# Creatine Kinase in Serum: 1. Determination of Optimum Reaction Conditions

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To establish optimum conditions for creatine kinase (EC 2.7.3.2) activity measurement with the creatine phosphate → creatine reaction, we re-examined all kinetic factors relevant to an optimal and standardized enzyme assay at 30 and 25 °C. We determined the pH optimum in various buffers, considering the effect of the type and concentration of the buffer, as well as the influence of various buffer anions on the activity. The relation between activity and substrate concentration was shown and the apparent Michaelis constants of creatine kinase for creatine phosphate and ADP were evaluated. We tested the effect on creatine kinase measurement of the concentration of substrates (glucose and NADP$^+$) in the auxiliary and indicator reactions, especially the influence of the added auxiliary (hexokinase) and indicator (glucose-6-phosphate dehydrogenase) enzymes on the lag phase, at different temperatures. The NADP$^+$ concentration proved to be the factor limiting the duration of constant reaction rate. We studied the inhibition of creatine kinase and adenylate kinase by AMP and established a convenient AMP concentration. For reactivation of creatine kinase, N-acetyl cysteine as sulfhydryl compound was introduced. Finally, we examined the relationship between activity and temperature.

Creatine kinase (ATP:creatine N-phosphotransferase; EC 2.7.3.2) in serum is of outstanding clinical significance in the enzymology of heart and skeletal muscle because of its high sensitivity and relative specifity. According to recent investigations, only skeletal and heart muscle contain large amounts of creatine kinase (1). Thus, considerable increases in this activity in serum almost exclusively reflect diseases and damage of skeletal or heart muscle.

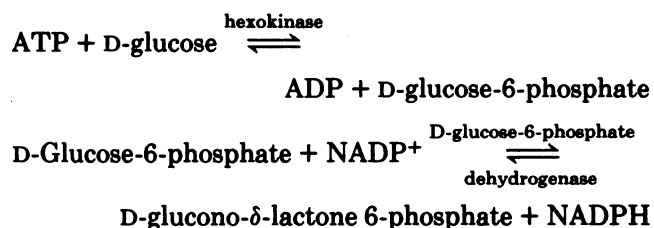Creatine kinase catalyzes the reversible phosphorylation of creatine by ATP:
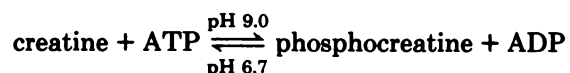
$$\text{creatine} + \text{ATP} \underset{\text{pH 6.7}}{\overset{\text{pH 9.0}}{\rightleftharpoons}} \text{phosphocreatine} + \text{ADP}$$

Because phosphocreatine (creatine phosphate) has a significantly higher energy than ATP, the equilibrium is largely shifted into the reverse direction. The equilibrium position depends also upon the pH. At pH optimum the reverse reaction proceeds four to six times faster than the forward reaction and should be preferred therefore in the development of an assay method. On the other hand, for the most desirable method only measurement techniques should be considered that follow the progress of the reaction directly and continuously (2). Such requirements are met only by the procedure in which the ATP formed is measured by means of an auxiliary and indicator enzyme:

$$\text{ATP} + \text{D-glucose} \overset{\text{hexokinase}}{\rightleftharpoons}$$

$$\text{ADP} + \text{D-glucose-6-phosphate}$$

$$\text{D-Glucose-6-phosphate} + \text{NADP}^+ \overset{\text{D-glucose-6-phosphate}}{\underset{\text{dehydrogenase}}{\rightleftharpoons}}$$

$$\text{D-glucono-}\delta\text{-lactone 6-phosphate} + \text{NADPH}$$

The course of the reaction can be monitored spectrophotometrically by measuring the conversion of NADP$^+$ to NADPH, which is done by following the increase in absorbance at 340 or 334 nm.

The method was described first by Oliver (3) and modified later by Rosalki (4) and Hess et al. (5) and others. We report here our re-examination of all kinetic factors relevant to the optimization and standardization of the creatine kinase assay at 30 °C (2) and 25 °C (6). A preliminary presentation[3] was given in 1974. In forthcoming papers we shall describe details of methodology, reference values, problems of inactivation and reactivation, the phenomenon of the "dilution effect," and the various interferences with the measurement that may be encountered.

Materials and Methods

Creatine phosphate, ADP, AMP, NADP$^+$, hexokinase (EC 2.7.1.1), and D-glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (both enzymes from baker's

[3] Szasz, G., Bernt, E., Staehler, F: Proposal for a selected method for creatine kinase in serum. Sixth International Symposium on Clinical Enzymology, Venice, 1974.

Table 1. Assay Conditions for Creatine Kinase Activity Measurement in Human Serum[a]

	This paper	Rosalki (4) 1967	Hess (5) 1968	German Standard Method (16) 1970	Warren (7) 1972
				Author	
Buffer	100	50	50	100	70
	(Imidazole)	(Tris)	(Triethanolamine)	(Triethanolamine)	(Imidazole)
pH	6.7	6.8	6.9	7.0	6.5
Creatine phosphate	30	10	20	35	15
ADP	2	1	1	1	3
Mg^{2+}	10	30	10	10	37.5
D-Glucose	20	20	20	20	10
$NADP^+$	2	0.8	0.8	0.6	2
Hexokinase	2500	600	1000	1200	4000
Glucose-6-phosphate dehydrogenase	1500	300	500	1200	2000
AMP	5	10	none	10	5
Thiol compound	20	5	10	9	5
	(N-acetyl cysteine)	(Cysteine)	(Cysteine)	(Glutathione)	(DTT)

[a] The final reagent concentrations in the reaction mixture are given, in mmol/liter for the chemicals and in U/liter for the enzymes.

yeast) were from Bio-Dynamics/bmc, subsidiary of Boehringer Mannheim, Indianapolis, Ind. 46250. 2-(N-Morpholino)ethane sulfonic acid (MES), morpholinopropane sulfonic acid (MOPS), and 1,4-piperazinediethanesulfonic acid (PIPES) were from Calbiochem, San Diego, Calif. 92112. N-Acetyl cysteine was from Diamalt AG., Munich. All other compounds, in the purest quality available, were from E. Merck, Darmstadt. Fresh solutions of the reagents were prepared each working day.

Sera of patients with skeletal muscle lesions or myocardial infarction served as the source of creatine kinase.

Table 1 lists the final reagent concentrations in the reaction mixture. The serum sample comprised about 0.02 of this mixture by volume at the assay temperature of 30 °C and about 0.04 at 25 °C. After a 3- to 5-min pre-incubation the reaction was usually started with creatine phosphate. Taking into consideration the 2-min lag phase, we continuously monitored the increase in absorbance for several minutes at 334 nm. If the reaction was started with serum instead of creatine phosphate the time required for reactivation and lag phase amounted to 5 min. These "standard" conditions were used throughout except where specifically mentioned in the text or figure legends.

Results

Buffer and pH

In the optimum pH range for creatine kinase—between pH 6.5 and 7.0—only a few buffers are effective, especially PIPES (pK, 6.8), imidazole (pK, 7.1) and MOPS (pK, 7.2). In comparison, the pK values for triethanolamine (pK, 7.9) and Tris (pK, 8.2) are too high and that for MES (pK, 6.15) is too low. With imidazole acetate buffer, a broad pH optimum was observed be-
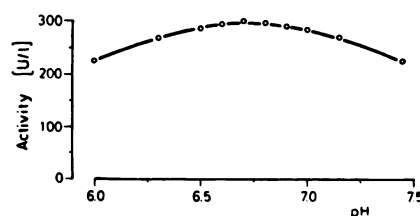

Fig. 1. Dependence of creatine kinase activity on pH (100 mmol/liter imidazole acetate buffer)

Table 2. Effect of Type of Buffer on Creatine Kinase Activity

	Imidazole acetate	Tri- ethanol- amine acetate	Sodium MOPS	Sodium MES	Sodium PIPES
U/liter[a]	166	165	163	159	150

[a] Mean activity of 12 sera, duplicate assays. Concentration of the buffers was 100 mmol/liter, the pH was 6.7.

tween pH 6.5 and 7.0 (Figure 1). The evaluation of pH activity curves of 16 different sera showed the pH optimum to be at pH 6.7, but the difference in activity between pH 6.7 and 6.8 was only 2%. The pH–activity relationship seemed to be independent of the assay temperature (30 or 25 °C) and the type of the buffer used (imidazole or triethanolamine acetate).

Comparison among five different buffers (Table 2), all at 100 mmol/liter concentration and pH 6.7, showed almost identical creatine kinase activity in imidazole and triethanolamine, whereas in MOPS, MES, and PIPES buffers slightly to significantly lower activity was

Table 3. Effect on Creatine Kinase Activity of the Acid Used to Adjust the pH

Acid:	Formic	Hydro-chloric	Acetic	Nitric
U/liter[a]	137	135	134	133

[a] Mean activity of 12 sera, duplicate assays. Imidazole buffer, 100 mmol/liter, pH 6.7.

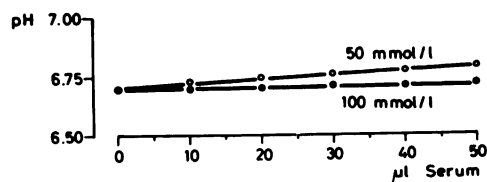Fig. 4. Dependence of pH upon the volume fraction of the serum in the assay solution (0 to 0.9), in imidazole acetate buffer

Fig. 2. Effect of some salts on creatine kinase activity

Fig. 5. Dependence of creatine kinase activity on creatine phosphate concentration
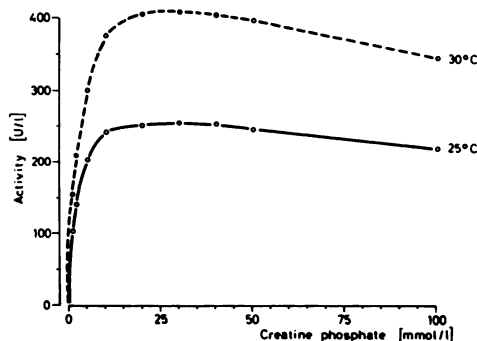

Fig. 3. Dependence of creatine kinase activity on buffer concentration
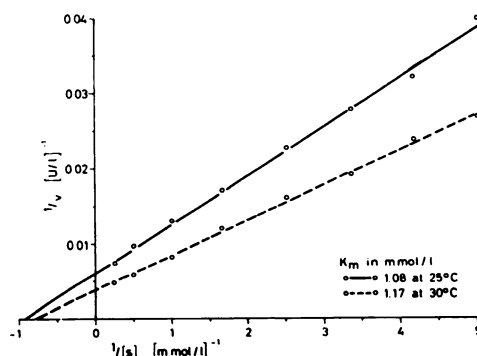
Fig. 6. Michaelis constants of creatine kinase for creatine phosphate—Lineweaver–Burk plot

measured. Thus the buffers of Good et al. (8) are not the best to use in measuring creatine kinase activity.

The type of acid used to adjust the pH only slightly affected the creatine kinase activity in imidazole buffer (Table 3). Acetic acid is the most practicable.

The effect of the buffer anions was additionally tested by adding increasing amounts of the corresponding sodium salt (Figure 2). Both sodium acetate and chloride inhibited creatine kinase markedly and to about the same extent.

Creatine kinase was inhibited by both imidazole and triethanolamine buffers (Figure 3). The activity without buffer, calculated by extrapolating the curves to 0 mmol/liter buffer concentration, is about 5% higher than at 50 mmol/liter, and an additional loss in activity of as much as 10% was observed on increasing the buffer concentration to 100 mmol/liter.

A larger proportion of serum in the assay mixture leads to an increase in pH (Figure 4). At 50 mmol/liter imidazole acetate buffer the increase in pH (ΔpH) per 10 μl of added serum to 500 μl reagent is 0.02, but only

0.005 at 100 mmol/liter. This is true also for freeze-dried sera with high pH values, up to pH 9.2. Moreover, the starting reagent, containing creatine phosphate in high concentration, may also cause a shift in pH. To maintain a constant pH in the reaction mixture, a buffer concentration of 100 mmol/liter is necessary.

Creatine Phosphate

Creatine kinase activity increases up to 30 mmol of creatine phosphate per liter; at greater concentrations we began to see inhibition (Figure 5). The relation between activity and creatine phosphate concentration is similar at both 25 and 30 °C.

The apparent Michaelis constants were calculated according to Lineweaver and Burk by means of regression analysis (Figure 6). For three different sera, the mean Michaelis constants of creatine kinase in human
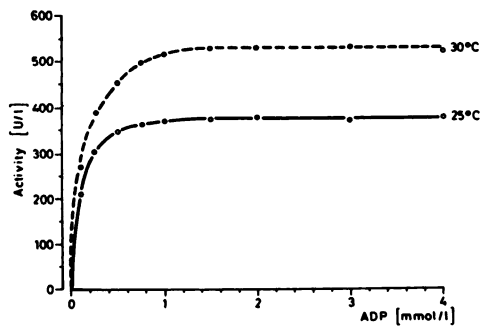
Fig. 7. Dependence of creatine kinase activity on ADP concentration
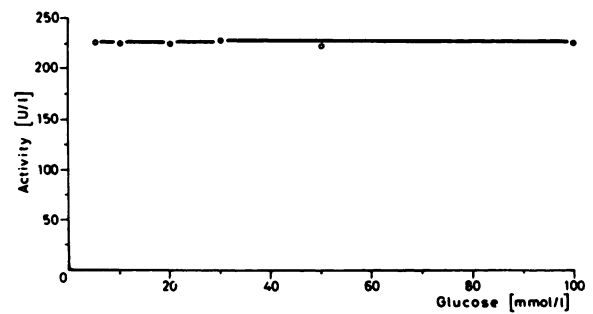

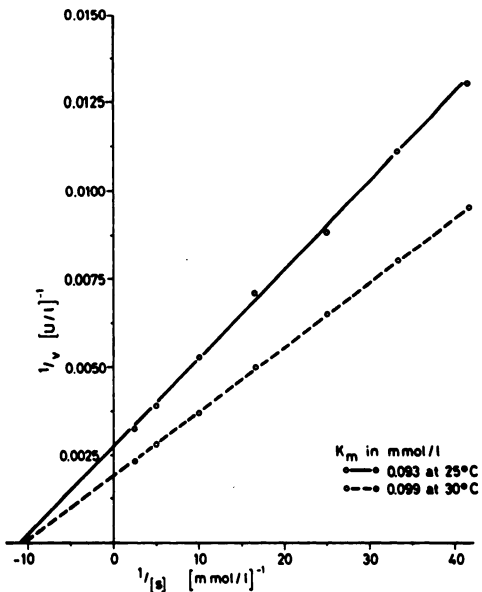Fig. 10. Effect of glucose concentration on the value obtained for creatine kinase activity

Fig. 8. Michaelis constants of creatine kinase for ADP—Lineweaver–Burk plot

Fig. 11. Dependence of the value obtained for creatine kinase activity on the NADP$^+$ concentration at a volume fraction of sample of 0.02
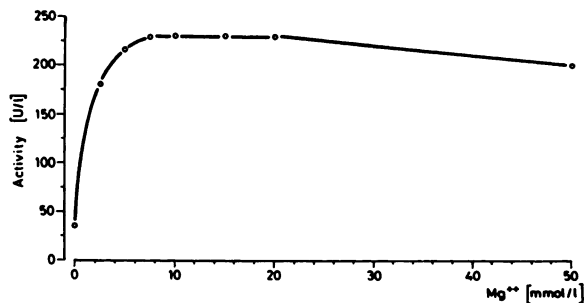

Fig. 9. Effect of magnesium ions on creatine kinase activity

serum for creatine phosphate were 1.08 mmol/liter at 25 °C and 1.17 mmol/liter at 30 °C.

Adenosine Diphosphate

For maximum creatine kinase activity, 2 mmol of ADP per liter was required (Figure 7). For ADP concentrations between 1 and 2 mmol/liter an increase was observed, especially at 30 °C, but there was no inhibition of activity with concentrations up to 4 mmol/liter.
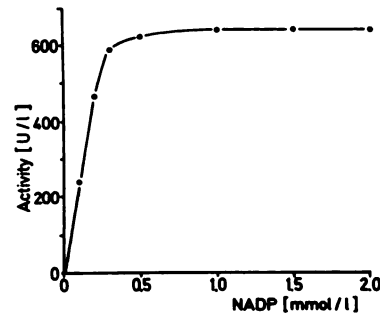
The necessity to use 2 mmol of ADP per liter was corroborated by the K_m values. For ADP, the mean Michaelis constants for creatine kinase in three human sera were 93 μmol/liter at 25 °C and 99 μmol/liter at 30 °C (Figure 8). Thus the twentyfold K_m value is reached just at 2 mmol of ADP per liter.

Magnesium Salts

Magnesium ions are essential for creatine kinase activity. Without added magnesium salt only 10% of the maximum activity was observed (Figure 9). The highest activity will be obtained with concentrations of 10 to 20 mmol/liter. Magnesium acetate and chloride were equally effective.

Glucose

At glucose concentrations between 5 and 100 mmol/liter, creatine kinase activity was the same (Figure 10). Because the reading for the reagent blank—i.e., the assay solution without sample—was very small and independent of glucose concentration, we saw neither contaminant activity nor side activity of the auxiliary or indicator enzyme.

NADP$^+$

Creatine kinase activity is indicated by the production of NADPH from NADP$^+$. Between 1.0 and 2.0 mmol/liter, measurement of the creatine kinase activity, up to 600 U/liter, is independent of NADP$^+$ concentration for 10 min (Figure 11).
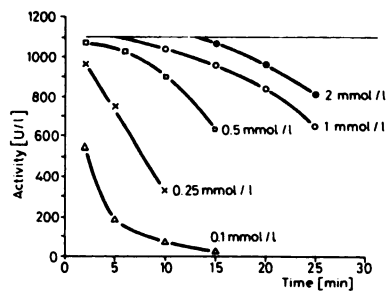
Fig. 12. Period of constant reaction rate at different NADP⁺ concentrations

Fig. 12. Period of constant reaction rate at different $NADP^+$ concentrations

Volume fraction of sample: 0.02

Fig. 14. Lag phase as a function of hexokinase activity (U/ml at 25 °C) in an assay mixture containing 2 U of glucose-6-phosphate dehydrogenase per milliliter
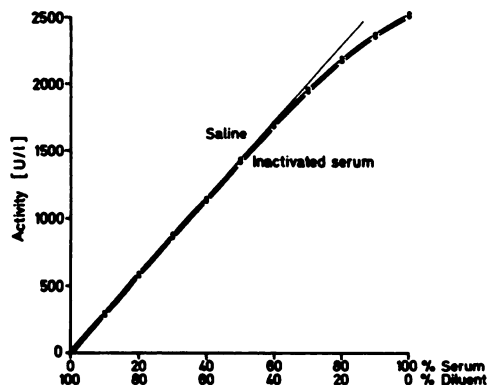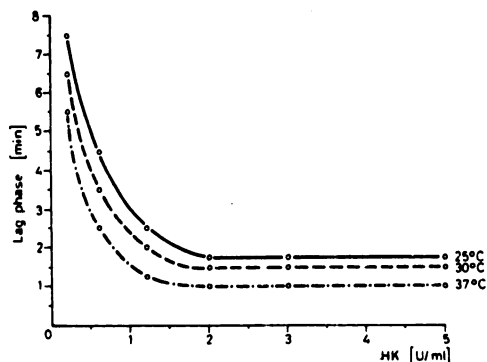

Fig. 13. Creatine kinase activity as a function of the amount of enzyme in the assay solution
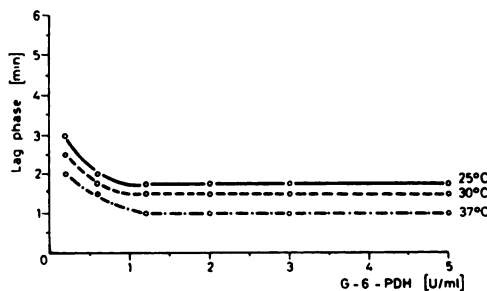
Volume fraction of sample: 0.02

Fig. 15. Lag phase as a function of glucose-6-phosphate dehydrogenase activity (U/ml at 25 °C) in an assay mixture containing 2 U of hexokinase per milliliter

The duration of constant reaction rate, however, depends greatly on $NADP^+$ concentration (Figure 12). Including a lag phase of 2 min, the reaction rate decreases at 1.0 mmol of $NADP^+$ per liter in the presence of sera with activity of 1100 U/liter after 10 min total reaction time, although only a tenth of the $NADP^+$ in the assay solution has been reduced to NADPH. At 2.0 mmol of $NADP^+$ per liter the reaction rate remains constant for almost 15 min.

Enzyme Concentration

The relationship between enzyme concentration (i.e., the amount of serum in the assay solution) and enzyme activity remained linear up to 1500 U/liter whether physiological saline or inactivated serum was used as diluent (Figure 13). The data were obtained immediately after a lag phase of 2 min and at a volume fraction of sample of 0.02. At higher enzyme concentrations, the actually measured activities remain under the expected values, although—e.g., at 2000 U/liter—only a tenth of the $NADP^+$ that originally was present in the assay mixture is transformed within 5 min.

Hexokinase

The duration of the lag phase depends on the activity of the added auxiliary and indicator enzymes. The "true" lag phase—after 3 min of pre-incubation and starting the reaction with creatine phosphate—could

be shortened by increasing the hexokinase activity to 2000 U/liter of reaction mixture. A greater hexokinase activity did not affect the duration of the lag phase (Figure 14).

As with every chemical reaction, the duration of the lag phase also depends on temperature. At 37 °C the lag phase is 1 min, at 25 °C it is 2 min, but it does not disappear at any temperature.

Glucose-6-phosphate Dehydrogenase

The lag phase is less influenced by glucose-6-phosphate dehydrogenase (Figure 15). Because the reagent blank increases gradually with added indicator enzyme, being 1.6, 4, and 8 U/liter at 1000, 2000, and 4000 U of glucose-6-phosphate dehydrogenase per liter, respectively, it is advisable to use a relatively low glucose-6-phosphate dehydrogenase activity.

In this study we used only baker's yeast glucose-6-phosphate dehydrogenase linked with $NADP^+$. The combination Leuconostoc mesenteroides enzyme and NAD^+ is now being studied.

N-Acetyl Cysteine

Creatine kinase in serum is rapidly inactivated (9, 10) but can be reactivated by adding sulfhydryl reagents (4, 7, 11-14). Such a sulfhydryl compound must quickly reactivate the enzyme without interfering with the measurement. Not only efficiency but also practicability
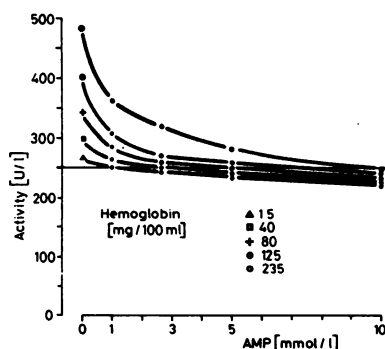
Fig. 16. Inhibition of adenylate kinase and creatine kinase by AMP

Apparent creatine kinase activity of human serum with added hemolysate, in the presence of 2 mmol of ADP per liter

Fig. 17. Creatine kinase activity as a function of reaction temperature

should be considered: solubility, stability (even in solution), odor, and price.

By these criteria, of 27 thiol compounds tested, N-acetyl cysteine was the most suitable. At a concentration of 20 mmol of N-acetyl cysteine per liter, a constant creatine kinase activity was achieved within 3 min of pre-incubation and an additional 2 min for lag phase after starting the reaction with creatine phosphate. Prolonging the pre-incubation up to 6 h did not further increase the activity. If the reaction is started with serum, a total of 5 min is necessary. N-Acetyl cysteine was stable in the assay solution at pH 6.7 for at least 24 h at 25 °C and a week at 4 °C, as assessed both by determination of free sulfhydryl groups and by analytical recovery of creatine kinase activity.

We have reported on our investigations into the problem of inactivation and reactivation of creatine kinase in a preliminary paper,[4] and details will soon be published in this series.

AMP

ADP is also acted upon by adenylate kinase (EC 2.7.4.3), generating additional amounts of ATP. The normal range of adenylate kinase activity in human sera lies between 0–50 U/liter at 25 °C (unpublished observation), but is markedly greater in hemolytic specimens.

Figure 16 illustrates the extent of this interference. The apparent creatine kinase activity proportionately increased with the amount of hemolysate added to the serum. Such interference could be diminished by adding AMP. Because not only adenylate kinase but also creatine kinase will be inhibited by AMP, the AMP concentration must be a compromise. The inhibition is competitive and therefore depends on the ADP concentration. In the presence of 2 mmol of ADP per liter, 3 mmol of AMP per liter is sufficient in the case of sera with slight to moderate hemolysis. Markedly hemolyzed sera require 5 mmol of AMP per liter, but 10 mmol/liter is too much even for grossly hemolytic sera. Creatine kinase is inhibited by 5 to 10% by the presence of 5 mmol of AMP per liter.

[4] Bernt, E., Gruber, W., and Szasz, G., Vergleich von Reaktivatoren der Creatinkinase im Serum. Z. Klin. Chem. Klin. Biochem. 12, 256 (1974) (Abstract).
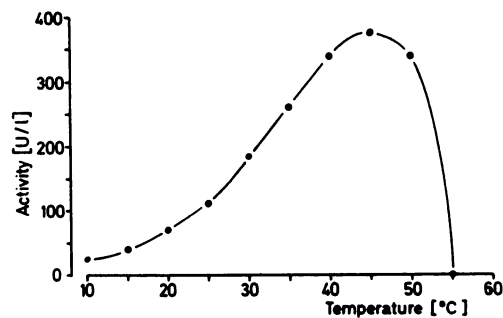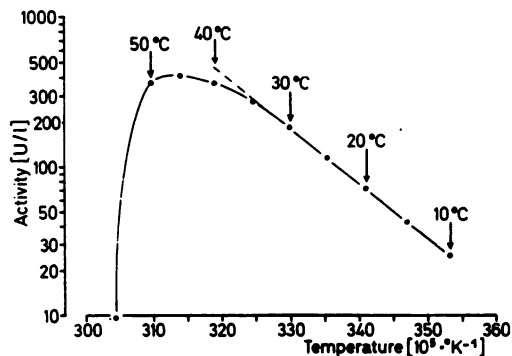
Fig. 18. Dependence of creatine kinase activity upon reaction temperature, plotted according to Arrhenius

Temperature

Creatine kinase activity increases with increasing reaction temperature up to 45 °C. At higher temperatures the activity rapidly decreases, and at 55 °C there is no detectable activity (Figure 17).

The activity–temperature relationship, plotted according to Arrhenius (Figure 18), was linear for temperatures between 10–35 °C; for higher temperatures the curve slopes downward. In an earlier study (15) in which a suboptimized method (16) was used, there was a deviation from linearity even at 30 °C. We used our "standard" conditions, except that the pH had to be adjusted because of the significant effect of temperature on the pH of the imidazole acetate buffer. If the pH is 6.7 at 25 °C, it will be, e.g., 7.2 at 10 °C or 6.4 at 55 °C.

Discussion

The total creatine kinase activity in serum can be a contribution from different isoenzymes. In cases of skeletal muscle damage the activity in serum is attributable almost exclusively to the presence of the MM isoenzyme, whereas in patients with myocardial infarction not only the MM, but also a variable MB isoenzyme activity (0–30%) is also present. The kinetic properties of the creatine kinase isoenzymes differ significantly (17).

With respect to the affinity of the enzyme for its substrates or in the temperature activity relationship,

we could find no differences between sera from patients with lesions of skeletal muscle or myocardial muscle. The MM isoenzyme obviously predominates in every serum, overshadowing the MB isoenzyme almost completely. The conditions we describe here are therefore optimum for the MM isoenzyme.

Table 1 compares the conditions described here and those formerly published by others (4, 5, 7, 16). It shows that almost all conditions we found to be optimal have already been used previously, although in none of the methods cited are all the conditions simultaneously optimal. Rosalki (4) recommends too-low concentrations of ADP, creatine phosphate, and NADP⁺, and a too-low auxiliary and indicator enzyme activity. The Standard Method of the American Association for Clinical Chemistry by Swanson and Wilkinson (18) corresponds exactly to the method of Rosalki (4). The method of Hess et al. (5) is better, but still not optimal. Apart from the too-low ADP and (especially) NADP⁺ concentrations, the main problem of the German Standard Method (16) arises from the use of reduced glutathione as reactivator. There may be an interference with creatine kinase activity measurement if the reagent contains considerable amounts of oxidized glutathione and the sample has a relatively high glutathione reductase (EC 1.6.4.2) activity. In this case NADPH, the product of the creatine kinase assay, will react with oxidized glutathione and the results for creatine kinase activity are incorrectly low.

Closest to the optimum are the conditions of Warren (7), but in that method the pH and the creatine phosphate concentration are too low and the magnesium ion concentration is too high.

The assay conditions we recommend are optimum at assay temperatures of either 30 °C (3) or 25 °C (6), probably even at 37 °C.⁵ There are now only two possibilities for further enhancement of creatine kinase activity at a fixed temperature: to use a lower buffer and (or) AMP concentration. In fact, in almost all methods buffer concentrations lower than 100 mmol/liter are specified. A decrease to 50 mmol/liter would increase the activity by about 5%, but there would be problems in maintenance of a constant pH, especially when the reaction is started with creatine phosphate. A still-justifiable decrease of the AMP/ADP molar ratio from 2.5 to 1.5 would increase the creatine kinase activity by only 2–3%.

How long the reaction rate remains constant definitely depends on the NADP⁺ concentration. With 2 mmol/liter, the linear range of activity could be extended to 1500 U/liter at a volume fraction of serum of

0.02. Because there is a slight deviation from linearity even at 2000 U/liter, although only 10% of the NADP⁺ in the assay solution has been consumed, the deflection from zero-order kinetics obviously is due to the inhibition by NADPH formed during the reaction. Evidence for this statement will appear in a subsequent paper.

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⁵ W. Gerhardt, personal communication.