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THE SYNTHESIS AND BREAKDOWN OF PHOSPHOCREATINE
IN ANIMAL TISSUES

A study of creatine phosphokinase

By

Harry Rosenberg

Thesis submitted for the degree of Doctor of Philosophy in the Australian National University
July 1955
In compliance with the regulations of the Australian National University, and since the work described in this Thesis has been carried out in collaboration with my supervisor, Professor A. H. Ennor, my contribution to this work may best be described by quoting from Professor Ennor's letter of June 18 to the Acting Registrar:

"...In the earlier part of the work, i.e. part of that discussed in Chapter II, and all of that discussed in Chapter III, the candidate's contribution was that of a junior worker, but in the work described in Chapter I the candidate's contribution was that of a co-worker, for he was equally responsible with myself for the planning and carrying out of the work. The work described in the Appendices is almost entirely the candidate's own contribution."

Candidate's signature:

H. Rosenberg.
This thesis embodies work carried out in the Department of Biochemistry, Australian National University, during the tenure of a Research Scholarship for which I am indebted to the Council of the University.
ACKNOWLEDGEMENTS

It is a pleasure to express my sincere thanks to my supervisor, Professor A. H. Ennor, for his constant interest and valuable advice during the course of this work. I would also like to thank Dr. J. F. Morrison, of the Department of Biochemistry, for devoting much of his time in discussion of various aspects of this work and for offering many helpful suggestions. Mr. E. J. Hannan, of the Department of Statistics, kindly carried out the statistical analyses.

I also wish to thank Dr. K. H. Fausacker, of the Department of Chemistry, and Dr. F. J. H. Hird, of the Department of Biochemistry, University of Melbourne, for gifts of p-iodosobenzoic acid and N-ethylmaleimide, respectively.

Thanks are also due to Miss D. Roberts for her skilful technical assistance in the later stages of this work and to Mr. V. Faral, of the Department of Physiology, for his help with the photographic work. Finally, I would like to express my thanks to Betty Rosenberg for her help in many ways, particularly in arranging and checking the references.
Throughout this work, in referring to the reaction catalysed by creatine phosphokinase the term 'forward reaction' is applied to the transfer of phosphate from phosphocreatine to ADP, and the term 'reverse reaction' is used for the transfer of phosphate from ATP to creatine:

\[
\text{PC} + \text{ADP} \xrightarrow{\text{forward}} \text{ATP} + \text{Cr}
\]

\[
\text{ATP} + \text{Cr} \xrightarrow{\text{reverse}} \text{PC} + \text{ADP}
\]

The above terms have also been used when discussing the work of other authors.

Temperatures are expressed in °C.

Figures and tables are presented on separate pages, a particular figure or table following immediately the page on which first reference to it has been made.

The following abbreviations will be used:

- **ADP** Adenosine diphosphate
- **AMP** Adenosine monophosphate (adenylic acid)
- **ATP** Adenosine triphosphate
- **CMBA** \(p\)-Chloromercuribenzoic acid
- **CPK** Creatine phosphokinase
- **Cr** Creatine
- **DA** Diphenylchloroarsine
- **G-1-P** Glucose-1-phosphate
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<td>G-1,6-di-P</td>
<td>Glucose-1,6-diphosphate</td>
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<td>IDP</td>
<td>Inosine diphosphate</td>
</tr>
<tr>
<td>ITP</td>
<td>Inosine triphosphate</td>
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<tr>
<td>I.S.</td>
<td>Ionic strength</td>
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<tr>
<td>Lewisite</td>
<td>Dichloro(2-chlorovinyl)arsine</td>
</tr>
<tr>
<td>Negmine</td>
<td>N-ethylglycocyamine (N-ethylguanidoacetic acid)</td>
</tr>
<tr>
<td>Negmidine</td>
<td>N-ethylglycocyamidine</td>
</tr>
<tr>
<td>-P</td>
<td>High-energy Phosphate Group (cf. Lipman, 1941)</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphocreatine</td>
</tr>
<tr>
<td>S.A.</td>
<td>Specific radioactivity (counts/min./μg P.)</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>Tosyl-</td>
<td>p-Toluenesulphonyl-</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>Versene</td>
<td>Ethylenediamine tetraacetic acid (Na salt)</td>
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GENERAL INTRODUCTION
GENERAL INTRODUCTION

(a) Historical

Nearly half a century ago the element phosphorus was introduced into biochemistry with the observation of Harden and Young (1905) that inorganic phosphate added to fermenting yeast could not, after a time, be precipitated with either magnesia mixture or silver nitrate. The subsequent work of Harden and Young (1906, 1908) produced evidence that the phosphate was esterified and largely appeared in a form of hexose phosphate. At that time the compound was considered to be merely a by-product of fermentation and its general importance was not realised until eight years later when Embden, Griesbach and Schmitz (1914) found hexose diphosphate in skeletal muscle.

The five decades that passed witnessed the advent of phosphorus to a position of major importance in the metabolic reactions of living matter and it has been stated that

"among the mineral elements essential to life none plays a more central role than phosphorus." (Glass, 1951).

The function of phosphate in the economy of vertebrates can be divided into two main parts:

The first, and quantitatively the larger, has a structural function; about 80% of the body phosphorus can be found in the skeleton in the form of bone. It is the remaining and quantitatively the
lesser part of the body phosphorus that has attracted the attention of the biochemist occupied with the metabolic reactions of the cell. Less than 20% of the body phosphorus can be found in the so-called acid-soluble fraction which contains, apart from orthophosphate, a multitude of soluble organophosphates. The distribution in the body of these compounds varies. About half of the total acid soluble fraction is found in the muscles; the remainder is distributed to a varying extent in other tissues and its presence is probably essential in every living cell.

After the discovery by Harden and Young in 1906 of hexose phosphate, knowledge of the role of phosphorus was not appreciably increased until about 1926. The decade that followed 1926 brought into this field a number of new contributions of which the most outstanding were:

The fact that hexose phosphate was formed not as the result of a side reaction but was the first of a series of phosphorylated intermediates which arose as a result of the stepwise degradation of glucose. This series of reactions, all of which were reversible, became known as the hexose, or glycolytic cycle.

It was also observed that metabolic reactions depended upon the presence of complex compounds, named nucleotides, all containing phosphate groups and which were required in catalytic amounts. To this group belonged the adenylic system (phosphorylation), phosphoglyceride nucleotides (oxidation and reduction) and
diphosphothiamine nucleotide (decarboxylation). As the result of study of phosphorylation reactions it was found that in many of these the transfer of phosphate was direct from compound to compound without the intermediate liberation of inorganic phosphate.

One of the most interesting aspects of the work involving phosphorylated intermediates was the discovery that some of these compounds contained phosphate bonds of a far higher energy content than those of ordinary phosphate esters. This last observation was of prime importance for the understanding of the fundamental reactions involving liberation of energy in various forms by living tissues.

In this group of compounds belong the adenine nucleotides and the phosphagen of vertebrate and invertebrate muscle - phospho-creatine and phosphoarginine.

(b) The discovery of phosphocreatine

The discovery of phosphocreatine took place in the beginning of 1927. It was preceded by a number of reports dealing with the diffusion rates of some compounds from muscle observed during rest and fatigue.

Embden and Adler (1922) found that stimulation of muscles suspended in fluid medium caused orthophosphate to diffuse from the
muscle into the medium. They considered this to be due to an alteration in the permeability of the tissue induced by stimulation.

Tiegs (1925) found that stimulation of a similar muscle preparation caused an increase in the rate of diffusion of creatine. Oxygenation of the muscle was found to produce a marked decrease in the diffusion rate observed, and this led Tiegs to the conclusion that under the influence of oxygen the "diffusible creatine had to a large extent been converted back into the non-diffusible form". In an attempt to explain the phenomenon Tiegs proposed that in the intact muscle creatine existed in some tautomeric form which exercise changed into diffusible creatine. The significance of this change lay in the maintenance of muscle pH.

No attempt was made, however, to correlate the results obtained with phosphate and creatine.

The actual discovery of phosphocreatine was closely connected with the methods of phosphate estimation employed in the period.

The method of Briggs (1922) depends on the reduction of phosphomolybdic acid by quinol and sulphite in acid solution, and using this method Eggleton and Eggleton (1927a) encountered a phenomenon of delayed colour development when estimating the phosphate content of muscle extracts. They concluded that this was due to some unknown substance present in muscle which broke down with the liberation of inorganic phosphate during the estimation. They
named the substance "phosphagen", a name which is still applied to phosphocreatine and related substances. The chemical nature of this substance was not investigated but it was appreciated that it existed in larger amounts in resting than in fatigued muscle and that it was absent from muscle which had passed into heat-induced rigor. It also became obvious that the presence of this substance in muscle extracts led to very significant errors in the estimation of the inorganic phosphate content. The authors also found that the "phosphagen" content of striated muscle was higher than that of cardiac, while the content of smooth muscle was very low and thus were "...temuted to correlate 'phosphagen' content of a muscle in its resting condition with its ability to respond to sudden demands for violent energy".

These results were elaborated upon in a further communication in which Eggleton and Eggleton (1927b) described the experiments in which the delayed colour reaction was observed. By an extrapolation technique they calculated the true inorganic phosphate content of muscle and found it to be about one fifth of the value obtained by the Briggs method (100 mg.%); the difference was considered to be the phosphate derived from the breakdown of phosphagen. These figures applied to resting muscle, and in fatigued muscle the phosphagen was lower and the inorganic phosphate correspondingly higher.

In the same year Fiske and Subbarow (1927a) presented a
preliminary report in which they described the existence in muscle of a very labile compound which appeared to be composed of creatine and phosphate. This compound accounted for the bulk of the inorganic phosphate as measured by the existing methods employing molybdate in acid solution.

In a more detailed publication Fiske and Subbarow (1927b) reported that of the 100 mg.% of phosphate believed to be present in muscle as orthophosphate only 20 mg.% was in actual fact inorganic phosphate, the remainder having originated from the substance under investigation.

Fiske and Subbarow, having developed a technique for its purification through a precipitation with copper in alkaline solution, isolated an amorphous substance which they identified to be almost entirely a compound of creatine and phosphate. The molecular ratio of creatine to phosphate was found to be very close to unity (0.97 - 1.03), this ratio being the same for samples obtained from differently treated muscles (where contents varied), provided the copper precipitation was included in the purification procedure.

These authors immediately suggested that the compound was responsible for some of the earlier results on diffusion rates. They also envisaged the important role that this compound was to play in the future knowledge of muscle biochemistry.

In a somewhat later communication Eggleton and Eggleton (1927c) still failed to identify phosphagen with phosphocreatine.
They claimed it to be "...of a nature of a hexosephosphate (unpublished results)" and postulated a scheme to explain the role of phosphagen in muscle which ran as follows:

(a) Phosphagen $\rightarrow$ lactic acid + inorganic phosphate

(b) Inorganic phosphate + glycogen $\rightarrow$ X

(c) X $\rightarrow$ phosphagen

While the above paper was in press Eggleton and Eggleton (1927d) reported a number of observations which indicated that phosphagen was not a hexosephosphate since the amount of the latter decreased with the purification of phosphagen. In the same communication they referred to the work of Fiske and Subbarow (1927b) having been brought to their notice. In the light of the latter work the Eggletons re-examined their product and found it to contain creatine and phosphorus in a molar ratio which approached unity in the process of purification. They considered that it was "...reasonable to suppose that creatine is associated intimately with the complex which we have designated 'phosphagen'...".

From the historical viewpoint then it would seem that whilst the Eggletons appreciated the chemical instability of phosphagen and indeed were the first to do so, the credit for the identification of the compound as phosphocreatine should go to Fiske and Subbarow. These workers (1929a) described the purification of phosphocreatine by a procedure involving multiple precipitations with both copper and calcium and which resulted in the
preparation of a crystalline calcium salt. The product gave analytical figures corresponding to $C_4H_8O_5N_3P\text{Ca} \cdot 4H_2O$ and contained bound phosphate and creatine in the ratio of 1.0.

The following structural formula for the compound was proposed:

\[
\begin{array}{c}
\text{NH-P(OH)₂} \\
\text{HN-C} \\
\text{N(CH₃)-CH₂-COOH}
\end{array}
\]

The newly discovered phosphocreatine was recognised quite early as an unusual compound. Its breakdown to varying degrees as a result of muscular exercise suggested some connexion with the supply of energy. This suggestion gained credence when Meyerhof and Suranyi (1927) found that unexpectedly large amounts of heat were liberated during its enzymic decomposition. These authors obtained a value of 150 cal./g. of $H_3P_4O_7$ which corresponds to ca. 15,000 cal. per Mole.

A lower figure, about 12,000 cal./Mole, was obtained by Meyerhof and Lohmann (1928b) who investigated the heat of hydrolysis in acid. The difference in results was apparently due to an error introduced by the blank in the experiments of Meyerhof and Suranyi (1927). This was finally cleared up by the work of Lohmann (1934a) who used a less crude system than that employed by Meyerhof and Suranyi (1927), namely an extract of frog muscle inactivated by dialysis and age and subsequently reactivated by the
addition of ATP. The heat of decomposition of phosphocreatine was found in these experiments to be about 12,000 cal./Mole which coincided with the figure obtained by acid hydrolysis (Meyerhof and Lohmann, 1928b). This figure was later confirmed by the same authors in another series of enzymic experiments (cf. Meyerhof and Lohmann, 1932).

While the data available at the end of the third decade of this century suggested a role of phosphocreatine in energy exchanges, it was not until the classical work of Lundsgaard (1930-31) that the first experimental evidence was actually afforded.

In the first of six papers, published in a series over two years, Lundsgaard (1930a) reported a number of interesting observations. He found that when 40 mg./Kg of sodium iodoacetate (IAA) was injected into the dorsal sac of a frog its muscles passed, after an interval, into a state of rigor which was irreversible and which often ended in the animal's death. Immediate post mortem examination showed that the pH of the poisoned muscles was slightly higher than that of the normal ones. If the muscles were allowed to "autolyse" at room temperature after death, the pH of the tissue was not markedly affected in the case of the poisoned muscle but in the normal muscle it fell very sharply under the same conditions.

These differences in acidity were due to the lactic acid content of the respective muscles. Thus in one case it was found that in normal muscle the lactic acid content increased as a result of autolysis
from 57 to 649 mg.%, while in poisoned muscle a slight decrease in lactic acid content actually occurred (39 to 27 mg.%).

In view of such results Lundsgaard immediately proceeded to work on isolated muscles from frogs poisoned with iodoacetate. He applied a short series of tetanic stimuli to the cut ends of the nerves leading into the muscles and analysed the latter for lactic acid content. It was shown that stimulation of the muscle from the poisoned animal did not lead to an increase in the lactic acid content.

So strong were the concepts of the lactic acid doctrine in the minds of physiologists at that time that Lundsgaard's first reaction was to suspect his methods and the possible interference of iodoacetate with the estimation of lactate. Having satisfied himself that his figures were reliable Lundsgaard proceeded to carry out a systematic investigation into the physiology of iodoacetate-poisoned muscle. A frog was poisoned with a large dose of the compound (400 mg./Kg.) and gastrocnemii were removed with the sciatic nerves. Both muscles were suspended in the same way and, while one was stimulated through the nerve, the other was kept as a control. At the same time a similar experiment was carried out on a normal frog. The experiments confirmed the original finding. While the lactic acid content increased several fold following stimulation of normal muscle, the iodacetate poisoned muscle showed no increase in lactic acid under the same conditions.
A number of other very interesting observations arose in the course of this work:

(1) While in normal muscle the stimulation induced uniform contractions throughout the duration of the experiment, the same period of stimulation of the poisoned muscle produced a number of normal contractions of uniform length after which the amplitude of the contractions rapidly fell to zero and the muscle itself passed into rigor.

(2) The phosphagen content of the normal muscle fell only slightly as a result of exercise, but in the poisoned muscle it was completely depleted.

(3) The disappearance of phosphagen in the poisoned muscle always coincided with the onset of rigor.

(4) The phosphate liberated by the breakdown of the phosphagen did not appear as inorganic orthophosphate but in the hexose phosphate fraction.

Thus the theory that the production of lactic acid was an essential condition for contraction needed revision, for contraction had taken place without its formation. It seemed clear that the energy for contraction was supplied by a compound which, as far as its actual amount allowed, could sustain the work for some period without lactic acid formation. The "revolution in muscle physiology" (Hill, 1932) was completed and can be summed up in Lundsgaard's statement:

"...Das Phosphagen ist der bei Muskelkontraktion
Lundsgaard (1930a) put forward the hypothesis that the breakdown of phosphocreatine was the event which supplied the energy for muscular contraction in the poisoned muscle and that it presumably did so in normal muscle as well. The experimental fact that the phosphocreatine content of normal muscle fell only to a slight extent on stimulation was explained by Lundsgaard as being due to its regeneration as a result of glycolysis.

Lundsgaard (1930b) then established a linear relation between the work performed and phosphocreatine breakdown, and repeated his statement on the role of phosphocreatine but somewhat more cautiously proceeded to state:

"...die Phosphagen spaltung der Kontraktion sozuzagen eine Stoffe naher steht als die Milchsaurebildung und von den im Moment bekannten energieerzeugenden Prozessen dem Kontraktion nachsten liegt."

Viewed in modern light it was perhaps by coincidence that the discussion turned immediately to ATP, but this compound was considered only from the point of view of its neutralising ability in connexion with ammonia production due to transformation into inosine derivatives. It took nearly four years to bring ATP into the place it now occupies in the picture.

Another interesting aspect of the early experiments of
Lundsgaard emerges from the effect of iodoacetate on the enzyme creatine phosphokinase in vitro. This will be discussed in detail later (see page 72).

In a further paper Lundsgaard (1931a) found, in experiments with claws of the crustacean Maia squinado, that the behaviour of phosphagen in iodoacetate-poisoned muscles was not confined to vertebrates since similar results could be observed with the crustacean phosphagen, phosphoarginine, which had been described previously by Meyerhof and Lohmann (1928a).

The work was finalised by Lundsgaard (1931b) when he checked the Tl/H (Tension x length/heat) factor for both poisoned and normal muscle and found them to be identical, accepting the known heat of hydrolysis of phosphocreatine. With those combined findings of Lundsgaard "...the applicability of the phosphate bond energy for the driving of the muscle machine was established" (Lipmann, 1941), and phosphocreatine and phosphoarginine were introduced into an important position in muscle biochemistry.

(c) The discovery of ATP

The role of PC in muscle metabolism cannot be discussed without the mention of ATP.

Work which led to the discovery of ATP began with that
of Liebig (1847) who reported that a crystalline mass was obtained when muscle extracts from which creatine had been removed were evaporated and treated with ethanol. This crystalline mass contained a number of compounds from which inosinic acid was isolated as a crystalline barium salt. It was appreciated by Kaiser (1895) that the molecule contained phosphoric acid and Neuberg and Brahn (1907) recognised the substance to be a nucleotide, believing its structure to be purine:phosphate:1-xylose.

The structure was finally established by Levene and Jacobs (1908, 1909a, 1909b, 1911). These authors showed that the sugar moiety was neither xylose nor arabinose as claimed before (Neuberg and Brahn, 1907); when the barium salt of inosinic acid was heated barium phosphate was one of the products, the remainder being neither a sugar nor free hypoxanthine, as expected from the formula of Neuberg and Brahn (1907), but a combination of the two. With the identification of the sugar as ribose the structure of inosinic acid was established as hypoxanthine:ribose:phosphate.

Adenylic acid (AMP) was discovered a few years later. Embden and Lacquer (1914) reported that the hexose diphosphate fraction of horse muscle extracts contained another substance which consisted of phosphate, adenine and a pentose. This substance was later isolated by Embden and Zimmermann (1927) who found that it could be easily purified by recrystallisation from hot water. The composition of the compound was found to be adenine:ribose:phosphate.
It was not until 1929 that it was realised that adenylic acid was a breakdown product and did not exist in a free state in muscle but was in the form of a compound in which there were two additional labile P atoms.

Reports to this effect came in the same year from Fiske and Subbarow (1929b) and from Lohmann (1929). The former authors started with the observation that, although the calcium salt of ATP was water soluble, a large proportion of the muscle purine could be found in the precipitate obtained as a result of the treatment of a muscle extract with calcium at alkaline pH. The authors realised that the precipitated purine compound could not be AMP and proceeded to purify it using precipitation of a mercury salt followed by the precipitation of the acid calcium salt with ethanol. The final product was converted into a silver salt of the composition $\text{C}_{10}\text{H}_{13}\text{O}_{13}\text{N}_{5}\text{P}_{3}\text{Ag}_3$. Therefore the compound contained three phosphate groups; two of these were very easily hydrolysed by dilute acid, while the third was far more stable.

The approach of Lohmann to the problem was from the pyrophosphate moiety. In his earlier work Lohmann (1923a) had expressed the belief that the labile phosphate found in the so-called "lact-acidogen" fraction (mixed Ba-salts) of Embden was inorganic pyrophosphate and that this pyrophosphate was hydrolysed by "...some muscle enzyme..." to orthophosphate. A number of methods were subsequently described by Lohmann (1928b) for the isolation of pyrophospha
from muscle and its estimation. The observation that pyrophosphate diffused only from autolysed muscle was interpreted by Lohmann (1928c) as indicating that pyrophosphate formed a complex with protein and thus could not diffuse out of the muscle.

Lohmann (1929) recast his previous theories when he found that the products of hydrolysis of "lactacidogen" with baryta were pyrophosphate and adenylic acid. Heating in dilute acid for a short time produced adenylic acid and orthophosphate in a molar ratio of one to two. Lohmann then tested his material for pyrophosphate and came to the conclusion that free pyrophosphate did not exist in muscle in significant amounts and therefore that the compound under investigation was adenylyl-pyrophosphate (ATP).

Later Lohmann (1931a) reported the isolation of the barium salt of ATP and its purification. The analytical figures show that Lohmann had obtained a practically pure substance. The procedure he recommended for the preparation of ATP from rabbit muscle by the alternate precipitation of the barium and mercury salt (with or without ethanol) is still the accepted basic method for the isolation of this compound and most of the subsequently published methods are only slight modifications of Lohmann's original preparation (cf. Kerr, 1941a; Needham, 1942; LePage, 1945; Dounce et al., 1948; Bielschovsky 1950).

Having ascertained the identity of the compound the team from the Kaiser Wilhelm Institute embarked upon a series of
"autolyzed" muscle did not contain AMP and therefore it could activate dialysed muscle extracts only together with ATP. In the case of the dialysed fresh extract ATP and muscle ash were sufficient for activity since the divalent ions were provided in the ash. In case of the "autolyzed" extract only adenylic acid was required and therefore the addition of ATP alone restored activity, Mg$^{++}$ and phosphate being present. At that time these facts were unknown to Lohmann and it took a long period of experimentation to establish them.

In a subsequent communication Lohmann (1931b) reported the results of many more experiments on a larger scale and finally came to the conclusion that the "true coenzyme" of lactic acid formation was the combination of ATP, magnesium ions and inorganic phosphate. He also reported that ATP induced the fermentation of dialysed macerated yeast. Lohmann (1931b) also searched for other enzymic systems in muscle which might be dependent upon ATP. He found that in the case of glyoxalase, ATP did not have any effect. In the case of the respiration rate of muscle suspensions, Lohmann found that, although ATP did have an activating effect, it was only to the extent of about 40% of the activating power of "Kochsaft". He then concluded that in those systems other activators, with or without ATP, played a role.
(d) ATP and PC – enzymic relationship

The next system tested by Lohmann (1934a) was that responsible for the enzymic cleavage of phosphocreatine into creatine and phosphate. From the previous results of Meyerhof and Suranyi (1927), who employed enzymic methods for the determination of the heat of decomposition of phosphocreatine, it was known that muscle extracts would catalyse the reaction. Also Meyerhof and Lohmann (1928a) reported that both minced muscle and muscle extracts could decompose phosphocreatine, but that the activity of the muscle extracts did not persist for any length of time and subsided to zero after several hours at room temperature. The results were similar to those obtained previously for the lactic acid formation (see above), and Lohmann (1934a) decided to check the effect of ATP on the reaction. At first "Kochsaft" was tried and the activity was immediately restored. Lohmann then tried the addition of ATP and found that it was as effective as "Kochsaft" even when added in very small amounts. Adenylic acid was also found to be very effective and, as reported by Lohmann, a mole of AMP could catalyse the disappearance of 1000 moles of phosphocreatine. This finding prompted him to postulate the theory that the disappearance of phosphocreatine involved the transfer of phosphate radicles from PC to AMP as a first step.

The following reactions were proposed:

(1) $AMP + 2 PC \rightarrow ATP + 2 Cr.$
(2) \[ \text{ATP} \rightarrow \text{AMP} + 2 \text{PO}_4^{3-} \].

(Reaction (1) above became known as the "Lohmann Reaction")

Thus the existence of creatine phosphokinase in muscle was established and the correct mechanism of the reaction proposed, with one exception: AMP, as shown by Ennor and Rosenberg (1954b), cannot take part in the reaction. This aspect of the reaction will be discussed in greater detail below, but it should be pointed out that Lohmann did not overlook the possibility of AMP not reacting for in the same communication he made the following statement:

"...The possibility exists, that the de-phosphorylation of ATP does not proceed to AMP but, with the removal of only one P, to ADP, which in turn reacts with one only mole of PC to form ATP."

Lohmann (1934a) also found that the creatine phosphokinase activity was not destroyed by dialysis whereas ATP'ase activity was lost. Thus, after dialysis, due to the absence of the ATP'ase the effect of AMP was no longer catalytic, and a finite amount of PC disappeared after the addition of a certain amount of AMP. It was also found that neither fluoride nor iodoacetate inhibited the enzyme. These data have been shown to be partially incorrect by Ennor and Rosenberg (1954b) and Rosenberg and Ennor (1955) since these workers showed that the enzyme was inhibited \textit{in vitro} by iodoacetate and contained a monothiol as part of the active centre(s). (See also Chapter I.)

In the discussion of the results Lohmann (1934a) postulated
a system in vivo whereby the ATP in muscle was dephosphorylated, probably during contraction, and then re-formed at the expense of phosphocreatine, the latter being resynthesised in the processes of aerobiosis and glycolysis. While at that time Lohmann did not investigate the possibility of the creatine phosphokinase reaction being reversible some proof was already available. Thus Meyerhof and Lohmann (1932) had reported that creatine was actively phosphorylated by muscle extracts and that this process could be accelerated by the addition of ATP. The appearance of phospho- creatine, moreover, was found to be related to the dephosphorylation of ATP.

The phosphorylation of creatine was studied further by Parnas, Ostern and Mann (1935), who found that in muscle extracts and "brei" creatine was actively phosphorylated during the breakdown of phosphoglycerate. This reaction, referred to at that time as the "Parnas reaction", was interpreted by the authors to be part of a scheme whereby creatine was the primary acceptor of phosphate from various donors (predominantly phosphopyruvate and phosphoglycerate). The phosphocreatine thus formed passed the phosphate on to adenylic acid in the Lohmann reaction.

Later Ostern, Baranowski and Reis (1935) expressed the view that the adenylic system could be the original acceptor of phosphate from the phosphopyruvate and that creatine was phosphorylated through the adenylic system.
Almost simultaneously with the latter group Needham and van Heyningen (1935a) published a preliminary report of their results, which supported the same view, and which showed that the presence of adenylic acid was obligatory for the Parnas reaction. In its absence the phosphoglycerate broke down almost entirely to inorganic phosphate. These results led the authors to the assumption that adenylic acid was an intermediate link in the phosphorylation of creatine.

At this time work on the phosphorylation of creatine was also in progress at the Kaiser Wilhelm Institute. The preliminary results of Meyerhof and Lehmann (1935) confirmed that adenylic acid was an essential component of the system concerned with the phosphorylation of creatine. Moreover, they showed that the effect of ATP was catalytic; a small amount of ATP could catalyse the phosphorylation of one thousand times the amount of creatine in the presence of a suitable donor system.

More detailed reports from both teams appeared later. Needham and van Heyningen (1935b) reported a synthesis of phospho-creatine from creatine and ATP by dialysed muscle extracts. The synthesis took place in the absence of phosphate donors other than ATP. The reaction could be made almost quantitative if the activity of ATPases in the extract was inhibited. The authors postulated that the synthesis occurred as a result of the Lehmann reaction working in reverse. However, they expressed doubt as to whether the reaction
could take place in one step since this would involve two molecules of creatine reacting with one of ATP and such a tri-molecular reaction was considered unlikely. They proposed a two-step reaction, viz.:

\[
\begin{align*}
(a) & \quad \text{ATP} + \text{Cr} & \quad \leftrightarrow & \quad \text{ADP} + \text{PC} \\
(b) & \quad \text{ADP} + \text{Cr} & \quad \leftrightarrow & \quad \text{AMP} + \text{PC}
\end{align*}
\]

This scheme found support in the already known fact that the Lohmann reaction could take place between ADP and PC. An analogy was also drawn with the observation of Lohmann (1935) that in a similar reaction catalysed by crayfish muscle extract phospho-arginine did not react with AMP but with ADP only. The latter observation may have been due to the absence of adenylate kinase activity from the crayfish muscle preparation.

In a simultaneous communication Lehmann (1935) elaborated on the previous work of Meyerhof and Lehmann (1935). He showed that Mg\(^{++}\) and AMP were essential for the phosphorylation of creatine by phosphoglycerate and that the reaction proceeded best between pH 8 and 9. Under these conditions it was possible to convert phosphoglycerate into phosphocreatine in such good yields (99%) that Lehmann (1935) suggested this as a method for the preparation of phosphocreatine. Under the same conditions arginine was phosphorylated in the presence of a crayfish muscle extract as an enzyme source. Lehmann put forward the view that the phosphorylation of creatine proceeded at the expense of the pyrophosphate fraction of ATP and
that the reaction was reversible with a pH-dependent equilibrium. This was proved by the synthesis of phosphocreatine using creatine and ATP only in the presence of Mg$^{++}$. Lehmann also found that the latter reaction could be forced further by the removal of the adenylic acid formed by the addition of adenylic acid deaminase. (Some remarks concerning the latter procedure will be offered below - p. 82). The final proof of the reversibility of the Lohmann reaction was the demonstration that the equilibrium could be reached from each direction and that the composition of the reaction mixture at equilibrium could be altered reversibly by alternate changes in the pH of the medium from alkaline to neutral.

At that stage Lehmann still favoured the view that the reaction proceeded in one step with the simultaneous exchange of two phosphate radicles. However, in a subsequent communication (Lehmann, 1936) he reported the results of kinetic studies which could only be explained in terms of a bi-molecular reaction and hence concluded that the reaction must take place in two steps (see above). Although he did not study the second step (between ADP and creatine), Lehmann assumed that this would also be a bi-molecular reaction.

No attempt at the purification of the enzyme responsible for the Lohmann reaction was made at that stage and in the eight years that followed very little work was done on the system.

The first attempt to obtain a purified preparation of the
enzyme was made by Banga (1943a) who described a partially purified enzyme preparation which catalysed the reaction between creatine and ATP. The enzyme responsible for this reaction was named ATP-creatine phosphophosphatase. Banga claimed that a second enzyme, ADP-creatine phosphophosphatase, catalysed the reaction between creatine and ADP. She found that during the fractionation procedure a fraction, which catalysed the second reaction, disappeared together with an enzyme which she termed ADP-isomerase. The latter enzyme was shown by Banga (1943b) to modify ADP with the result that the product was capable of releasing P when attacked by actomyosin. The ADP-isomerase was stable to heat in acid solution and could be precipitated with trichloroacetic acid without loss of activity. These properties indicate that ADP-isomerase is identical with myokinase (Kalckar, 1942; Colowick and Kalckar, 1943; Kalckar, 1943), although at that time Szent-Györgyi (1944) expressed doubt as to the identical nature of these two enzymes. The fact that ADP-creatine phosphorylase activity disappeared at the same stage as did an enzyme with myokinase-like activity and that it could not be isolated seems to indicate that ADP-creatine phosphorylase did not, in fact, exist; the activity observed was probably due to an integrated action of CPK and myokinase. As shown recently by Amor and Rosenberg (1954b) these enzymes together are capable of catalysing the "in vitro" transfer of phosphate from PC to ADP. Banga (1943a) further showed that the pH optimum of the reaction:
was near 9.0 in borate buffer and that the composition of the equilibrium mixture was pH-dependent. The reaction:

\[ \text{Cr} + \text{ATP} \rightarrow \text{PC} + \text{ADP} \]

was not studied.

The next important contribution on creatine phosphokinase came from Szecsenyi and Degtyar (1948) who reported that in muscle the enzyme existed in two forms, namely, as a free, water-soluble enzyme and as a bound component in a CPK-actomyosin complex. The catalytic properties of the two forms were similar. The enzyme catalysed the forward reaction at about pH 7 and the reverse reaction at about pH 9. Mg\(^{++}\) or Ca\(^{++}\) were necessary for enzymic activity and had a profound effect on the direction in which the reaction proceeded. Thus for the forward reaction a far higher concentration of the cation was necessary than was the case with the reverse reaction. In case of the reverse reaction, where reaction velocity was highest at pH 9, the reaction could be forced back if the cation (Ca\(^{++}\) or Mg\(^{++}\)) concentration was raised at any stage. The communication also included some kinetic studies.

A further purification of CPK involving the fractionation of muscle extracts with solvents at low temperatures was reported by Askonas (1951a). The solvents tested were ethanol, n-propanol, methanol and acetone. Of those tested n-propanol proved most suitable in the case of CPK. With this method a six-fold purification
of the enzyme in about 60% yield was obtained. Using the protein purified by prepanol precipitation, Askonas (1951b) investigated the action of thyroxine on the reverse reaction and found it to be inhibitory \((40 - 50\% \text{ at } 2.5 \times 10^{-5} \text{ M})\). The in vivo action of thyroxine was also tested by comparing the CPK activity of tissues taken from rats which had received a subcutaneous injection of thyroxine with that of tissues from normal controls. While a significant difference was observed in experiments of short duration (9 hours), this did not obtain in long term experiments (24 hours and longer). The effect observed in the short term experiments could have been due to some of the injected thyroxine having been carried with the tissues into the enzyme assays and thus exerting in vitro effect.

Askonas also reported the effect of other metabolic inhibitors on CPK. No inhibition was found with 2,4-dinitrophenol in concentrations up to \(1 \times 10^{-3} \text{ M}\), and only a very slight inhibition was observed with either iodine or iodosobenzoate at a concentration of \(1 \times 10^{-4} \text{ M}\).

Narayanaswami (1952) reported the presence of creatine phosphokinase in rat and guinea pig brain. The reaction was studied using a non-dialysed, crude saline extract of brain tissue as the source of the enzyme. It was found that Mg\(^{++}\) was essential but it could be substituted (in decreasing order of activity) by either Ca\(^{++}\) or Mn\(^{++}\). The effect of pH on the reaction was similar to that described for muscle CPK and it was claimed that the acceptor of phosphate from
phosphocreatine was adenylic acid and not ADP since in the forward reaction the rates were higher in the presence of ATP as compared with ADP. The fact that ADP acted as an acceptor was explained by Narayanaswami in terms of the assumption that adenylate kinase was present in the system and converted the ADP present into AMP in the usual fashion. The effect of a number of inhibitors on the system was also tested but only with iodoacetate was a positive result obtained, 100% inhibition resulting at a concentration of $1 \times 10^{-3}$ M.

Contrary to the findings of Narayanaswami (1952), Zeytlin (1953) reported that aqueous extracts of brain dialysed for 48 hours contained a powerful phosphoamidase, capable of breaking down phosphocreatine in the absence (as presumed by the efficiency of dialysis) of the adenylic system. On testing the adenylate-dependent CPK activity of brain Zeytlin (1953) found that phosphocreatine synthesis proceeded at a much slower rate than in muscle. It was concluded that a relatively important pathway existed in brain for the independent breakdown, and possibly synthesis, of phosphocreatine.

The possibility is not excluded that in the system used by Zeytlin the adenine nucleotides were not completely removed by dialysis. Even in the presence of traces of ADP, creatine phosphokinase coupled with an ATPase can decompose phosphocreatine at very high rates, as recently demonstrated by Ennor and Rosenberg (1953) in vitro. This reaction could have been observed by Zeytlin and mistaken for the action of phosphoamidase, particularly as no mention is
made by him of any attempt to control ATPases or to ensure their absence.

After publication of the major portion of the work described in the experimental section of this thesis a number of further communications appeared on the subject.

In a short communication Kuby, Noda and Lardy (1953) reported having obtained CPK as a single protein which was homogeneous as judged by the criteria of sedimentation rates and electrophoresis. Later Noda, Kuby and Lardy (1954a) reported that the enzyme was obtained in the crystalline form. This was followed by detailed reports dealing with the preparation of the crystalline enzyme (Kuby et al., 1954a) and its physical properties and homogeneity studies (Noda et al., 1954b).

The results of further studies on CPK by the same team were reported in two later communications dealing with kinetics (Kuby et al., 1954b) and equilibria (Noda et al., 1954c). The findings reported in those communications included a study of the effect of various buffer systems on the enzymic activity. The reaction rates decreased in the following order: histidine-HCl, glycyl-glycine, β-glycerophosphate, Tris.

The effect of substrate concentration on the reaction velocity was also investigated and the Km values were calculated. In the reverse reaction it was found that ADP was a competitive inhibitor relative to ATP but a non-competitive one relative to
creatine. The divalent cations Mg\(^{++}\), Ca\(^{++}\) and Mn\(^{++}\) were found capable of activating the enzyme to a varying extent; these results will be discussed below.

The action of several inhibitors was tested and the observation of Askonas (1951b) on the inhibitory effect of thyroxine was confirmed. An explanation of this effect was offered on the basis of a complex formation between thyroxine and Mg\(^{++}\) and the theory was supported by data of the dissociation constants of such complexes. The authors also found that the enzyme was very sensitive to p-chloromercuribenzoate.

Dealing with the equilibria Noda et al. (1954c) determined the equilibrium constant of the reaction and confirmed the finding of Szorenyi and Dextyar (1948) of the effect of Mg\(^{++}\) concentration on the apparent equilibrium constant. The connexion between this phenomenon and the respective dissociation constants of the Mg\(^{++}\) salts of ATP and ADP at various pH were discussed.

Chappell and Ferry (1954) reported a number of findings on the enzyme creatine phosphokinase which were in accordance with the (then already published) results described in the experimental section of this thesis. Later Oliver (1954) likewise confirmed the observation of Ennor and Rosenberg (1954b) that phosphocreatine reacted only with ADP. These communications will be discussed in further detail below.

Thus in the last twenty years a comprehensive study has been
made of the enzyme creatine phosphokinase and the enzyme itself has been crystallised. At present the knowledge of creatine phosphokinase reaction constitutes the entire information concerning enzymic reactions in which phosphocreatine participates. This compound is believed to be the source of urinary creatinine, the latter arising from phosphocreatine by a non-enzymic reaction (cf. Borsook and Dubnoff, 1947). However in a recent communication Carutto (1954) claimed that the enzymic conversion of phosphocreatine to creatinine in the presence of glucose-1-P was catalysed by extracts of washed muscle according to the following reaction:

\[ \text{PC} + \text{G-1-P} \rightarrow \text{G-1:6-di-P} + \text{creatinine}. \]

Unfortunately no details of this reaction are available at present.

The problem of an alternative pathway in the metabolism of phosphocreatine is not entirely hypothetical and the possibility of such pathways will be discussed below together with some circumstantial evidence pointing towards their existence.

Until further proof can be presented the CPK system must be regarded as the only one in which phosphocreatine participates and its significance in vivo apparently lies in the speed with which high energy phosphate may be transferred to ADP if and when required. While this does not explain the presence of either PC or CPK in tissues other than muscle it seems a fairly satisfactory theory for the latter. It seems possible, however, that even this theory might
require revision in the light of the recent observation that single muscle contractions can take place without the expenditure of either ATP or phosphocreatine (cf. Monnaerts, 1954; Fleckenstein, Janke, Davies and Krebs, 1954; Fleckenstein, Janke, Lechner and Bauer, 1954).

If another intermediate be postulated, closer even than ATP to the actual energy converting reaction, it becomes difficult to understand why this intermediate does not draw directly on the supply of energy available from phosphocreatine. If there is no other intermediate some other explanation is necessary as to the source of energy for the contractions which have taken place.

The solution of these problems may cast a new light on the metabolic reactions connected with muscular activity including those concerned with the metabolism of phosphocreatine in muscle, an understanding of which might be expected to help in the study of the function of phosphocreatine in other tissues.
CHAPTER I

THE PROPERTIES OF CREATINE PHOSPHOKINASE
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INTRODUCTION

As an aid to the better appreciation of the part played by phosphocreatine it seemed of some importance to determine, in detail, the properties of the creatine phosphokinase system. Consequently the experimental work described in this chapter deals with the various conditions affecting the reaction:

$$\text{PC} + \text{ADP} \rightleftharpoons \text{ATP} + \text{Cr.}$$
(a) **Materials**

**Creatine phosphokinase (CPK).** The enzyme was prepared according to Askonas (1951a). It was found that the protein fraction precipitated between 50 and 60\%(v/v) \(\alpha\)-propanol was most active. The protein solution was freeze-dried and the dry material stored at \(-15^\circ\). It was found that under these conditions no loss of activity occurred after two years.

Paper electrophoresis of the enzyme preparation at three different pH levels showed (Fig. 1) that the preparation contained two main protein components. In order to ascertain which fraction contained the enzyme, several strips were run in a single experiment in phosphate buffer pH 7.4 and at the end of the run one of the strips was developed with the protein staining solution described below (p. 43), while the other was carefully dried in vacuo at room temperature and sprayed with a mixture containing 1 ml. of 7 \(\times\) \(10^{-3}\) M. ADP, 1 ml. of \(1 \times 10^{-2}\) M. F6 and 0.5 ml. of \(1 \times 10^{-2}\) M. MgCl\(_2\). The damp strip was allowed to incubate for two min. at 37\(^\circ\) and then dried at 90\(^\circ\). It was then sprayed with a freshly prepared mixture of 1.0 ml. of 5 N NaOH and 3.0 ml. of a solution containing 2\% \(\alpha\)-naphthol and 0.05\% diacetyl in 50\%(v/v) ethanol. The pink colour which appeared where creatine had been liberated by the action of CPK showed the position of the
Figure 1.

Electrophoresis of the CPK preparation on paper.

Conditions: Strip I represents a 16 hour run in citrate buffer pH 5.7. Strip II - 16 hours run in borate buffer pH 10.0. Strips III and IV were run simultaneously for 22 hours in phosphate buffer pH 7.4. All buffers were adjusted with respect to their ionic strength so that their conductivity on paper was equal to that of a 0.1 M. solution of NaCl (assumed to have I.S. = 0.1).

Development: Strips I-III were developed with the protein staining solution (p. 43). Strip IV was incubated with a mixture of ADP, PC and CaCl₂ and developed for creatine.
enzyme. A comparison of strips III and IV (Fig. 1) shows clearly that the main component possessed enzymic activity.

In order to determine the proportion of the total protein represented by the enzyme the coloured zones were eluted separately from the strip (Fig. 1-III) in 0.01 M NaOH and the concentration of the Bromphenol blue with which the protein had been stained was determined in a Spekker (Hilger) photometer using a Spectrum Yellow 606 filter. Comparison of the extinctions showed that the enzyme constituted about 75% of the total protein present. This value is based on the assumption that the two proteins present have the same binding capacity for the dye on a weight basis (cf. however Franglen and Martin, 1954).

Attempts to obtain a more complete separation of the enzyme from the contaminating protein were then made using ammonium sulphate fractionation and heat denaturation. This work was however suspended following the announcement of Kuby et al. (1953) that the enzyme had been obtained in a homogeneous form, and a later personal communication that it had been crystallised.

The CPK preparation in hand was tested for enzymic activity with other substrates and it was found that it was unable to release inorganic phosphate from ATP, ADP or inorganic pyrophosphate when incubated for periods up to one hour at 37° in 0.025 M. TRIS buffer, pH 7.2 or 9.0. When tested under the same conditions in the presence of ADP and myosin the enzyme failed to
release inorganic P which indicated that the preparation was free from myokinase. It was then decided to use the preparation without further purification.

**Myosin.** This was prepared by the method of Bailey (1942). After four re-precipitations it was free from myokinase. The enzyme was stored in solution at 30° in 0.5 M KCl (pH 7.5) and remained active for several weeks. A drop of toluene was added as a preservative.

**Myokinase.** The enzyme was prepared from rabbit skeletal muscle as described by Kalckar (1943), using the modification of Rowles and Stocken (1950). The enzyme was freeze-dried and stored in sealed bottles at -15°.

**Amylase.** This was prepared from potatoes as described by Krishnan (1949). The enzyme was stored in the freeze-dried state at -15°.

Both in the case of amylase and myokinase, preparations stored for three years showed no loss of activity.

**Adenylic acid deaminase.** This enzyme was prepared from rabbit skeletal muscle using procedure A as described by Kalckar (1947). The enzyme, which is particulate, was stored in suspension as recommended by Kalckar and was used on the first and second day after preparation.

**Phosphocreatine.** PC was prepared as the crystalline sodium salt as described by Ennor and Stocken (1948c). Solutions
were made up as required.

**Adenosine and inosine polyphosphates.** ATP was prepared from rabbit skeletal muscle by either the method of LePage (1945) or of Kerr (1941a). ADP was prepared from ATP by incubation of the latter with myosin; the product was isolated from the digest by the method of Dounce, Rothstein, Beyer, Meier and Freer (1943). ITF and IDP were prepared from the corresponding adenosine derivatives by the method of Kleinzeiler (1942).

All the above-mentioned nucleotides were prepared as the Ba-salts and were over 95% pure as judged by chemical and enzymic analysis. The following procedure was used in order to convert the Ba-salts into the corresponding sodium salts:

The requisite amount of the Ba-salt was suspended in water and the pH adjusted to 7.5. An excess of cation exchange resin in the sodium form (Amberlite IR-100-Na or Dowex-50) was then added to the suspension and the tube shaken for 30 min. At the end of this time the supernatant which was free of Ba++, was poured off and the resin washed several times with water. The washings were combined with the original supernatant and made to volume. This method was more satisfactory than the conventional precipitation of the Ba++ as BaSO₄ since losses due to adsorption were avoided. It also eliminated the risk of hydrolysis which may have occurred if the Ba-salts were dissolved in acid. When pure samples of either ATP or ADP were required the solutions of the
Na-salts were fractionated on an anion exchanger by the method of Cohn (1953). The samples purified in this manner were homogeneous as judged by paper ionophoresis.

Acrylic acid. ATP was prepared from ATP by the action of apyrase and was purified as described by Kerr (1941b). It was obtained as the crystalline, free acid and stored as such. Solutions were made up as required and adjusted to pH 7.2 by the addition of 1 N NaOH.

Creatine. The creatine used was a commercial preparation which was thrice recrystallised as described by Hunter (1923). Its purity was checked by analysis of the nitrogen content. Only freshly prepared solutions were used.

Other guanidine compounds. The preparation of these compounds and of their phosphorylated derivatives is described in Appendix III.

Cystine ethyl ester hydrochloride. This compound was prepared by the method of Friedmann (1903) as described by Ennor and Stocken (1943b). All solutions were made up immediately before use and were adjusted to pH 7.2 by the addition of 0.1 N NaOH. In some cases solutions of the required pH were obtained by dissolving the compound in 0.1 M TRIS buffer.

Inhibitors. p-Chloromercuribenzoic acid (CMBA) was synthesised as described by Whitmore and Woodward (1932); o-iodosobenzoic acid, N-ethyl maleimide, lewisite and diphenyl-
chloroarsine (DA) were obtained as gifts from various sources, as acknowledged elsewhere. The other inhibitors used were obtained commercially and were recrystallised before use. Solutions of the inhibitors were made up in water and the pH adjusted to the required value by the addition of 0.1 M NaOH or HCl. In the case of diphenylchloroarsine (DA) a stock solution (1 x 10^-2 M) was made up in ethanol and dilutions were made with water. When this stock solution was diluted 1:10 the solution was slightly opalescent and a precipitate usually appeared after several hours; dilutions were therefore made just prior to use. A solution obtained as a result of higher dilutions remained clear. In tests involving DA appropriate controls were set up to check on any inhibition which may have been produced by the ethanol present.

All other reagents were A.R.

Buffer system. In the initial stages of the work several buffer systems were employed but, with one exception, all either proved inhibitory to the enzyme (phosphate and borate) or interfered with the colorimetric estimation of creatine (glycine and glycyld-glycine). TRIS proved relatively free from these objections and was used in the pH range 7.2 - 9.0. In the bulk of the work TRIS supplied by the Sigma Chemical Co., U.S.A., was used and the particular grade supplied ("SIGMA 7-9") was purified by recrystallisation from 90% ethanol.
(b) Methods

Creatine estimations. The estimation of creatine was carried out as described by Ennor and Stocken (1948b). Whenever such estimations were carried out on a sample containing alkaline earth metals a sufficient quantity of sodium ethylenediamine-tetraacetate (Versene) was added to prevent the precipitation of the carbonates by the alkaline α-naphthol reagent.

Determination of enzymic activity. The procedure varied depending upon the reaction studied. The complete system in case of the forward reaction consisted of the following mixture:

- TRIS buffer, pH 7.2, 0.1 M. 1.0 ml.
- CaCl₂, 4 x 10⁻² M. 0.2 ml.
- Phosphocreatine, 2 x 10⁻³ M. 0.5 ml.
- CPK solution (0.1 mg. protein/ml.) 0.2 ml.
- ADP, 1 x 10⁻³ M. 1.0 ml.
- Water to 4.0 ml.

In practice all components with the exception of the ADP were mixed and brought to temperature (370) in the water bath. The reaction was then started by the addition of 1.0 ml. of the ADP solution. Incubation times varied with the particular experiment, but the standard incubation time was 3 min.

When only one component of the reaction mixture varied,
the others were combined in a stock solution and samples added to each tube. This ensured uniform composition of the reaction mixture.

In the case of the reverse reaction the following standard mixture was employed:

- TRIS buffer, pH 9.0, 0.1 M. 1.0 ml.
- CPK solution (1.0 mg. protein/ml.) 0.5 ml.
- CaCl₂, 4 x 10⁻³ M. 0.1 ml.
- Creatine, 2 x 10⁻³ M. 0.5 ml.
- ATP, 1 x 10⁻³ M. 1.0 ml.
- Water to 4.0 ml.

As in the case of the forward reaction the components were added as a stock solution wherever possible. The ATP was usually omitted from the mixture and added at the beginning of the incubation period. Incubation times varied and are specifically referred to in the text. The incubation was usually carried out at 30° to avoid possible denaturation of the enzyme at the high pH employed.

In the early experiments and in both the forward and the reverse reactions the reaction was stopped by the addition of 1.0 ml. of 25% (w/v) trichloroacetic acid (TCA). In the case of the forward reaction the amount of protein present did not produce a visible precipitate, consequently the mixture was not filtered. Samples were immediately withdrawn (for creatine estimation).
and transferred to tubes containing a sufficient amount of 5 M NaOH to render the samples alkaline. In the case of the reverse reaction where the amount of enzyme was twentyfive times higher a small precipitate did form on the addition of TCA. This was coagulated by shaking, the suspension filtered and then treated as in the case of the forward reaction. All reaction mixtures were cooled in an ice-salt mixture after the addition of TCA.

Later it was found that the reaction could be stopped by the addition of 1.0 ml. of a mixture containing 4 ml. of 4 x 10^{-3} M Na-p-chloromercuribenzoate, 5 ml. of 0.2 M Versene and 1.0 ml. of 5 M NaOH. The two inhibitors stopped the reaction instantly and the alkali ensured that the protein present remained in solution. All solutions thus treated remained clear. The amount of protein in solution did not interfere with the estimation of creatine.

**Paper electrophoresis and ionophoresis.** This was performed using a commercial apparatus (L.K.B., Sweden) with direct current supplied by dry cells. Ionophoresis of nucleotides was carried out in citrate buffer pH 3.5 on Whatman No. 1 paper and the location of the bands determined with the use of a ultraviolet lamp fitted with a suitable filter. Electrophoresis of proteins was carried out on Schleicher and Schull paper No. 2043 B. The buffers employed were: borate, pH 10.0, veronal-citrate, pH 3.6, phosphate, pH 7.4 and citrate, pH 5.7. All buffers were
adjusted to ionic strength 0.1 (cf. legend, Fig. 1). Care was taken to keep the current constant at 0.5 M.A./cm. of front. In case of proteins the run varied from 4 to 22 hours. The location of the bands was determined by staining the dried strip with a solution containing 0.05% bromphenol blue, 1.0% HgCl₂ and 2.0% acetic acid in water. The stained strips were washed free of excess dye with 0.5% aqueous acetic acid, then several times with water and once with ethanol (Dr. W. E. Van Heyningen, personal communication).
RESULTS

(a) The forward reaction

The initial experiments involved the determination of the effect of cysteine on the initial reaction velocities. As it was known from previous work (Askenas, 1951b) that the pH optimum of the forward reaction lay in the vicinity of pH 7, this pH value was used in the first instance. The results shown in Fig. 2 indicate that cysteine, at a concentration of $1 \times 10^{-4}$ M, produces an acceleration of the reaction velocity with little or no shift in the equilibrium. The presence of cysteine, however, is not essential. It will also be noted that in the absence of cysteine the reaction is linear within the first 3 min. This reaction period was therefore adopted as the standard incubation time. It was decided to omit cysteine from the reaction mixture.

The effect of pH on the reaction was then determined. Sodium succinate buffer was used for the pH range 4.5 - 6.5 and TRIS buffer for the range of pH 7.0 - 9.0.

An appreciable hydrolysis of phosphocreatine occurred under the more acid conditions and in these cases appropriate control tubes were run. The results (Fig. 3) indicate a well-defined optimum at pH 7.2 and subsequent experiments were carried out at that pH.
The effect of cysteine on the CPK-catalysed reaction.

The experimental mixture contained all the components of the complete system for the forward reaction. The points •••• and •••• represent the means of duplicate analyses from reaction mixtures in the absence and presence of cysteine ($1 \times 10^{-4}$M) respectively. Incubation at $37^\circ$, pH 7.2.
Figure 3.

Effect of pH on CPK activity (forward reaction).

The experimental mixture contained all the components of the complete system with the exception that the pH of the buffers varied in each case, as indicated. The experimental points marked •• and ○○ were obtained using 0.025 M. sodium succinate and TRIS respectively. Initial rates were measured; 3 min. incubation at 37°.
Although the speed of the reaction was shown to vary considerably in different buffers (Kuby et al., 1954b) it would seem from the shape of the curve (Fig. 3) that the difference was not very pronounced in the case of TRIS and succinate buffers.

The activating effect of cations on the forward reaction. The effect of cations (mainly Ca\(^{++}\) and Mg\(^{++}\)) on CPK has been divided into their effect on the reaction rate and on the equilibrium.

The effect of Ca\(^{++}\) and Mg\(^{++}\) on the forward reaction rate was investigated in the presence of all the components of the complete system as described in methods, but the cation concentration was varied. The reaction was carried out at two enzyme levels with each cation as an activator.

The results (Fig. 4) were found to be identical for both Ca\(^{++}\) and Mg\(^{++}\) within the limits of experimental error, so that Fig. 4, although specifically referring to Ca\(^{++}\), equally well represents the results obtained with Mg\(^{++}\). It will be seen from the graph that the enzyme is inactive in the absence of the cation and that the activity increases sharply with increasing concentration of the cation until a maximum is reached, i.e. at a concentration of about 3 \(\times 10^{-3}\) M.

A slight inhibitory effect became apparent when the concentration of the cation reached \(10^{-2}\) M., and at a concentration of \(5 \times 10^{-2}\) M. the inhibition was 66 and 70\% in the case of Ca\(^{++}\)
Figure 4.

The effect of Ca^{++} concentration on CPK activity.

The experimental mixture contained all the components of the complete system for the forward reaction with the exception that Ca^{++} concentration was varied as indicated and two concentrations of enzyme were used: 3.5 μg. protein/ml. (○-○) and 7.0 μg. protein/ml. (●-●). 3 min. incubation at 37°.
and Mg\(^{++}\) respectively.

In order to elucidate the role of cations in the action of CPK a more detailed study was made of the effect of the concentrations of Ca\(^{++}\), Mg\(^{++}\) and Mn\(^{++}\) on the enzyme activity at different enzyme levels. The observed relationships between Ca\(^{++}\), Mg\(^{++}\) and Mn\(^{++}\) concentrations and the reaction velocity, shown in Figs. 5, 6 and 7, conform to a simple Michaelis-Menten relation, that is, to the equation:

\[
v = \frac{V_x}{K_x + x}
\]

where \(v\) = initial velocity at standard concentration of substrate,

\(V\) = maximum velocity,

\(x\) = concentration of cation,

\(K_x\) = dissociation constant of the enzyme-metal complex formed according to the equation:

\[
Me + E \rightleftharpoons Me - E
\]

These results are consistent with the hypothesis that each of the cations tested combines with CPK in the ratio of one cation to one active centre to form the active enzyme-cation complex.

Table 1 lists the dissociation constants which were
Figure 5.

The effect of Ca$$^{++}$$ concentration on CPK activity.

Experimental conditions employed were identical with those described in Fig. 4. The concentrations of enzyme used were: 5 μg. protein/ml. (○○) and 10 μg. protein/ml. (●●). $[\text{Ca}^{++}]$ refers to the molar concentration of Ca$$^{++}$.
Figure 6.

The effect of Mg\(^{++}\) concentration on CPK activity.

Experimental conditions employed were identical with those described in Fig. 4 with the exception that Mg\(^{++}\) was substituted for Ca\(^{++}\). The enzyme concentrations used were 5 μg. (○○○) and 10 μg. (●●●) protein/ml. [Mg\(^{++}\)] refers to the molar concentration of Mg\(^{++}\).
The effect of Mn$^{++}$ concentration on CPK activity.

The experimental conditions were identical with those described in Fig. 4 with the exception that Mn$^{++}$ was substituted for Ca$^{++}$. The enzyme concentrations employed were 7.5 µg. (○○○) and 10 µg. (●●●) protein/ml. [Mn$^{++}$] refers to the molar concentration of Mn$^{++}$. 

Figure 7.
Table 1.

CPK-metal dissociation constants and maximal velocities obtained with Ca++, Mg++, and Mn++.

(Experimental conditions were identical with those described in Figs. 4 - 6. Vmax is expressed in arbitrary units.)

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>CPK concentration (μg protein/ml)</th>
<th>K found (M)</th>
<th>Vmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca++</td>
<td>5.0</td>
<td>1.3 x 10^-4</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>1.3 x 10^-4</td>
<td>22.2</td>
</tr>
<tr>
<td>Mg++</td>
<td>5.0</td>
<td>2.3 x 10^-4</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>1.3 x 10^-4</td>
<td>22.7</td>
</tr>
<tr>
<td>Mn++</td>
<td>7.5</td>
<td>6.5 x 10^-5</td>
<td>28.4</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>5.9 x 10^-5</td>
<td>44.5</td>
</tr>
</tbody>
</table>
calculated for each cation at each enzyme level, using the method of Lineweaver and Burk (1934). They are based on the assumption that the enzyme preparation was free of metal ions. This would seem reasonable as the enzyme was subjected to prolonged dialysis before use and moreover no activity was observed in the absence of the cations (cf. Fig. 4). Table 1 also lists the relative Vmax values obtained. It can be seen from these results that the activation of CPK by Ca++ is almost identical with that by Mg++, i.e., similar values were obtained for the dissociation constants of the two complexes and both maximal velocity values were the same. On the other hand, Mn++ was far more effective than either Ca++ or Mg++ as an activator of CPK. The dissociation constant of the Mn-enzyme complex was significantly lower, and the relative maximal velocity was twice as great.

Effect of substrate concentration on initial velocity.

The effect of increasing concentrations of phosphocreatine on the initial velocity of the forward reaction is shown in Fig. 8. The Km value of CPK for FC was found to be $2 \times 10^{-2}$ M.

In the system employed the effect of ADP differed from that of FC in that, within the concentration range $1 \times 10^{-4}$ to $10^{-3}$ M. ADP showed a marked inhibition of the initial velocity. At first this was thought to be due to the co-ordination of the Ca++ necessary for the enzymic activity. The inhibition, however, persisted when this possibility was eliminated by increasing the Ca++
The effect of PG concentration on CPK activity.

The experimental mixture contained all the components of the complete system for the forward reaction with the exception that PG concentration was varied as indicated, and that three concentrations of CPK were employed:

- 0.75 μg. protein/ml. (○—○)
- 1.0 μg. protein/ml. (●—●)
- 1.25 μg. protein/ml. (×—×)

2 min. incubation at 37°. [PC] refers to the molar concentration of phosphocreatine.
concentration simultaneously with that of ADP so as to keep a constant Ca\textsuperscript{++}:ADP molar ratio. Fig. 9 shows the effect of ADP concentration on the initial velocity both at a constant Ca\textsuperscript{++} concentration and at a constant Ca\textsuperscript{++}:ADP molar ratio. The experiment was repeated with several different batches of ADP in order to eliminate the possibility that the results were due to the presence of an inhibitor, but in all cases the pattern was similar to that shown in Fig. 9. Finally one sample of ADP was reprecipitated as a Ba-salt, and, after the removal of the barium, the solution was chromatographed on Dowex-1 according to the procedure described by Cohn (1953). The resulting solution containing ADP was shown to be homogeneous by paper ionophoresis at pH 3.5. When the experiment was repeated with this solution the result obtained was identical with that shown in Fig. 9.

An increase of the initial velocities with increasing concentration of ADP was finally obtained at considerably lower concentrations of ADP and with a Ca\textsuperscript{++}:ADP molar ratio of 3. A plot of the inverse of the reaction velocity against the inverse of the ADP concentration failed, however, to show a Michaelis-Menten relation because apparently at the lower ADP concentrations initial velocities were no longer measured in the time interval employed for incubation and with the amount of enzyme used. It was not practicable, however, to test this explanation by using lower enzyme concentrations or shorter incubation times,
Figure 9.

Effect of increasing ADP concentration on CPK activity.
Forward reaction: 0.025 M. TRIS, pH 7.2; PC, 1.25 x 10^{-3} M. CPK, 5 μg./ml. 2 min. incubation, 37°. The ADP concentration was varied at a constant Ca^{++} concentration of 2 x 10^{-3} M. (•••) or together with the Ca^{++} concentration in such a way as to keep a constant Ca^{++}/ADP molar ratio of 2.0 (•••).
for both procedures would have resulted in the release of amounts of creatine too small to permit accurate estimation.

(b) The reverse reaction

The effect of pH on the reverse reaction. The optimum pH for the reverse reaction has already been found by other workers to lie in the vicinity of 9.0. Thus Banga (1943a) reported an optimum at pH 9.05 in borate buffer. Szöregyi and Degtyar (1943) employed glycine buffer pH 9.1, but no mention was made of the determination of the optimum. Using glycine, glycylglycine and histidine HCl Kuby et al. (1954b) found the optimum to lie between pH 8.3 and 9.0. These data were confirmed in the present work using TRIS buffer in the range pH 7.0 - 9.0 and it was found that the initial velocities were maximal at pH 9.0 which was the highest pH value tested. It is seen from the graph (Fig. 10) that the initial velocities were unlikely to increase with further rise in pH and pH 9.0 was therefore chosen as the standard pH for the reverse reaction.

The requirement for enzyme in the reverse reaction. It was found that in the reverse reaction a far higher concentration of enzyme was necessary in order to achieve initial rates comparable with those of the forward reaction. The standard mixture
Figure 10.

Effect of pH on CPK activity (reverse reaction).

The experimental mixture contained all the components of the complete system with the exception that the pH of the buffer varied in each case as indicated. 5 min. incubation at 37°.
Effect of added cysteine on the reverse reaction. It was found that the addition of cysteine had no appreciable effect on the reverse reaction when added in concentrations ranging from $1 \times 10^{-5}$ to $2 \times 10^{-3}$ M. Cysteine was therefore omitted from the reaction mixture.

The activating effect of cations on the reverse reaction.

The effect of increasing cation concentration on the reverse reaction rate is shown in Fig. 11. It will be seen that the reaction does not proceed in the absence of cations and that the effects of Ca$^{++}$ and Mg$^{++}$ are similar. The effect of the cations was tested only up to a concentration of $1 \times 10^{-4}$ M., since it was shown (see below) that under the experimental conditions employed higher concentrations of the cations exert a considerable effect on the equilibrium of the reaction with the result that at the higher concentrations initial velocities would no longer be measured.

In order to determine the dissociation constant of the enzyme-metal complex under the experimental conditions employed for the reverse reaction the inverse of the initial velocities was plotted against the inverse of the cation concentrations according to the method of Lineweaver and Burk (1934) and the result is shown in Fig. 12. It will be seen from the plot that initial rates were measured at the concentrations employed since
Figure 11.

The effect of Ca$^{++}$ and Mg$^{++}$ on CPK activity.

The experimental mixture contained all the components of the complete system for the reverse reaction with the exception that Ca$^{++}$ and Mg$^{++}$ concentrations were varied as indicated. 5 min. incubation at $30^\circ$. (Ca$^{++}$: $\bullet$; Mg$^{++}$: $\circ$.)
Figure 12.

The effect of Ca$^{++}$ and Mg$^{++}$ concentrations on CPK activity.

For details of experimental procedure, see Fig. 11. Points $\circ$ and $\bullet$ represent results obtained with Ca$^{++}$ and Mg$^{++}$ respectively. $[\text{Me}^{++}]$ refers to the molar concentrations of the metal ions.
a straight line was obtained. The values of the dissociation constants for both Ca\textsuperscript{++} and Mg\textsuperscript{++} calculated on this basis were found to be identical - 0.9 x 10\textsuperscript{-4} M. This value is not greatly different from that obtained under the conditions of the forward reaction.

**Effect of substrate concentration on initial velocity.**

The effect of increasing the concentrations of creatine and ATP in the reverse reaction is shown in Figs. 13 and 14 respectively. A Michaelis-Menten relationship was obtained. The $K_m$ value for creatine was found to be 7 x 10\textsuperscript{-4} M.

In the case of ATP the Lineweaver and Burk (1934) plot did not give a straight line when the concentration of ATP was varied and the concentration of Ca\textsuperscript{++} held constant. This was presumably due to the co-ordination effect of ATP. When the initial velocities obtained were plotted directly against the ATP concentrations (Fig. 15) a curve was obtained with a maximum at an ATP:Ca\textsuperscript{++} molar ratio of about 2.5. Using ATP and Ca\textsuperscript{++} at this ratio in subsequent reactions, it was possible to obtain a straight line plot (Fig. 14) and the $K_m$ value for ATP was calculated to be 2.3 x 10\textsuperscript{-4} M.
The effect of creatine on CPK activity.

The experimental mixture contained all the components of the complete system for the reverse reaction with the exception that the concentration of creatine was varied as shown. 2 min. incubation, 30°. \([\text{Creatine}]\) refers to the molar concentration of creatine.
Figure 14.

The effect of ATP concentration on CPK activity.

The complete system for the reverse reaction was employed with the following exception: the concentration of ATP was varied as indicated and the concentration of Ca$$^{++}$$ adjusted in each case to give a molar ratio Ca$$^{++}$$/ATP of 0.4. Two enzyme concentrations were employed: 125 μg. protein/ml. (○○) and 75 μg. protein/ml. (△△). Incubation: 2 min. at 30°. [ATP] refers to the molar concentration of ATP.
The effect of ATP concentration on CPK activity.

Experimental conditions were identical with those described in Fig. 14 with the exception that the concentration of Ca**+** was kept constant throughout at $1 \times 10^{-4}$ M. Enzyme concentration: 125 μg. protein/ml. Incubation: 5 min. at 30°.
(c) **The effect of various substances on creatine phosphokinase activity.**

The experiments described in this section were carried out using mainly the forward reaction because of the greater convenience in determination of initial velocities as compared with the reverse reaction. The accuracy of the activity measurements is also higher in the forward reaction since activity is measured directly as the amount of creatine released and not by difference.

The effect of anions capable of chelation on the forward reaction. The effect of these ions is shown in Table 2. The extent to which these ions inhibit the CK activity probably depends on the efficiency with which they can compete with the enzymic system for the cation under the given conditions. Thus the most powerful chelating agent of those tested, Versene, produces an inhibition of 80% at a concentration of $1 \times 10^{-4} \text{ M}$, the concentration of Ca$^{++}$ in the mixture being $2 \times 10^{-3} \text{ M}$. The effect of phosphate, citrate and borate is of interest since they are frequently employed as buffers in biological systems.

The effect of the alkaline earth metals. The effect of Ba$^{++}$ and Sr$^{++}$ on the forward reaction was tested in the presence and absence of Ca$^{++}$ in optimal amounts. The results (Table 3) show that while Ba$^{++}$ produced a slight inhibition in the presence of Ca$^{++}$, it was capable of producing a slight activation of
Table 2.

The effect of certain cations on CFK activity.

(Experimental mixture contained all the components of the complete system for the forward reaction. The various anions were added as sodium salts in the concentrations indicated. 2 min. incubation at 37°.)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (M)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF</td>
<td>$1 \times 10^{-3}$</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>$6.5 \times 10^{-2}$</td>
<td>100</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>$1.3 \times 10^{-3}$</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>$1.3 \times 10^{-2}$</td>
<td>87</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>$1.3 \times 10^{-3}$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$1.3 \times 10^{-2}$</td>
<td>95</td>
</tr>
<tr>
<td>Na$_2$B$_4$O$_7$</td>
<td>$5.0 \times 10^{-3}$</td>
<td>12</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>$1.3 \times 10^{-3}$</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>$1.3 \times 10^{-2}$</td>
<td>59</td>
</tr>
<tr>
<td>Sodium oxalate</td>
<td>$1.3 \times 10^{-3}$</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>$1.3 \times 10^{-2}$</td>
<td>70</td>
</tr>
<tr>
<td>Versene</td>
<td>$1.0 \times 10^{-5}$</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>$5.0 \times 10^{-5}$</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>$1.0 \times 10^{-4}$</td>
<td>80</td>
</tr>
</tbody>
</table>
Table 3.

The effect of Ca++ and Sr++ on the activity of CPK.

(The experimental mixture contained all the components of the complete system for the forward reaction except where Ca++ was omitted as shown below. BaCl₂ and SrCl₂ added as indicated. Incubation, 3 min., 37°.)

<table>
<thead>
<tr>
<th>Concentration BaCl₂ (M)</th>
<th>Concentration BaCl₂ (M)</th>
<th>Concentration SrCl₂ (M)</th>
<th>Umoles creatine released</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 x 10⁻³</td>
<td></td>
<td></td>
<td>0.65</td>
</tr>
<tr>
<td>2 x 10⁻³</td>
<td>2 x 10⁻³</td>
<td></td>
<td>0.53</td>
</tr>
<tr>
<td>2 x 10⁻³</td>
<td></td>
<td>2 x 10⁻³</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>2 x 10⁻³</td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 x 10⁻³</td>
<td>0</td>
</tr>
</tbody>
</table>
the enzyme when added alone. Strontium, on the other hand, was not only unable to activate the enzyme, but was completely inhibitory to the system in the presence of Ca++. The effect of metabolic inhibitors. The effect of some of the commonly used metabolic inhibitors is shown in Table 4. The compounds with the most pronounced inhibitory effect are those generally used for their reactivity with -SH groups either by virtue of their ability to oxidise them or to form a complex. An exception is dinitro-2-cresol. These observations indicate that a free -SH group is essential for enzymic activity. The nature of this group was therefore investigated with the use of various -SH inhibitors.

CMBA. The reversal of CMBA inhibition by cysteine was studied on the complete system and at two inhibitory levels of CMBA. The results (Table 5) indicate that the inhibition by CMBA can be partially reversed by a two-fold molar excess of cysteine, the reversal being more pronounced at the lower inhibitory level.

Levisite. The effect of levisite was tested on the complete system when it was found that, at concentrations up to 2.5 x 10⁻⁴ M, it did not produce inhibition.

Diphenylchloroarsine (DA). The inhibitory effect of DA and its reversal by cysteine is shown in Table 6, from which it is clear that DA at a concentration of 2.5 x 10⁻⁴ M gives rise to an inhibition of 32%. Moreover, this inhibition can be
Table 4.

The effect of certain metabolic inhibitors on the activity of 

CPK.

(For experimental conditions see Table 2.)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (M)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium azide</td>
<td>1.3 x 10^{-3}</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1.3 x 10^{-2}</td>
<td>18</td>
</tr>
<tr>
<td>Sodium 2-chloromercuribenzoate</td>
<td>1.0 x 10^{-8}</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^{-7}</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^{-6}</td>
<td>100</td>
</tr>
<tr>
<td>Sodium iodoacetate</td>
<td>1.0 x 10^{-6}</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^{-5}</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>1.0 x 10^{-4}</td>
<td>100</td>
</tr>
<tr>
<td>Dinitro-2-cresol</td>
<td>1.3 x 10^{-3}</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6.5 x 10^{-3}</td>
<td>95</td>
</tr>
<tr>
<td>N-Ethyl maleimide</td>
<td>1.0 x 10^{-6}</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>1.3 x 10^{-5}</td>
<td>100</td>
</tr>
<tr>
<td>Sodium iodosobenzoate</td>
<td>1.0 x 10^{-3}</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>1.3 x 10^{-7}</td>
<td>100</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>2.5 x 10^{-5}</td>
<td>11</td>
</tr>
</tbody>
</table>
The inhibition of CPK by CHBA and its reversal by cysteine.

(For experimental conditions see Table 2. In all cases the experimental mixture (with ADP omitted) was preincubated with the inhibitor (as shown below) for 5 min. Whenever cysteine was added at the end of this period, the mixture was preincubated for another 5 min. before the start of the reaction.)

<table>
<thead>
<tr>
<th>Concentration of p-chloromercuribenzoate (M)</th>
<th>Concentration of cysteine (M)</th>
<th>Creatine released (μmoles)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>3.66</td>
<td>0</td>
</tr>
<tr>
<td>$1 \times 10^{-7}$</td>
<td>-</td>
<td>6.35</td>
<td>27.7</td>
</tr>
<tr>
<td>$1 \times 10^{-7}$</td>
<td>$1 \times 10^{-7}$</td>
<td>7.97</td>
<td>8.0</td>
</tr>
<tr>
<td>$1 \times 10^{-7}$</td>
<td>$2 \times 10^{-7}$</td>
<td>8.55</td>
<td>4.0</td>
</tr>
<tr>
<td>$1 \times 10^{-6}$</td>
<td>-</td>
<td>1.53</td>
<td>82.4</td>
</tr>
<tr>
<td>$1 \times 10^{-6}$</td>
<td>$1 \times 10^{-6}$</td>
<td>1.45</td>
<td>83.4</td>
</tr>
<tr>
<td>$1 \times 10^{-6}$</td>
<td>$2 \times 10^{-6}$</td>
<td>1.83</td>
<td>78.3</td>
</tr>
</tbody>
</table>
Table 6.
The effect of lewisite and dichlorophenyldichloroarsine (DA) on CPK activity, and the reversal of the inhibition by cysteine.
(For experimental conditions and preincubation times see Table 5.)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Cysteine concentration</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lewisite</td>
<td>2.5 x 10^{-4}</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>DA</td>
<td>2.0 x 10^{-5}</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>DA</td>
<td>1.5 x 10^{-4}</td>
<td>-</td>
<td>66</td>
</tr>
<tr>
<td>DA</td>
<td>2.5 x 10^{-4}</td>
<td>-</td>
<td>82</td>
</tr>
<tr>
<td>DA</td>
<td>2.5 x 10^{-4}</td>
<td>1.25 x 10^{-3}</td>
<td>0</td>
</tr>
</tbody>
</table>
completely reversed by cysteine at a concentration of $1.25 \times 10^{-3}$ M.

The effect of carnosine on the activity of CPK. The tests with carnosine were carried out as a result of the claims of Severin and Meshkova (1952) and of Meshkova and Zaitseva (1953) that the addition of carnosine to a suspension of minced pigeon breast muscle caused an intensification of the phosphorylation of creatine.

In order to check the effect of carnosine on the urea enzyme system this compound was added to the complete system in both the forward and the reverse reaction. The concentrations of carnosine employed were comparable to those used by the Russian workers and ranged from 1.0 to 5.0 mg. carnosine/ml. of reaction mixture.

The result obtained in case of the forward reaction is shown in Fig. 16. It will be noted that an inhibitory effect was obtained, the inhibition being roughly linear with the concentration of carnosine.

In the case of the reverse reaction no effect was observed. In both cases special controls had to be carried out since it was found that carnosine exerted a slight inhibition on the colour formation in the estimation of creatine.
Figure 16.

The effect of carnosine on CPK activity (forward reaction).
The effect was tested on the complete system under standard conditions. Concentrations of carnosine are given per ml. of the experimental mixture. 3 min. incubation at 37°.
(d) **The specificity of creatine phosphokinase**

**Specificity for purine nucleotide.** The observations of other workers on the specificity of CPK for the nucleotide were discussed in the General Introduction. The present work indicates that the enzyme is specific for ADP.

The tests which were carried out were made possible by the fact that the enzyme preparation was completely free from myokinase activity.

Several experiments were carried out in order to demonstrate specificity for ADP. A bulk reaction mixture was made up which contained all the components of the complete system described for the forward reaction (see Methods) except that ADP was replaced by ATP. Samples were removed at intervals for creatine estimation. At zero plus 10 min. a small amount of ATP was added followed at zero plus 20 min. by the addition of myokinase. Sampling was then continued for further 25 min. The result (Fig. 17) shows that in the first ten minutes no creatine was liberated and consequently no transfer of phosphate from PC to AMP had been effected. Immediately following the addition of ATP there was some liberation of creatine which, however, did not continue, and which was therefore presumably due to a small contamination of ADP in the ATP preparation. As was to be expected the reaction ceased as soon as this had been phosphorylated, but recommenced on the addition of myokinase.
The experimental mixture at zero time contained all the components of the complete system for the forward reaction except that ADP was substituted by AMP. At 10 min. a small amount of ATP (1/10 of the amount of AMP present) was added (A) and at 20 min. (B) myokinase solution equivalent to 0.5 mg. of the freeze dried protein. The mixture was sampled for creatine determination at the points indicated. Temperature: 37°C.
It is, of course, necessary to have a small amount of ATP or ADP in order to "trigger off" the reaction in the presence of myokinase, but this quantity can be very small indeed, since the reaction is self-accelerating.

The reactions involved when ATP is used are:

\[
\begin{align*}
ATP + AMP & \quad \iff \quad 2 \text{ ADP (myokinase)} \\
2 \text{ ADP} + 2 \text{ PC} & \quad \iff \quad 2 \text{ ATP (CPK)} \\
2 \text{ ATP} + 2 \text{ AMP} & \quad \iff \quad 2 \text{ ADP (myokinase)}, \text{ etc.}
\end{align*}
\]

If ADP is used to start the reaction it will be converted to ATP by the action of CPK in the presence of PC and the cycle repeated. As each full cycle doubles the amount of ADP present in the system it must remain in excess as long as the system contains AMP and myokinase.

Using the same components in a different order of addition it is possible to produce an artificial system probably similar to that employed by Narayanaswami (1952) who used a crude, dilute, non-dialysed brain extract. Such a system could reasonably be expected to contain CPK, myokinase and a small amount of either ATP and/or ADP. It was with such a system that Narayanaswami claimed transfer of phosphate from PC to AMP when these two compounds were added. In order to reproduce similar conditions an experimental mixture was made up containing all the components of the complete system for the forward reaction in the proportions described, except that ADP was omitted and myokinase as well as a
trace of ATP was added. Samples were removed for creatine analysis for 10 min. at the end of which period sufficient AMP was added to bring its concentration to $1.6 \times 10^{-4}$ M. Sampling was continued for further 40 min. The result is shown in Fig. 18 and it will be seen that in the first 10 min. no transfer of phosphate occurred as indicated by the absence of free creatine. The addition of ATP led to the liberation of a very small amount of creatine in the first minute. This was almost certainly due to the presence of a trace (confirmed by ionophoresis) of ADP in the sample of ATP. A short lag-period followed during which the reaction rate was quite slow but it then increased rapidly as the reaction entered the self-accelerating phase shown by the concave-upwards part of the curve. The final part of the curve represents the approach to the equilibrium of the CPK reaction.

The initial lag-period can be explained as being due to the very small amount of ATP present and until such time as sufficient ADP was formed to saturate the active centres of the enzyme, the concentration of ADP was the limiting factor for activity.

The ability of inosine diphosphate to substitute for ADP in the reaction was tested and it was found that this compound could not accept phosphate from PC in the presence of CPK. In incubations up to 30 min. it was found that no significant transfer occurred. When present together with the adenine derivative IDP did not influence the normal reaction. This result indicated that
Substrate specificity in the CPK-catalysed reaction.

The experimental mixture contained all the components of the complete system for the forward reaction except that ADP was omitted and myokinase (2.5 \( \mu \text{g. protein/ml.} \)) was present in the mixture. A trace of ATP (conc. \( 1.6 \times 10^{-6} \text{ M.} \)) was also present. AMP was added at point marked (A) to a concentration of \( 1.6 \times 10^{-4} \text{ M.} \). Temperature: \( 37^\circ \).
the enzyme preparation was free from nucleotide diposphokinase described by Berg and Joklik (1954).

Specificity for creatine. A number of guanidine-compounds were tested for their ability to act as a substrate in the CPK catalysed reaction. Several were phosphorylated (see Appendix III) and the phosphorylated derivatives tested in the forward reaction for their ability to substitute for phospho-creatin. With the exception of one case (nacmine) none were able to act as substrates in the reaction. The compounds tested with negative results were: glycoxyamine (guanidinoacetic acid), β-guanidinopropionic acid and taurocyanine (guanidinotaurine); the corresponding three phosphorylated compounds were also tested. Neither of the substances mentioned above reacted in the system catalysed by CPK, nor did they exert any inhibition on the reaction in the presence of the normal substrate (creatinine or FC) when tested in concentrations up to six times the concentration of the latter.

All the three compounds tested produce a colour similar to that of creatine with the alkaline α-naphthol - diacetyl reagent, the molecular extinction being slightly different with each compound but generally was about one fifth that of creatine. In all experiments involving those guanidine compounds special controls were therefore employed in order to blank out the colour due to their presence.
N-ethylguanidinoacetic acid (nagmine) was found capable of accepting phosphate from ATP in the CPK reaction. The test with this substance was carried out in the complete system for the reverse reaction but creatine was substituted by nagmine. Since preliminary tests showed that the reaction rate was much slower in the presence of nagmine as compared with creatine a longer incubation time was employed and the reaction progress followed for a period of 18 hours. A parallel experiment was carried out with creatine under the same conditions. The progress of the reaction was followed by the disappearance of creatine and nagmine respectively, using the same colour reaction for both compounds. (It was previously established that the molecular extinction of nagmine in the colour reaction was practically identical with that of creatine.)

The result (Fig. 19) showed that nagmine was phosphorylated by the ATP-CPK system, but at a much slower rate than creatine. On the other hand, the equilibrium reached in each case was the same for both compounds, since after an 18 hour period both compounds were phosphorylated to the same extent.

In order to ensure that the disappearance of nagmine and creatine from the solution was actually due to their phosphorylation, samples were withdrawn from the solutions after the 18 hour incubation period and hydrolysed for 9 min. in 0.1 N HCl at 65°. Free creatine and nagmine were estimated in the respective solutions.
Figure 19.

CPK-catalysed phosphorylation of creatine and negmine.

The experimental mixture contained all the components of the complete system for the reverse reaction in the case of creatine (●●). In the case of negmine (□□) the latter was substituted for creatine in the same concentration. Incubation at 36°C.
prior to, and after, hydrolysis. The analytical figures (Table 7) showed that as a result of the enzymic reaction negmine was converted into a compound from which it could be liberated under the same conditions which cause the hydrolysis of phospho-creatine. Moreover, the same conditions have been later found to cause the hydrolysis of synthetic phosphonegmine to negmine and phosphate. It is therefore reasonable to assume that phosphonegmine was the product of the enzymic reaction.

(e) **Equilibrium studies on CPK**

The effect of various factors on the equilibrium was studied under the conditions described for the reverse reaction. This was chosen because of the ease with which the effect of cations could be demonstrated. Thus it will be shown that a shift in the equilibrium occurs when the cation concentration is increased by a certain factor compared with the optimal concentration. If the forward reaction were studied it would have been necessary to decrease the concentration of cation already present in the equilibrium mixture in order to demonstrate the same effect. While this would have been possible by the use of chelating agents, accurate quantitative data would not have been obtained.

The effect of pH on equilibrium. The effect of pH is
Table 7.

Recovery of creatine and magnesium by hydrolysis for 9 min. at 65° in 0.1 N hydrochloric acid after reaction with creatine phosphokinase – adenosine triphosphate for 18 hr.

(For experimental details refer to Fig. 19.)

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>Final (18 hr.)</th>
<th>Final (after hydrolysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatine (µmoles)</td>
<td>20.0</td>
<td>7.2</td>
<td>19.6</td>
</tr>
<tr>
<td>Magnesium (µmoles)</td>
<td>20.0</td>
<td>8.0</td>
<td>19.6</td>
</tr>
</tbody>
</table>
shown in Fig. 20. It is clear that the amount of creatine which is phosphorylated at equilibrium depends on the pH of the experimental mixture, the reverse reaction being favoured at higher pH. These results conform with the findings of other workers which have been referred to above.

The effect of Ca$^{++}$ and Mg$^{++}$ on the equilibrium at pH 9.
The amount of creatine phosphorylated at pH 9 in 30 min. in the presence of varying concentrations of the two cations is shown in Fig. 21. It will be seen that the curve exhibits a peak at a concentration of the cations of $1 \times 10^{-4}$ M. The effect of the two ions is very similar. The first part of the curve indicates the very low activity of the enzyme in the presence of low concentrations of the cations and it is quite reasonable to assume that the reaction rates under the conditions were too low for the equilibrium to be reached in the time interval employed.

The second part of the curve, however, represents the distinct effect of the cations on the equilibrium. The cation concentration in that case was equal to, or greater than, that required to produce the maximal initial rates under the given conditions. Under these conditions the equilibrium is reached in about 15 min. Thus the result was presumably due to a shift in equilibrium.

In order to demonstrate this point the following experiment was carried out:
Figure 20.

Effect of pH on the equilibrium of CPK.

The complete system for the reverse reaction was employed with the exception that the pH of the buffer (TRIS) varied as indicated.

30 min. incubation at 30°.
The effect of Ca\textsuperscript{++} and Mg\textsuperscript{++} concentration on the equilibrium of creatine phosphokinase.

The experimental mixture contained all the components of the complete system for the reverse reaction with the exception that the concentration of either Ca\textsuperscript{++} (○○○○) or Mg\textsuperscript{++} (●●●●) varied as indicated. 30 min. incubation at 30\textdegree C.
A bulk experimental mixture was prepared containing the components of the complete system for the reverse reaction. The reaction was allowed to proceed for 30 min. in order to reach equilibrium, samples being withdrawn for creatine estimation at various time intervals. At that stage the mixture was divided and, while one part was left unchanged, the other received an addition of CaCl₂ sufficient to increase the concentration of the cation to $2 \times 10^{-3}$ M. Sampling was then continued in both portions at intervals for 30 min.

The results of this experiment (Fig. 22) show that equilibrium was reached after the first 20 min. when 55% of the total creatine present was phosphorylated and that in the experimental mixture to which no additional Ca⁺⁺ was added there was no further change. In the remaining portion of the mixture following the addition of Ca⁺⁺ at 31 min. there occurred a sharp fall in the phosphocreatine level which at 45 min. was such that only 23% of the creatine present was now phosphorylated. The effect of the increased Ca⁺⁺ concentration is therefore, as Szonenyi and Uertyar (1943) claimed, to produce a shift in the equilibrium of the reaction.

The effect of Ba⁺⁺ and Sr⁺⁺ on the equilibrium.

Since the effect of Ca⁺⁺ and Mg⁺⁺ on the equilibrium was thought to be due to the formation of co-ordinate complexes with ATP and ADP it was of interest to check the effect of Ba⁺⁺ and Sr⁺⁺ which
Figure 22.

The effect of Ca$^{++}$ on the equilibrium of CPK.

The points ○ ○ represent results obtained on a mixture containing all the components of the complete system for the reverse reaction in proportions described in "methods". At (A) the concentration of Ca$^{++}$ was increased in a portion of the mixture to $2 \times 10^{-3}$ M., and the points ○ ○ represent analytical figures obtained on this portion. Temperature: 30°.
could reasonably be expected to form similar complexes to a lesser or higher degree. The experimental test system was similar to that described in the case of Ca^{++} except that the course of the reaction was not followed and only final equilibrium levels of creatine were estimated.

A number of tubes were set up, all containing the complete system including Ca^{++} at optimal concentration. One tube was incubated for 60 min. without further additions. Three tubes were incubated for 30 min. without additions and at the end of this period the tubes received an addition of Ca^{++}, Ba^{++} or Sr^{++}. The tubes were then incubated for a further period of 30 min. Three more tubes received the Ca^{++}, Ba^{++} or Sr^{++} in the same amount as the tubes above, but the addition was made from the start and incubation was carried out for 60 min. The result (Table 3) shows that under optimum conditions (tube 1) 49% of the creatine present was phosphorylated. The effect of the addition of Ca^{++} and Ba^{++} after equilibrium had been reached (tubes 2 and 3 respectively) was to shift the equilibrium to such an extent, that the amount of phosphocreatine present was decreased from 49% to 19.5% and 28% respectively. The results were similar when these ions were present at the same concentrations from the beginning of the reaction (tubes 5 and 6). It may thus be inferred that Ba^{++} exerts an effect similar to that of Ca^{++} on the equilibrium. The effect of Sr^{++}, on the other
Table 8.

Effect of the concentration of Ca++, Ba++ and Sr++ on the equilibrium of CPK.
(The complete system for the reverse reaction was employed except that the cations were present in the concentrations indicated:

"optimum concentration" = 1 \times 10^{-4} \text{ M},

"excess" represents a final concentration = 2 \times 10^{-3} \text{ M}.

60 min. incubation at 30°.)

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Treatment</th>
<th>% Creatine phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Optimum Ca++ concentration at zero time</td>
<td>49.0</td>
</tr>
<tr>
<td>2</td>
<td>Optimum Ca++ concentration at zero time, excess of Ca++ added at zero + 30 min.</td>
<td>19.5</td>
</tr>
<tr>
<td>3</td>
<td>Optimum Ca++ concentration at zero time, excess of Ba++ added at zero + 30 min.</td>
<td>28.0</td>
</tr>
<tr>
<td>4</td>
<td>Optimum Ca++ concentration at zero time, excess of Sr++ added at zero + 30 min.</td>
<td>42.0</td>
</tr>
<tr>
<td>5</td>
<td>Excess of Ca++ added at zero time</td>
<td>16.5</td>
</tr>
<tr>
<td>6</td>
<td>Excess of Ba++ added at zero time</td>
<td>27.5</td>
</tr>
<tr>
<td>7</td>
<td>Excess of Sr++ added at zero time</td>
<td>4.7</td>
</tr>
</tbody>
</table>
hand, was at the best very slight but was very pronounced on the actual reaction rate which is almost entirely inhibited in both directions (tubes 4 and 7). These results are in agreement with those obtained on the effect of \(Sr^{++}\) and \(Ba^{++}\) on the forward reaction:

**The effect of myokinase on the equilibrium.** The addition of myokinase to the system at equilibrium can be expected to have the effect of increasing the concentration of PC due to the partial removal of ADP and its conversion to ATP.

The results of an experiment to test this expectation (Fig. 23) show that at equilibrium, about 50% of the creatine present was phosphorylated and that this value was increased to about 70% when myokinase was added. An attempt to obtain still higher yields by the use of adenylic acid deaminase (Kalokar, 1947) was not successful due to the fact that this enzyme has a very sharp pH optimum at pH 5.9. This result will be discussed below in connexion with certain practical applications of the reaction and some earlier results of Lehmann (1935).

**Validity of analytical method.** Throughout the experiments described above the rate at which CPK could transfer phosphate from PC to ADP or from ATP to creatine was determined on the basis of analytical figures for the amount of creatine present at the end of the experimental period. Clearly the concentration(s) of any one or all of the reaction components could
Figure 23.

The effect of myokinase on the equilibrium of CPK.

All the components of the complete system for the reverse reaction were present at zero time and myokinase (500 μg. protein) was added at equilibrium at the point marked with the arrow. Temperature: 30°.
have been determined but creatine was chosen because of the ease with which it could be accurately estimated. Thus in order to estimate either ATP or ADP in the presence of each other enzymic methods must be employed since there is no chemical method to distinguish between the two compounds. The enzymic estimation of ATP and ADP in the reaction mixture with the use of myokinase and myokinase or aspartase (cf. Roules and Stocken, 1950) would be complicated by the presence of PC and CPK in the system and by the inevitable liberation of phosphate from PC by the combined action of the enzymes present. Apart from this, enzymic analysis is very cumbersome compared with a chemical method, particularly when the chemical method is very simple as in the case of creatine. Another advantage of the method lies in that the estimation of creatine is carried out in alkaline solution in which medium PC is perfectly stable and does not react with a-naphthol and diacetyl.

In the forward reaction phosphocreatine is present at the beginning of the reaction and it is the only possible source of creatine. Thus the appearance of free creatine can be reasonably taken as an estimate of enzymic activity. It can, however, be argued that the disappearance of creatine in the case of the reverse reaction does not constitute proof of the formation of PC. In order to prove this point some experiments were carried out in which a "balance sheet" was prepared. A number of tubes were set
up, each containing all the components of the complete system for the reverse reaction with the exception of the enzyme. After equilibration at 30° free and total (9 min. hydrolysis in 0.1 N HCl at 65°) creatine was estimated in the first tube. CPK was then added to the second tube and the mixture was allowed to reach equilibrium. A third tube was incubated for the same length of time but without the addition of the enzyme. Free and total creatine was then estimated in the tubes. The results (Table 9) show the soundness of the analytical method employed and provide the proof for the assumption, inherent in the experiments, that a fall in creatine content can be attributed to a phosphorylation reaction.

The complete recovery of the bound creatine after mild hydrolysis points towards phosphocreatine as the product of the reaction.
Table 9.

"Balance sheet" of the reverse reaction of CPK.

(All tubes contained the components of the complete system for the reverse reaction with the exception of the enzyme which was omitted in some cases as indicated below. Total creatine is the sum of free creatine and that bound as phosphocreatine. 20 min. incubation at 30°C.)

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Treatment</th>
<th>Creatine, µg.</th>
<th>Free</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CPK omitted; not incubated.</td>
<td>136.0</td>
<td></td>
<td>136.0</td>
</tr>
<tr>
<td>2</td>
<td>CPK added; incubated.</td>
<td>104.5</td>
<td></td>
<td>185.5</td>
</tr>
<tr>
<td>3</td>
<td>CPK omitted; incubated.</td>
<td>136.2</td>
<td></td>
<td>136.2</td>
</tr>
</tbody>
</table>
DISCUSSION

There is some disagreement between the present data and work published by other authors. It was found in this work that the dissociation constants for Ca\textsuperscript{2+} and Mg\textsuperscript{2+} in the forward reaction were 1.3 and 2 \times 10^{-4} M, respectively whilst Kuby et al. (1954b) found a value of 2 \times 10^{-3} M for Mg\textsuperscript{2+}. The value for Ca\textsuperscript{2+} was not quoted by these authors. Similar differences arise in the case of the dissociation constant for Mg\textsuperscript{2+} in the reverse reaction. More marked divergence between the results is found with Mn\textsuperscript{2+} which in the present work gave a dissociation constant of 6 \times 10^{-5} M, whereas the value found by Kuby et al. (1954b) was 2 \times 10^{-3} M. The data on relative maximal velocities are also in disagreement. Unlike Kuby et al. (1954b) who found that Ca\textsuperscript{2+} would produce only one half the initial velocity which was obtained with Mg\textsuperscript{2+}, it was found in the work presented above that no significant difference existed between these metal activators.

These discrepancies are too great to be accounted for by experimental error, particularly as the Lineweaver and Burk (1934) plots, in all cases, are plotted on a significant number of points. The probable cause of these discrepancies lies in the concentration of ADP employed in the experiments. Thus in the present experiments the concentration of ADP for the forward reaction was kept at 2.5 \times 10^{-4} M, while Kuby et al. (1954b) used a
concentration of $2 \times 10^{-3}$ M. At the higher ADP concentration the amount of Ca$^{++}$ or Mg$^{++}$ added to achieve a finite concentration of freely available cation would necessarily be higher than at a lower concentration of ADP due to complex formation between ADP and the cation. Thus with an ADP concentration eight times higher than in the present work Kuby et al. (1954b) found a dissociation constant for Ca$^{++}$ which was ten times higher than quoted here. As would be expected this phenomenon is not confined to the CPK system alone. Thus a similar observation was made by Green and Mommaerts (1954) in their study of a Ca$^{++}$-activated muscle ATPase. They found that the $K_m$ value for ATP varied with the concentration of Ca$^{++}$, although in each case a rectilinear Lineweaver-Burk plot was obtained.

Another possible explanation lies in the nature of the buffering system employed in the experiments. Of the systems tested by them Kuby et al. (1954b) found that TRIS gave the lowest reaction velocity and Noda et al. (1954c) have expressed the opinion that interaction between the buffer and metal activator might affect the observed value of the dissociation constants. The large differences between the values quoted in the work described above (using TRIS buffer) and those of Kuby et al. (1954b) (using glycyl-glycine) might then be partially due to such interaction. The fact that in the present work the affinity of the enzyme for Mg$^{++}$ was several times higher than for Ca$^{++}$ or
Mg\textsuperscript{++}, while in the results of Kuby et al. (1954b) it was identical with that for Mg\textsuperscript{++}, could be explained in terms of the relative affinity of the buffers employed for either of the ions used.

Nevertheless the high initial velocities together with the apparently greater affinity of the enzyme for Mn\textsuperscript{++} suggests that this metal might possibly be the true "in vivo" activator of CPK. It should be remembered, however, that the CPK used in the present experiments was not homogeneous, so that it may not be permissible to compare the absolute initial velocities with those obtained by Kuby et al. (1954b).

In the present work it was found that the presence of a cation was obligatory and that the reaction did not proceed at all in its absence. This finding, which has been confirmed by Kuby et al. (1954b), is in disagreement with the claim of Szondy and Degtjär (1943) that the reverse reaction proceeded in the absence of Ca\textsuperscript{++} or Mg\textsuperscript{++}. It would seem that the activity found by the latter authors in the absence of added Ca\textsuperscript{++} was due to the presence of traces of metal in the system employed. These traces were capable of activating the reverse reaction only since the requirement for cation in this reaction is much lower than in the forward reaction.

The case of ADP inhibition of the forward reaction remains difficult to explain, particularly in view of the fact that
this was not observed by other workers. Thus Kuby et al. (1954b) found that a normal Michaelis-Menten relation held for ADP. Also Chappell and Perry (1954) found that the initial velocity of the reaction increased with increasing ADP concentration. It was in view of these results that the experiment was repeated many times under different conditions and with the Ca++ concentration adjusted in various combinations relative to the ADP concentration. All possibility of some extraneous inhibitor in the ADP was carefully excluded and the material was, in one case at least, chromatographed and checked for homogeneity by paper ionophoresis. In all cases, however, the result was similar and the ADP proved inhibitory.

An interesting feature of the reverse reaction is the relatively slow rate at which it progresses in comparison with the forward reaction. In the present work it was necessary in case of the reverse reaction to increase the enzyme concentration to twenty-five times the amount used for the forward reaction in order to obtain comparable reaction velocities. A similar result was obtained by Kuby et al. (1954b) who compiled the net maximal velocity of transphosphorylation in the two directions and found that the rate was six times higher in case of the forward reaction.

This observation suggests a possible explanation from the point of view of "in vivo" conditions:

In the living muscle the forward reaction takes place
during exercise when the muscle depletes the ATP store and it becomes necessary to draw on the energy stores in the phosphagen. This is probably a very urgent demand and the reaction must take place sufficiently rapidly in order to keep the ADP phosphorylated to an extent sufficient to ensure relaxation. The chemical process of recovery of the phosphagen which takes place at a later stage can probably occur at a slower pace.

The experiments on the effect of various substances on CPK have shown that a free -SH group(s) within the CPK molecule is necessary for enzyme activity. The fact that added cysteine is unnecessary, together with the finding that the enzyme retains maximal activity for long periods even in high dilutions, indicates a high degree of stability of the -SH groups. This property of the enzyme enables preparations to be kept for long periods either in solutions or in the dried state.

The experimental results obtained with the arsenicals, lewisite and DA, are of interest, for they give some indication of the proximity of the -SH groups on the enzyme molecule. CPK is not sensitive to the action of lewisite, and it may be thus inferred that the essential -SH groups cannot form a stable $\text{As}^\text{V}$ ring (cf. Lotespeitch and Peters, 1951). DA, on the other hand, proved to be an effective inhibitor, presumably giving a stable monothiocarsenite, and the inhibition could be reversed quantitatively by the addition of cysteine in the molar ratio
of 5 : 1. On the basis of existing theories and the work done on this subject by the Oxford school (cf. Peters, 1952), it may be presumed that this is a monothiol attack.

Amongst the inhibitors tried (Table 4) iodoacetate was shown to produce 100% inhibition at a concentration $1 \times 10^{-4}$ M. While this result is not as striking as those obtained with other inhibitors it gains interest in view of the results of Lundsgaard (1933a) (General Introduction p. 11). According to Lundsgaard, in the iodoacetate-poisoned frog muscle phosphocreatine broke down as a result of stimulation. The dose employed by Lundsgaard was 400 mg./kg. of the sodium salt which, assuming even distribution, corresponds to a mean body concentration of about $2 \times 10^{-3}$ M. This value is twenty times larger than the concentration which "in vitro" produces complete inhibition. The important factor, however, in extrapolation of "in vitro" results to the intact tissue is the knowledge of the concentration of the inhibitor at the actual site of the enzyme. It is impossible to deduce this concentration from the data available, but certain comparisons can be made.

Thus Krebs (1931) reported that in suspension of various rat tissues iodoacetate inhibited glycolysis to over 90% at a concentration of $3 \times 10^{-4}$ M. At a concentration of $3 \times 10^{-3}$ M, the inhibition was almost complete. Meyerhof and Boyland (1931) found that "in vitro" $5 \times 10^{-4}$ M, iodoacetate...
completely inhibited glycolysis in muscle. A similar figure was
given by Peters, Rydin and Thompson (1934). It would thus seem,
at least on the basis of "in vitro" studies, that the glycolytic
system and CPK are inhibited by the same order of concentration
of iodoacetate. While it is difficult to extrapolate these data
to "in vivo" conditions, it is not unreasonable to assume, that
a concentration of iodoacetate sufficient to block glycolysis
"in vivo" would also inhibit CPK activity.

Yet in Lundsgaard's experiments CPK was decomposed, so
that if the above assumption is correct, this decomposition
could not have been brought about by CPK. It may be possible to
explain the phenomenon on the basis of an alternative pathway of
phosphocreatine dephosphorylation without the participation of
the CPK system, but so far no direct proof for such a pathway
exists.

It is possible that the resistance of CPK "in vivo" to
iodoacetate poisoning may be due to the peculiarity of the dis-
tribution of this enzyme within the muscle cell. Thus Szorosnyi
and Degtyar (1943) found that apart from the CPK present in the
soluble fraction in muscle, there is a CPK bound very strongly
to the actomyosin complex in muscle. This CPK could only be ex-
tracted from the acetone powders. This fraction constitutes
about 4% of the total actomyosin in muscle. They have also re-
ported that CPK was found in a protein complex with the actin
These findings have since been confirmed by Wollemann and Feuer (1952) who found CPK activity in actin. This fraction could not be separated from the actin by multiple washing and constituted about 10-25% of the total CPK activity of muscle.

It was shown since by Chappell and Perry (1953) that the glycolytic reactions in muscle are associated with the sarcomeres, the bulk of the CPK being found in solution in the sarcoplasm. It is possible then that in the iodoacetate poisoned muscle the soluble and particulate systems (including the sarcoplasmic CPK and the glycolytic system) are more accessible to the action of the inhibitor and that the small fraction of the actin- or actomyosin-bound CPK is protected by some mechanism of the myofibril which prevents the access of the inhibitor to the site. Whichever explanation may be true, the problem requires further clarification.

The case of carnosine is of interest. Carnosine and its methyl derivative, anserine, are found in the muscles of most vertebrates but their function is not known. An investigation into the role of carnosine and anserine in the intermediate metabolism of muscle was carried out by the Russian team of S.E. Severin. A number of interesting results were recorded. It was found (Severin and Bashkova, 1950) that the addition of carnosine or anserine to muscle suspensions caused a marked
increase in the esterification of phosphate, particularly under aerobic conditions. The addition of either compound was accompanied by an increase in oxygen consumption. It was found later (Severin and Neshkova, 1952; Neshkova and Laitseva, 1953) that if creatine was added to the same system it was phosphorylated and that the amount of PC formed depended on the concentration of carnosine or anserine. The results obtained in the present work with the pure system exclude the possibility of any influence of carnosine at the level of phosphate transfer from ATP to creatine. The more likely explanation for the results obtained by the Russian team is that the effect of carnosine is, by some unknown mechanism, to intensify oxidation and oxidative phosphorylation. This results in the production of an excessive amount of high energy phosphate mainly in the form of ATP.

Creatine can under these conditions be regarded as a convenient "trap" and the addition of an excess of creatine provides an avenue into which the \( \text{P} \) can be channelled. The inhibition of the forward CK reaction by carnosine might tend to prevent the decomposition of the PC formed, but it is doubtful if this applies "in vivo". The exact mode of action of the two peptides will have to be known before any conclusions can be drawn about their connexion with creatine metabolism.

The results dealing with the specificity of the enzyme for substrate leave little doubt as to the nature of the phosphate
acceptor in the CPK system employed, i.e. the water-soluble enzyme from muscle. In the work previously published by various workers (see General Introduction) there were indications that the phosphate could be transferred from PC to ATP as well as to ADP. In one case (Narayanaswami, 1952) it was claimed that the speed of the reaction was greater with AMP than with ADP. This can be explained considering the reaction:

\[ \text{PC} + \text{ADP} \rightarrow \text{ATP} + \text{creatinine} \]

In the presence of AMP and myokinase one of the products of this reaction (ATP) is continuously removed from the system, while one of the reactants (ADP) is continuously replaced in double amounts. This must therefore result in a net increase of reaction rate. The model system described above was designed to reproduce the conditions as probably obtained in Narayanaswami's (1952) experiments and provides proof of the above explanation (see figs. 17 and 18).

The claim of Banga (1943a) that two forms of CPK existed in muscle, one capable of phosphorylating ADP and the other transferring P to AMP must also be attributed to contamination with myokinase, particularly since this second enzyme, "ADP-creatinine phosphorase", was never separated by Banga from the myokinase fraction. The latter view is supported by Colowick (1951).

It seems that apart from the requirement for at least two phosphate groups on the adenosine molecule the enzyme also
requires the free amino-group, since it does not react with inosine derivatives; a similar observation was made by Berg and Joklik (1954) and it seems that the claims of Kleinzeller (1942) that phosphate can be transferred from PC to IDP, were based on results obtained with an enzyme preparation contaminated with nucleoside diphosphokinase.

Specificity studies on the guanidine component of the reaction do not include a large range of compounds but from results available it seems that the enzyme requires a second substitution on the α-N of the guanidino group, such as provided in the case of creatine and negmine:

Creatine: \[
\begin{array}{c}
\text{CH}_3 \\
N \text{- CH}_2 \text{- COOH} \\
\text{HN}=O \\
\text{NH}_2
\end{array}
\]

Negmine: \[
\begin{array}{c}
\text{C}_2\text{H}_5 \\
N \text{- CH}_2 \text{- COOH} \\
\text{HN}=O \\
\text{NH}_2
\end{array}
\]

From these results it is not possible to deduce what function the alkyl group has in the reaction, but no activity was observed with the guanidino compounds tested which lacked this substitution. In the latter group belongs glycocyanine (guanidino acetic acid) and both glycocyanine in the reverse reaction and phosphoglycocyanine in the forward reaction proved completely non-reactive. However, Fawaz and Seraidarian (1946) claimed that
dialysed extracts of rabbit muscle catalysed the transfer of phosphate from phosphoglycocyamine to ATP, although no transfer could be observed with ATP and glycocyamine. The authors concluded that the reaction, unlike that with creatine, was not reversible. The latter observation alone makes it difficult to accept the results, since if glycocyamine can participate in the CPK system it should be able to do so both in the reverse and forward reaction. It is therefore likely that the results of Fawaz and Seraidarian (1946) arose as a result of some fault in the methods employed. Indeed, the methods used for the estimation of phosphoglycocyamine and ATP lacked specificity since hydrolysis in H acid for various periods was employed for the estimation of the two compounds with the use of a number of corrections. These facts, as well as the crudity of the enzymic preparation employed render their results open to criticism.

The results with negmine are of interest. In the present work it was found that, apart from creatine, negmine alone of the guanidino compounds tested was phosphorylated by the CPK system. This observation has a certain bearing on some previously published work relating to the origin of creatinine in the body.

Thus Lipmann (1941) suggested that the "...constantly occurring decay of creatine to creatinine might be related to the linking of creatine to phosphate in the body."
Later Borsook and Dubnoff (1947) expressed the view that urinary creatinine originated from phosphocreatine through a spontaneous breakdown of the latter. This theory was supported by the authors on the basis of the study of the distribution of creatine, phosphocreatine and creatinine in muscle and urine respectively and of the equilibria between the compounds under the physical conditions within the animal body. Secondly, Borsook and Dubnoff (1947) based their theory on the fact that, although an extensive search had been made by many workers for an enzymic system capable of converting either creatine or phosphocreatine into creatinine, no such system had been found (cf. however Carutto, 1954).

Recently Armstrong (1953) made an interesting observation that negmidine injected into rats was excreted in the urine in the form of the amidine, N-ethylglycocoyamidine (negmidine), which is the analogue of creatinine:

Creatinine:  
\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{CH}_2 \\
\text{HN}=\text{C} & \quad \text{C}=\text{O} \\
\text{NH} & \\
\end{align*}
\]

Negmidine:  
\[
\begin{align*}
\text{H}_3\text{C} & \quad \text{CH}_2 \\
\text{HN}=\text{C} & \quad \text{C}=\text{O} \\
\text{NH} & \\
\end{align*}
\]

If the assumption be made that similar mechanisms control the formation of negmidine and creatinine and that the mechanism suggested by Borsook and Dubnoff (1947) is correct,
then neamine would have to be phosphorylated in the body before neamidine could be formed. In the present work the CPK system was found capable of phosphorylating neamine and it was inferred that neamidine arose from phosphoneneamine. Had neamine been found non-reactive in the CPK system, it would be necessary to assume an alternative pathway for its phosphorylation or to revise the theory of the origin of urinary creatinine. So far the "in vitro" phosphorylation of neamine by the CPK system has provided the only experimental evidence, however circumstantial, that the phosphorylated compound may be the precursor of the amidine.

The theory of Borsok and Dubnoff (1947) may, however, require some revision in the light of the results of Caputio (1954) which have been mentioned above (General Introduction, p. 31). It is possible that the reaction observed by Caputto (transfer of phosphate from PG to glucose-1-phosphate with the formation of creatinine) could be responsible for the formation of urinary creatinine. Proof of this point could perhaps be obtained by testing phosphoneneamine in the system of Caputto (1954) of which, however, no details are yet available.

The observations concerning the effect of changes in cation concentration and pH on the equilibrium of the CPK system suggest that the enzyme is very sensitive to those changes and that the equilibrium depends both on the concentration of
the cation and on pH of the mixture. The most likely mechanism for this effect is the influence of the two factors on the concentration of the free substrate, whether this be ATP (ADP) or the corresponding Ca\(^{++}\) or Mg\(^{++}\) complexes. Since the degree of dissociation of these complexes varies with pH of the medium and with the concentration of free cation (cf. Noda et al., 1954c) it is clear that the two latter factors will affect the composition of the equilibrium. The effect of cations is probably more pronounced than that of pH since at pH 9 and higher the reaction can be reversed by increasing the Ca\(^{++}\) concentration.

The response of the equilibrium to changes in pH is not confined to the CPK system alone and is probably typical of other systems containing phosphate esters and activated by divalent metal ions. Thus a pH-equilibrium curve for potato and animal phosphocreatine activated by Mg\(^{++}\) which is given by Hanes and Haskell (1942) is essentially identical in shape with that in Fig. 20 above.

With reference to the results obtained with myokinase it should be noted that the yield of PC can be increased from about 50% to about 70% by the addition of this enzyme due to the fact that it enables the utilisation of the \(\beta\)-P of ATP in the FC synthesis. The preparation of phosphocreatine from ATP using the CPK system is not generally practicable and could not
be preferred to synthetic methods (cf. Ennor and Stocken, 1945). However occasion may arise when the preparation of F3 from ATP becomes desirable and indeed necessary (see Appendix I, p. 116 below) in which case the higher yield consequent on the addition of myokinase is of advantage. It is for this reason that attempts were made to repeat the work of Lehmann (1935) who claimed that increased yields of F3 from ATP could be obtained if the system (already containing CPK and myokinase) was supplemented with Schmidt's deaminase. The adenylic acid deaminase prepared according to Kalckar (1947) and used in the present work was essentially Schmidt's deaminase and it is doubtful whether a slight modification in the preparative procedure could have any effect on the pH optimum of an enzyme. Yet the addition of deaminase to the reverse reaction system had no effect whatsoever. Separate tests showed that the enzyme was completely inactive above pH 7. Figs. 10 and 26 (above) show that no phosphorylation of creatine can take place below pH 7. It is therefore highly doubtful whether the integration of CPK and adenylic deaminase is possible at all.
SUMMARY

(1) The enzyme creatine phosphokinase was prepared from rabbit skeletal muscle. The purified preparation contained two protein components, the larger of which (75%) was active.

(2) The properties of the enzyme were described and the optimal conditions for its activity in both the forward and reverse reaction were studied.

(3) The effect of various substances, including some metabolic inhibitors, on the activity of CPK was investigated. Some information as to the nature of the active-SH groups on the enzyme was gained.

(4) The specificity of the enzyme for the nucleotide and the guanidino compound was studied and it was found that the enzyme was specific for ADP. With the exception of L-ethylglycocyamine, guanidino compounds other than creatine could not act as a substrate for CPK.

(5) The results obtained with L-ethylglycocyamine were taken as providing indirect evidence for the formation of urinary creatinine from phosphocreatine.

(6) The effect of a number of factors on the CPK-catalysed equilibrium was investigated.

(7) The findings were discussed in the light of results
obtained by other authors and with reference to the functions of
the enzyme "in vivo".
CHAPTER II

THE DISTRIBUTION OF PHOSPHOCREATINE AND OF CREATINE
PHOSPHOGLICASE IN ANIMAL TISSUES
CHAPTER II

THE DISTRIBUTION OF PHOSPHOCREATINE
AND CPK IN ANIMAL TISSUES

INTRODUCTION

It was generally believed, until recently, that phosphocreatine was confined to muscular and nervous tissue (Lippmann, 1941). In their first communication on phosphagen, Eggleton and Eggleton (1927a) reported the presence of phosphagen in skeletal muscle and, to a lesser extent, in cardiac muscle. Smooth muscle (rabbit's stomach and uterus) was found to contain only traces of the compound, while its content in testis was doubtful.

Gerard and Wallen (1929) found 'labile phosphate' in fresh nerve and assumed it to be phosphocreatine on the basis of its lability in acid molybdate (cf. also Gerard and Tupikova, 1939).

It was not until recently that phosphocreatine was identified in liver by Barker and Ennor (1951).

This was followed by a report of part of the work described in this chapter (Ennor and Rosenberg, 1952b) in which
phosphocreatine was shown to be present in a number of tissues including spleen, kidney and testis.

In the same year Alexeeva and Kok (1952) confirmed the results of Emnor and Rosenberg (1952b). The method employed by Alexeeva and Kok (1952) involved the estimation of creatinine in the tissue extract after hydrolysis in the presence of dilute acid and molybdate, under which conditions phosphocreatine is converted almost quantitatively into creatinine and phosphate (Barker, Emnor and Harcourt, 1950).

Little information is available concerning the presence of creatine phosphokinase activity in tissues other than muscle on which all the original work with CPK has been carried out. The only other tissues where its presence has been demonstrated are brain (Narayanaswami, 1952) and kidney (Lohmann, 1934a).

The studies on the distribution of both FC and CPK in various tissues were undertaken as part of a study (as yet incomplete) designed to determine the possible existence of function for FC other than that of a 'storehouse' of energy for the adenylic acid system (see p. 97).
EXPERIMENTAL

(a) Materials

All materials used were as described above (see Materials, Chapter I).

(b) Methods

Removal of tissues. In order to simplify the rapid removal of tissues and minimise the losses of phosphocreatine all tissues were removed under nembutal anaesthesia. The kidney, spleen and testis were exposed, with minimal handling, and loose ligatures placed around the vascular supply of each of these organs. The ligature was then tightened, the organ simultaneously excised and immediately dropped into liquid N$_2$. In the case of muscle (gastrocnemius), ligatures were applied at both ends before excision. The abdominal incision was then temporarily closed. An incision was then made in the neck and the trachea exposed, a canula was inserted and artificial respiration commenced. The cerebrum was then exposed, a sample of brain obtained and immediately dropped into liquid N$_2$. In some cases the brain was frozen "in situ" and samples obtained as described by Kerr (1935).
The abdomen was then reopened, the incision extended into the thoracic cavity and the heart exposed. A lobe of liver and the whole heart were then removed and dropped into liquid N₂.

**Preparation of tissue extracts.** With the exception of muscle all tissues were ground in mortars previously cooled to the temperature of liquid N₂. The powdered material was weighed into a Potter-Elvehjem homogeniser tube (previously cooled in a mixture of ice and salt) and homogenised with ice-cold 10% (w/v) TCA. The product was then centrifuged and re-extracted with cold 5% (w/v) TCA. In the case of muscle a cutting type homogeniser (Nalco) was used. After centrifugation the extracts were combined and diluted to 50 ml. Immediately 40 ml. was withdrawn and, after adjustment of pH to 7-7.5, made to 50 ml. Estimations were carried out on such solutions after appropriate dilution. During all stages of extraction the solutions were kept at 0° and the neutralised extracts were stored at -10°.

In most cases the estimations of PC were carried out within 24 hours of extraction.

**Determination of 'free' and 'bound' creatine.** 'Free' creatine refers to creatine determined directly on a sample of the neutralised extract by the method described by Ennor and Stocken (1943b). Since PC has no free amino group it is non-reactive with the alkaline diacetyl-α-naphthol reagent employed. Moreover, it is quite stable under the alkaline conditions under
which the development of colour occurs. 'Total' creatine is
the sum of 'free' creatine and that which is released following
acid hydrolysis. The difference between the 'total' creatine
and 'free' creatine gives the 'bound' creatine which is assumed
to have its origin in phosphocreatine.

For the determination of total creatine, a sample was
hydrolysed under mild conditions (9 min. in 0.1 N HCl at 65°)
to hydrolyse the PC with minimum formation of creatinine (cf.
Barker, Turner and Harcourt, 1950). The solution was then neu-
tralised by the addition of the theoretical amount of 0.4 N
NaOH and cooled rapidly to room temperature by immersion in
ice. 1.0 ml. of 4 x 10⁻² M TBA was then added to each tube
followed by the alkaline α-naphthol reagent and diacetyl solu-
tion. The volume was adjusted to 10 ml. and the colour allowed
to develop in the dark for 20 min. (It was found that under
the influence of daylight the solutions rapidly acquired a blue
colour, the intensity of which increased with time. This reac-
tion did not occur in artificial light. The phenomenon was
finally traced to a reaction, activated by ultraviolet light,
between alkaline α-naphthol and trichloroacetate. The reaction
appears sufficiently sensitive to form the basis of a method for
the estimation of the latter.)

Some TBA extracts, particularly of liver, contained
glycerol and as a result the solutions, in which the colour due
to free creatine was estimated, were slightly opalescent. In this case blank determinations, from which the diacetyl was omitted, were employed. In the samples which were hydrolysed for the determination of total creatine sufficient breakdown of glycogen occurred to render the solutions clear. Special blanks were therefore not necessary.

After development the colour intensities were measured at the same intervals as the standards, which contained 10 and 20 μg of creatine. Using the above method, which was shown by Barker and Ennor (1951) to result in excellent recoveries of both creatine and PC added to tissue extracts, analyses have been carried out on the PC content of various tissues from a number of different laboratory animals.

Preparation of tissue extracts for the estimation of CK activity. The required tissues were removed from rabbits under nembutal anaesthesia. The tissues were wrapped in polythene sheets and placed in crushed ice.

Aqueous extracts of the tissues were made at first but in later experiments 0.1 M phosphate buffer (pH 7.5) was used as the extractant. With the exception of muscle the weighed tissues were extracted in a Potter-Elvehjem homogeniser with four volumes of the buffer. A Molco type homogeniser was used for muscle. The suspensions were centrifuged and 5 ml. of the extract dialysed with stirring for 22 hours against 100 volumes of 0.01 M phosphate
buffer (pH 7.5). The buffer was replaced four times during the course of dialysis. The dialysed extracts were made to 10.0 ml. and filtered. For the assay of enzymic activity the following stock solution was made up:

- TRIS, pH 7.2, 0.1 M. 30.0 ml.
- F3, 2 x 10^{-3} M. 15.0 ml.
- CaCl2, 1 x 10^{-1} M. 3.0 ml.
- Water to 60.0 ml.

The reaction mixture contained 2.0 ml. of the stock solution, the required amount of tissue extract and water to 3.0 ml. The tubes were placed in the water bath and allowed to reach 37°C. The reaction was started by the addition of 1.0 ml. of 1 x 10^{-3} M. ADP. Depending on the tissue employed the reaction was stopped 5 to 65 min. later by the addition of 1.0 ml. of the mixed SMEA-Versene inhibitor described above (see Methods, Chapter I). Samples were then taken for the estimation of creatine. As free creatine could also arise from PC as a result of the action of phosphatases, a sample of each tissue extract was also incubated with the stock solution but the ADP was omitted and in its place 1.0 ml. of water added.

Assay of CPK activity. All extracts were appropriately diluted with 0.01 M. phosphate buffer pH 7.5 so that with the amount of extract added to the reaction mixture initial rates were measured. The unit of CPK activity was defined as µg. creatine
liberated per min. per g. of tissue under the conditions of the experiment.
RESULTS

Distribution of PC in animal tissues. The results of the assay of PC in various tissues of a number of animals are shown in Table 10. It will be noted that PC was found in all tissues examined, although the absolute amounts varied considerably. As found by other workers (Eggleton and Eggleton, 1927a; Alexeeva and Sok, 1952) skeletal muscle contained a higher amount than any other tissue. There was a notable variation in the PC content in cardiac muscle in separate experiments and, since the hearts were beating vigorously when removed from the animals, it is possible that even in the short interval between excision and freezing a portion of PC became dephosphorylated.

A comparison of the PC concentrations in particular organs shows that there is also a species variation. Thus the PC content in certain tissues of the cat is higher than in the rabbit or guinea pig. This is evident in the spleen, kidney and liver.

In view of the large variation from organ to organ and sometimes from animal to animal it was of interest to compare the PC and creatine contents of the tissues. The result of this comparison is shown in Table 11 where the ratio of 'bound' to 'total' creatine is given for each tissue. The variation is
considerable and it is difficult to compare the results, because of the number of very low values which might be explained as being due to decomposition of PC during the treatment of the tissue. However, if one neglects the values lower than 10%, the figures suggest that, irrespective of the tissue, or species, the proportion of creatine phosphorylated is about 20-25% of the total amount present.

**Distribution of CPK activity in various tissues.** The distribution of CPK activity in some tissues of the rabbit is given in arbitrary units in Table 12. It is clear that the tissues fall into two groups. To the first belong the muscular tissues (striated, cardiac and smooth muscle) and brain, all of which contain a far higher amount of activity than the tissues in the second group, namely kidney, spleen and liver. Included in Table 12 is a single result obtained with the guinea pig liver where considerably higher activity was found than in the case of the rabbit. The value obtained from the guinea pig liver is comparable with those obtained for rabbit spleen and kidney.
Table 12.

GFK activity in various tissues of the rabbit.

(With the exception of skeletal muscle all tissues were extracted with 0.1 M. phosphate buffer (pH 7.5) and activity estimated in the dialysed extract. Activity measurements on muscle were carried out on the crude dialysed extract prepared according to Ackonos (1961a). The unit is defined as μg. creatine liberated/min./g. of tissue under standard experimental conditions.)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Units/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>4000</td>
</tr>
<tr>
<td>Heart</td>
<td>1300</td>
</tr>
<tr>
<td>Brain</td>
<td>900</td>
</tr>
<tr>
<td>Uterus</td>
<td>600</td>
</tr>
<tr>
<td>Spleen</td>
<td>30</td>
</tr>
<tr>
<td>Kidney</td>
<td>12</td>
</tr>
<tr>
<td>Liver</td>
<td>1</td>
</tr>
<tr>
<td>(Liver - guinea-pig)</td>
<td>(27)</td>
</tr>
</tbody>
</table>
DISCUSSION

The method for the estimation of phosphocreatine employed in the present work has a number of advantages over the existing methods and these have been discussed elsewhere (cf. Ennor and Rosenberg, 1952b). The high degree of sensitivity and specificity of the method employed made it possible to demonstrate that phosphocreatine had a much wider distribution than was generally supposed. Of great interest is the fact that, whilst the concentration varies greatly from tissue to tissue, the proportion of the total creatine present in the form of phosphocreatine shows much less variation. This suggests that an equilibrium exists in the tissue between the creatine and phosphocreatine present. The belief that the presence of phosphocreatine is almost exclusively confined to muscular and nervous tissue (Eggleton and Eggleton, 1929; Gerard and Tupikova, 1939) gave rise to the concept that there was a relation between the presence of phosphocreatine in an organ and the "...speed of action required by these organs" (Lipmann, 1941). The occurrence of phosphocreatine in organs such as liver, spleen and kidney on which no sudden demands for energy are made suggests the need for some modification of this concept.

It was shown above that creatine phosphokinase activity...
was present in all organs examined. It is of interest to note that in the group of organs which are characterised by a high PC content the CPK activity is also high while low CPK activity was found in the group of tissues with a low PC content.

The above mentioned comparisons rest upon the assumption that the enzymic activity measured in the tissue extracts under the conditions employed actually represented a proportional fraction of the total CPK activity of each tissue. This may not necessarily be the case in some instances. The extracts employed contained the soluble extractable enzyme. While it is known (cf. p.73-4) that in muscle about 75% of the total CPK activity is present in the soluble fraction, this may not be the case in liver or kidney where a large number of the enzymes present are associated with the particles. If CPK in these tissues belongs in this group, an estimate of its activity in an aqueous extract may be subject to error. An investigation into the distribution of CPK activity within the cell would therefore be of great interest.

Another point that must be mentioned is that the estimations of enzymic activity were performed under conditions which were found to be ideal for the muscle CPK described in Chapter I. It is possible that the same conditions are not ideal for the CPK from other tissues. This may, in part, explain the very large difference in the amount of activity found in the
two groups mentioned above.

The fact, however, that CPK activity was found in all the organs examined indicates that even in organs where sudden large changes in the metabolic rate cannot be clearly visualized, the adenylic acid system seems to depend upon a reservoir of readily available chemical energy in the form of phosphocreatine. On the other hand, this reservoir can also serve for the purpose of shunting off all excess of energy which may arise on some occasions. Such a phenomenon was demonstrated in muscle in the experiments with carnosine and anserine discussed in Chapter I (p. 74), and a similar effect will be demonstrated below (Chapter III) when dealing with fatty livers induced by CS\textsubscript{4} (cf. also Monor and Stocken, 1942a and Monor, 1950).

The possibility is not excluded, however, that phosphocreatine has a function other than that of acting as a reservoir of readily available energy for the adenylic acid system and that it may contribute energy to certain endergonic reactions directly and without the mediation of the latter system.
SUMMARY

(1) A method for the estimation of phosphocreatine has been described.

(2) The distribution of phosphocreatine in various tissues of a number of laboratory animals was studied and phosphocreatine was found in all tissues examined.

(3) Creatine phosphokinase activity was found in a number of tissues of the rabbit and the amount of enzyme was proportional to the concentration of phosphocreatine.

(4) The role of creatine phosphokinase in the metabolism of creatine was discussed.
CHAPTER III

ATP AND PHOSPHOCREATINE IN FATTY LIVERS
CHAPTER III

ATP AND PHOSPHOCREATINE IN FATTY LIVERS

INTRODUCTION

The ability of both carbon tetrachloride (CCl₄) and anterior pituitary extracts (APF) to produce a marked infiltration of fat into the liver is a well-known phenomenon (cf. Best and Campbell, 1936; Cameron and Karunaratne, 1936). However, it was not until the work of Ennor and Stocken (1948a) that the distribution of some acid soluble phosphates in the fatty liver was studied. These authors found that the total acid soluble phosphorus content of the livers of guinea-pigs treated with CCl₄ increased by about 25-30% of the normal value and that the greater part of this increase occurred in the organic fraction. It was further shown that the increase in the organic acid-soluble P took place in the acid labile (7 min. in N-HCl at 100°C) compounds of both the barium-insoluble and barium-soluble, ethanol-precipitable fractions. An increase was also observed in the molybdate-labile P. On the basis of these results the authors postulated that in the fatty livers induced by CCl₄ there was an increase in the concentration of high energy phosphates,
namely, ATP, ADP and PC. It was later confirmed by Ennor (1950) that treatment with CCl₄ led to an increase in PC concentration of the liver up to 15 mg%. The theory proposed by Ennor as an explanation of the phenomena was that, before any cellular damage can be seen under the microscope in the CCl₄-poisoned livers, there has been produced a 'biochemical lesion' (cf. Garrileescu and Peters, 1921) which is an undefined change in the enzymic relationships within the cell. In order to correct this lesion energy is necessary, and energy in the form of fat is brought into the cell by unknown mechanisms. However, the cellular enzymic mechanisms are by that time sufficiently damaged to be unable to utilise the energy produced by the oxidation of the fat. The excess energy is therefore funnelled off and bound in the form of high energy phosphate.

The findings of Ennor and Stocken (1948a) were also confirmed by Rowles (1952), by Tsuboi and Stowell (1951) and by Richter (1951). The latter author also explained the accumulation of high energy phosphates in the fatty livers by the inability of the damaged cells to utilise the large excess of energy available as a result of the intensive oxidation of fat.

Hove and Hardin (1952) found that the ability to synthesise creatine from glycocyamine was greatly reduced in liver slices obtained from rats on a vitamin E deficient diet which
have been treated with CCL₄ in amounts insufficient to cause necrosis. They also showed that there was a slight rise in the creatine content of the liver and a marked fall in the creatine content of muscle. Urinary creatine increased three-fold. A parallel was drawn between the action of CCL₄ and vitamin E deficiency which produced very similar results. It was also found that administration of vitamin E prevented or markedly lessened the effects of treatment with CCL₄.

Morgan (1951) investigated the effect of various hormones, including those from the anterior pituitary, on the phosphocreatine content of the guinea-pig liver. No significant effect was observed with either crude anterior pituitary extract or purified thyrotrophic hormone and only a slight increase in PC was found as a result of treatment with ACTH.

The present work deals with the effect of administration of carbon tetrachloride and anterior pituitary extracts on the concentration of ATP and phosphocreatine in the liver of the guinea-pig.
EXPERIMENTAL

(a) Materials

Anterior pituitary extracts. The saline extracts of anterior lobes of ox pituitary glands were prepared as described by Morrison (1952).

Carbon tetrachloride solution. A 50% solution of CCl₄ in arachis oil was used throughout.

All other materials employed in the work described in this Chapter have been mentioned elsewhere (Chapter I).

(b) Methods

Treatment of animals. Animals receiving CCl₄ were treated as described by Emor and Stocken (1948a). APE-treated animals received a single injection of 1.0 ml/100 g. of the pituitary extract described above. Free access to water was given but food was withdrawn after the injection. The animals were killed 24 hours later.

All animals were killed by cervical dislocation, the thoracic cavity opened and the livers removed. A large portion of the liver was immediately dropped into liquid N₂. The
remainder was used for estimation of the fat, water and nitrogen content.

The liquid N2-frozen portion was then extracted as described in Chapter II. A portion of the extract was neutralised and the remainder treated with ether as described below for the estimation of ATP.

**Phosphorus estimation.** Inorganic phosphate was estimated directly on the TCA extract by the method of Ennor and Stocken (1950).

**Nitrogen.** Duplicate samples of fresh liver were asched with the digestion mixture described by Campbell and Hanna (1937). The digest was made to a suitable volume and samples were distilled in the apparatus of Markham (1942). The distillate was collected in a boric acid buffer containing a mixed indicator described by Conway and O'Malley (1942) and was then titrated with C.01 N HCl.

**Creatine.** Creatine was estimated by the method of Ennor and Stocken (1943b).

**Phosphocreatine.** The PC content of the liver was determined as the difference between the bound and free creatine as described in Chapter II above.

**ATP.** In order that the enzymic determination of ATP could be carried out directly the non-neutralised portion of the extract was extracted three times with peroxide-free ether
cooled to -15°. The aqueous phase was then filtered, the pH adjusted to 7.5 and the dissolved ether removed with a stream of air. In extracts thus treated the concentration of TCA was sufficiently low to exert less than 2% inhibition on myosin ATPase. Enzymic estimation of ATP using myosin ATPase was then carried out on the TCA-free extract using the procedure of Rowles and Stocken (1950).

**Water content.** In order to determine the water content weighed duplicate samples of fresh liver were dried to constant weight in the oven at 110°.

**Fat content.** In the present work 'fat' refers to the material extracted from the liver by means of a mixture of ethanol-diethyl ether in the ratio of 7:3.

**Expression of results.** It was found that the variation in both fat and water content was too large to permit calculation of analytical figures on the basis of the fresh liver weight (cf. Ennor and Stocken, 1948a). All results were therefore expressed on the basis of dry, fat-free weight.
RESULTS

The results of a number of experiments are presented in Table 13. It is clear from the table that both AFE and CCl₄ treatment caused an accumulation of fat in the livers to an approximately equal extent. It is also evident that the nitrogen content as expressed on the dry, fat-free basis does not change significantly and this provides a basis for the expression of results which is independent of the variation in water and fat content.

It is clear from the data concerning the acid soluble phosphates of the fatty livers produced by both AFE and CCl₄ that there are metabolic differences between the two conditions. Thus a five-fold increase in inorganic phosphate is produced by CCl₄ while no significant difference is found after treatment with AFE. The increase in ATP and PC is also greater with the CCl₄-induced fatty liver. It will be noted that after AFE treatment there is a significant increase in both the total and free creatine and also in phosphocreatine.

In the case of CCl₄ treatment the free creatine showed a slight fall while the total creatine showed a slight rise. However, statistically neither figure is significant even at the 5% level. Yet a very significant increase in phosphocreatine was found. Statistical analysis of the data obtained
Table 13.
The effect of AFB and CCl₄ treatment on the distribution of acid soluble phosphates in liver.

(Figures in brackets in heading denote number of animals in group. The first line of figures for each variable is the mean of the group. 'Difference' denotes the difference between the means of the particular group and the controls. The significance refers to this difference. *= significant at 5% level; **= significant at 1% level; NS = not significant.)

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>Controls (14)</th>
<th>AFB (15)</th>
<th>CCl₄ (11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat, % (dry basis) Mean</td>
<td>24.8</td>
<td>41.6</td>
<td>44.5</td>
</tr>
<tr>
<td>Difference (+, -)</td>
<td>-</td>
<td>+12.8</td>
<td>+19.7</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Nitrogen, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>15.3</td>
<td>14.6</td>
<td>14.6</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>-0.7</td>
<td>-0.7</td>
</tr>
<tr>
<td>S</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Inorganic P, mg.%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>67.8</td>
<td>73.6</td>
<td>377</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>+5.8</td>
<td>+309</td>
</tr>
<tr>
<td>S</td>
<td>-</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>ATP, mg.%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>224</td>
<td>290</td>
<td>566</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>+66</td>
<td>+342</td>
</tr>
<tr>
<td>S</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Free Cr. mg.%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>154</td>
<td>226</td>
<td>143</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>+72</td>
<td>-11</td>
</tr>
<tr>
<td>S</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Total Cr. mg.%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>164</td>
<td>245</td>
<td>191</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>+81</td>
<td>+27</td>
</tr>
<tr>
<td>S</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>PO, mg.%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>15.2</td>
<td>41.5</td>
<td>77.1</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>+26.3</td>
<td>+61.9</td>
</tr>
<tr>
<td>S</td>
<td>-</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>
shows that the degree of variance in both the free and total creatine is approximately equal and is about 15 times the variance in phosphocreatine. Moreover, the correlation coefficient between the free and total creatine is very high (over 0.9) and as a result the increase in PC is highly significant.
DISCUSSION

The mechanism of action of CCl₄ in causing the production of fatty livers is not known, and various theories have been advanced. Not all, however, can satisfactorily explain the phenomenon of the actual mobilisation of the fat, the increased oxidation rates and the increase in ATP and PC found in the fatty livers. According to Christie and Judah (1954) the phenomena observed in the fatty livers produced by CCl₄ are explained on the basis of a derangement of enzymic systems and impairment of the activity of a number of enzymes, particularly those associated with mitochondria. It was shown that a loss in diphosphopyridine nucleotide occurred thus causing the inhibition of a number of oxidative processes. This is difficult to reconcile with the fact that the fatty liver slices exhibit markedly elevated oxidation rates (Ennor, 1943).

Other aspects of CCl₄ toxicity are of interest, namely the connexion between CCl₄ and vitamin E deficiency. Although some of the effects of CCl₄ poisoning are similar to those produced by vitamin E deficiency and have been lessened by the administration of the vitamin, there is no indication at present as to the nature of the reaction(s) affected by CCl₄.
and which also involve vitamin E. (cf. Heve and Martin, 1952; Sellars, Zen and Lucas, 1950).

The action of AFS is similarly not understood, perhaps because AFS is a complex mixture of factors. Thus Morrison (1952) reported that both AFS and growth hormone caused an increase in the fat content of mouse livers. Levin and Parbor (1956) confirmed the lipogenic action of both hormones in some cases, but also found that certain preparations of either growth hormone or AFS failed to produce fatty livers. They also found that while the effect of anterior pituitary extracts was completely abolished by adenectomy, adrenal hormones alone failed to produce a fatty liver. It was therefore concluded that some corticoesteroids together with some unknown factor from the anterior lobe of the pituitary were required for the production of a fatty liver.

The findings produced in Table 13 as well as the results of other workers suggest that the metabolic patterns of the fatty livers produced by SGL, and AFS differ and thus quite distinct mechanisms are perhaps involved. Anterior pituitary extract, unlike SGL, does not produce any cellular damage so that the two types of fatty livers described in the present work are similar only in their fat content and in the elevation of the levels of high energy phosphates. On the basis of this observation it seems that the most acceptable theory is that
proposed by Sennor (1950) and corroborated by Lichter (1951), namely that the intensive oxidation of fat which is accumulated in the liver leads to an increase in the production of high energy phosphate in the form of ATP. The damaged cell cannot utilise the large amount of energy produced and this causes a rise in the level of phosphorylation of ADP. The result is that a portion of the high energy P is transferred to creatine, presumably as a result of the CPK-catalysed equilibrium between ATP and creatine.

It is of interest to note the difference between the CCl₄- and APF-produced fatty livers in so far as the concentration of inorganic phosphate is concerned. Since CCl₄-produced fatty livers have markedly increased concentrations of inorganic phosphate, whereas fatty livers produced by APF treatment contain normal amounts, it may be inferred that the presence of inorganic phosphate in large amounts occurs as a consequence of the cell damage which is visible microscopically. The release of phosphatases as a result of such damage is a possibility. Histochemical assay of phosphatase has, in fact, shown that this enzyme in the normal liver is localised in the blood vessels and bile ducts, whereas in the CCl₄-produced fatty liver it can also be demonstrated on the periphery of the damaged liver cells. No such damage occurs in the APF-produced fatty liver. (Dr. H. Kramer, personal communication.)
SUMMARY

(1) It was shown that in livers which have become fatty as a result of treatment with either AFB or CCl₄ there was a rise in the levels of ATP and phosphocreatine.

(2) The inorganic phosphate content of the fatty livers produced by CCl₄ was raised five-fold, but no increase occurred in the AFB-produced fatty livers.

(3) The possible mechanism of these changes is discussed.
APPENDIX I

THE USE OF CREATINE PHOSPHOKINASE FOR THE PREPARATION OF $^{32}$P-LABELLED NUCLEOTIDES
APPENDIX I

THE USE OF CREATINE PHOSPHOKINASE IN THE
PREPARATION OF $^{32}$P-LABELLED NUCLEOTIDES

INTRODUCTION

In investigations involving the adenylic acid system it is sometimes desirable to use ATP or ADP labelled with $^{32}$P in one or more positions. Lindberg and Ernster (1952) made use of ADP labelled in either the P atom attached to the ribose ($\alpha$-P) or terminally ($\beta$-P), and Hems and Bartley (1953) prepared ATP labelled in both the middle ($\beta$-P) and terminal ($\gamma$-P) atoms for the investigation of transphosphorylations involving inosine and adenosine polyphosphates. $^{32}$P-labelled ATP was prepared by Eggleston (1954) who used the product for the preparation of labelled inosinic acid.

The method for the preparation of $^{32}$P-labelled ATP and ADP described below differs from those mentioned above in that "in vitro" reactions catalysed by highly purified enzymes are employed. The method offers a means of introducing a label into any one or more of the P atoms in the ATP or ADP molecule.
(a) **Principle of method.**

The following reactions are involved:

1. \[ \text{ATP} \rightarrow \text{ADP} + \text{PO}_{4}^{3-} \quad \text{(Myosin ATP'ase)} \]  
   
2. \[ \text{ATP} + \text{glucose} \rightarrow \text{ADP} + \text{glucose-6-P} \quad \text{(Hexokinase)} \]  
   
3. \[ \text{ATP} + \text{AMP} \rightarrow 2 \text{ADP} \quad \text{(Myokinase)} \]  
   
4. \[ \text{ATP} \rightarrow \text{AMP} + \text{PO}_{4}^{3-} \quad \text{(Apyrase)} \]  
   
5. \[ \text{ADP} + \text{PC} \rightarrow \text{ATP} + \text{creatine} \quad \text{(CPK)} \]  
   
6. \[ \text{AMP} + 2\text{PC} \rightarrow \text{ATP} + 2\text{creatine} \quad \text{(CPK + Myokinase)} \]

Reaction IV is reversible, but, as described in Chapter I above, it proceeds almost quantitatively from L to R at pH 7.2. Reaction V is catalysed by CPK and myokinase and does not proceed in the presence of CPK alone (see Chapter I, p. 55). A catalytic amount of either ATP or ADP is necessary to start the reaction, which then becomes self-accelerating.

From the tabular representation of the reactions employed for the synthesis of specifically labelled compounds (Table 14) it may be seen that the various procedures are similar and consist mainly in the transfer of a P group from PC to either ADP or AMP using CPK or CPK and myokinase respectively. In some cases myosin ATP'ase or apyrase were used when one or two P atoms respectively were to be removed from ATP either before or after
Table 14.

Scheme for the preparation of $^{32}$P-labelled compounds.

(The following symbols are used: $^*$ for isotopically labelled P group; APP, APP, AP, P refer to ATP, ADP, AMP, PO$_4$, respectively. For enzymic systems indicated, see text.)

<table>
<thead>
<tr>
<th>Compound \ Reactant (and source)</th>
<th>Reaction</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) $\text{AP}^<em>$ $\text{AP}^</em>$ (animal)</td>
<td>III</td>
<td>$\text{AP}^<em>$ + $2\text{P}^</em>$</td>
</tr>
<tr>
<td>(2) $\text{AP}^<em>$ $\text{AP}^</em>$ (animal)</td>
<td>$I_a$ (or $I_b$)</td>
<td>$\text{AP}^<em>$ + P$^</em>$ (or Gluc. 6-$^*\text{P}$)</td>
</tr>
<tr>
<td>(3) $\text{AP}^<em>$ $\text{AP}^</em>$ (1 above) + P$^*$</td>
<td>V</td>
<td>$\text{AP}^*$ + Creatine</td>
</tr>
<tr>
<td>(4) $\text{AP}^<em>$ $\text{AP}^</em>$ (3)</td>
<td>I</td>
<td>$\text{AP}^*$ + P</td>
</tr>
<tr>
<td>(5) $\text{AP}^<em>$ $\text{AP}^</em>$ (4) + P$^*$ (animal)</td>
<td>IV</td>
<td>$\text{AP}^*$ + Creatine</td>
</tr>
<tr>
<td>(6) $\text{AP}^<em>$ $\text{AP}^</em>$ (animal)</td>
<td>V</td>
<td>$\text{AP}^*$ + Creatine</td>
</tr>
<tr>
<td>(7) $\text{AP}^<em>$ $\text{AP}^</em>$ (6 above)</td>
<td>I</td>
<td>$\text{AP}^<em>$ + P$^</em>$</td>
</tr>
<tr>
<td>(8) $\text{AP}^<em>$ $\text{AP}^</em>$ (7 above) + P$^*$</td>
<td>IV</td>
<td>$\text{AP}^*$ + Creatine</td>
</tr>
<tr>
<td>(9) $\text{AP}^<em>$ $\text{AP}^</em>$ (animal)</td>
<td>IV</td>
<td>$\text{AP}^*$ + Creatine</td>
</tr>
<tr>
<td>(10) $\text{AP}^<em>$ $\text{AP}^</em>$ (2 above) + P$^*$</td>
<td>IV</td>
<td>$\text{AP}^*$ + Creatine</td>
</tr>
</tbody>
</table>
transphosphorylation with CPK. It may also be seen from Table 14 that ATP labelled in all three positions and $^{32}P$-labelled PC are the only $^{32}P$-labelled compounds required for the syntheses.

Because of the similarity of the reactions it was not thought necessary to carry out all procedures, and only two will be described in detail.

(b) Materials and methods

$^{32}P$-labelled ATP and PC. Phosphocreatine labelled with $^{32}P$ was isolated in the initial experiments from muscles of animals previously injected with $^{32}P$-orthophosphate, but later it was obtained by incubation of creatine and $^{32}P$ with a muscle suspension as described below. Labelled ATP was generally isolated from animals injected with $^{32}P$-orthophosphate. ATP thus obtained was shown to be labelled in all three P groups, though not to the same extent (Innor and Rosenberg, 1954).

$^{32}P$. $^{32}P$ was obtained from the Atomic Research Establishment, Harwell, and was used in the form of $\text{Na}_2^{32}\text{PO}_4$. All samples were heated in $\text{HNO}_3$ at 100° for 1-2 hours to convert any pyrophosphate to orthophosphate. The samples were then adjusted to the desired pH by the addition of $\text{NaOH}$. All samples
contained a small amount of added 'carrier' orthophosphate.

Estimation of $^{32}\text{P}$ and $^{31}\text{P}$. $^{31}\text{P}$ and $^{32}\text{P}$ were estimated as described by Emor and Stocken (1950) and Emor and Rosenberg (1952a). The specific radioactivity ($SA$) (counts/min/μg) of the P groups of ADP and of phosphocreatine were estimated using enzymic and non-enzymic methods as described by Emor and Rosenberg (1954a).

Other materials and methods employed in this work were described in Chapter I.
RESULTS

Preparation of phosphocreatine labelled with $^{32}$P.

200 g. of muscle was taken from the hind limbs of a rabbit under nembutal anaesthesia. The muscles were immediately homogenised under an atmosphere of O$_2$ in a Waring Blendor in 300 ml. of 0.1 M TRIS buffer, pH 7.2, containing 6.7 mmoles of creatine and 1.5 mmoles of NaN$_2$H$_2$$^{32}$PO$_4$ (equivalent to 1 mc. of $^{32}$P).

The suspension was stirred under O$_2$ for 3 min., 100 ml. of 25% (w/v) trichloroacetic acid were then added and immediately mixed in the blendor. The precipitated protein was rapidly removed by filtration and the extract immediately adjusted to pH 7 by the addition of 5 N NaOH. PC estimations on the neutralized extract showed that approx. 1.2 mmoles of the compound were present. Separation of the PC was effected by the following procedure:
Repeated precipitations with carrier $^{32}P_{O_4}^{3-}$ were carried out in order to remove traces of highly labelled $^{32}P_{O_4}^{3-}$. Ba$^{++}$ was removed from the final solution by adding Na$_2$SO$_4$ until only a trace of Ba$^{++}$ could be detected by Na rhodizionate. The Ba$_2$SO$_4$ was then filtered off and the remaining Ba$^{++}$ removed by shaking the solution with amberlite I.R.100 (Na form). The yield was 0.25 mmoles of PC with a specific activity of 300 counts/min./µg P.

Isolation of the isotopically labelled PC from the aqueous solution was unnecessary since the following step was carried out immediately.
Preparation of (β-32P)ATP

Step (1) – Preparation of (β:Y-32P)ATP (Table 14).

Reaction (1). 0.5 mmoles of PC (Na salt) was added to the 32PC above thus reducing the 5A to 103.

The reaction:

\[ \text{AMP} + 2\text{PC} \rightleftharpoons \text{APP} + 2 \text{creatinine} \]

was then carried out as follows:

The reaction mixture contained 100 ml. of 0.1 M Tris buffer pH 7.2; 50 ml. of PC solution (0.75 mmoles); 20 ml. of AMP solution (0.75 mmoles) adjusted to pH 7.2; 2 ml. of 1 x 10^{-3}M ADP and 12 ml. of 1.0 N CaCl_2. The mixture was brought to temperature (37°C) in the water bath and then GFK (20 mg. protein) and myokinase (5 mg. protein) were added. The progress of the reaction was followed by determining the increase in free creatinine (Fig. 24). Equilibrium was reached after 20 min. and the mixture was cooled and treated with 50 ml. of 25% w/v TCA.

The nucleotides were isolated from the TCA filtrate by precipitation as Ca salts, which were then dissolved by the addition of glacial acetic acid and reprecipitated by the addition of 20% mercurochrome acetate in 2% acetic acid. The mercury precipitate was decapped with HgS, and the HgS removed by filtration. The HgS was expelled from the filtrate by aeration, and the solution adjusted to pH 7 by addition of 5 N NaOH.
Figure 24.

Liberation of creatine during the incubation of AMP with PC in the CPK-myokinase system.

Samples were withdrawn from the mixture for the estimation of creatine at the times indicated. Incubation at 37°, pH 7.2.
Step (2) - Preparation of (β²³²)ADP (Table 14, Reaction 7). The above solution was incubated with myosin in 0.025 M glycine buffer, pH 7.0, at 37° for 30 min. The reaction was stopped by the addition of 10% HCl, and the ADF isolated from the filtrate as described above.

Analysis of the ADF solution showed that the yield obtained was 0.13 mmoles of ADF with an S4 in the βP of 100.

Step (3) - Preparation of (β³²³P)ATP (Table 14, Reaction 8). The reaction involved in this step is:

\[ \text{ATP}^{32P} + \text{PC} \rightarrow \text{ATP}^{32P} + \text{creatine}. \]

The reaction mixture contained 0.12 mmoles of the (β³²³P)ADP (above) in 40 ml. of water, 20 ml. of 0.1 M TRIS buffer pH 7.2, 0.24 mmoles of NaPC in 10 ml. of 0.05 M TRIS buffer pH 7.2, and 4 ml. of 1.0 M CaCl₂. The mixture was brought to 37° in the water bath and CPK solution (7 mg. protein in 6 ml. of water) was added. The progress of the reaction was followed as in step (1) above by the determination of free creatine. When equilibrium was reached the solution was deproteinized and the ATP was isolated as described in step 1.

The reaction yielded 0.06 mmoles of ATP with the following specific activities: α-P = 9.2, β-P = 104, γ-P = 0. The product was contaminated with 0.1 mmoles of orthophosphate with an S4 of 3.7. No further purification was carried out.
Preparation of (\(\gamma^{-32P}\))ATP

This is a one-step reaction only (Table 14, reaction 9):

\[
\text{APP} + P^{32}C \rightarrow \text{APP}^2 + \text{creatine}
\]

Isotopically labelled phosphocreatine for this reaction was prepared as described above.

The experimental mixture contained 30 ml. of 0.05 M. TRIS buffer pH 7.2, 0.8 mmoles of \(P^{32}C\) (SA of 147) in 100 ml. of water, 0.6 mmoles of ADP in 50 ml. of water, 12 ml. of 1.0 M. CaCl\(_2\), and 8 ml. of CK solution containing 20 mg. of protein. The mixture was incubated at 37\(^\circ\) and the progress of the reaction followed by the liberation of creatine. Equilibrium was reached after 20 min. The mixture was then incubated for a further 15 min. and deproteinised by the addition of TCA as described above. The nucleotides were isolated by the precipitation of the Ba-salts followed by the precipitation of the mercury salt. The yield was 0.2 mmole of ATP with a specific radioactivity of \(\alpha-P = 9.2, \beta-P = 4.2\) and \(\gamma-P = 137\).
DISCUSSION

The experiments quoted in this Chapter were carried out to illustrate the practical application of the method. Since the labelled substances were not required for immediate use, it was not considered worthwhile to carry out any further purification which could have been effected by the use of anion exchange resins (cf. Cohn and Carter, 1950).

It will be noted that in the two samples of ATP prepared, namely APP\textsuperscript{32}P and APPF\textsuperscript{32} radioactivity was found in the α-P atom. The activities were low, and since the reactions involved are known, it is unlikely that this could represent true activity in this atom. It is, on the other hand, very likely that the result obtained was due to the methods employed for the estimation of the SA of the α-P. The α-P was estimated as the difference between the total organic (wet ashed) P and the P hydrolysed by N HCl in 10 min. at 100°. If, therefore the 10 min. hydrolysis was incomplete, some of the β-P or γ-P would have been estimated as α-P.

An SA of about 4 was also found in the β-P of APPF\textsuperscript{32}. Here β-P was estimated as the difference between the P released (in two separate tests) by apyrase and myosin ATP'ase respectively. Thus if myosin did not completely hydrolyse the γ-P while apyrase
completely hydrolysed both the \( \gamma \)- and \( \beta \)-P, this difference, taken for \( \beta \)-P only, would include some radioactivity.

Actual labelling of the \( \beta \)-P would be possible in the system employed if myokinase activity were present during the incubation in the preparative stage. However it was definitely shown that both the myosin used as well as the CPK preparation were completely free from myokinase activity.

The high specificity of the enzymes used and the simplicity of the reactions involved point to the fact that the low SA obtained for the \( \alpha \)- and \( \beta \)-P atoms of the labelled ATP preparations must have arisen as a result of analytical error. A specific 5'-nucleotidase would permit correct analysis and assessment of the SA of the \( \alpha \)-P, but this was not available.

ATP labelled with \( ^{32}P \) in all three P atoms was not required for the preparations described in this Chapter, although ATP labelled in this fashion has been isolated from muscles of animals previously injected with \( ^{32}P \) (Ennor and Rosenberg, 1954a). These workers found the label to be present in all three groups, but predominantly in the \( \beta \)- and \( \gamma \)-P. However, in long-term experiments when the tissues were obtained from animals 40-90 hours after injection with \( ^{32}P \), the \( \alpha \)-P was also labelled, but still to a lesser degree than were the \( \beta \) and \( \gamma \) atoms (Ennor and Rosenberg, unpublished). Similar observations have already been reported by Sacks and Altshuler (1942) using cat muscle, by Flock and Bollman
(1944) using rat muscle and by Sacks (1951) using rat liver.

It seems, however, that the preparation of labelled AMP from labelled ATP isolated from animals as described above is rather wasteful and the product would necessarily have a low SA. The methods recently suggested by Albaum, Pankin and Harvill (1953) and by Eggleston (1954) for the preparation of labelled AMP by the use of a fermentation procedure seem more acceptable.
(1) A method for introduction of $^{32}$P into any one or more of the P atoms of ATP is described.

(2) The method depends upon the use of "in vitro" enzymic systems.
APPENDIX II

THE USE OF CREATINE PHOSPHOKINASE FOR THE DETERMINATION
OF THE SPECIFIC RADIOACTIVITY OF PHOSPHOCREATINE—P
IN TISSUE EXTRACTS
APPENDIX II

THE USE OF CREATINE PHOSPHOKINASE FOR THE DETERMINATION
OF THE SPECIFIC RADIOACTIVITY OF PHOSPHOCREATINE-P
IN TISSUE EXTRACTS

INTRODUCTION

In studies involving the determination of the turnover rate of phosphocreatine by means of $^{32}$P, it is almost universal practice to employ hydrolysis at 37° or room temperature in the presence of dilute acid and molybdate in order to release the isotopically labelled P atom. This method, however, lacks specificity as it is known (Weil-Malherbe and Green, 1951; Lutwak and Sacks, 1953) that many phosphorus compounds undergo hydrolysis under these conditions. The low concentrations of PC present in tissues preclude its isolation in a chemically pure state. It was thus desirable to find a method which is both specific and sensitive. The high specificity of creatine phosphokinase suggested a possible solution and it was thought that if this reaction could be integrated with myosin ATP'ase which releases the terminal P of ATP only, then a method would be available
for the specific release of the phosphocreatine $P$ as inorganic phosphate.

The reactions involved are:

1. $\text{P}^{32}\text{C} + \text{APP} \rightleftharpoons \text{APP}^\ast + \text{creatine} \quad (\text{CPK})$

2. $\text{APP}^\ast \rightarrow \text{APP} + \text{P}^\ast \quad \text{(myosin ATPase)}$

where APP and APP represent ATP and ADP respectively.

Although reaction (1) is reversible, the availability of the myosin 'trap' as well as the suitable pH conditions and Ca$^{++}$ concentration (see Chapter I) will favour the forward reaction. Subsequent determination of the $^{31}\text{P}$ and $^{32}\text{P}$ released as inorganic $P$ in the reaction by the method of Emmor and Rosenberg (1952a) enables the determination of the specific radioactivity (SA) of the PC--P with a high degree of specificity.
EXPERIMENTAL

Materials.

All materials used were as described above (see Materials, Chapter I).

Preparation of tissue extracts.

TCA extracts of tissues were prepared as described in Chapter II. The tissues were taken from animals which were previously injected with $^{32}$P-orthophosphate so that the TCA extracts contained a number of organophosphates labelled with $^{32}$P. The extracts were not immediately neutralised but were extracted with ether as described in Chapter III (p. 104) to remove partially the TCA. Four extractions were employed with a result that the concentration of TCA in the extracts was lowered to 0.06% at which concentration myosin is not inhibited and CPK is inhibited only to the extent of about 4%.

Even though peroxide-free ether was used for the extraction a certain amount of peroxides usually appeared after the ether was removed by aeration. Such peroxides are strongly inhibitory to CPK but the inhibition was removed by the addition of
cysteine ethyl ester until a positive nitroprusside reaction was obtained.

$^{32}P$ and $^{31}P$.

The estimation of $^{32}P$ and $^{31}P$ was carried out as described in Appendix I.
RESULTS

Recovery of added PC. In order to check the efficiency of the method recoveries of known amounts of PC were carried out using CPK and myosin. Preliminary experiments on the coupled reaction of CPK and myosin showed that the reaction proceeded readily at pH 7.2. The conditions employed were therefore similar to those used for the forward CPK reaction. The reaction mixture contained all the components of the complete system for the forward reaction described above (see 'Methods', Chapter I), except that myosin was also present and that the amounts of PC added varied from 1.35 to 5.4 μmoles.

The results (Table 15) show that under the conditions of the experiment almost 100% of the phosphocreatine P is released as inorganic P. The small amounts of P found after incubation with myosin only (column 2, Table 15) are partially due to the presence or traces of ATP in the ADP employed. These values will also include all the inorganic P originally present in the mixture and most probably introduced with the PC solution. This explains the increasing values of blank when larger amounts of PC were added.

Determination of the SA of phosphocreatine-P. The method described above was then applied to the estimation of the
Table 15.
Recovery of added phosphocreatine in the coupled reaction of CPK and myosin.
(The reaction mixture contained all the components of the complete system for the forward reaction (Chapter I) with the exception that the concentration of FC was varied as shown below and myosin was added to the mixture in the concentration of 30 μg. N/ml. 5 min. incubation at 37°.)

<table>
<thead>
<tr>
<th>μM FC added</th>
<th>μM P found</th>
<th></th>
<th>μM P released</th>
<th></th>
<th>% released in 5 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With myosin</td>
<td>with myosin and CPK</td>
<td>released by CPK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.35</td>
<td>0.25</td>
<td>1.65</td>
<td>1.40</td>
<td>103.3</td>
<td></td>
</tr>
<tr>
<td>2.70</td>
<td>0.50</td>
<td>3.0</td>
<td>2.50</td>
<td>92.5</td>
<td></td>
</tr>
<tr>
<td>4.05</td>
<td>0.57</td>
<td>4.20</td>
<td>3.63</td>
<td>89.5</td>
<td></td>
</tr>
<tr>
<td>5.40</td>
<td>0.70</td>
<td>5.75</td>
<td>5.05</td>
<td>93.5</td>
<td></td>
</tr>
</tbody>
</table>
Specific radioactivity of PC phosphorus determined on identical samples by chemical and enzymic methods.

(For experimental detail see Table 15.)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Determined with CPK and myosin</th>
<th>Determined by acid molybdate hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>114</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>3.4</td>
<td>6.6</td>
</tr>
<tr>
<td>4</td>
<td>10.0</td>
<td>10.4</td>
</tr>
<tr>
<td>5</td>
<td>7.3</td>
<td>8.0</td>
</tr>
<tr>
<td>6</td>
<td>6.0</td>
<td>7.4</td>
</tr>
<tr>
<td>7</td>
<td>6.7</td>
<td>9.7</td>
</tr>
</tbody>
</table>
and other organophosphates which are partially hydrolysed under the conditions of the acid molybdate hydrolysis. Comparison of the data in Table 16 suggests that some compound (or compounds) with an SA higher than that of PC is present in the extract and that it is, partially at least, hydrolysed by the acid molybdate reagent. It is also clear that the use of the chemical method for the determination of the SA of PC-P is subject to error.
DISCUSSION

The enzymic method for the determination of the specific radioactivity of the phosphorus of phosphocreatine described above has a number of advantages over the chemical method using acid molybdate. The prime advantage is its specificity, since creatine phosphokinase is not known to transfer phosphate to ADP from any other naturally occurring compound except phosphocreatine. Also, it is without action on ATP, ADP or AMP (cf. Chapter I). Its effect on other phosphates was not tested, but it was found that the amount of phosphate released by CPK and myosin from samples of TCA extracts was never higher than the theoretical amount expected on the basis of determination of 'free' and 'total' creatine (cf. Chapter II). On the other hand, molybdate hydrolysis frequently gave rise to amounts of phosphate higher than those expected on the basis of creatine analysis (Emnor and Rosenberg, 1952b). This indicates that in the case of the enzymic method it is unlikely that phosphate not associated with creatine had been released.

Another advantage of the enzymic method lies in the fact that the phosphorus of PC is released as orthophosphate and can then be estimated as such by the procedure of Emnor and Stocken (1950). In this procedure the sample is treated with the acid-molybdate reagent and the phosphomolybdic acid formed is immediately extracted into isobutanol, the entire operation lasting 10 sec. In
the case of the acid-molybdate hydrolysis the sample is allowed
to stand in the presence of the reagent for 30 min. at room tem-
perature or 37° in order to effect the hydrolysis of PC. The
phosphomolybdic acid formed is extracted into isobutanol at the
end of this period. It was found that the colour intensity fol-
lowing reduction of the phosphomolybdic acid in standards thus
treated was approximately 18% higher than in similar standards
treated according to the procedure of Ennor and Stocken (1950).
It is doubtful whether the use of these standards permitted
accurate estimation of phosphate in samples which have been sub-
jected to acid molybdate hydrolysis since on occasions it led to
negative values for the PC phosphorus as determined by the above
method. This was often the case with solutions where the amount
of PC present was low compared with the inorganic phosphate.

While the enzymic method offers the means for the
estimation of the SA of the PC phosphorus, it is not sufficiently
absolute for the quantitative estimation of phosphocreatine,
since the recovery of PC by the coupled reaction (Table 15 above)
is generally below 100%. The method described in Chapter II is
therefore preferable for the quantitative estimation of phospho-
creatine. The incompleteness of the release of the phosphorus
of PC by the coupled enzymic reaction does not, however, affect
the value of the SA found, for in those cases it is a ratio and
not an absolute quantity which is being determined.
SUMMARY

(1) A method for the estimation of the specific radioactivity of the phosphorus of phosphocreatine has been described.

(2) The method depends upon the use of creatine phosphokinase and myosin ATP'ase "in vitro".

(3) It was shown that the SA determined by the enzymic method differs from that determined by a hydrolysis method employing acid molybdate.

(4) The advantages of the enzymic method over the chemical one were discussed.
APPENDIX III

THE PREPARATION OF SUBSTITUTED GUANIDINES
APPENDIX III

THE PREPARATION OF SUBSTITUTED GUANIDINES

INTRODUCTION

In the experiments involving the specificity of creatine phosphokinase a number of guanidino compounds other than creatine were employed as test substrates. The synthesis of those compounds and of their phosphorylated derivatives is described below. In all cases the substituted guanidines were prepared from the corresponding amines by guanylation with either S-methyl- or S-ethylthiourea.

The amino compounds required for the guanylation were in most cases commercially available with the exception of N-ethylglycine, the preparation of which is described below.
EXPERIMENTAL AND RESULTS

Materials

All materials employed for synthesis were commercial preparations of varying degree of purity and were recrystallised from suitable solvents before use. S-Ethylthiourea was prepared by the method of Brand and Brand (1942) and was isolated as the hydrobromide.

Preparation of N-Ethylguanidoacetic acid.

This compound was prepared from N-Ethylglycine and S-methylthiourea sulphate according to the equation:

\[
\begin{align*}
\text{CH}_3\text{CH}_2 & \quad \text{HN-CH}_2\text{-COOH} \\
\text{S-CH}_3 + \text{HN=C} & \quad \text{NH}_2 + \text{HN=C} \\
\text{NH}_2 & \quad \text{NH-CH}_2\text{-COOH}
\end{align*}
\]

The method is similar to that described by Schütte (1943) for the preparation of creatine. N-Ethylglycine which was required for the above reaction and which was not commercially available was prepared by the method of Fischer and Bergmann (1913) in three steps:
Step 1. N-α-Toluenesulphonylglycine was prepared according to the reaction:

\[
\text{CH}_3\text{Ph-SO}_2\text{Cl} + \text{H}_2\text{N-CH}_2\text{COOH} \rightarrow \text{CH}_3\text{Ph-SO}_2\text{NH-CH}_2\text{COOH} + \text{HCl}
\]

50 g. of glycine (BDH) was dissolved in 600 ml. of water at room temperature and the solution placed in a square glass jar. 152 g. of toluenesulphonyl (tosyl) chloride was added together with 10 ml. of ether in order to depress the melting point of the tosyl chloride. The mixture was stirred at 1000 RPM using a cutting type stirrer from a Waring-Blendor. Throughout the reaction 5 N NaOH was allowed to drop into the vessel at a rate sufficient to keep the solution alkaline, but below pH 10, in order to avoid hydrolysis of the tosyl chloride. When the reaction was completed the solution was filtered, cooled and acidified to pH 3 by the addition of 10 N HCl. The bulky precipitate was filtered off and washed on a Buchner funnel firstly with dilute HCl and then with cold water. Recrystallisation from hot water yielded 146 g. (95% of theory) of colourless platelet-shaped crystals which melted at 142°.

Step 2. N-α-toluenesulphonyl:N-ethylglycine was prepared according to the reaction:

\[
\text{CH}_3\text{Ph-SO}_2\text{NH-CH}_2\text{COOH} + \text{CH}_3\text{CH}_2\text{I} \rightarrow \text{N-CH}_2\text{COOH} \\
\text{CH}_3\text{Ph-SO}_2 + \text{HI}
\]
65 g. of tosylglycine dissolved in 343 ml. of 3 N NaOH and 25 ml. of ethyl iodide were placed in a sealed tube. The tube was rocked in a water bath at 95°, until only one liquid phase was apparent, and was then left in the bath for a further 50 min. The tube after cooling was opened and the contents acidified; the resultant precipitate was filtered off and washed with water. The product was then redissolved in water by addition of solid K₂CO₃ and reprecipitated with HCl. The solid was washed free of acid with water and then washed with a solution of Na₂S₂O₃ to remove traces of iodine. It was finally washed free of thiosulphate with water. Recrystallisation from hot water yielded 60 g. (77% of theory) of colourless needle-shaped crystals which melted at 120-121°.

Step 3. N-Ethylglycine hydrochloride was prepared by acid hydrolysis of N-\(\beta\)-tosyl:N-Ethylglycine:

\[
\begin{align*}
\text{CH}_3\text{CH}_2
\end{align*}
\]
\[
\begin{align*}
\text{N-CH}_2\text{COOH} \quad \text{HCl} \quad \text{CH}_3\text{CH}_2\text{NH-CH}_2\text{COOH} + \text{CH}_3\text{Ph SO}_3\text{H}
\end{align*}
\]

10 g. of tosylethylglycine was refluxed with 7 N HCl for 16 hrs., by which time the material had dissolved completely. Upon cooling toluene sulphonic acid crystallised and was removed by filtration. The residue was evaporated to near dryness and the residual HCl removed in a stream of hot air. The residue was
extracted with hot ethanol and the extract treated with ether. The latter was added slowly until crystallisation commenced and then more rapidly, four volumes being added in all. The product was redissolved in ethanol and recrystallised several times by the addition of ether. The final yield was 2.9 g. (54% of theory) of very fine, needle shaped crystals. The product melted at about 149° and decomposed at about 200°.

N-Ethylguanidinoacetic acid (negmine) was then prepared according to the reaction given above (p.137).

7 g. of ethylglycine hydrochloride and 12 g. of S-methylthiourea sulphate were dissolved in 15 and 40 ml. of ammonia (c.g. 0.912) respectively. The solutions were mixed at room temperature and allowed to stand. Crystals appeared after 3.5 hr. and the solution was filtered after standing for further 16 hours. The product was recrystallised from hot water, washed with ethanol and ether and air-dried. The yield was 2.6 g. The product melted with decomposition at 230°. It gave a positive reaction with diacetyl and α-naphthol indicating the presence of a substituted guanidine and a negative reaction with ninhydrin, indicating the absence of ethylglycine. Nitrogen analysis showed that the product was impure since it contained 19% N (theory 30%). Several other batches were prepared but all proved to be impure. At this stage further purification was abandoned as a sample of pure N-ethyl-
guanidinoacetic acid was obtained from Dr. Armstrong (University of
Utah, Salt Lake City). This compound had been prepared from N-ethylglycine and cyanamide by a method which has been submitted for publication (Armstrong, 1955).

Preparation of \( \beta \)-guanidinopropionic acid.

This compound was prepared from \( \beta \)-alanine and S-ethyl-thiocurea hydrobromide according to the reaction:

\[
\begin{align*}
\text{S-CH}_2\text{CH}_3 & + \text{H}_2\text{N-CH}_2\text{CH}_2\text{COOH} \rightarrow \text{NH}_2 + \text{H}_2\text{N-CH}_2\text{CH}_2\text{GOCCH} \\
\text{NH}_2 & + \text{HS-CH}_2\text{CH}_3
\end{align*}
\]

The method was similar to that described by Brand and Brand (1942) for the preparation of guanidinoacetic acid.

49.2 g. (0.55 moles) of \( \beta \)-aminopropionic acid (\( \beta \)-alanine) and 92.5 g. (0.5 mole) of S-ethylthiocurea were dissolved in a total volume of 126 ml. of 2 \( \text{M} \) NaOH and allowed to stand at room temperature for one hour. The oily layer (ethyl mercaptan) which appeared on the surface was carefully removed. 50 ml. of ether was added and the solution allowed to stand for 60 hr. After that period the solution was slightly cloudy and contained a small amount of flocculent material which was filtered off. The solution was then extracted several times with ether in order to remove the dissolved mercaptan, and the dissolved ether was
removed by aeration. After adjusting to pH 7 the solution was treated with 4 volumes of ethanol and 2 volumes of acetone to yield a precipitate. This product was recrystallised several times from hot water until free from mercaptan. The yield was 14.6 g. Nitrogen analysis showed 32.5% N (theory 32.1). The product gave a positive test with diacetyl and α-naphthol and a negative test with ninhydrin. No further purification was carried out.

Preparation of 2-guanidinoethanesulphonic acid (Taurocyamine).

This compound was prepared from taurine (2-aminoethanesulphonic acid) and S-ethylthiourea according to the reaction:

\[
\text{HN}=\text{C} \quad \text{NH}_2
\]

\[\text{S-CH}_2\text{CH}_3 + \text{H}_2\text{N-CH}_2\text{CH}_2\text{SO}_3\text{H} \rightarrow \text{HN}=\text{C} \quad \text{NH}_2\quad + \quad \text{HS-CH}_2\text{CH}_3\]

37.5 g. of taurine (mixed BDH and Lights, recrystallised) dissolved in 100 ml. of water at 80° and 55 g. of S-ethylthiourea hydrobromide in 150 ml. of 2 N NaOH, cooled to 0°, were mixed together. The mixture was allowed to stand overnight at room temperature after which crystals appeared in the solution. The crystals were removed and the solution treated as described for guanidinopropionic acid. The solid obtained after precipitation
with acetone was combined with the crystals obtained originally from the reaction mixture and the combined product recrystallised from water. The yield was 27 g. (53% of theory) of colourless pyramidal shaped crystals. The melting point was $257-260^\circ$. This was in disagreement with the results of Thoai and Robin (1954) who prepared the compound and found a M.P. of $223-230^\circ$. A melting point of $224-226^\circ$ was also quoted by Dittrich (1878). The sample was then analysed and the following results were obtained:

<table>
<thead>
<tr>
<th>Element</th>
<th>C</th>
<th>H</th>
<th>O</th>
<th>N</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found(%)</td>
<td>21.1</td>
<td>5.5</td>
<td>29.0</td>
<td>25.1</td>
<td>19.0</td>
</tr>
<tr>
<td>Calculated(%)</td>
<td>21.5</td>
<td>5.4</td>
<td>28.7</td>
<td>25.1</td>
<td>19.1</td>
</tr>
</tbody>
</table>

At that stage a communication of Engel (1875) describing the preparation of taurocyamine came to hand. The melting point of this compound quoted there was $260^\circ$. As this agreed with the melting point obtained in the present work, and since the analytical results were satisfactory, further purification was considered unnecessary.

Preparation of the phosphorylated derivatives of the substituted guanidines.

The phosphorylation of the guanidino compounds was carried out in a similar fashion in all instances and according to the
finally isolated as a Ba-salt. The Ba-salt was reprecipitated several times from an aqueous solution by the addition of four volumes of ethanol, washed and dried. The yield was 10.4 g. of a white amorphous material. Analysis showed that the product contained four mols. of water of crystallisation. The nitrogen content was found to be 9.45% (theory 10.05%); the product contained 7.0% of organic phosphorus (theory 7.4%). The molar ratio N/org. P was 2.99 (theory 3.00). The product contained some inorganic phosphate which constituted 8.3% of the total P. Assuming that this phosphorus was present in the form of barium orthophosphate and on the basis of the other analytical figures the compound was 93-94% pure with an admixture of 6-7% of barium orthophosphate. For the purpose for which the compound was required this impurity was of no consequence and the product was used without further purification.

The phosphorylation of other substituted guanidines was carried out using the method described above for the preparation of phosphoguanidinopropionic acid. Experimental details of those preparations will therefore not be given with the exception of starting materials, fractionation, yields and analytical data that were obtained.
Phospho-guanidinoacetic acid.

Commercially available glycocyamine recrystallised from water was used for the reaction. 5.9 g. of glycocyamine, 100 ml. of 10 N NaOH and 15 ml. of POCI₃ were reacted. Fractionation with ethanol yielded an amorphous precipitate which was precipitated between two and four volumes of ethanol. The product was redissolved in water and treated with an equal volume of ethanol. After standing overnight at room temperature a fine crystalline precipitate appeared and was filtered off, washed consecutively with 50%, 75% and 95% ethanol, then ether and dried "in vacuo". The yield was 1.5 g. Treatment of the mother liquor with barium acetate and ethanol yielded a crop of the Ba salt which was set aside. Analysis of the crystalline Na-salt showed the presence of four mols of water of crystallisation. The product was found to contain 10.4% of organically bound P (theory 9.9%) and 13.1% N (theory 13.4). The molar N/P ratio was 2.8 (theory 3.0). The compound was used without further purification.

Phospho-guanidinoethanesulphonic acid (phosphotaurocyamine).

20 g. of taurocyamine, 55.5 g. (38 ml.) of POCI₃ and 160 ml. of 15 N NaOH were used for the reaction. The final volume
of the combined filtrate and washings was 250 ml. This solution
was fractionated with ethanol and it was found that the product
precipitated as an oil between 1.5 and 3.0 volumes of ethanol.
Attempts to crystallise the Na-salt were not successful and the
product was isolated as a Ba-salt by precipitation from aqueous
solution with four volumes of ethanol in the presence of barium
acetate. The barium salt was purified by reprecipitation and
dried "in vacuo" over CaCl₂. The yield was 11.6 g. Water analysis
showed that 3 mols of water were present. The formula
C₃H₅O₆N₃PSBa.3H₂O demands 8.8% N and 31.4% Ba. Found -8.6% N
and 31% Ba. No further purification was carried out.
SUMMARY

The synthesis of the following compounds has been described:

N-Ethylglycine
N-Ethylguanidinoacetic acid (ethylglycocyamine, negmine)
β-Guanidinopropionic acid
2-Guanidinoethanesulphonic acid (taurocyamine)
Phosphoguanidinoacetic acid (phosphoglycocyamine)
Phospho-β-guanidinopropionic acid
Phospho-2-guanidinoethanesulphonic acid (phosphotaurocyamine)
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