counted for in residual lymph, implying that the remainder of the radioactivity present was in the form of protein which had lost its original specific immunological configuration.

3. It is concluded that transfused antibody entered cells in certain organs which became highly labelled, notably in liver, kidney, spleen, bone marrow, lung and gut, without being broken down to the amino acid level, and was rapidly changed into some other kind of intracellular protein. The level of radioactivity in the plasma free amino acid fraction at death, however, was high enough to be consistent with the idea that the protein radioactivities of other poorly labelled tissues, notably muscle, could be due to incorporation of labelled free amino acids.

4. Evans Blue uptake may be a convenient guide to plasma protein assimilation by tissues. This dye is found in high concentration in the highly labelled tissues and hardly at all in the poorly labelled ones, supporting the view that the cells of the latter are impervious to proteins.

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REFERENCES

- Bradley, J. E. S., Holloway, R. C. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 192.
 Dovey, A., Holloway, R. C. & McFarlane, A. S. (1952). *Biochem. J.* **51**, xxvi.
 Drinker, C. K. & Yoffey, J. M. (1941). *Lymphatics, Lymph and Lymphoid Tissue*, pp. 149-53. Cambridge, Mass: Harvard University Press.
 Heidelberger, M., Treffers, H. P., Schoenheimer, R., Ratner, S. & Rittenberg, D. (1942). *J. biol. Chem.* **144**, 555.
 Humphrey, J. H. & McFarlane, A. S. (1954). *Biochem. J.* **57**, 186.
 Keppie, J. & Macfarlane, M. G. (1948). *Brit. J. exp. Path.* **29**, 458.
 Keston, A. & Dreyfus, J. C. (1951). *Fed. Proc.* **10**, 206.
 Khesin, R. V. (1952). *C.R. Acad. Sci. U.R.S.S.* **84**, 1209. From *Chem. Abstr.* **46**, 10,229.
 Rawson, R. A. (1943). *Amer. J. Physiol.* **138**, 708.
 Yuile, C. L., Lamson, B. G., Miller, L. L. & Whipple, G. H. (1951). *J. exp. Med.* **93**, 539.

Some Properties of Creatine Phosphokinase

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The enzyme creatine phosphokinase responsible for catalysing the reaction

was first described by Lohmann (1934), who showed that in extracts of skeletal muscle the two labile phosphate groups of adenosine triphosphate were transferred to creatine. The reversibility of the reaction was demonstrated by Lehmann (1935), and it was claimed by both of these authors that the dephosphorylation of adenosine triphosphate proceeded to adenylic acid with the result that 2 molecules of phosphocreatine were formed. Banga (1943) effected a partial purification of the enzyme, and the preparation was able to transfer only the terminal phosphate group of adenosine triphosphate to creatine with the formation of phosphocreatine. In addition, Banga (1943) claimed that another enzyme was responsible for the transfer of phosphate from

adenosine diphosphate to creatine. This was named adenosine diphosphate-creatine phosphotransferase in contrast to adenosine triphosphate-creatine phosphotransferase. Later Soreni & Degtyar (1948) achieved a partial purification of creatine phosphokinase and described certain of its properties. Recently Askonas (1951) has described a purification technique depending upon propanol fractionation. The properties of creatine phosphokinase present in cerebral cortical tissue were described by Narayanaswami (1952), but in this work no attempt was made to effect any purification. The present communication is concerned with a more detailed investigation into the properties of creatine phosphokinase than has hitherto been reported.

Abbreviations

The following abbreviations will be used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine 5'-phosphate; PC, phosphocreatine; CPK, creatine phosphokinase; THAM, aminotris(hydroxymethyl)methane.

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EXPERIMENTAL

Materials

Creatine phosphokinase. The CPK used in these studies was prepared from sheep skeletal muscle by a slight modification of the method described by Askonas (1951). It was found that the protein fraction precipitated between 50 and 60% (v/v) propanol was most active. The enzyme was stored at 1° in 0.001M-THAM, pH 7.2, to which was added a drop of toluene. The protein content was such that 1 ml. of the preparation contained 4 mg. N. This preparation was unable to release inorganic P from ATP, ADP, AMP or PC when incubated for periods up to 1 hr. at pH 7.2 or 10.5 at 37° in 0.025M-THAM.

The preparation was also free from myokinase activity, since no inorganic P was released from ADP in the presence of myosin and CPK.

The experiments were carried out over a period of 4 months and during this time no deterioration in activity was observed. In experiments concerned with the forward reaction it was the practice to prepare hundredfold dilutions immediately before use, and in all the experiments described such freshly made dilutions were used because of the fear that oxidation of SH groups of the enzyme may occur if solutions containing only 40 µg. N/ml. were kept for any length of time. Recently, however, activity tests have been carried out on some hundredfold diluted solutions which have been kept at +1° and at -15° for over 3 months, but no evidence of a decrease in activity was observed. One preparation of CPK has been freeze-dried and stored at -15° for 15 months without any loss of activity.

Myosin. This was prepared by the method of Bailey (1942) from rabbit skeletal muscle; after four precipitations it was free from myokinase.

Myokinase. This was prepared from rabbit skeletal muscle as described by Kalckar (1943).

Apprase. This was prepared according to Krishnan (1949) from potatoes.

Adenosine triphosphate and adenosine diphosphate. ATP was prepared from rabbit muscle as described by LePage (1945). ADP was prepared from ATP after incubation with myosin and was separated from the digest by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). Both nucleotides were prepared as the Ba salts and were over 95% pure as judged by chemical and enzymic analysis. They were converted into the Na salts before use in the following way. The requisite amount of the Ba salt was weighed out and suspended in water. The slightly alkaline (pH 7.5-8.0) suspension was then shaken with an excess of Amberlite ion-exchange resin (1R-100 Na form) until solution was effected. In practice 100 mg. dibarium ATP suspended in 10 ml. water was shaken with 5 g. of resin and was completely dissolved in about 5 min. The supernatant, after having been tested with Na rhodizonate to ensure absence of Ba²⁺, was combined with several aqueous washes of the resin and made up to the required volume with water. This method was more satisfactory than the conventional precipitation of Ba as BaSO₄, since losses due to adsorption were avoided. It also eliminated the risk of hydrolysis, which may have ensued if the Ba salts were dissolved in acid.

Adenylic acid. AMP was prepared from ATP by the action of apyrase and was purified as described by Kerr (1941). 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 0.1N-NaOH.

Phosphocreatine. PC was prepared as the Na salt as described by Ennor & Stocken (1948b). Solutions were made up as required and adjusted to pH 7.2 by the addition of 0.1N-NaOH.

All solutions of ATP, ADP and PC when not in use were stored at -15°.

Cysteine ester hydrochloride. This was prepared by the method of Friedmann (1903) as described by Ennor & Stocken (1948a). All solutions were made up immediately before use and were adjusted to pH 7.2 by the addition of 0.1N-NaOH.

Creatine. The creatine used was a commercial preparation which was thrice recrystallized as described by Hunter (1928), and its purity checked by analysis of nitrogen. All solutions were freshly prepared before use.

Inhibitors. Most inhibitors used were commercial preparations and were of the highest purity obtainable. Exceptions were *p*-chloromercuribenzoic acid, which was synthesized as described by Whitmore & Woodward (1932), and *o*-iodosobenzoic acid and *N*-ethylmaleimide which were gifts from Drs K. Pausacker and F. Hird of Melbourne University.

Buffer system. In the initial experiments several buffer systems were employed but, with one exception, all either proved inhibitory to the enzyme (phosphate and borate) or interfered with the estimation of creatine (glycine). THAM proved free from these objections and was used over the range pH 7.2-10.5. Two samples of THAM were used, one being an Eastman product kindly provided by Dr C. Long of the University of Aberdeen, and the other having been obtained from Sigma Chemical Co., U.S.A. Both were recrystallized before use from 90% (v/v) ethanol.

Methods

The technique adopted to determine the activity of the enzyme was as follows.

Forward reaction. Test tubes containing 2.0 ml. 0.1M-THAM of appropriate pH, 0.2 ml. of CPK ($\approx 8 \mu\text{g. N}$), 1.0 ml. of 0.002M-PC, and the activators or inhibitors under study in a total volume of 6.0 ml., were equilibrated in a water bath at 37°. The reaction was started by the addition of 2.0 ml. of 0.001M-ADP and stopped at the requisite time by the addition of 2.0 ml. of 25% (w/v) trichloroacetic acid. The tubes were then quickly cooled in an ice/salt mixture and, as filtration was unnecessary, 2.0 ml. samples were withdrawn for analysis of the creatine released. These were transferred to 10 ml. graduated tubes containing sufficient 5N-NaOH to render the sample alkaline. The creatine present was determined as described by Ennor & Stocken (1948a) except for one modification. Since many of the enzyme digests contained alkaline-earth metals these, when present in the higher concentrations, formed precipitates when the alkaline (NaOH and Na₂CO₃) solution of α -naphthol was added to the sample taken for creatine determination. Such precipitations were avoided by the addition of ethylenediaminetetraacetic acid (Versene).

Reverse reaction. Similar volumes of 0.1M-THAM and enzyme were added as before, but in this case the enzyme concentration was increased 50-fold, 2.0 ml. of 0.001M creatine plus activators and/or inhibitors in a total volume of 6.0 ml. After equilibration at 37° the reaction was started by the addition of 2.0 ml. of 0.001M-ATP. The subsequent

treatment was as described above except that here filtration after the addition of trichloroacetic acid was necessary.

In some experiments determinations were made of the PC remaining or synthesized, and in these cases the method described by Ennor & Rosenberg (1952) was employed. In no case was ATP or ADP measured.

RESULTS

Forward reaction: ADP + PC → ATP + creatine

pH optimum. Determination of the pH optimum (Fig. 1) was made in the presence of optimum amounts of Ca^{2+} (see below). The enzyme digests were incubated for 3 min. at 37° .

In these experiments, THAM buffers were used to cover the range pH 7.2–9.5, while succinate buffers at a final concentration of 0.005M were employed to cover the range pH 4.5–6.5. Under acid conditions there was an appreciable hydrolysis of PC and in all cases appropriate control tubes were incubated to determine the necessary corrections.

The results indicate a well-defined optimum at pH 7.2 and all subsequent experiments were carried out at this pH.

Reaction rate and the effect of cysteine. To permit an adequate investigation of the effect of activators and inhibitors the progress of the reaction with time was determined.

For this purpose two bulk solutions were made up, both containing Ca^{2+} in optimum amounts (see below) but only one with cysteine ester (10^{-4}M). In addition both contained 5 μmoles of ADP and of PC. After equilibration at 37° the reaction was started by the addition of CPK (final concentration, 1 $\mu\text{g. N/ml.}$). Samples were withdrawn at intervals and transferred to tubes containing sufficient 25% (w/v) trichloroacetic acid to bring the final concentration to 5%. Analyses for creatine were then carried out as described above.

Under the conditions of the experiment it is apparent (Fig. 2, lower curve) that of the total amount of creatine released up to the time when equilibrium was reached (about 25 min.), 50% is released in 3 min. Moreover, it will be noted that the speed of the reaction is constant up to this time. As a consequence of this result the effect of the addition of various ions, etc., has been studied following the 3 min. period.

The effect of cysteine (Fig. 2, upper curve) is to produce an acceleration in the reaction rate but no shift in the equilibrium.

Effect of Ca^{2+} and Mg^{2+} . To test the influence of Ca^{2+} and Mg^{2+} , two enzyme concentrations were chosen, corresponding to 0.5 and 1.0 $\mu\text{g. N/ml.}$ of the digest. Constant amounts of enzyme in each tube were ensured by the preparation of two stock solutions each containing PC, CPK, THAM and water, and varying only in their content of CPK. Samples of stock solutions were pipetted into test

tubes containing varying amounts of Ca^{2+} or Mg^{2+} and water to bring the volume to 6.0 ml. After equilibration at 37° the reaction was started by the addition of 2.0 ml. of 0.001M-ADP and stopped, 3 min. later, by the addition of 2.0 ml. of 25% (w/v) trichloroacetic acid.

The results of this experiment (Fig. 3) show that at both enzyme concentrations the activity of the enzyme increased rapidly with increasing Ca^{2+} concentration, and that there was no activity in the

Fig. 1. Effect of pH on CPK activity. The experimental points marked ●—● and ○—○ were obtained using 0.025M sodium succinate and THAM, respectively. For experimental details see text.

Fig. 2. Reaction rate in presence and absence of cysteine. The points ○—○ and ●—● represent analytical data from reaction mixtures in the absence and presence of cysteine ($1 \times 10^{-4}\text{M}$), respectively.

Fig. 3. Creatine phosphokinase activity in the presence of varying Ca^{2+} concentration. Two concentrations of CPK were used; $0.5 \mu\text{g./ml.}$, $\bullet-\bullet$; and $1.0 \mu\text{g./ml.}$, $\circ-\circ$. Abscissa: $\mu\text{g. Ca}/\mu\text{g. CPK nitrogen}$.

absence of Ca^{2+} . A maximum was reached when the ratio of $\mu\text{g. Ca}/\mu\text{g. N}$ (Ca/N) was between 100 and 150, and with increasing Ca^{2+} concentration the activity fell slightly. The results obtained with the highest Ca/N ratio (2000) investigated are not shown, but at this level the amounts of creatine released with 0.5 and $1.0 \mu\text{g. N/ml.}$ digest were 28.0 and $42.2 \mu\text{g.}$, respectively, corresponding to respective inhibition of 48 and 66% . Since the maximum enzyme activation at each of the two enzyme levels investigated occurred over the same range of Ca/N ratios, it may be inferred that it is not the concentration of Ca^{2+} but the ratio of this concentration to that of the enzyme that is important.

The influence of Mg^{2+} was investigated under precisely the same conditions, and the results were practically identical with those obtained with Ca^{2+} . Thus no activity was apparent in the absence of Mg^{2+} , and when the Mg/N ratio was increased to 2000 the amounts of creatine released in the presence of 0.5 and $1.0 \mu\text{g. N/ml.}$ digest were 32.5 and $38.5 \mu\text{g.}$, representing respective inhibitions of 54 and 70% .

An additional experiment has been carried out wherein tubes containing the usual experimental mixture were incubated for 6 min. , at which time equilibrium was reached. The Ca^{2+} concentration was then increased to give a Ca/N ratio of 2000, and the tubes incubated for a further 6 min. The appropriate controls were also set up and the results (Table 1) indicate that the effect of a high Ca/N ratio is inhibitory and that increased amounts of Ca^{2+} had no effect similar to that described in the case of the reverse reaction (see below).

Table 1. The inhibitory effect of Ca^{2+} on CPK activity in the forward reaction

All tubes contained ADP and PC in the usual concentrations. Temp. 37° .

Tube no.	Treatment	Creatine liberated (μmoles)
1	6 min. incubation, $\text{Ca/N}=100$	1.16
2	12 min. incubation, $\text{Ca/N}=100$	1.16
3	6 min. incubation, $\text{Ca/N}=4000$	0.26
4	12 min. incubation, $\text{Ca/N}=4000$	0.40
5	6 min. incubation, $\text{Ca/N}=100$, followed by 6 min. incubation $\text{Ca/N}=4000$	1.17

Table 2. The effect of various substances on CPK activity in the forward reaction

All experiments were carried out under optimum conditions (see text) and tubes incubated for 3 min. at 37° .

Compound	Concentration (M)	Inhibition (%)
NaF	1.0×10^{-3}	21
	6.5×10^{-2}	100
I_2 in KI	1.3×10^{-5}	30
Sodium citrate	1.3×10^{-3}	6
	1.3×10^{-2}	87
Na_2HPO_4	1.3×10^{-3}	0
	1.3×10^{-2}	95
MnCl_2	1.0×10^{-2}	46
$\text{Na}_2\text{B}_4\text{O}_7$	5.0×10^{-3}	12
Sodium azide	1.3×10^{-3}	12
	1.3×10^{-2}	18
Sodium <i>p</i> -chloromercuribenzoate	1.0×10^{-8}	6
	1.0×10^{-7}	29
	1.0×10^{-6}	100
Sodium iodoacetate	1.0×10^{-6}	12
	1.0×10^{-5}	30
	1.0×10^{-4}	100
Dinitro- <i>o</i> -cresol	1.3×10^{-3}	0
	6.5×10^{-3}	95
<i>N</i> -Ethyl maleimide	1.0×10^{-6}	38
	1.3×10^{-5}	100
Sodium iodosobenzoate	1.0×10^{-8}	13
	1.3×10^{-7}	100
Thyroxine	2.5×10^{-5}	11
Ethylenediaminetetraacetic acid	1.0×10^{-5}	12
	5.0×10^{-5}	73
	1.0×10^{-4}	80

On the basis of the above results all experiments on the forward reaction were carried out in the presence of such amounts of Ca^{2+} as gave a Ca/N ratio = 100, and this is referred to as the optimum ratio.

The effect of inorganic ions other than Ca^{2+} and Mg^{2+} . It will be seen from Table 2 that at a concentration of 0.013 M , phosphate produces an inhibition. A similar remark applies to citrate. Varying degrees of inhibition were produced by the other ions tested. The effects of Ba^{2+} and Sr^{2+} were somewhat different, and in view of the influence of

Table 3. *The effect of the alkaline earth metals on CPK activity*

With the exception of alkaline earth metals all tubes contained the usual experimental mixture (see text).

Concentration CaCl_2 (M)	2×10^{-3}	2×10^{-3}	2×10^{-3}	—	—
Concentration BaCl_2 (M)	—	2×10^{-3}	—	2×10^{-3}	—
Concentration SrCl_2 (M)	—	—	2×10^{-3}	—	2×10^{-3}
$\mu\text{moles creatine released}$	0.65	0.53	0.20	0.09	0

Table 4. *The reversal of the p-chloromercuribenzoate inhibition by cysteine*

Experiments carried out on the forward reaction under optimum conditions (see text). 3 min. incubation at 37° .

Concentration of <i>p</i> -chloromercuri- benzoate (M)	Concentration of cysteine (M)	Creatine released (μmoles)	Inhibition (%)
—	—	8.66	0
1×10^{-7}	—	6.35	27.7
1×10^{-7}	1×10^{-7}	7.97	8.0
1×10^{-7}	2×10^{-7}	8.55	4.0
1×10^{-6}	—	1.53	82.4
1×10^{-6}	1×10^{-6}	1.45	83.4
1×10^{-6}	2×10^{-6}	1.83	78.8

Ba^{2+} on the reverse reaction (see below) the results are dealt with separately (Table 3).

In this experiment BaCl_2 and SrCl_2 were added to reaction mixtures in the presence and absence of optimum amounts of Ca^{2+} , and the mixtures incubated for 3 min. at 37° . The results (Table 3) show that whilst Ba^{2+} produced a small degree of activation of the enzyme in the absence of Ca^{2+} , it is somewhat inhibitory when Ca^{2+} is present in optimum amounts. Sr^{2+} , on the other hand, was not only unable to activate the enzyme but was definitely inhibitory to the complete system.

The effect of metabolic inhibitors. The compounds with the most pronounced inhibitory effects are seen to be (Table 2) those generally used for their reactivity with SH groups either by virtue of their ability to oxidize or to form a complex. An exception is dinitro-*o*-cresol.

In the case of *p*-chloromercuribenzoate some experiments have been carried out to determine the degree to which the inhibition produced by this compound can be reversed by the addition of cysteine. In these experiments the inhibitor was added to the system 5 min. before the cysteine, which was added 5 min. before the reaction was started by the addition of ADP. The results (Table 4) indicate that the *p*-chloromercuribenzoate inhibition can be partially counteracted by the added cysteine.

The role of AMP as a P acceptor in the CPK-catalysed reaction. Several experiments have been carried out to determine the ability of AMP to accept phosphate from PC in the presence of CPK, but in no case was AMP phosphorylated. It seemed therefore reasonable to suspect the presence of myokinase in the impure systems hitherto used (Lohmann, 1934; Lehmann, 1935; Banga, 1943; Narayanaswami, 1952), and the effect of this enzyme when added to the CPK system has been determined.

Fig. 4. Substrate specificity in the CPK-catalysed reaction. PC and CPK present and AMP added as test substrate at zero time, ATP added in trace amounts at A and myokinase at B. For details see text.

20.0 ml. 0.1 M-THAM, pH 7.2, 5.0 ml. 0.001 M-PC, 1.0 ml. 0.2 M- CaCl_2 , 0.2 ml. CPK ($\approx 80 \mu\text{g}$. N), and 43.6 ml. water were pipetted into a flask and equilibrated at 37° . 10.0 ml. of 0.001 M-AMP was added at zero time and 5.0 ml. samples were withdrawn at intervals up to 10 min. and transferred to tubes containing 1.5 ml. of 25% (w/v) trichloroacetic acid. 1.0 ml. 0.001 M-ATP was added at zero plus 10 min. and samples withdrawn at intervals up to zero plus 20 min. At this time 0.5 ml. of myokinase (≈ 0.5 mg. of the freeze-dried preparation) was added and the mixture sampled for a further 25 min. The samples, as in all other experiments, were rapidly cooled, made alkaline and prepared for creatine analysis.

As seen from the first portion of the time curve in Fig. 4, and up to 10 min., 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 a liberation of creatine which, however, did not continue, and which was 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.

It is of course necessary to have a small amount of ATP or ADP present to start the reaction in the presence of myokinase, but this quantity can be very small indeed, since the reaction is self-accelerating. Thus the reactions involved if ATP is used are:

If ADP is used as the starter in the reaction it will be converted into ATP by CPK and the cycle repeated. As each full cycle doubles the amount of ADP in the system, it must remain in excess as long as the system contains AMP and myokinase.

Such a system must, however, differ from that which is most probably present in a suspension of cerebral cortex, and therefore in the type of system described by Narayanaswami (1952), which would be expected to contain CPK, myokinase, and small amounts of ATP.

An experimental mixture was prepared containing 0.025M-THAM pH 7.2, Ca^{2+} in optimum amounts, myokinase, 10 μ moles of PC and 0.1 μ moles of ATP. Incubation was carried out at 37° and samples were withdrawn for

Fig. 5. Substrate specificity in the CPK-catalysed reaction. ATP, PC, CPK and myokinase present and AMP added as test substrate at A. For details see text.

creatine determination at the times indicated in Fig. 5. CPK was added at zero time, and analysis of samples, withdrawn at intervals up to zero plus 10 min., indicated that no creatine had been liberated. At zero plus 10 min., 0.1 ml. of 0.1M-AMP was added, which brought the concentration of AMP to 1.6×10^{-4} M. It will be seen that this was followed by an immediate liberation of a small amount of creatine. This was almost certainly due to the presence of a small amount of ADP in the sample of AMP. A short lag period then followed during which the reaction rate was quite slow, but this rapidly increased until the reaction neared equilibrium. The lag period which followed the initial small increase in creatine was probably due to the fact that only a small amount of ATP was present. Until such time, therefore, as sufficient ADP was formed to saturate the active centres of the enzyme, the concentration of ADP would be the limiting factor for its activity.

Reverse reaction: $\text{ATP} + \text{creatine} \rightarrow \text{PC} + \text{ADP}$

pH optimum. Determination of the pH optimum for the reaction was carried out using optimum amounts of Ca^{2+} (see below) and the enzyme mixtures were incubated at 37° for 15 min. The amount of creatine which was phosphorylated increased with increasing alkalinity up to pH 10.5, which was the highest pH at which the experiment was carried out. All subsequent experiments were carried out at pH 10.5 in 0.025M-THAM.

Reaction rate and the effect of cysteine. The speed with which creatine is phosphorylated in the presence of CPK was determined by withdrawing samples at various intervals up to 60 min. from a reaction mixture at 37° containing Ca^{2+} in optimum amounts (see below), ATP, THAM and

Fig. 6. The influence of excess Ca^{2+} on the reverse reaction. The points O—O represent analytical figures obtained on a mixture containing CPK, creatine, ATP and Ca^{2+} (in optimum amounts) in 0.025M-THAM pH 10.5 incubated at 37°. At A the Ca^{2+} concentration was increased in a portion of the mixture and the points ●—● represent analytical figures obtained on this portion. For details see text.

creatine, and after the reaction had been started by the addition of CPK at zero time.

It will be seen (Fig. 6) that equilibrium was reached in 30 min., by which time 55 % of the creatine present was phosphorylated. The addition of cysteine ester at concentrations up to 0.002M was found to be without effect on either the speed or the equilibrium position of the reverse reaction.

Effects of Ca^{2+} and Mg^{2+} . These were studied as follows.

A stock solution containing 30.0 ml. THAM pH 10.5, 7.5 ml. $2 \times 10^{-3}\text{M}$ creatine, 1.5 ml. of CPK ($\equiv 6\text{ mg. N}$) and 21.0 ml. water was prepared. 2.0 ml. samples of this were transferred into a series of tubes containing varying amounts of CaCl_2 or MgCl_2 and water to make a final volume of 3.0 ml. The tubes and contents were then equilibrated in a water bath at 37° and the reaction started by the addition of 1.0 ml. of 0.001M-ATP. The reaction was allowed to continue for 15 min. and was interrupted by the addition of 1.0 ml. of 25% (w/v) trichloroacetic acid. The tubes were immediately cooled, the contents filtered, and 2.0 ml. samples transferred to tubes containing sufficient 5N-NaOH to render them alkaline. The degree to which creatine was phosphorylated was determined by measurement of the residual creatine and comparison of this with a control tube to which 1.0 ml. of ATP had been added after the addition of trichloroacetic acid.

The results (Fig. 7) show that there is a well-defined optimum relationship between the enzyme activity and the concentration of Ca^{2+} or Mg^{2+} . As the concentration of ions is increased there is a sharp increase in the degree to which the creatine is phosphorylated and a maximum is reached when $\log(\text{Ca}^{2+}/\text{N})$ or $\log(\text{Mg}^{2+}/\text{N}) = 2.9$. As the Ca^{2+} or Mg^{2+} concentration was increased there occurred a fall in the percentage of creatine which was phosphorylated. It would thus seem that this decrease

Fig. 7. The influence of Ca^{2+} and Mg^{2+} on the reverse reaction. Reaction carried out in 0.025M-THAM pH 10.5 and at 37° . Experimental mixture contained CPK, ATP, creatine and varying amounts of Ca^{2+} (O---O) and Mg^{2+} (□---□). For details see text. '% phosphorylation' refers to the extent to which the added creatine was phosphorylated.

was due simply to an inhibition of enzyme activity, as was the case in the experiments dealing with the forward reaction. Since, however, Soreni & Degtyar (1948) found that Ca^{2+} and Mg^{2+} influenced the equilibrium of the reaction, additional experiments were carried out.

A bulk reaction mixture was prepared containing 20.0 ml. 0.1M-THAM pH 10.5, 10.0 ml. $2 \times 10^{-3}\text{M}$ creatine, 2.0 ml. $4 \times 10^{-3}\text{M}$ - CaCl_2 , 20.0 ml. 10^{-3}M -ATP and 27.0 ml. water. The temperature of the mixture was brought to 37° in a water bath and a 4.0 ml. sample taken for analysis of the initial creatine content. The reaction was then started by the addition of 1.0 ml. of CPK ($\equiv 4\text{ mg. N}$) at zero time. At zero plus 2, 5, 15 and 30 min., 4.0 ml. samples were withdrawn and treated as before, and immediately after the last sample was removed 16.0 ml. of the mixture was transferred to another vessel in the water bath, and at zero +31 min., 0.1 ml. of 0.8M- CaCl_2 was added to the remaining 40 ml. (see Fig. 6A). Sampling of both mixtures was then continued and samples were withdrawn from the latter at zero plus 32, 35, 45, 50 and 60 min. The fraction to which no additional Ca^{2+} was added was sampled at zero +33 and 58 min.

The results of this experiment (Fig. 6) show that up to 30 min., 55 % of the added creatine had been phosphorylated and that in the experimental mixture to which no additional Ca^{2+} was added there was no further change. In the remaining portion of the mixture following the addition of Ca^{2+} at 31 min. there occurred a sharp fall in the phosphocreatine level which at 45 min. was such that only 23 % of the creatine was now phosphorylated. The effect of increased Ca^{2+} concentration is therefore, as Soreni & Degtyar (1948) have claimed, to produce a shift in the equilibrium of the reaction.

The effect of Ba^{2+} and Sr^{2+} . The experimental arrangement was similar to that described in the preceding section except that the course of the reaction was not followed.

All tubes contained optimum concentrations of Ca^{2+} and, in addition to the control tube to which no ATP was added, three tubes contained an excess of Ca^{2+} , Sr^{2+} and Ba^{2+} , respectively. The total volume in these tubes was 8.0 ml. and, with the amount of CPK used, the optimum concentration of Ca^{2+} was 10^{-4}M . To the tube requiring excess Ca^{2+} sufficient CaCl_2 was added to bring the final concentration to 0.002M. A similar concentration was adopted in the case of those tubes containing Ba^{2+} and Sr^{2+} . These tubes were incubated for 60 min. at 37° . Another series of tubes was set up simultaneously, but these were incubated for the first 30 min. in the presence of optimum amounts of Ca^{2+} only and at the end of this time, after the addition of the Ca^{2+} , Sr^{2+} and Ba^{2+} in similar amounts to the first series, the reaction was allowed to continue for a further 30 min.

The results (Table 5) show that under optimum conditions (tube 1) 49 % of the creatine was phosphorylated. The effect of the addition of Ca^{2+} and Ba^{2+} (tubes 2 and 3, respectively) is to shift the equilibrium to such an extent that 19.5 and 28.0 % of the creatine was phosphorylated, respectively.

Table 5. *The effect of the addition of Ca^{2+} , Ba^{2+} and Sr^{2+} on the reverse reaction*

All incubations were for 60 min. at 37°. For details of the experiments see text.

Tube no.	Treatment	Creatine phosphorylated (%)
1	Optimum Ca^{2+} concentration at zero time	49.0
2	Optimum Ca^{2+} concentration at zero time, excess of Ca^{2+} added at zero + 30 min.	19.5
3	Optimum Ca^{2+} concentration at zero time, excess of Ba^{2+} added at zero + 30 min.	28.0
4	Optimum Ca^{2+} concentration at zero time, excess of Sr^{2+} added at zero + 30 min.	42.0
5	Excess of Ca^{2+} added at zero time	16.5
6	Excess of Ba^{2+} added at zero time	27.5
7	Excess of Sr^{2+} added at zero time	4.7

Table 6. *'Balance' sheet of the reverse reaction*

Total creatine is the sum of free creatine and that bound as phosphocreatine. For details of experimental procedure see text.

Sample	Creatine ($\mu\text{g.}$)	
	Free	Total
Sample 1 (before addition of CPK)	186.0	186.0
Sample 2 (15 min. after addition of CPK)	104.5	185.5
Control sample	186.2	186.2

It will be noted that much the same result was achieved when these ions were present in excess from zero time (tubes 5 and 6). The effect of Sr^{2+} , on the other hand, is not to shift the equilibrium but to inhibit enzyme activity (tubes 4 and 7), which is in agreement with its observed effect (see above) on the forward reaction.

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 have been determined, but creatine was chosen because of the ease with which it could be estimated. The choice had another advantage in that the estimation was carried out in an alkaline solution, in which medium PC was perfectly stable and did not react with α -naphthol and diacetyl. It may, however, be argued that the disappearance (in the case of the forward reaction) or the appearance (in the case of the reverse reaction) of creatine does not constitute proof of the respective formation or breakdown of PC. Some experiments have been carried out in which a 'balance sheet' was prepared. Thus in the 'reverse reaction' a reaction mixture was prepared containing 7.5 ml. THAM pH 10.5, 3.8 ml. 0.002M creatine, 7.5 ml. 0.001M-ATP, 0.75 ml. 0.004M- CaCl_2 , and 10.2 ml. water. After equilibration at 37°, sample 1 (Table 6) was withdrawn for determination of free and total creatine. 0.38 ml. of CPK (≈ 1.52 mg. N) was then added and the mixture incubated for 15 min., when sample 2 (Table 6) was taken for analysis as before. In

addition, a control sample (Table 6) containing 0.002M creatine was diluted with water in the same proportions as in the reaction mixture, and analysed for free and total creatine.

The results (Table 6) show the soundness of the analytical methods employed and provide proof of the assumption, inherent in these experiments, that a fall in creatine content can be attributed to a phosphorylation reaction.

DISCUSSION

The present data are not in entire agreement with published work. Thus the pH optimum for the reverse reaction was found by Banga (1943) to lie at pH 9.05, and at pH 9.1 by Soreni & Degtyar (1948). In our hands the reaction rate in the presence of optimum concentrations of Ca^{2+} was still increasing at pH 10.5, which was the most alkaline condition investigated. This difference in results is probably explained by the fact that neither Banga (1943) nor Soreni & Degtyar (1948) appear to have appreciated the critical nature of the enzyme requirement of Ca^{2+} . The use of borate buffer by Banga (1943) is another possible explanatory factor, since borate is somewhat inhibitory to the enzyme. In agreement with Soreni & Degtyar (1948) it has been found that Mg^{2+} may replace Ca^{2+} , but whilst these workers have found that the reverse reaction may proceed in the complete absence of Ca^{2+} , the present results indicate an obligatory requirement for this ion.

Thus not only was the enzyme completely inactive in the absence of Ca^{2+} or Mg^{2+} but the inhibition produced by ethylenediaminetetraacetic acid was removed by the addition of Ca^{2+} or Mg^{2+} . The activity found by Soreni & Degtyar (1948) in the absence of added Ca^{2+} would therefore seem to be due to traces of Ca^{2+} in the system employed. In the present experiments a well-defined maximum enzyme activity was obtained with a Ca^{2+} concentration of 10^{-4} M, or when $\log \text{Ca}/\text{N} = \bar{2}.903$ (see Fig. 7).

The phosphorylation of ADP reaches maximal values at pH 7.2 with a Mg^{2+}/N or Ca^{2+}/N ratio of

100–200. An increase of this ratio to 2000 results in a much lowered degree of phosphorylation which is due not to a shift in the equilibrium but to an inhibition of enzymic activity.

The complete enzyme system, whether at pH 7·2 or 10·5, had no essential requirement for added cysteine, although, if present, cysteine had a slight activating effect in the case of the forward reaction. No such effect was observed with the reverse reaction.

It will be noted that 50 times as much enzyme was used in the case of the reverse reaction as in the forward reaction and in spite of many experiments it was not possible to reduce this factor by an appreciable amount and yet retain a convenient reaction rate.

Evidence has been adduced to show that a free SH group or groups within the CPK molecule is or are 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 dilution, 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 solution or in the dried and frozen state.

The inhibitory effect of phosphate, fluoride and citrate is presumably due to the reaction of these anions with the Ca^{2+} necessary for enzyme action. Because of the widespread use of phosphate as a buffer in *in vitro* systems its inhibitory effect would seem of especial interest, particularly in those cases in which PC and the adenylic acid system play a part.

There seems now little doubt as to the nature of the phosphate acceptor in the CPK system in so far as CPK separated from skeletal muscle is concerned. In the purified system investigated here only ADP could function as an acceptor. Moreover, in the reverse reaction it has been shown (Ennor & Rosenberg, unpublished observations) that ADP cannot serve as a phosphate donor in the phosphorylation of creatine except in the presence of myokinase. It would therefore seem, in agreement with Colowick's (1951) suggestion, that Banga's (1943) observation that two forms of CPK were present in skeletal muscle can be explained by the presence of myokinase in the rather crude preparations.

In the experiments described by Narayanaswami (1952) the position may be somewhat different, since cerebral cortex was used. Since the preparations were not dialysed ADP and ATP may have been present. Whether or not myokinase is present in cerebral tissue is a matter for speculation, but nevertheless is a reasonable assumption which Narayanaswami (1952) did in fact make. Narayanaswami's (1952) observation that the speed of the

reaction was greater in the presence of AMP than ADP can be explained by considering the reaction

In the presence of AMP and myokinase one of the products (ATP) is continuously removed while one of the reactants (ADP) is continuously replaced in doubled amount. The net result therefore must be an increase in the reaction rate.

Although the preparation of CPK used throughout the experiments reported here was active in extreme dilution, attempts were made to effect a more complete separation from the contaminating proteins known to be present as a result of paper electrophoretic analysis. Whilst these experiments were successful in as much as a considerable purification was achieved, work on this problem was suspended following the work of Kuby, Noda & Lardy (1953), in which it was announced that the enzyme had been obtained in a homogeneous form, as judged by electrophoretic and sedimentation analysis.

SUMMARY

1. The enzyme creatine phosphokinase requires SH groups for its activity, but has no obligatory requirement for added cysteine.

2. The enzyme has a well-defined optimum pH = 7·2 and 10·5 for catalysing the forward and reverse reactions, respectively, as expressed in the equation

3. The presence of Ca^{2+} or Mg^{2+} is obligatory for enzyme action, but the absolute amounts required depend upon the nature of the reaction under investigation (i.e. forward or reverse above). Excess amounts of Ca^{2+} or Mg^{2+} are inhibitory in the case of the forward reaction and alter the equilibrium in the case of the reverse reaction.

4. AMP could not serve as a phosphate acceptor.

5. *p*-Chloromercuribenzoate, *N*-ethylmaleimide and iodoacetate were inhibitory. Inhibition was also produced by dinitro-*o*-cresol. Phosphate, citrate, fluoride and ethylenediaminetetraacetic acid were also inhibitory.

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REFERENCES

- Askonas, B. A. (1951). *Biochem. J.* **48**, 42.
- Bailey, K. (1942). *Biochem. J.* **36**, 121.
- Banga, I. (1943). *Stud. Inst. med. Chem. Univ. Szeged*, **3**, 59.
- Colowick, S. P. (1951). *The Enzymes*, eds. J. B. Sumner and K. Myrback. New York: Academic Press, Inc.
- Dounce, A. L., Rothstein, A., Beyer, G. T., Meier, R. & Freer, R. M. (1948). *J. biol. Chem.* **174**, 361.

- Ennor, A. H. & Rosenberg, H. (1952). *Biochem. J.* **51**, 606.
 Ennor, A. H. & Stocken, L. A. (1948a). *Biochem. J.* **42**, 557.
 Ennor, A. H. & Stocken, L. A. (1948b). *Biochem. J.* **43**, 190.
 Friedmann, E. (1903). *Beitr. chem. Physiol. Path.* **3**, 1.
 Hunter, A. (1928). *Creatine and Creatinine*. London: Longmans Green and Co.
 Kalckar, H. M. (1943). *J. biol. Chem.* **148**, 127.
 Kerr, S. E. (1941). *J. biol. Chem.* **139**, 131.
 Krishnan, P. S. (1949). *Arch. Biochem.* **20**, 261.
 Kuby, S. A., Noda, L. & Lardy, H. A. (1953). *Fed. Proc.* **12**, 234.
 Lehmann, H. (1935). *Biochem. Z.* **281**, 271.
 LePage, G. A. (1945). *Manometric techniques and Related Methods for the Study of Tissue Metabolism*, eds. W. W. Umbreit, R. H. Burris and J. F. Stauffer. Minneapolis: Burgess Publishing Co.
 Lohmann, K. (1934). *Biochem. Z.* **271**, 264.
 Narayanaswami, A. (1952). *Biochem. J.* **52**, 295.
 Soreni, E. T. & Degtyar, R. G. (1948). *Ukr. biochem. J.* **20**, 234.
 Whitmore, F. C. & Woodward, G. E. (1932). *Org. Synth., Collective*, **1**, 153.

Studies in the Biochemistry of Micro-Organisms

92. THE COLOURING MATTERS OF *PENICILLIUM ISLANDICUM* SOPP PART 4. IRIDOSKYRIN, RUBROSKYRIN AND ERYTHROSKYRINE

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In our first communication concerning the pigments of *Penicillium islandicum* Sopp (Howard & Raistrick 1949) it was shown that the dried mycelium of laboratory cultures of one strain of this species, no. N.R.R.L. 1036, contained about 20% of its weight of a complex mixture of colouring matters. One constituent of this mixture was isolated and identified as the hitherto undescribed islandicin (1:4:5-trihydroxy-2-methylanthraquinone, I). This substance was isolated from the mycelium of five out of six morphologically typical strains of *P. islandicum*, and the presence in the mycelium of several other colouring matters was indicated by a number of colour reactions. Three of these colouring matters have now been isolated in a pure crystalline state and it is the purpose of the present communication to describe, *inter alia*, their isolation, properties and reactions, and to discuss their relationship to other fungal colouring matters. None of them appears to have been described previously. Their chemical constitutions, at present uncertain, are under investigation in this laboratory.

When the dried and ground mycelium of strain N.R.R.L. 1036 was extracted for a long period in a percolator with boiling light petroleum, a bright red solid accumulated in the solvent. Islandicin was separated from this solid by sublimation in high vacuum at 180°. The unsublimed residue, on crystallization from benzene, gave small amounts of a substance, crystallizing as deep red plates or rods, m.p. 358–360°, for which we propose the name iridoskyrin because the crystals exhibit a striking green iridescence. Further extraction of the mycelial powder with chloroform removed a complex mixture of pigments, and when the extract was chromatographed on calcium carbonate, a further

small amount of iridoskyrin appeared in the eluate together with much islandicin. The chromatogram consisted of a number of bands of different colours. From one of them, a broad green band, there was obtained a substance crystallizing as dark red plates, m.p. 289–290° (decomp.), for which we propose the name rubroskyrin. The losses involved in the separation of these two colouring matters make it impossible to state with certainty the proportions present in the mould. It is estimated, however, that the dried mycelium of *P. islandicum* strain N.R.R.L. 1036 contains about 0.2% of iridoskyrin and about 2.5% of rubroskyrin.

An ether extract of the wet mycelium of the same strain of *P. islandicum*, N.R.R.L. 1036, was shaken out with aqueous sodium carbonate. Acidification of this alkaline extract yielded a dark red, amorphous powder which was fractionated by chromatographing an acetone solution on magnesium oxide. Development of the chromatogram with methanol gave an eluate containing the magnesium salt of a third colouring matter which was obtained as dark red micro-needles, m.p. 108–110°, for which we propose the name erythroskyrine. It was found later that the same substance constitutes practically the whole of the colouring matter present in the mycelium of a different strain, L.S.H.T.M. no. BB 233, of *P. islandicum*. It is estimated that the dried mycelium of this strain contains about 10% of erythroskyrine, while strain N.R.R.L. 1036 contains approximately 3.5%.

Iridoskyrin

Iridoskyrin, $C_{30}H_{18}O_{10}$, is dimorphic, crystallizing as thin rods or hexagonal plates of a deep red colour and exhibiting a green iridescence. It melts at