cori et al. (1958) were able to obtain these reactions. DPN (and probably DPNH₂) does not appear to function as an intact molecule, but rather by virtue of its non-enzymic hydrolysis to ADP ribose (or as a result of its contamination by this compound), which in turn is enzymically converted to AMP. The experimental results indicate that AMP is converted to ATP, presumably via ADP, in the presence of either PEP or PC. (1:3-DPGA may be involved directly in the conversion of AMP to ATP, but in any event, this compound would be converted non-enzymically to 3-PGA which would give rise to PEP). As ATP is formed and the appropriate glycolytic enzymes are present in the enzyme preparation, the synthesis of PC from creatine and 1:3-DPGA and the degradation of PC in the presence of 3-PGA can be accounted for by well established reactions. Only
catalytic amounts of adenine nucleotide need be formed because of
the nature of the reactions.

It would seem that the failure of Cori et al. (1956) to
obtain this overall reaction with heart muscle preparations might
well be ascribed to the absence of one or more of the enzymes
required. Some preliminary experiments have shown that ATP is
formed as a result of incubation of DPN (or DPNH₂) in the presence
of PC and partially purified preparations from rabbit and pig heart
muscle, but no studies have been made of the overall reaction. It
is also surprising that these authors failed to obtain PC synthesis
when DPN, and 3-PGA were incubated with a rat skeletal muscle
preparation.

The inability of Cori et al. (1956) to detect the presence
of ADP and ATP in their incubation mixtures can be accounted for by
the insensitivity of their assay procedure. The minimum amount of
ADP (or ATP) which could be estimated by the method used was 0.005
μmoles. As the sample taken for estimation was equivalent to 0.3
ml. of the reaction mixture, then the presence of ADP (or ATP) at a
concentration of 1.7 x 10⁻⁵ M would escape detection. Such a
concentration of ADP was shown by these authors to be effective in
increasing the synthesis of PC from creatine (Table 5 of their paper).
It might also be mentioned that the values quoted by Cori et al.
(1958) for the CPT activity of the muscle extracts are very low and
in a number of experiments the values given for the amount of PC
synthesized in a 20' incubation period are taken to represent the
rate of synthesis - they should however be regarded as the amount of PC formed at equilibrium.

Strong evidence in favour of the non-participation of adenine di- and triphosphate was the finding by Cori et al. (1958) that whereas the synthesis of PC from creatine and ATP was inhibited by the addition of glucose and hexokinase, that from creatine and 1,3-DPGA was not. Because of the lack of kinetic studies, the interpretation of the results obtained is open to criticism. There are no grounds for assuming that the affinity of hexokinase for ATP is greater than that of CPT. Indeed, if the reverse were true then with high concentrations of CPT (and very large amounts of CPT are present in extracts of skeletal muscle), no inhibition of PC synthesis would be obtained. It would seem that the supposed inhibition of the CPT activity of the extracts could be explained as being due to the displacement of the equilibrium of the CPT catalysed reaction by the addition of glucose and hexokinase.

The conversion of AMP to ATP in the presence of either PEP or PC implies that an enzyme(s) is present in the muscle extract which can convert AMP to ADP. The further conversion of ADP to ATP would be catalysed either by pyruvic kinase or CPT, but these enzymes have been shown to be inactive with AMP. Experiments with charcoal-treated preparations have shown that myokinase plus CPT catalyse the overall reaction

\[
\text{AMP} + 2 \text{PC} \xleftrightarrow{\text{myokinase + CPT}} \text{ATP} + 2 \text{creatinine}
\]

although either enzyme alone is without effect. This reaction has been investigated and will be discussed in Chapter 3.
SUMMARY

1. The reaction

\[
3\text{-PGA} + \text{PC} \leftrightarrow 1,3\text{-DPGA} + \text{Creatine}
\]

does not involve a direct phosphoryl group transfer catalysed by a single enzyme.

2. The overall reaction in both directions is catalysed by preparations from rat and rabbit skeletal muscle in the presence of either DPN or DPNH₂.

3. The pyridine nucleotides function by virtue of their non-enzymic conversion to, or as a result of their contamination by, ADP ribose which undergoes enzymic hydrolysis to AMP.

4. Skeletal muscle preparations, treated with charcoal to remove ADP and ATP, catalyse the conversion of highly purified samples of AMP to ATP in the presence of either PC or PEP.

5. Rabbit skeletal muscle contains an enzyme which hydrolyses ADP ribose to AMP, and appears to be specific for this compound, but which does not hydrolyse either oxidised or reduced DPN.
CHAPTER 3.

A STUDY OF THE PATHWAY OF PHOSPHORYL GROUP

TRANSFER FROM N-PHOSPHORYLCREATINE TO ADENOSINE MONOPHOSPHATE
INTRODUCTION

The observation that the addition of AMP and PC to a rabbit muscle preparation, treated with charcoal and Dowex-1 (Cl⁻) to remove nucleotides, results in the formation of ATP and the release of creatine (Chapter 2), raises once again the question of whether or not the phosphoryl group of PC can be transferred directly to AMP. The phosphorylation of AMP by PC in the presence of muscle extracts was first demonstrated by Lohmann (1934) and subsequently Lehmann (1935) suggested that the reaction observed occurred in two steps with the intermediate formation of ADP. Banga (1943) claimed that the reaction:

\[ \text{PC} + \text{AMP} \xrightarrow{\text{ADP}} \text{ADP} + \text{Creatine} \]  

was catalysed by an enzyme which she partially purified from rabbit muscle extracts and called ADP-creatine phosphopherase. However, because the above authors took no precautions to exclude ATP and ADP from their reaction mixtures and with the subsequent discovery that myokinase is present in muscle extracts (Colowick & Kalokar, 1943), it became generally accepted that the phosphorylation of AMP by PC occurs via the
following reactions:-

$$\text{AMP} + \text{ATP} \rightleftharpoons_{\text{myokinase}} 2 \text{ADP} \quad (2)$$

$$\text{ADP} + \text{PC} \rightleftharpoons_{\text{CPT}} \text{ATP} + \text{Creatine} \quad (3)$$

It is clear that only low levels of either ADP or ATP would be required to prime the above reactions and that the initial rate of formation of the products would be autocatalytic. Under conditions where both enzymes are saturated with their respective substrates, the overall reaction velocity will be determined by the concentration of one of them. The final equilibrium of the reaction will be a function of the concentrations of AMP and PC. In the presence of excess PC, AMP would be quantitatively converted to ATP with the release of 2 moles of creatine per mole of AMP as shown in the following equation:

$$2 \text{PC} + \text{AMP} \rightarrow \text{ATP} + 2 \text{Creatine}$$

Chappell and Perry (1954) have shown that if purified samples of CPT and myokinase were added together to their incubation mixture, these enzymes catalysed the above reactions without the addition of either ADP or ATP. They proposed that the reaction was due to the contamination of the dialysed enzymes by ADP or ATP. Recently, Molnar and Lorand (1960) made similar observations using crystalline preparations of CPT and myokinase which had been exhaustively dialysed.
The latter authors presumed that prolonged dialysis would completely remove contaminating nucleotides but, in view of results reported in this chapter, it would appear that such a conclusion is untenable.

Both myokinase and CPT occur in the muscle preparations in which the phosphorylation of AMP was observed (Chapter 2) but as the preparation had been extensively dialysed and then treated with Dowex-1 (Cl⁻) and Norit A to remove nucleotides it was expected that the ADP or ATP would have been removed. Thus there appeared to be two possible explanations of the observed reaction.

1. The reaction mixture still contained ADP or ATP either as a result of incomplete removal from the muscle preparation or as a contaminant of one of the components of the reaction mixture.

2. ADP may arise enzymically as the result of the direct transfer of a phosphoryl group from PC to AMP catalysed by CPT, myokinase or some other enzyme present in the muscle extract.

Although doubt has been cast on the report by Banga (1943) that an enzyme in muscle catalyses the direct phosphorylation of AMP by PC the possibility that such an enzyme exists must be considered. Indeed, this enzyme may easily go undetected in preparations containing myokinase. It
has been reported that CPT is specific for ADP and does not
catalyse the phosphorylation of AMP (Ennor & Rosenberg, 1954 b;
Oliver, 1954; Chappell & Perry, 1954). However, the methods
used by these authors to detect ADP formation depended on the
stoichiometric assay of the nucleotide and it is possible that
a low level of ADP, sufficient to prime reactions (2) and (3)
may have escaped detection (Chapter 1).

The studies reported here have shown that purified
preparations of CPT and myokinase catalyse the conversion of
AMP to ATP in the presence of PC even after repeated treatment
of the enzyme with charcoal and Dowex-1 (Cl−) to remove
nucleotides and rigorous precautions to exclude ADP and ATP
from the reaction mixtures. It appears that some ATP firmly
bound to CPT is not completely removed by this treatment.

EXPERIMENTAL

Chemicals. Nucleotides were obtained from Sigma Chemical Co.,
St. Louis, Mo. AMP was purified by chromatography as described
in Chapter 1. Bovine albumin obtained from Armour Laboratories,
London was treated with charcoal by the following procedure.
The protein solution (25 mg./ml.) was stirred with acid washed
Norit A (1 g./10 ml.) for 30 min. at 0°, filtered and then
stirred overnight with the same level of Norit A. Both
treatments were then repeated and the final traces of charcoal
were removed by high speed centrifugation. The preparation of PC, NEM buffers and the acid treatment of charcoal have been described in Chapter 1. Ammediol buffers and calcium phosphate gel were prepared as described in Chapter 2.

**Enzymes.** Preparative methods for myosin and CPT have been described in Chapter 1 and for the rabbit muscle preparation (Fraction II) and apyrase in Chapter 2. Crystalline CPT prepared by Dr. J. Morrison according to method A of Kuby, Noda and Lardy, (1954 b) was used to study the nucleotide contamination of the enzyme. Myokinase was prepared by a number of methods. For the preliminary experiments it was prepared according to the method of Colowick and Kalckar (1943) with the exception that only the 0.5 - 0.8 saturated ammonium sulphate fraction was collected. This fraction was dialysed free of ammonium sulphate stirred with Norit A (1 g./25 ml.) for 30 min. at 0°, filtered, and the charcoal treatment repeated with stirring overnight. The final filtrate was stirred for 10 min. with Dowex-1 (Cl⁻) (100 mg./ml.) and the resin removed on a Buchner funnel. Further purification was achieved by diluting the protein to 10 mg./ml., adding, with stirring, half a volume of calcium phosphate gel (25 mg./ml.), allowing to stand for 5 min. and centrifuging at 1,500 g. This removed more than half of the protein. The enzyme remained in the supernatant and was stored at 1°. Myokinase was also prepared
by the method of Callaghan (1957) but the final chromatography on a calcium phosphate gel column was omitted. Although this preparation showed a single protein peak when subjected to electrophoresis at pH 8.6 and pH 5.2, a number of minor components were detected at pH 7.2. The preparation was, however, free of CPT activity. Before use, the enzyme was treated with acid washed charcoal as described for CPT (Chapter 1). Crystalline myokinase was prepared by the method of Noda and Kuby (1957). Myokinase was stored in the ammonium sulphate solution from which it was crystallised (0.61 sat.) and the enzyme spun down as required. The myokinase was diluted to a concentration of 1 mg./ml. in a solution containing 0.01 M cysteine (pH 7.2), 0.01 N NEM buffer (pH 7.2) and albumin (1mg./ml.). The enzyme was stable to storage at this dilution for 1 week. Subsequent dilutions were carried out stepwise in albumin (1 mg./ml.).

**METHODS**

Methods for the estimation of creatine and protein and for the detection of low levels of ADP and/or ATP have been described in Chapter 1.

**Electrophoresis.** This was carried out by Mr. R.F. Adams of this department, using a Perkin Elmer model 38A apparatus.

**Chromatography.** Nucleotides were chromatographed on Whatmann 3MM paper by ascending chromatography in isobutyric acid: NH₃.
(0.88 s.gr.): water (66:1:33; by vol.) and were detected by visual inspection in ultraviolet light.

RESULTS

Preliminary Experiments.

In view of the report by Jandorf, Klemplerer and Hastings (1941) that it is necessary to stir muscle extracts with charcoal for 12 hr. to completely remove DPN, preliminary experiments were undertaken to determine if prolonged treatment of Fraction II with charcoal would remove the nucleotides which prime the reactions (2) and (3). Fraction II was stirred with charcoal (1gm./10 ml.) for 12 hr., filtered and the treatment repeated. The ability of the preparation to catalyse the phosphorylation of AMP in the presence of PC remained unchanged.

A similar investigation was undertaken to determine if treatment of purified preparations of myokinase and CPT with charcoal and Dowex-1 (Cl⁻) to remove contaminating nucleotides would inhibit the formation of ATP from AMP and PC in the presence of these enzymes. During this investigation it was found that myokinase prepared by an adaptation of the method of Colowick and Kalckar (1943) and treated to remove contaminating nucleotides catalysed the phosphorylation of AMP in the presence of PC without the addition of CPT. However, it was found that, although 37.5 µg. of this myokinase preparation brought about a rapid release of creatine, the activity dropped rapidly at lower protein levels and
at a fifth of the above level there was no release of creatine. As this preparation of myokinase was subsequently shown to contain CPT it would seem as though the complete loss of activity was due to the dilution of the contaminating CPT to a level at which no activity was detected under the test conditions. When CPT was included in the reaction mixture only 0.5 µg. of myokinase was required to catalyse the release of creatine.

Stability of Myokinase.

Myokinase was found to be unstable in dilute solution, due presumably to the oxidation of the SH-groups which are essential for activity. For this reason cysteine, KCN and protein were tested in the diluting solution and the reaction mixture for ability to protect this enzyme. The most satisfactory results were obtained when protein was included in both the diluting solution and the reaction mixture. Callaghan (1957) suggested the use of purified CPT as a stabilizing protein for the dilution of myokinase and while this enzyme gave the required protection, charcoal treated bovine albumin gave the same results. The use of albumin rather than CPT would allow of a study of the effect on the reaction velocity of varying the concentration of the two enzymes independantly. Myokinase diluted in albumin (1 mg./ml.) to a final concentration of 12 µg./ml. was shown to suffer only a slight loss of activity during storage for 3.5 hr. at 0°. However, for the studies reported here the enzyme was diluted freshly from a
stock solution (1 mg./ml.) and used within 30 min.

**Effect of Metal Ion and pH on the Velocity of the Reaction.**

In order to determine the conditions under which a maximum rate of creatine release was obtained, the concentration of both myokinase and CPT was fixed. The rate of creatine release was markedly increased by the addition of Mn\(^{++}\), Mg\(^{++}\), Ca\(^{++}\) or Co\(^{++}\) (Table 3.1) but was not affected by the other metal ions tested. Although Mn\(^{++}\) gave the greatest apparent activation of the reaction, this metal ion interferes with the colorimetric procedure employed for the estimation of creatine and Mg\(^{++}\) was used for subsequent experiments. The effect of varying the concentration of this ion is shown in Table 3.2.

The pH optimum for the reaction was pH 7.2.

**Product of the Phosphorylation of AMP.**

The nucleotide resulting from the phosphorylation of AMP in the presence of PC, CPT and myokinase has been identified chromatographically as ATP. Similar results had been obtained when AMP and PC were incubated with the rabbit muscle preparation (Fraction II) (Chapter 2). A study of the stoichiometry of the reaction under conditions where PC is in excess has shown that 2 moles of creatine are released for each mole of AMP phosphorylated.

**Rate of Creatine Release from PC in the Presence of AMP, CPT and Myokinase.**

In the work reported below myokinase prepared by the
TABLE 3.1

Effect of Metal Ions on the Release of Creatine from N-Phosphorylcreatine

The reaction mixture contained; NEN buffer (pH 7.2), 50 μmoles; PC, 5 μmoles; AMP, 1 μmole; albumin, 1 mg.; CPT, 2 μg.; myokinase (prepared by an adaptation of the method of Colowick and Kalckar, 1943), 1 μg.; and 2.5 μmoles of the test metal ion in a total volume of 1 ml. Incubation time, 5 min. Temp. 38°.

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>Release of Creatine (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.04</td>
</tr>
<tr>
<td>Ca^{++}</td>
<td>0.55</td>
</tr>
<tr>
<td>Co^{++}</td>
<td>0.51</td>
</tr>
<tr>
<td>Mg^{++}</td>
<td>0.61</td>
</tr>
<tr>
<td>Mn^{++}</td>
<td>0.79 *</td>
</tr>
</tbody>
</table>

No activity was shown by the following:–

Al^{+++}, Ba^{++}, Be^{++}, Cd^{++}, Cu^{++}, Fe^{+++}, Li^{++}, Ni^{++} and Sr^{++}.

* This metal ion interferes in the method for the estimation of creatine.
TABLE 3.2

Effect of Magnesium Ion Concentration on the Release of Creatine from N-Phosphorylcreatine

The reaction mixture was the same as described for Table 3.1 but with varying levels of Mg$^{2+}$ ions added.

Incubation time, 4 min. Temp. 38°.

<table>
<thead>
<tr>
<th>Magnesium Ion Concentration</th>
<th>Release of Creatine (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( M )</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.040</td>
</tr>
<tr>
<td>( 1 \times 10^{-6} )</td>
<td>0.066</td>
</tr>
<tr>
<td>( 1 \times 10^{-5} )</td>
<td>0.095</td>
</tr>
<tr>
<td>( 1 \times 10^{-4} )</td>
<td>0.444</td>
</tr>
<tr>
<td>( 1 \times 10^{-3} )</td>
<td>0.702</td>
</tr>
<tr>
<td>( 2.5 \times 10^{-3} )</td>
<td>0.630</td>
</tr>
<tr>
<td>( 1 \times 10^{-2} )</td>
<td>0.301</td>
</tr>
</tbody>
</table>
method of Callaghan (1957) or a crystalline preparation of the enzyme prepared as described by Noda and Kuby (1957) has been used. These were preferred as myokinase prepared by the adaptation of the method of Colowick and Kalckar (1943) was shown to be contaminated with CPT.

The nature of the time curve of the creatine release (Fig. 3.1.) is similar to that obtained by Chappell and Perry (1954), although as previously pointed out these authors made no efforts to remove contaminating nucleotides. The results are consistent with the formation of ADP which, as it increases, causes an overall increase in the reaction velocity. Thus the reaction is autocatalytic until a level of ADP is reached where this substrate no longer effects the reaction velocity. The final reaction rate will be a function of the concentration of one of the enzymes. Extrapolation of the linear part of the curve to the base line gives a measure of what shall subsequently be called the lag period.

The results are consistent with the formation of ADP; but do not give any indication of the source of the ADP or ATP required to prime reactions (1) and (2). Experiments were therefore carried out to determine whether ADP was produced enzymically by direct phosphorylation of AMP by PC or whether ADP or ATP was added as a contaminant of one of the components of the reaction mixture.
Fig. 3.1. Rate of Creatine Release from PC in the Presence of AMP, CPT and Myokinase. The reaction mixture contained; NEM buffer (pH 7.2), 50 µmoles; PC, 2.5 µmoles; MgSO₄, 1 µmole; AMP, 1 µmole; albumin, 1 mg.; myokinase (Callaghan preparation), 1 µg. and CPT, 2 µg. in a total volume of 1.0 ml. Temp. 38 °.
Effect of Added ATP on the Length of the Lag Period.

In order to gain some indication of the level of nucleotide which would be required to prime the overall reaction the level of added ATP required to reduce the length of the lag was determined. It was found (Fig. 3.2) that ATP at a final concentration of $5 \times 10^{-8}$M did reduce the length of the lag period without affecting the rate of creatine release over the linear part of the curve. It was found that the addition of $1 \times 10^{-8}$M ATP to the reaction mixture caused no change in the time curve of the creatine release. This level of nucleotide could not be detected by the sensitive assay method described in Chapter 1.

Attempt to Demonstrate Direct Phosphorylation of AMP.

In an attempt to demonstrate direct phosphorylation of AMP this compound was incubated under the conditions described in Fig. 3.1 but with 0.5 mg. of either CPT or myokinase. It was found that there was no detectable release of creatine after incubating for 3 hr. even when apyrase was added to overcome the possibility of an unfavourable equilibrium. In addition the incubated reaction mixture was assayed to detect low levels of ADP or ATP using the linked CPT-myosin system (Chapter 1). The sensitivity of this detection method was limited by the presence of AMP but it did establish that less than 1 nmole of ADP had been formed during a 3 hr. incubation period. Thus it must be
Fig. 3.2. Effect of Added ATP on the Length of the Lag Period. The reaction mixture contained; NEM buffer (pH 7.3), 200 μmoles; albumin, 2 mg.; PC, 5 μmoles; magnesium acetate, 5 μmoles; AMP, 2 μmoles; CPT, 20 μg.; myokinase (Crystalline Prep.), 1.2 μg. and added ATP in a total volume of 2 ml. 0.2 ml. samples were taken for the estimation of creatine at the times indicated. Temp. 37°.

●-●, No additions or ATP (1 x 10⁻³ M); ▲-▲, ATP (5 x 10⁻³ M); ■-■, ATP (3 x 10⁻⁷ M).
assumed that during the relatively short period of the lag and at the lower enzyme concentrations used (Fig. 3.1) direct phosphorylation would not account for the concentration of ADP required to prime reactions (2) and (3).

Effect of Pre-Incubating the Reaction Mixtures with Apyrase.

As AMP was not phosphorylated by PC in the presence of either myokinase or CPT it was assumed that the nucleotide which primed the autocatalytic formation of ADP was present as a contaminant of one of the components of the incubation mixture. An attempt was therefore made to remove ADP or ATP by pre-incubating the reaction mixture with apyrase. A reaction mixture similar to that described in Fig. 3.1 but without PC was incubated with apyrase (6 µg.) for 10 min. at 38° and PC, which is a synthetic product and free of nucleotides, was then added. No creatine release was obtained under these conditions and the effect of apyrase was the same without a period of pre-incubation. This suggested either that the contaminating nucleotide is very rapidly destroyed or that the apyrase inhibition is due to successful competition of apyrase with CPT and myokinase for ADP and ATP formed as a result of reactions (2) and (3).

It was found that the inhibition caused by apyrase could not be overcome by increasing the level of CPT in the reaction
mixture, but that when the myokinase was increased five-fold there was a release of creatine from PC even with a pre-incubation time of 30 min. As no pre-incubation was required for inhibition by apyrase in the presence of 1 µg. of myokinase and as the only change in the components of the reaction mixture was a five-fold increase in the level of myokinase, it seems unlikely that the failure of apyrase to inhibit under the latter conditions could be due to insufficient time for destruction of contaminating nucleotide. It also appears that the inhibition by apyrase at the lower myokinase concentration is not due to removal of the nucleotide which primes the reaction but rather, the apyrase must successfully compete with CPT and myokinase for ADP and ATP produced by reactions (2) and (3). It must be assumed, however, that myokinase and CPT react with these nucleotides under conditions where apyrase is unable to react with them. The apyrase apparently maintained the nucleotide concentration at a constant level so that the reaction does not become autocatalytic and no creatine release is observed. When the level of myokinase is increased ADP and ATP are formed faster than they can be hydrolysed and creatine is released though the rate of release is reduced by the presence of the apyrase.

The effect of added ATP on the length of the lag period (Fig. 3.2) indicated that the level of ADP or ATP required to start
the overall reaction was less than $5 \times 10^{-8}$ M. It is not known whether the failure of apyrase to hydrolyse the contaminating nucleotide during the prolonged pre-incubation is due to the inability of the enzyme to hydrolyse the very low level of substrate present or the fact that the contaminating nucleotide present is bound to protein in a form which cannot be hydrolysed by apyrase but presumably is available to myokinase or CPT.

**Attempt to Detect Contamination of AMP by ADP or ATP.**

The sensitivity of the linked CPT-myosin system for the detection of ADP or ATP is limited by the presence of AMP (Chapter 1) to 1 part in 2,500 and the method is therefore inadequate for the detection of the level of nucleotide required to prime reactions (2) and (3). It was expected that under conditions where the enzymes were saturated with AMP it may be possible to detect the presence of ADP or ATP as a contaminant of this substrate by studying the effect of increased levels of AMP on the length of the lag. It was found, however, that a five-fold increase in AMP increased rather than decreased the lag (Fig. 3.3) and that there was a reduction in the final linear rate of creatine release. Presumably this effect is due to inhibition of one or both of the enzymes involved in the release of creatine. A sample of AMP was twice repurified by the chromatographic procedure previously described and the product
Fig. 3.3. Effect of AMP Concentration on the Velocity of the Reaction. The reaction mixture was as described for Fig. 3.1 but with the AMP concentrations indicated.

- - 1 µmole AMP
- - 5 µmoles AMP
isolated in a crystalline form. Using this sample of AMP the results were identical with those obtained with the once purified material. Thus, it seems reasonable to conclude that AMP is not the source of the contaminating nucleotides.

**Effect of the Albumin Concentration on the Velocity of the Reaction.**

Because albumin has been used to stabilise myokinase it was possible that it might have been the source of the nucleotide contaminant, in spite of the fact that it had been charcoal treated. An attempt was made to reduce the level of the albumin used in the reaction mixture but this reduced the velocity of the reaction (Fig. 3.4), presumably as a result of the instability of the myokinase. A five-fold increase in the level of the albumin failed to affect the length of the lag but higher levels of protein have not been tested because of the increase in the creatine blank reading. No ADP or ATP was detected in 5 mg. of albumin assayed in the CPT-myosin system (Chapter 1). For all subsequent studies the level of albumin in the reaction mixture was maintained at 1 mg./ml.

**Effect of the Concentration of CPT and Myokinase on the Velocity of the Reaction.**

Crystalline myokinase was used in this study but it was not treated with charcoal to remove nucleotide as there is considerable loss of protein during such treatment, particularly
Fig. 3.4. Effect of Albumin Concentration on the Velocity of the Reaction. The reaction mixture contained:

NEM buffer (pH 7.5), 100 μmoles; PC, 2.5 μmoles; AMP, 1 μmole; magnesium acetate, 2.5 μmoles; albumin, 0.1 mg.; CPT, 10 μg.; and myokinase, 0.6 μg. (Crystalline Prep.); in a total volume of 1 ml. Temp. 37°.

○ - ○; No additions. ● - ●; 1 mg. Albumin.
at low protein concentrations. However, when 1 mg. of the crystalline enzyme was tested in the CPT-myosin system (Chapter 1), neither ADP or ATP was detected. A sample of the crystalline enzyme which had been diluted in albumin to give a protein concentration of 10 mg./ml. was treated with charcoal as described for CPT. This preparation of myokinase when combined with CPT catalysed the phosphorylation of AMP by PC but was not used extensively due to loss of activity on storage.

The reaction velocity has been studied under conditions where the concentration of one of the enzymes is fixed and the other varied.

(a) **Effect of Varying the Myokinase Concentration at a Constant Level of CPT.**

The rates of creatine release under these conditions are shown in Fig. 3.5. As the myokinase was increased the length of the lag was reduced and the rate of creatine release over the linear part of the curve increased. The reaction velocity was proportional to the enzyme concentration (Fig. 3.6) up to a level of 6.4 μg. of myokinase after which the rate fell off. Presumably this is due to the fact that the reaction rate is not determined by the concentration of CPT. When the line joining the points representing the linear rate of creatine release is
Fig. 3.5. Release of Creatine from PC at a Constant Level of CPT and Varying Levels of Myokinase.

The reaction mixture contained: NEM buffer (pH 7.3), 200 μmoles; albumin, 2 mg.; PC, 5 μmoles; magnesium acetate, 5 μmoles; AMP, 2 μmoles; CPT, 10 μg. and varying levels of myokinase in a total volume of 2 ml. Samples (0.2 ml.) were taken for the assay of creatine at the times indicated. Temp. 37°. The following amounts of a crystalline preparation of myokinase were added; • - • , 0.4 μg.; △ - △ , 0.48 μg.; ○ - ○ , 0.64 μg.; Δ - Δ , 0.8 μg.
Fig. 3.6. The Effect of Myokinase Concentration on the Velocity of the Reaction at a Fixed Concentration of CPT. The rates were calculated from the linear part of the curves obtained in Fig. 3.5.
extrapolated back, it does not pass through the origin but rather cuts the abcissa. This result indicated the presence of a small amount of some highly toxic impurity, which was poisoning a constant amount of the enzyme added to the reaction mixture.

(b) **Effect of Varying the CPT Concentration at a Constant Level of Myokinase.**

The time courses of the release of creatine when the level of myokinase was maintained constant and the level of CPT varied are shown in Fig. 3.7. At a concentration of 2.5 μg./ml. of CPT the rate of creatine release was limited by the level of that enzyme since a two-fold increase in CPT reduced the length of the lag period and increased the linear rate of creatine release. The results show that the concentration of myokinase present is limiting the rate of creatine release above a concentration of 5 μg./ml. of CPT as a five-fold increase in the latter enzyme did not change the rate of creatine release, although, the length of the lag was reduced. These results are consistent with the fact that ADP or ATP were added to the reaction mixture when the CPT level was increased.

It has been shown that under the conditions described in Fig. 3.2 that more than $1 \times 10^{-8}$ M ATP was required to reduce the length of the lag. If the 20 μg. of CPT were contaminated with sufficient ADP or ATP to give a final concentration of more than $1 \times 10^{-8}$ M it should be possible to detect the presence of
Fig. 3.7. Release of Creatine from PC at a Constant Level of Myokinase and Varying Levels of CPT. The reaction mixture was as used in Fig. 3.5 with 1.2 μg. of myokinase and varying levels of CPT in a total volume of 2 ml. Samples (0.2 ml.) were taken for the estimation of creatine at the times indicated. Temp. 37°. The following amounts of CPT were added; O - O, 5 μg.; Δ - Δ, 10 μg.; ⋄ - ⋄, 50 μg.
nucleotide by incubating 5 mg. of the CPT in the CPT-myosin system previously described (Chapter 1). No nucleotide could be detected either before or after boiling the sample of enzyme using a method which could detect 0.1 μmoles of added ATP. In addition, these nucleotides could not be detected in the supernatant from the precipitation of CPT by perchloric acid which had subsequently been neutralized with KOH and corresponded to 1 mg. of the original protein. It must thus be assumed that either the nucleotide bound to the enzyme reduced the lag at a much lower concentration than does nucleotide added to the reaction mixture or that the nucleotide is bound to the enzyme in a form which could not be detected by the methods used.

Detection of the Nucleotide Contaminant of Crystalline CPT.

The above results suggest that either ADP or ATP is firmly bound to CPT, so that it is not removed by the charcoal treatment employed and though still able to act as a substrate for myokinase or CPT. Consequently, efforts were directed towards the detection of either ADP or ATP in samples of crystalline CPT which had not been treated with charcoal.

Spectrophotometric Method. When the method of Warburg and Christian (1941) for the detection of nucleic acids in protein was applied to a solution of crystalline CPT it was found that the ratio of E 260μ/260μ corresponded to 0.25% contamination by some nucleic acid like compound (Table 3.3). Treatment of the
<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>SOLVENT</th>
<th>RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystalline CPT (Sample 1)</td>
<td>0.01 M Phosphate buffer (pH 7.4)</td>
<td>1.65</td>
</tr>
<tr>
<td>Sample 1 (10 mg./ml.) stirred with Norit A (1 g./10 ml.) for 100 min.</td>
<td>&quot;</td>
<td>1.77</td>
</tr>
<tr>
<td>Sample 1 (10 mg./ml.) stirred with Norit A (1 g./10 ml.) for 100 min. and then for 16 hr.</td>
<td>&quot;</td>
<td>1.77</td>
</tr>
<tr>
<td>Sample 1 dialysed against $10^{-5}$ M versene in $10^{-3}$ M NEM (pH 8.0) for 3 days and against $10^{-3}$ M NEM (pH 8.0) for 3 days.</td>
<td>&quot;</td>
<td>1.74</td>
</tr>
<tr>
<td>Crystalline CPT (Sample 11).</td>
<td>H$_2$O</td>
<td>1.64</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.01 M Phosphate buffer (pH 7.0)</td>
<td>1.63</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.01 M Acetate buffer (pH 5.0)</td>
<td>1.70</td>
</tr>
<tr>
<td>Freeze Dried CPT Prep. (Charcoal Treated before drying).</td>
<td>H$_2$O</td>
<td>1.76</td>
</tr>
<tr>
<td>Albumin.</td>
<td>0.01 M Phosphate buffer (pH 7.4)</td>
<td>1.76</td>
</tr>
<tr>
<td>Albumin (10 mg./ml.) stirred overnight with charcoal (100 mg./ml.).</td>
<td>&quot;</td>
<td>1.76</td>
</tr>
</tbody>
</table>
enzyme with charcoal brought about an increase in the ratio of $E_{280}/E_{260}$. This was consistent with the removal of nucleotide from the enzyme. The reduction in the absorption of the charcoal treated CPT at 260 m$, assuming the contaminant to be an adenine nucleotide, corresponded to the removal of 1-2 $\mu$moles of nucleotide per gram. Prolonged dialysis of crystalline CPT against versene brought about an increase in the ratio, indicating the removal of nucleotide. A control experiment in which CPT was replaced by albumin showed that the ratio of $E_{280}/E_{260}$ for this protein remained unchanged after treatment with charcoal. During these experiments it was also shown that the pH of the solvent affected the ratio of the absorption of CPT at 280m$ and 260m$.

The ratios obtained for two samples of crystalline CPT were much lower than the value of 1.85 reported by Noda, Kuby and Lardy (1954). Even after prolonged treatment with charcoal the ratio did not rise above 1.77.

CPT-Myosin Method. The crystalline enzyme was tested in the CPT-myosin system previously described (Chapter 1). A very slow release of creatine was obtained when 5 mg. of the crystalline enzyme was incubated in the test system. The level of ADP or ATP was less than 0.1 $\mu$ mole/gm. of protein which is much lower than the level of nucleotide detected by the spectrophotometric assay. Thus either the material removed from the CPT which
absorbs at 260 μl may not be entirely ADP or ATP, or the method for the detection of low levels of these nucleotides may not be giving a true estimate of the level present.

**Isolation.** Material which absorbs in ultraviolet light has been recovered from the charcoal which had been stirred with crystalline CPT. 200 mg. of crystalline CPT was dissolved in 5 ml. of cold distilled water and stirred for 100 min. with 500 mg. of acid washed Norit A at 1°. The charcoal was removed by filtration and was washed with water. The charcoal was stirred three times for 1 hr. with 5 ml. of a 10:90 (v/v) mixture of isoamyl alcohol and water. The aqueous layer from the combined extracts was taken to dryness under reduced pressure and the material which absorbed in ultraviolet light applied to 3 MM Whatman paper and chromatographed as described in the methods. The material isolated from the crystalline CPT was found to have the same $R_F$ as ATP and was isolated from the paper but the concentration precluded any spectrophotometric studies being made.

**DISCUSSION**

The results in this section show that the phosphorylation of AMP by rabbit muscle extracts at the expense of PC can be accounted for by the combined action of CPT and myokinase. It seems probable that the overall reaction is dependant on the presence of traces of ATP to start the reaction rather than a direct phosphorylation of AMP. The fact that the activity is not destroyed by rigorous treatment of the enzyme with charcoal.
or Dowex-1 (Cl\textsuperscript{−}), which might be expected to remove all nucleotide, can probably be correlated with the observation that crystalline CPT contains bound nucleotide, tentatively identified as ATP. The ability of protein-bound ADP and ATP to take part in enzymic reactions has been discussed by Yagi and Noda (1960).

It was observed by Molnar and Lorand (1960) that crystalline preparations of CPT and myokinase, which had been dialysed exhaustively, could catalyse the phosphorylation of AMP by PC, without further addition of ADP or ATP. They presumed that prolonged dialysis would completely remove contaminating nucleotides from the proteins and reported that pre-incubation of the reaction mixture with apyrase did not stop the reaction. These conclusions are untenable and the reaction would seem to be due to the difficulty of completely removing adenine nucleotides from proteins by dialysis (Martonosi, Gouvea and Gergely, 1960) or by treatment with charcoal, Dowex-1 (Cl\textsuperscript{−}) or with apyrase. The observation by Molnar and Lorand (1960) that dialysed CPT catalysed an exchange between \textsuperscript{14}C-creatine and PC is consistent with the presence of trace amounts of ATP in the crystalline enzyme.

It is interesting to note that pyruvic kinase can catalyse the conversion of AMP to ATP in the presence of PEP and myokinase (Bucher & Pfleiderer, 1955; Molnar & Lorand, 1960). It might well be that crystalline pyruvic kinase, like CPT, is also contaminated by either ADP or ATP.
The results presented here underline the difficulties associated with completely removing bound nucleotide from protein. This emphasises the need for caution in assessing evidence that a reaction proceeds by a route which does not involve participation of these compounds.

SUMMARY

1. It has been shown that AMP is phosphorylated by PC in the presence of purified CPT and myokinase even when these enzymes have been treated with charcoal in an attempt to remove contaminating nucleotides.

2. The reaction does not involve direct phosphorylation of AMP by PC by myokinase or CPT.

3. Crystalline CPT is contaminated with a nucleotide, tentatively identified as ATP, which is not completely removed by treatment with charcoal, Dowex-1 (Cl^-) or apyrase.

4. The low level of ATP bound to the charcoal treated CPT is able to prime the phosphorylation of AMP by PC as a result of the combined action of CPT and myokinase.
CHAPTER 4.

THE HYDROLYSIS OF ADENOSINE DIPHOSPHATE RIBOSE BY A

PYROPHOSPHATASE PRESENT IN RABBIT MUSCLE EXTRACTS
CHAPTER 4.

THE HYDROLYSIS OF ADENOSINE DIPHOSPHATE RIBOSE BY A PYROPHOSPHATASE PRESENT IN RABBIT MUSCLE EXTRACTS

INTRODUCTION

Although it has been shown that cleavage of the nicotinamide ribose bond of DPN by DPNases results in the formation of ADP ribose (Kaplan, Colowick & Nason, 1951) little is known of the metabolic role of this nucleotide beyond the fact that it may be further hydrolysed at the pyrophosphate bond. The relatively non-specific pyrophosphatases from potatoes (Kaplan et al., 1951) and snake venom (Kaplan & Stolzenbach, 1957) as well as liver DPNH$_2$ pyrophosphatase (Jacobson & Kaplan, 1957) have been shown to catalyse this hydrolysis of ADP ribose. On the basis that this latter enzyme attacks ADP ribose at twice the rate at which DPNH$_2$ is hydrolysed it should possibly be regarded as an ADP ribose pyrophosphatase and it has been suggested (Jacobson & Kaplan, 1957) that the metabolic function of this pigeon liver enzyme may be to hydrolyse ADP ribose produced by the action of DPNase on DPN.

Earlier in this work (Chapter 2) it was reported that rabbit muscle preparations catalyse the hydrolysis of ADP ribose to give AMP whilst there is no hydrolysis of DPN or DPNH$_2$. Since there had been no previous report of an enzyme which
specifically hydrolyses ADP ribose, further investigations have been undertaken. More detailed studies have confirmed the earlier finding that DPN and DPNH$_2$ are not hydrolysed by the rabbit muscle preparation and furthermore, it has been shown that the hydrolysis of ADP ribose can be represented by the equation:

\[
\text{ADP ribose} \rightarrow \text{AMP} + \text{ribose-5'-phosphate}
\]

Thus the enzyme responsible for this reaction may be termed an ADP ribose pyrophosphatase.

**EXPERIMENTAL**

**Chemicals.** The preparation of buffers, PC, sodium pyruvate and acid washed charcoal and the purification of AMP have been described in Chapter 1. ADP ribose was obtained from Pabst Laboratories, Milwaukee, Wis. and other nucleotides were supplied by Sigma Chemical Co. St. Louis, Mo. Commercial samples of DPN (purity 95%) and β-DPNH$_2$ (purity 98%) were used without further purification. Albumin was treated with charcoal as described in Chapter 3.

**Enzymes.** CPT was prepared and treated with Norit A as described in Chapter 1. Alcohol and lactic dehydrogenases were obtained commercially and adenylic acid deaminase and apyrase were prepared as described in Chapter 2. Myokinase was a crystalline preparation (Noda & Kuby, 1957) which was dialysed against 0.01 M succinate buffer (pH 6.5) and freeze dried in ampoules. Each ampoule contained 0.5 mg. of protein which, before use, was
dissolved in 0.2 ml. of a solution containing 0.01 M cysteine (pH 7.4), 0.01 N NEM buffer (pH 7.4) and charcoal treated albumin (1 mg./ml.). Subsequent dilutions of the myokinase were carried out in charcoal treated albumin (1 mg./ml.). The muscle preparation used for the study of the hydrolysis of ADP ribose was prepared as described in Chapter 2. Fraction II has been used for most of these studies, but the activity of Fraction I was also tested.

METHODS

Methods for the estimation of protein and for creatine (3 ml. volume) have been described in Chapter 1. Ribose and DPN were estimated as described in Chapter 2.

Detection of the Hydrolysis of the Pyrophosphate Bond of DPNH₂.
The method used for the detection of the hydrolysis of the pyrophosphate bond of DPNH₂ was basically that used by Jacobson and Kaplan (1957), but the oxidation of the coenzyme was carried out by the addition of lactic dehydrogenase and sodium pyruvate. The oxidation was carried out in cuvettes of 1 cm. light path which contained 0.3 ml. of 1 M ammediol buffer (pH 7.4) and the test sample in a total volume of 2.9 ml. The absorption at 340 mµ was measured and then 0.05 ml. of 0.1 M sodium pyruvate and 0.01 ml. of lactic dehydrogenase added. The concentration of the latter enzyme was such as to give complete oxidation of the DPNH₂ in less than 2 min. and was added routinely even though the muscle extract was found to contain this enzyme.
The absorption at 340 m\(^\lambda\) was measured after the addition of lactic dehydrogenase and the difference in the readings of the test and the control, which consisted of DPNH\(_2\) incubated in the absence of the muscle preparation, would give a measure of any absorption due to reduced NAD. Oxidation of DPNH\(_2\) or hydrolysis of the nicotinamide ribose bond of DPNH\(_2\) during the incubation with the muscle preparation would be detected as a drop in absorption at 340 m\(^\lambda\) prior to oxidation of the coenzyme.

**Determination of Enzymic Activity.** The rate of hydrolysis of ADP ribose was determined by following the rate of formation of AMP. Two methods for the determination of the activity of the enzyme have been used.

**Method 1.** The reaction was stopped by placing the incubation mixture in a boiling water bath for 2 min. and the protein was removed by centrifugation. Both AMP and ADP ribose were shown to be stable to the treatment used to stop the enzymic hydrolysis. AMP was estimated in the protein free supernatant using either adenylic acid deaminase (Kalckar, 1947) or by a method which takes advantage of the fact that in the presence of CPT, myokinase and PC each mole of AMP is converted to ATP with the release of 2 moles of creatine (Chapter 3).

**Method 2.** It has been found that when CPT, myokinase and PC are included in the reaction mixture in which AMP is being formed from ADP ribose there is a release of creatine from PC as a
result of the phosphorylation of AMP to ATP. Thus the rate of the creatine release was used as a direct measure of the enzymic hydrolysis of the ADP ribose. As Fraction II contained adequate amounts of CPT and myokinase to catalyse the release of creatine from PC and as no further fractionation of the enzyme preparation has as yet been attempted, it was necessary to add only PC to obtain a creatine release in the presence of ADP ribose, buffer, Mg$^{++}$ and the muscle preparation.

RESULTS

Comparison of the Two Methods for the Estimation of AMP.

Incubation of AMP, PC, CPT and myokinase gave a stoichiometric release of 2 moles of creatine per mole of AMP over the range tested (Fig. 4.1). Satisfactory recoveries of AMP from incubation mixtures were also obtained by this method of estimation and the results were in close agreement with those obtained using adenylic acid deaminase.

Kinetics of the Hydrolysis of ADP ribose.

The hydrolysis of ADP ribose by rabbit muscle preparations was linear over a 3 hr. incubation period at pH 8.5 (Fig. 4.2). Similar results were obtained at pH 7.0, at which pH there was no significant difference in the reaction rate. Mg$^{++}$ was included in the reaction mixtures as this metal ion was required for the activation of CPT and myokinase, but subsequently it was shown to be required also for the hydrolysis
Fig. 4.1. Stoichiometry of the release of creatine from PC in the presence of AMP, CPT and myokinase. The reaction mixture contained; NEM buffer (pH7.3), 50 μmoles; magnesium acetate, 1.25 μmoles; PC, 2.5 μmoles; albumin, 0.5 mg.; CPT, 5 μg.; myokinase, 0.5 μg. and AMP in a total volume of 0.5 ml. Tubes were incubated for 20 min. and the creatine release estimated. Temp. 38°.
Fig. 4.2. Rate of Hydrolysis of ADP ribose. The reaction mixture contained; ammoniobal buffer (pH 8.5), 100 µmoles; magnesium acetate, 5 µmoles; PC, 5 µmoles; ADP ribose, 2.5 µmoles and 6.6 mg. of Fraction II in a total volume of 1 ml. Creatine estimations were carried out at the times indicated. Temp. 38°.
of ADP ribose. The reaction was dependent on the presence of the muscle preparation and the rate of hydrolysis was proportional to the protein added (Fig. 4.3).

No attempt was made to determine the activity of the enzyme in unfractionated muscle extracts since they contain ATPases and adenylic acid deaminase which would interfere with the assay methods. However, a comparison was made of the activities of Fractions I and II. The results (Table 4.1) indicate that the second ammonium sulphate fractionation gave only a small increase in the specific activity of the enzyme.

**Effect of pH on the Reaction Velocity.**

A comparison of the effect of pH on the velocity of the hydrolysis of ADP ribose, as assayed by method 2, was carried out in two buffers. The initial velocity of the release of creatine was not markedly affected by the change in pH over the range from pH 6.9 to pH 8.5 (Table 4.2) and there was no significant difference in the results obtained in the two buffer systems. However, the release of creatine represents the sum of the activities of three enzymes and no conclusions regarding the effect of pH on the rate of hydrolysis of ADP ribose can be drawn from these results.

**Metal Ion Requirement.**

The effect of metal ions on the rate of hydrolysis of ADP ribose was studied by measuring the AMP formed after the
Fig. 4.3. Effect of Enzyme Concentration on the Rate of Hydrolysis of ADP ribose. The reaction mixture contained; ammediol buffer (pH 7.4), 50 μmoles; magnesium acetate, 0.5 μmoles; PC, 2.5 μmoles; ADP ribose, 0.5 μmoles and varying amounts of Fraction II in a total volume of 0.5 ml. The creatine release was estimated after incubation at 38° for 30 min.
TABLE 4.1

Comparison of the Activities of Fractions I and II

The reaction mixture contained; ammediol buffer (pH 7.0), 100 µmoles; PC, 5 µmoles; ADP ribose, 2.5 µmoles; magnesium acetate, 5 µmoles; and the enzyme in a total volume of 1 ml. Temp. 38°. Tubes were incubated for 1.5 hr. and the release of creatine estimated. A unit of activity is defined as the hydrolysis of 1 µmole ADP ribose/mg. of protein/hr.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml.)</th>
<th>Specific Activity</th>
<th>Total Activity</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>49</td>
<td>16.6</td>
<td>32,943</td>
<td>100</td>
</tr>
<tr>
<td>II.</td>
<td>20</td>
<td>28.4</td>
<td>23,752</td>
<td>71</td>
</tr>
</tbody>
</table>
TABLE 4.2

Effect of pH on the Hydrolysis of Adenosine Diphosphate Ribose

The reaction mixture contained: buffer, 100 μmoles; PC, 5 μmoles; magnesium acetate, 0.5 μmoles; ADP ribose, 0.5 μmoles and Fraction II, 4.1 mg. in a total volume of 1 ml. 0.2 ml. samples were taken for creatine assay after incubation at 38° for 1.5 hrs. The pH values reported were measured with a glass electrode in the remainder of the sample at the end of the incubation.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Creatine Release (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM-HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.9</td>
<td></td>
<td>0.346</td>
</tr>
<tr>
<td>7.2</td>
<td></td>
<td>0.365</td>
</tr>
<tr>
<td>7.5</td>
<td></td>
<td>0.340</td>
</tr>
<tr>
<td>7.95</td>
<td></td>
<td>0.423</td>
</tr>
<tr>
<td>8.05</td>
<td></td>
<td>0.430</td>
</tr>
<tr>
<td>Ammediol-HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td></td>
<td>0.354</td>
</tr>
<tr>
<td>7.2</td>
<td></td>
<td>0.352</td>
</tr>
<tr>
<td>7.7</td>
<td></td>
<td>0.342</td>
</tr>
<tr>
<td>8.15</td>
<td></td>
<td>0.40</td>
</tr>
<tr>
<td>8.55</td>
<td></td>
<td>0.38</td>
</tr>
</tbody>
</table>
reaction had been stopped by boiling (Method I). Recovery experiments showed that the presence of the test metals did not interfere with the estimation. It has been shown (Table 4.3) that the enzyme has an absolute requirement for metal ion and that this may be satisfied by Mn++ , Co++ or Mg++ but not by Ca++.  

**Effect of the Magnesium Concentration on the Rate of Hydrolysis of ADP ribose.**

As sulphate ions have been reported to inhibit CPT activity (Noda & Nihei, 1960) magnesium acetate rather than magnesium sulphate was used to study the level of Mg++ required to obtain the maximum rate of hydrolysis of ADP ribose. The rate of hydrolysis of ADP ribose has been followed by both Method 1 and 2 and as the samples taken for the estimation of AMP by the former method contain varying amounts of Mg++ the metal ion added for the estimation was adjusted to give a final concentration of $2.5 \times 10^{-3} \text{M}$. Similar results were obtained by the two methods (Table 4.4) and in the presence of $5 \times 10^{-4} \text{M}$ ADP ribose the rate of hydrolysis was a maximum at a concentration of $1.5 \times 10^{-3} \text{M Mg}^{++}$. High levels of Mg++ were inhibitory and at a concentration of $1 \times 10^{-2} \text{M}$ the inhibition reached 45%.

**Effect of Substrate Concentration on the Reaction Velocity.**

The initial rates of hydrolysis of ADP ribose at
Effect of Metal Ions on the Hydrolysis of Adenosine Diphosphate Ribose

The reaction mixture contained: ammediol buffer (pH 7.4), 100 μmoles; ADP ribose, 0.5 μmoles; Fraction II, 6.2 mg. and 0.5 μmoles of the test metal ion in a total volume of 1 ml. Incubation time, 30 min.; Temp. 36°. The reaction was stopped by boiling for 2 min. and the supernatant assayed for AMP release in the presence of CPT, myokinase and PC.

<table>
<thead>
<tr>
<th>Metal Ion</th>
<th>ADP ribose Hydrolysed (μmoles/ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Mg^{++}</td>
<td>0.024</td>
</tr>
<tr>
<td>Mn^{++}</td>
<td>0.0305</td>
</tr>
<tr>
<td>Co^{++}</td>
<td>0.0410</td>
</tr>
<tr>
<td>Ca^{++}</td>
<td>0</td>
</tr>
</tbody>
</table>
TABLE 4.4

Effect of Magnesium Ion Concentration on the Hydrolysis of Adenosine Diphosphate Ribose

Reaction mixture A contained: ammediol buffer (pH 7.4), 100 μmoles; ADP ribose, 0.5 μmoles; Fraction II, 8.3 mg. and Mg^{++} ions as indicated, in a total volume of 1 ml. Reaction mixture B was the same as A but contained in addition 5 μmoles of PC. Incubation time, 1 hr.; Temp. 38°. At the end of the incubation tubes containing reaction mixture A were boiled for 2 min. and the AMP present in the supernatant estimated using CPT, myokinase and PC. Hydrolysis of ADP ribose in reaction mixture B was estimated by direct assay of the creatine released (Method 2).

<table>
<thead>
<tr>
<th>Magnesium Acetate (mM)</th>
<th>ADP ribose Hydrolysed (μmoles/ml./hr.)</th>
<th>ADP ribose Hydrolysed (μmoles/ml./hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.011</td>
<td>0.005</td>
</tr>
<tr>
<td>0.5</td>
<td>0.093</td>
<td>0.105</td>
</tr>
<tr>
<td>1.0</td>
<td>0.103</td>
<td>0.111</td>
</tr>
<tr>
<td>1.5</td>
<td>0.105</td>
<td>0.116</td>
</tr>
<tr>
<td>2.0</td>
<td>0.100</td>
<td>0.110</td>
</tr>
<tr>
<td>2.5</td>
<td>0.099</td>
<td>0.097</td>
</tr>
<tr>
<td>5.0</td>
<td>0.077</td>
<td>0.088</td>
</tr>
<tr>
<td>10</td>
<td>0.054</td>
<td>0.064</td>
</tr>
</tbody>
</table>
different nucleotide concentrations have been plotted according to the method of Lineweaver and Burk (1934) and are shown in Fig. 4.4. The \( K_M \) for ADP ribose was calculated to be \( 3.8 \times 10^{-5} \)M.

**Substrate Specificity of the Enzyme.**

It had been established (Chapter 2) that DPN was not hydrolysed by the rabbit muscle preparation, but the results obtained with DPNH\(_2\) had been obtained by a direct spectro-photometric estimation which limited the level of both the nucleotide and the protein which could be added. Thus a small degree of hydrolysis could escape detection and further investigations were undertaken. The results of studies at higher nucleotide concentrations and in the presence of an increased level of protein indicate that there is no hydrolysis of the pyrophosphate bond of DPNH\(_2\) (Table 4.5) and that no detectable oxidation of the coenzyme or hydrolysis of the nicotinamide ribose bond occurred during the incubation. The sample of DPNH\(_2\) used for these studies contained 3% DPN but this remained constant during the period of incubation as would be expected from previous results (Chapter 2). No conversion of DPNH\(_2\) to AMP could be detected after incubation for 4.5 hrs. although 0.5 \( \mu \)moles of ADP ribose underwent complete hydrolysis in this time. It has also been shown that AMP and ATP are not hydrolysed by the rabbit muscle preparation.
Fig. 4.4. Effect of Substrate Concentration on the Reaction Velocity. The reaction mixtures contained; ammediol buffer, (pH 7.4), 200 μmoles; magnesium acetate, 2 μmoles; PC, 10 μmoles; Fraction II, 12.4 mg. and varying concentrations of ADP ribose in a total volume of 2 ml. The rate of hydrolysis was followed by taking 0.5 ml. samples for creatine estimation at 10, 20 and 30 min.

\[ \text{V} = \mu \text{moles ADP ribose hydrolysed/ml./hr.} \]
TABLE 4.5

Stability of $\beta$-DPNH$^2$ under Conditions Leading to the Hydrolysis of ADP Ribose

Reaction mixture A contained; ammediol buffer (pH 7.4), 100 μmoles; magnesium acetate, 2.5 μmoles and 5 μmoles of $\beta$-DPNH$^2$ in a total volume of 1 ml. Reaction mixture B contained the same components as reaction mixture A and in addition 8.3 mg. of Fraction II. The reaction mixtures were incubated at 38° and 0.1 ml. samples taken for the detection of the hydrolysis of the pyrophosphate bond of DPN$^2$ by the method described in the text.

<table>
<thead>
<tr>
<th>Time (Hrs.)</th>
<th>Reaction Mixture</th>
<th>Before Oxidation $E_{340\mu}$</th>
<th>After Oxidation $E_{340\mu}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A</td>
<td>0.995</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.978</td>
<td>0.037</td>
</tr>
<tr>
<td>1.5</td>
<td>A</td>
<td>1.010</td>
<td>0.052</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.978</td>
<td>0.062</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>0.985</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.970</td>
<td>0.065</td>
</tr>
</tbody>
</table>
Stability of the Enzyme.

Fraction II has been stored for as long as 2 months at 1-2° without loss of activity although protein precipitation occurred during this period of storage. The precipitated protein was routinely spun down and discarded.

Identification of the Products of Hydrolysis of ADP ribose.

As one of the products of the hydrolysis of ADP ribose is attacked by adenylic acid deaminase and gives rise to ATP, as judged by paper chromatography, in the presence of PC, CPT and myokinase (Chapter 2) it may be concluded that it is AMP. In view of this, it appeared likely that the other product was ribose-5'-phosphate.

Attempts to separate authentic samples of ribose-5'-phosphate and ribose from ADP ribose, AMP, ADP and ATP using solvent systems commonly employed for the separation of nucleotides, sugars and phosphorylated compounds were unsuccessful. No system gave complete separation although a number of systems did allow the separation of several of the test compounds (Table 4.6). Consequently, conditions were chosen so that ADP ribose underwent complete hydrolysis and ADP ribose, ADP ribose plus PC, and ribose-5'-phosphate were incubated for 4.5 hr. under conditions described in Table 4.3 with the Mg²⁺ concentration 1 x 10⁻³ M. Protein was removed by placing the incubation mixtures in a boiling water bath for 2 min. and
TABLE 4.6

*R*<sub>F</sub> Values of Adenine Nucleotides, Ribose and Ribose-5'-phosphate

Test compounds have been separated on Whatman 3 MM paper by ascending chromatography in the solvents listed below. Nucleotides were detected by visual inspection in ultraviolet light and sugars and derivatives detected by spraying with an aniline phthalate reagent (Partridge, 1949). Chromatograms were developed at 23-24°.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>S&lt;sub&gt;1&lt;/sub&gt;</th>
<th>S&lt;sub&gt;2&lt;/sub&gt;</th>
<th>S&lt;sub&gt;3&lt;/sub&gt;</th>
<th>S&lt;sub&gt;4&lt;/sub&gt;</th>
<th>S&lt;sub&gt;5&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>0.48</td>
<td>0.145</td>
<td>0.61</td>
<td>0.15</td>
<td>0.33</td>
</tr>
<tr>
<td>ADP</td>
<td>0.26</td>
<td>0.04</td>
<td>0.48</td>
<td>0.075</td>
<td>0.42</td>
</tr>
<tr>
<td>ATP</td>
<td>0.12</td>
<td>0.025</td>
<td>0.40</td>
<td>0.07</td>
<td>0.435</td>
</tr>
<tr>
<td>ADP ribose</td>
<td>0.24</td>
<td>0.06</td>
<td>0.40</td>
<td>0.155</td>
<td>0.44</td>
</tr>
<tr>
<td>Ribose</td>
<td>0.71</td>
<td>0.61</td>
<td>0.49</td>
<td>0.75</td>
<td>0.92</td>
</tr>
<tr>
<td>Ribose-5'-phosphate</td>
<td>0.59</td>
<td>0.26</td>
<td>0.33</td>
<td>0.255</td>
<td>0.97</td>
</tr>
<tr>
<td>Minor spot</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.23</td>
</tr>
</tbody>
</table>

S<sub>1</sub> Acetone; Acetic acid; H<sub>2</sub>O (50:15:30; by vol.)

S<sub>2</sub> Acetate buffer pH 3.6 0.1M; H<sub>2</sub>O; 95% ethanol (16:4:80; by vol.)

S<sub>3</sub> Isobutyric acid; IN NH<sub>4</sub>OH; 0.1N Versene (100:60:1:6; by vol.)

S<sub>4</sub> Ammonium acetate pH 7.4 1M; 95% Ethanol (30:70; v/v)

S<sub>5</sub> Saturated ammonium sulphate; H<sub>2</sub>O; Isopropanol (79:12:2; by vol.)
centrifuging. The supernatants were chromatographed in the solvents described in Table 4.6 and an attempt was made to detect ribose-5'-phosphate using an aniline phthalate spray (Partridge, 1949). The only component of the reaction mixture detected by the spray was the ammediol buffer and this could be readily distinguished from the sugars as it gave a yellow rather than a red spot. The ribose-5'-phosphate could not be detected by increasing the amount of material applied to the paper as the high salt concentration of the reaction mixtures affected the chromatographic behaviour of the components present. In addition, the presence of ammediol buffer changed the R_F values obtained for authentic samples of ribose and ribose-5'-phosphate chromatographed in the solvent system S_1 (Table 4.6). This difficulty was not encountered with the other solvents tested.

The presence of ribose-5'-phosphate in the three reaction mixtures was finally established by using a sensitive method for the detection, on chromatograms, of phosphorus containing compounds (Rosenberg, 1959). However, the only solvent system in which the ribose-5'-phosphate was clearly separated from P_i was the system S_5 (Table 4.6).

**Stoichiometry of the Reaction.**

An unsuccessful attempt was made to study the stoichiometry of the hydrolysis of ADP ribose. AMP was estimated
in the protein free supernatant from an incubation mixture similar to that described in Table 4.1 but without PC and the reaction stopped and protein removed as described in Method 1. A sample of the supernatant was passed through a column of acid washed Nuchar C to remove nucleotides and the combined eluate and washings, which did not absorb at 260 m\textmu, were assayed for ribose and ribose containing compounds. However, less than 10% of the expected level of these compounds was present. Subsequently it has been shown that ribose-5'-phosphate is retained on a charcoal column under the conditions used.

DISCUSSION

It is suggested that the enzyme present in rabbit muscle preparations which catalyses the hydrolysis of ADP ribose but which does not attack pyridine nucleotides, AMP or ATP could be called an ADP ribose pyrophosphatase. This enzyme differs from the other pyrophosphatases which have been reported to attack ADP ribose in its substrate specificity. The occurrence of both a DPNase and an ADP ribose pyrophosphatase in muscle may indicate that ADP ribose is involved in a pathway for the catabolism of DPN in that tissue.

ADP ribose has been isolated from perchloric acid and ethanolic extracts of chicken, rat and guinea pig liver at a level of 10 \textmu moles per 100 g. of fresh tissue (Hansen, Hageman, Freeland & Wilkin, 1956) and as DPN was not decomposed when subjected to the same treatment as the liver they concluded that ADP ribose occurred naturally. However, as it has subsequently
been shown (Forrest, Wilken & Hansen, 1960) that the
nicotinamide ribose bond of the reduced pyridine nucleotides
is hydrolysed as a result of the procedures employed for the
extraction and separation of nucleotides from tissues
(Hurlbert, Schmitz, Brumm & Potter, 1954) there appears to be
some doubt as to whether this nucleotide does in fact occur
in vivo. Recently the non-protein component of crystalline
glycerol-1-phosphate dehydrogenase (Ankel, Bucher & Czok, 1960)
was identified as ADP ribose. However, the nucleotide does
not seem to play any significant role in the activity of this
enzyme as it may be removed with charcoal and this treatment
does not affect the activity of the enzyme, the ability of the
protein to crystallize or the level of DPNH₂ which may be bound
by the protein although the level of titratable -SH groups is
higher when ADP ribose is removed.

ADP ribose occurs frequently as a contaminant or break­
down product of commercial samples of DPN and DPNH₂ even when
the pyridine nucleotides have been stored at -10° with a
dessicant. The addition of samples of pyridine nucleotides
containing ADP ribose to a rabbit muscle extract would result
in the formation of AMP. This reaction may be of considerable
importance in a system where the adenine nucleotides are to be
excluded but which require the addition of pyridine nucleotides
(Chapter 2). The recent report by Cori, Traverso-Cori, Marcus,
Tetas, Múnoz and Lagarrigue (1960) that DPN and ADP ribose can catalyse the transfer of a phosphoryl group from PEP to creatine may be readily explained if the rat skeletal muscle extract used contained an ADP ribose pyrophosphatase.

The specificity of ADP ribose pyrophosphatase would allow the use of this enzyme for the estimation of ADP ribose present as a contaminant of samples of DPN or DPNH₂. The DPNH₂ pyrophosphatase described by Jacobson and Kaplan (1957) could be used for the estimation of ADP ribose present in DPN but no other method is available for the estimation of ADP ribose present in DPNH₂.
SUMMARY

1. Rabbit skeletal muscle contains an enzyme, ADP ribose pyrophosphatase, which catalyses the reaction:

\[
\text{ADP ribose} \rightarrow \text{AMP + Ribose-5'}-\text{phosphate.}
\]

2. The enzyme does not hydrolyse DPN, DPNH₂, AMP or ATP.

3. The enzyme has an absolute requirement for metal ion and may be activated by Mg⁺⁺, Mn⁺⁺ or Co⁺⁺ but not by Ca⁺⁺. High levels of Mg⁺⁺ inhibit the enzyme.

4. The \( K_M \) of the enzyme for ADP ribose was found to be \( 3.8 \times 10^{-5} \text{M} \).
REFERENCES


