often been found that the thrombin formed in the presence of pyrocatechol was stable for periods up to 20 min., whilst in its absence, loss occurred after 4-5 min. On the other hand, in the case of a prothrombin concentration of approximately 20% of the normal value, or less, where the plasma is diluted 12 times, significant losses of thrombin occur even in the presence of pyrocatechol after 3 min. incubation. Despite this, a satisfactory assay can be carried out since complete conversion of prothrombin into thrombin occurs in that period. It is preferable to carry out the assay of prothrombin with citrated plasma, because calcium oxalate adsorbs prothrombin (Menghini, 1949). The assay both in a macro- and in a micro-form has been carried out in blood, plasma, and serum of man and other mammals and also in isolated plasma fractions.

SUMMARY

1. Phenols depress the antithrombic activity of serum.
2. This property, as well as the accelerating effect of phenols on the thrombin fibrinogen reaction, is utilized in a prothrombin assay.
3. Results of prothrombin assays in mammalian plasma in the presence of pyrocatechol are presented.

It is desired to acknowledge the financial assistance of the National Health and Medical Research Council, Australia. Many of the blood samples were provided by the Red Cross Blood Bank Transfusion Service, Melbourne. The fibrinogen preparation was a gift of Miss M. Nance, Lister Institute, London. Miss R. Donovan has given valuable technical assistance.

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Creatine Phosphokinase: Assay and Application for the Micro-determination of the Adenine Nucleotides

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(Received 29 August 1953)

The determination of creatine phosphokinase activity has been complicated by the fact that although creatine phosphate is extremely labile in acid solution, the assays of this enzyme which have been described in the literature (e.g. Banga, 1943; Askonas, 1951; Narayanaswami, 1952) have at some stage usually involved exposure to a pH well below 7. Creatine phosphate breakdown during deproteinization with trichloroacetic acid can be minimized by working at low temperature, but the subsequent determination of this substance by methods involving phosphate analysis presents

difficulties when the system contains other labile organophosphates or free phosphate. Usually creatine phosphate has been determined by phosphate analysis after fractionation (Meyerhof, 1930; LePage & Umbreit, 1945), or after hydrolysis with acid molybdate (Fiske & Subbarow, 1929). The latter method is not entirely satisfactory, however, as a number of workers, particularly Weil-Malherbe & Green (1951), have indicated that under the acid molybdate conditions, creatine phosphate may not be the only labile organophosphate. When creatine phosphate is estimated in systems containing

relatively higher concentrations of adenosine diphosphate or adenosine triphosphate the errors in the acid molybdate method may be considerable due to the slow breakdown of the latter substances. The method of Lowry & Lopez (1946) permits the estimation of phosphate in the presence of acid-labile organophosphates but even under these conditions there is a slow breakdown of creatine phosphate.

The estimation of creatine rather than phosphate as a means of following the reaction offers the advantage that determinations can be made under alkaline conditions, in which creatine phosphate is stable, by the method of Eggleton, Elsdon & Gough (1943). In general this method has not been widely used, but Ennor & Stocken (1948) have investigated some of the sources of error while Ennor & Rosenberg (1952) have adapted it for the estimation of the creatine phosphate content of tissues.

In the present paper details are given of a method for the assay of creatine phosphokinase activity by following the liberation of creatine. The interactions between the creatine phosphokinase system and the myokinase and hexokinase systems, respectively, have been studied and in particular the latter interaction has been adapted for the colorimetric estimation of extremely low concentrations of adenosine polyphosphates.

EXPERIMENTAL

Methods

Creatine phosphokinase. Minced rabbit muscle was treated for 30 sec. in a Waring Blendor with 3 vol. of a medium containing 0.025 M-KCl and 0.039 M borate buffer, pH 7.1. This medium extracts the minimum of protein from the myofibrils (Perry, 1953). After being stirred for 30 min. the extract was filtered through muslin, the residue re-extracted with 2 vol. of borate:KCl and again passed through muslin. The combined filtrates were centrifuged for 15 min. at 600 g and the preparation of creatine phosphokinase carried out either as described by Askonas (1951), employing butanol treatment followed by propanol fractionation, or by the Banga (1943) procedure up to the fourth stage. Preparations were finally dialysed with stirring for 14 hr. against glass-distilled water, and in some cases freeze-dried. Freeze-dried preparations were dissolved in glass-distilled water as they were required for use.

Crystalline yeast hexokinase. Hexokinase was prepared by Mr E. J. Morgan after Kunitz & Macdonald (1943) and stored at -15° as an $(\text{NH}_4)_2\text{SO}_4$ paste. For use the paste was dissolved in 1% (w/v) glucose and dialysed against 1% glucose for 24 hr.

Myokinase. Myokinase was prepared by the method of Kalckar (1947) and was stored at 0° as an $(\text{NH}_4)_2\text{SO}_4$ paste.

Muscle acetone-powder extracts. Dialysed extracts were prepared as described in the following paper (Perry, 1954).

Hexokinase activity. Hexokinase activity was assayed by following the disappearance of 10 min. P, i.e. inorganic P liberated on heating for 10 min. in π acid at 100° , when hexokinase was added to a reaction mixture containing 0.005 M- MgSO_4 , 60 μ moles glucose and 12–15 μ moles

adenosine triphosphate (ATP). The total volume was 2 ml. and incubation was at 30° . One unit of hexokinase activity was defined as that amount of enzyme which would transfer 1 μ mole of phosphate from ATP to glucose in 5 min.

Deproteinization. After incubation in phosphate buffer deproteinization was carried out using the alkaline reagents of Somogyi (1945). Usually after inactivation of the enzyme 0.2 ml. of 0.3 N- $\text{Ba}(\text{OH})_2$ was added followed by 0.2 ml. of the 5% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ reagent. When aminotrihydroxymethylmethane (Tris) buffer (Gomori, 1946), was used it was necessary to add NaOH to the $\text{Ba}(\text{OH})_2$ solution so that the deproteinized incubation mixture was just pink to phenolphthalein.

Creatine. Creatine as supplied by British Drug Houses Ltd. was recrystallized from hot water as the monohydrate.

Creatine phosphate. Sodium creatine phosphate hexahydrate was prepared synthetically by Mr E. J. Morgan using the method of Zeile & Fawaz (1938). It was stored in the cold over CaCl_2 , and 0.1 M solutions were made up as required. The creatine blank of these solutions was very low and did not increase appreciably during storage at -15° for periods up to 2 months.

Adenosine triphosphate. ATP was prepared by a method similar to that described by Needham (1942). The Ba salt was converted into the Na salt as described by Chappell & Perry (1953).

Adenosine diphosphate. ADP was prepared by Mr E. J. Morgan from ATP as described by Bailey (1942). It was stored as the Ba salt and converted into the Na salt by the same method as used for ATP.

Creatine estimation. The reagents were prepared and estimations carried out as recommended by Eggleton *et al.* (1943). The stock alkali was stored at 30° since at room temperature crystallization tended to occur. Colour intensities were measured using filter 303 in an EEL photoelectric colorimeter (Evans Electroselenium Ltd.). Some measurements were also made on a Beckman apparatus at 520 m μ .

Phenylmercuric acetate. This reagent was supplied by Hopkin & Williams. It was used in 0.05 M solution, made by dissolving 0.34 g. in 10 ml. dioxan and adding 10 ml. glass-distilled water. The *p*-chloromercuribenzoic acid used in some experiments was synthesized by Mr B. R. Slater by the method of Whitmore & Woodward (1932).

Adenylic acid. Dr A. L. Lehninger kindly provided the sample of adenylic acid (Bischof Ltd.) used in this investigation. No ATP or ADP could be detected in this sample by the chromatographic method of Eggleston & Hems (1952).

RESULTS

Essential requirements of the method were that the enzyme reaction should be stopped rapidly and effectively, and deproteinization be accomplished without the pH falling below 7 at any stage. The barium hydroxide and zinc sulphate reagents of Somogyi (1945), however, did not destroy completely the enzyme activity and were therefore employed after the enzyme had been inactivated as described below.

As there is considerable evidence suggesting that free sulphydryl groups are essential for creatine phosphokinase activity (Lehmann & Pollack, 1942;

Askonas, 1951; Bailey & Marsh, 1952), organomercury compounds were considered likely substances to inactivate this enzyme effectively. In particular *p*-chloromercuribenzoate and phenylmercuric acetate were examined and found to be highly satisfactory when used at a final concentration of 0.01M. *p*-Chloromercuribenzoate is conveniently soluble in alkali, but unlike phenylmercuric acetate was not readily available in the quantities required. Phenylmercuric acetate is

Table 1. *Inactivation of creatine phosphokinase by phenylmercuric acetate*

Each reaction mixture contained 0.005M-MgSO₄, 5 μ moles ADP, 10 μ moles creatine phosphate, 0.05M potassium phosphate buffer, pH 7.4; 0.5 ml. 0.05M phenylmercuric acetate was then added, followed by the enzyme. The total volume was 2.5 ml. The tubes were then incubated for 75 min. at 30°. In the control tube the enzyme was added at zero time and phenylmercuric acetate at the end of the incubation period.

Creatine phosphokinase (ml.)	Time of addition of phenylmercuric acetate	Creatine liberated (μ g.)
0	—	0
0.2	Zero time	2
0.4	Zero time	1
0.2	After 75 min.	300

Fig. 1. The recovery of creatine from incubation mixtures. Varying amounts of a standard creatine solution were added to a typical reaction medium, which contained 0.005M-MgSO₄, 10 μ moles creatine phosphate, 5 μ moles ADP, 0.36 mg. serum albumin, and either 0.1M potassium phosphate buffer, pH 7.4, or 0.05M Tris buffer, pH 7.4; total volume, 2.0 ml. To this medium 0.5 ml. 0.05M phenylmercuric acetate was added, and the deproteinization method appropriate to the buffer was carried out. Creatine was then estimated in the deproteinized solutions. +, direct estimation of creatine; □, recovery with phosphate buffer; ○, recovery with Tris buffer.

only sparingly soluble in water but was used as a 0.05M solution in 50% (v/v) aqueous dioxan in which it was readily soluble. It was apparent from many experiments, an example of one type being given in Table 1, that at the concentration used phenylmercuric acetate completely and instantaneously inactivated the enzyme.

An additional advantage of using organomercurials to inactivate the enzyme was that they also effectively removed sulphhydryl compounds (cysteine was added to the incubation medium in some assays) which might have inhibited the colour formation in subsequent creatine estimations (Ennor & Stocken, 1948).

Some care was necessary in the choice of buffer for the enzyme assays because the development of colour in the creatine estimation was inhibited by buffers such as glycine, glycyglycine and veronal. Phosphate and Tris buffers were found to be suitable. With the usual incubation mixture containing phosphate buffer to which phenylmercuric acetate was later added followed by alkaline deproteinization, recovery was quantitative; when Tris was used, however, the recovery was only about 80%, but the results were reproducible (Fig. 1). Similar results were obtained when enzyme tests were carried out in 0.01M cysteine or over a wide range of magnesium sulphate concentrations. Doubling the phenylmercuric acetate concentration or increasing the dioxan present during the colorimetric estimation to 20% had no effect on creatine recovery. Finally the procedure given below was adopted.

Measurement of creatine phosphokinase activity

The incubation mixture contained 0.05M phosphate or Tris buffer, pH 7.4, 0.005M-MgCl₂ or MgSO₄, 0.002–0.005M ADP, 0.01M cysteine (freshly neutralized before use), 0.005M creatine phosphate, enzyme solution and water to 2 ml. Incubation was carried out at 30°. The enzyme solution was diluted appropriately just before assay and added to the incubation tube, which was then allowed to equilibrate at 30° for 4 min. The reaction was started by the addition of the creatine phosphate and stopped after 3–5 min. by the addition of 0.5 ml. of 0.05M phenylmercuric acetate in 50% (v/v) aqueous dioxan. Creatine liberation was determined at several concentrations of enzyme. The results were plotted graphically, as in Fig. 2, and the activity of the preparation calculated from the slope.

Aqueous extracts of muscle acetone powder and purified enzyme preparations obtained by the methods of Banga (1943) and Askonas (1951), respectively, had extremely high creatine phosphokinase activity and needed to be diluted several hundred times before they could be satisfactorily

assayed. To avoid spontaneous inactivation, cysteine was an essential component of the incubation medium when these dilute preparations were used. This feature is well illustrated in Fig. 2 which compares the effect of enzyme concentration with and without cysteine present in the incubation mixture, on the amount of creatine liberated in a system containing ADP, creatine phosphate and Mg^{2+} . Fig. 3 shows that magnesium ion is an essential component of the system; in its absence

Fig. 2. The effect of enzyme concentration on creatine phosphokinase activity. The complete incubation mixture contained 0.005M- $MgSO_4$, 5 μ moles ADP, 10 μ moles creatine phosphate, 0.01M cysteine, 0.05M Tris buffer, pH 7.4. After temperature equilibration the enzyme was added and incubated for 5 min. at 30°. The total volume was 2.0 ml. $\times$, $\square$, duplicate determinations with the complete system; $\circ$, without cysteine.

Fig. 3. The effect of Mg^{2+} concentration on creatine phosphokinase activity. The conditions were as described in Fig. 2.

the activity was only about 3% of that obtained in the presence of 0.005M- $MgSO_4$, the optimum concentration for activation. Increasing the concentration of ADP resulted in an increase in the rate of creatine liberation up to a maximum at approximately 0.002M. Further increase in ADP concentration produced a very slight fall in activity (Fig. 4).

Under standard incubation conditions the method gave reproducible values for various creatine phosphokinase preparations; this is illustrated in Table 2 which contains the results of five independent determinations, carried out over a period of 13 days, of the creatine phosphokinase activity of an aged dialysed aqueous extract of muscle acetone powder.

Interaction of creatine phosphokinase and hexokinase: estimation of adenine nucleotides

When hexokinase and glucose are added to the creatine phosphokinase enzyme system the following sequence of reactions take place:

Fig. 4. The effect of ADP concentration on creatine phosphokinase activity. The conditions were as described in Fig. 2.

Table 2. *Creatine phosphokinase activity of aged dialysed aqueous extract of muscle acetone powder*

Incubation carried out at 30° in presence of 0.005M ADP and 0.005M- $MgCl_2$.

Date	Activity (μ g. creatine liberated/hr./ml. enzyme)	Dilution used for assay
30. i. 53	161 000	1 in 500
31. i. 53	173 000	1 in 500
3. ii. 53	161 000	1 in 1000
6. ii. 53	171 000	1 in 1000
11. ii. 53	164 000	1 in 1000

In the presence of excess hexokinase very little ATP will accumulate in the system for as rapidly as it is formed by the Lohmann reaction it will be reconverted to ADP. Thus when both ADP and hexokinase are present in excess relative to creatine phosphokinase, the amount of creatine liberated is directly proportional to the activity of the creatine phosphokinase. This was demonstrated experimentally under conditions in which doubling the amount of hexokinase present or halving the concentration of ADP did not appreciably affect the rate of creatine production at any of the concentrations of creatine phosphokinase used.

It might also be expected from the equations (1) and (2) that ADP would become the limiting factor in the presence of excess of creatine phosphokinase and a given hexokinase concentration. The results shown in Fig. 5 indicate that this indeed is the case. Purified enzyme preparations (Askonas, 1951) were used in this particular system and the amount of creatine liberated was in linear relation to the amount of added ADP. No creatine was liberated in the absence of added ADP.

A feature of this system was its extreme sensitivity to low concentrations of ADP, e.g. in the experiment illustrated in Fig. 6, 0.0056 μ mole ADP (i.e. 2.4 μ g.) gave rise to 47 μ g. creatine after 6 min. incubation. ATP behaved in a similar manner and when compared on a molar basis with ADP in the same system it was equally effective in catalysing the liberation of creatine (Fig. 6).

Purified creatine phosphokinase is specific for ADP and will not phosphorylate adenylic acid (Banga, 1943), and hence the latter substance did not catalyse the liberation of creatine at any of the concentrations tried. In the presence of myokinase, adenylic acid was almost as efficient as ADP, on a molar basis, in catalysing the liberation of creatine (Fig. 5). This system is of particular interest in that if it contained only creatine phosphokinase, hexokinase, myokinase, creatine phosphate, adenylic acid and glucose there would be no production of ADP, which is essential for the liberation of creatine by the creatine phosphokinase reaction. For the conversion of adenylic acid (AMP) into ADP by the myokinase reaction ATP is necessary (reaction 3).

Only a very small amount of ATP relative to the adenylic acid added need be present for the reactions (3) and (1) working together to bring about the autocatalytic conversion of adenylic acid to ADP. In the conditions employed in the experiment illustrated in Fig. 5 the hexokinase is tending to compete with the autocatalytic action of reactions (1)

and (3) by reconvert ATP into ADP, but the myokinase reaction can produce two molecules of ADP per molecule of ATP, whereas the hexokinase reaction can yield only one. The net result in presence of the creatine phosphokinase system is that there will be an autocatalytic production of ATP. This ensures that when excess of myokinase is present the adenylic acid will exist virtually as ADP. That this is so is shown by Fig. 5 where adenylic acid is practically as efficient as ADP or ATP in liberating creatine under the particular experimental conditions. This means that the

Fig. 5. The effect of varying the ADP and adenylic acid concentration in the presence of creatine phosphokinase (Askonas, 1951), hexokinase and myokinase. The incubation medium contained 0.05 M Tris, 0.005 M-MgSO₄, 10 μ -moles creatine phosphate, 50 μ moles glucose, 6 units hexokinase. The total volume was 2.0 ml. The reaction was started by adding creatine phosphokinase and stopped after 5 min. at 30°. x, ADP concn. varied; +, adenylic acid concn. varied; O, adenylic acid concn. varied in the presence of myokinase.

Fig. 6. Liberation of creatine by ADP and ATP in creatine phosphokinase-hexokinase system. Incubation medium contained 0.05 M phosphate buffer, pH 7.4, 0.005 M-MgCl₂, 10 μ moles creatine phosphate and 60 μ moles glucose. 6 min. incubation at 30°. O, ATP; x, ADP.

enzyme preparations must contain sufficient ADP or ATP to prime the myokinase reaction, though the concentrations of ADP or ATP needed are much lower than that of the added adenylic acid.

It is of interest to note that similar results with ATP and ADP on the one hand and adenylic acid on the other can be obtained if hexokinase is replaced by a magnesium-activated ATPase as is found in isolated myofibrils (see Perry, 1954).

Creatine phosphokinase and myokinase

When myokinase, creatine phosphokinase and the appropriate substrates are incubated together the reactions which occur are represented by equations (3) and (1). If excess of creatine phosphokinase and creatine phosphate are present the rate of creatine liberation will depend on the ADP concentration, i.e. in the coupled system on the rate of ADP formation by myokinase. It should be possible to arrange the conditions so that the rate of creatine liberation is a measure of myokinase activity, the assay of which by existing methods is by no means an easy matter.

In Fig. 7 is shown the progress curve when creatine phosphokinase, myokinase, creatine phosphate, adenylic acid and ATP are incubated together. The rate of creatine liberation is steady over a period of 10 min., but if ATP is omitted an initial lag phase appears in the progress curve. During this period the rate of creatine liberation starts very slowly and

builds up until after some minutes it is equal to the rate obtained when ATP is added initially. This lag phase is presumably due to the fact that the enzyme preparations contain enough ATP or ADP to prime the autocatalytic formation of ADP by the combined action of reactions (3) and (1). Ultimately enough ATP and adenylic acid are present so that the enzyme myokinase itself rather than its substrates become limiting and the rate of creatine liberation is identical with that obtained when adenylic acid and ATP were both present in excess initially. The results illustrated in Fig. 7 were obtained with myokinase and creatine phosphokinase (Askonas, 1951) which had been dialysed with constant stirring against distilled water for 24 hr. at 0°. They indicate too why it was possible to assay adenylic acid in the absence of added ATP by the creatine phosphokinase-hexokinase-myokinase systems described earlier. In this latter system (see Fig. 6) the added adenylic acid was present in about one-hundredth of the concentration used in the experiment illustrated in Fig. 7, and hence in the presence of high myokinase and creatine phosphokinase activities and traces of ADP or ATP, adenylic acid was virtually as effective in catalysing the liberation of creatine as was the same concentration of ADP.

DISCUSSION

The procedure described offers a satisfactory method of assaying creatine phosphokinase activity under the defined conditions, the only real limitation being in the choice of a buffer which does not interfere with the colorimetric determination of creatine. The method also lends itself to the study of coupled systems involving creatine phosphokinase and other enzymes which use ADP or ATP as substrate. By virtue of the fact that in these coupled systems the adenine nucleotides play coenzyme-like roles, small amounts of them will produce large overall changes in the system. The creatine phosphokinase-hexokinase system is particularly useful for the detection and estimation of small amounts of ADP and ATP. When both are present it does not distinguish between them as these substances are equally effective in catalysing creatine liberation. If the preparations are free of myokinase, ADP and ATP on the one hand can be readily distinguished from adenylic acid on the other. Usually, creatine phosphokinase prepared by the methods of Banga (1943) or Askonas (1951) are free of myokinase. The feature of this method of determination of the adenine nucleotides is its extreme sensitivity. As used in this and the following paper (Perry, 1954) the amounts of adenine nucleotide determined have been in the range 0.5–5.0 $\mu\text{g.}$, but by prolonging incubation times and decreasing the final volume used for colorimetric

Fig. 7. Progress curve obtained with dialysed myokinase in the presence of excess of dialysed creatine phosphokinase (Askonas, 1951) at 30°. The complete system contained 1.5 μmoles adenylic acid, 0.08 μmole ATP, 10 μmoles creatine phosphate, 0.005 M-MgSO₄, 0.05 M Tris buffer, pH 7.4; total volume, 2.0 ml. Creatine phosphokinase was added directly before the myokinase. ○, complete system; +, without ATP.

measurement it should be possible to increase the sensitivity by a considerable extent. The rate of creatine liberation by a particular nucleotide sample will vary with the enzyme preparations and it is necessary to determine the rate of creatine liberation with graded amounts of known standard nucleotide at the same time and under identical conditions.

The experiments indicate that even dialysed purified preparations of creatine phosphokinase are liable to be contaminated with very small amounts of ADP and ATP. It should be emphasized, however, that although these nucleotides contaminate the enzyme, the amounts must be extremely small as in the experiment shown in Fig. 6 none could be demonstrated. Here, in the absence of added ADP, using the same creatine phosphokinase as was used in the experiments illustrated in Fig. 7, there was no liberation of creatine. Nevertheless, such small amounts of ADP or ATP would be adequate to prime the autocatalytic generation of ATP from adenylic acid by the combined action of creatine phosphokinase and myokinase.

SUMMARY

1. A method for the assay of creatine phosphokinase is described. The method involves creatine estimation under conditions in which creatine phosphate is completely stable.

2. The coupled creatine phosphokinase-hexokinase system has been demonstrated to be an extremely sensitive means of determining 0.5–5.0 $\mu\text{g.}$ of ADP and ATP.

3. By the addition of myokinase the creatine phosphokinase-hexokinase system can be used to determine small amounts of adenylic acid.

4. Some aspects of the coupled creatine phosphokinase-myokinase system have been studied.

During this investigation one of us (J. B. C.) was supported by a Medical Research Council Studentship. For this award and a Research Expenses Grant made by the same body (to S. V. P.), we wish to express our gratitude.

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Creatine Phosphokinase and the Enzymic and Contractile Properties of the Isolated Myofibril

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(Received 29 August 1953)

Myofibrils from rabbit skeletal muscle which have been carefully isolated and freed from other intracellular components contain a small but constant amount of nucleotide (Perry, 1952, 1953). This nucleotide consists mainly of adenosine diphosphate (ADP), and in view of the importance of the adenosine polyphosphates in cell metabolism, and in the contractile process in particular, the close associa-

tion of ADP with the isolated contractile unit itself appears to be of some significance. Wherever ADP is found in the cell, enzyme mechanisms exist for its phosphorylation to adenosine triphosphate (ATP), and it is therefore reasonable to suggest, as was done in an earlier communication (Perry, 1952), that some system must be present in the muscle cell for the conversion of the myofibrillar ADP into ATP.