

[Chem. Pharm. Bull.]  
29(11)3350—3355(1981)

## The Inactivation Profile of Rabbit Muscle Creatine Phosphokinase in Tris-acetate Buffer Solutions<sup>1)</sup>

HSINGCHU HSU<sup>a</sup> and JUN WATANABE<sup>\*,b</sup>

Department of Pharmaceutics, Chia Nan Jr. College of Pharmacy,<sup>a</sup> 72-1, Pauan, Jenteh,  
Tainan, Taiwan, R.O.C. and Department of Biopharmaceutics, Faculty of  
Pharmaceutical Sciences, Nagoya City University,<sup>\*,b</sup> 3-1, Tanabe-dori,  
Mizuho-ku, Nagoya 467, Japan

(Received May 9, 1981)

The inactivation profile of rabbit muscle creatine phosphokinase (CPK) has been investigated in Tris-acetate buffer solutions under various conditions of pH and temperature.

The inactivation pattern follows apparent first-order kinetics at pH very close to 6.00 (isoelectric point of CPK) and 30 to 39°C and at pH 7.40, 39°C. The activation energy of the inactivation at pH very close to 6.00 is 31.8 kcal/mol. The inactivation half-lives of CPK are 13.6 h (pH 5.95, 30°C), 7.9 h (pH 5.93, 35°C), 3.0 h (pH 5.90, 39°C) and 11.9 h (pH 7.40, 39°C).

At pH 6.70, the inactivation profile follows a biexponential curve at 30°C and at 39°C. The time course consists of two types of inactivation: an initial rapid inactivation ( $\alpha$ -phase) and a gradual inactivation ( $\beta$ -phase).

Anomalous irregular inactivation profiles are also observed at pH 7.00, 7.40 and 8.00 at 30°C as well as at pH 8.00 at 39°C. Some possible explanations of these irregular inactivation profiles are presented.

**Keywords**—rabbit muscle creatine phosphokinase; Tris-acetate buffer; pH; temperature; first-order inactivation; biphasic inactivation; irregular inactivation

Muscle type creatine phosphokinase isoenzyme is known to be involved in the energy producing and utilizing mechanism of muscular activity. Since the isoenzyme is one of the tissue localized enzymes, elevation of the isoenzyme activity in blood has been used as an important diagnostic marker of various muscular diseases.<sup>2)</sup> In recent years, attempts have been made to correlate the serum creatine phosphokinase level with the muscular lesions due to intramuscular injections.<sup>3)</sup> Tris buffer systems have been widely used in the isolation and quantitation of the isoenzyme<sup>4)</sup> and Tris-acetate buffer solution has been reported to be a favorable system in terms of both stability and activity.<sup>5)</sup> However, the inactivation profile of the isoenzyme either *in vivo* or *in vitro* remains obscure.

This report presents the results of an *in vitro* study under various pH conditions and at various temperatures in 50 mM Tris-acetate buffer system and discusses possible patterns of inactivation of rabbit muscle creatine phosphokinase (CPK).

### Materials and Methods

**Materials**—Purified rabbit muscle creatine phosphokinase (CPK: 128.5 U/mg protein) and ADP were obtained from Sigma Co., U.S.A. Tris(hydroxymethyl)-aminomethane, glacial acetic acid, sodium chloride and magnesium acetate were supplied by Wako Co., Japan. All reagents were of biochemical or analytical grade and were used without further purification.

**Incubation Conditions**—The CPK was dissolved in 50 mM Tris-acetate buffer solutions of pH 6.00, 7.00, 7.40 and 8.00 with initial activity in the range of 1157.2—3989.4 U/l (indicated in each figure). The activity range is similar to that usually detected in serum of patients with muscular lesions. The resultant solutions were divided into small glass tubes with tight polyethylene caps. The tubes were incubated in a water bath at 30, 35, or 39 ± 0.1°C. The initial 15 min of incubation was taken as the time required for temperature equilibration, so that at the end of the 15th minute the zero time count was started. After certain periods

of incubation, samples were serially removed from the water bath and submerged in an ice bath. The activity determinations were carried out within two days.

**Activity Determination**—The CPK activity was determined at 25°C with a CK-NAC activated Monotest kit (Boehringer Mannheim, West Germany) which is based on the optimized Oliver-Rosalki method,<sup>6)</sup> using a spectrophotometer (model UV-210, Shimadzu, Japan). The activity was calculated from the equation:  $U/1 = \Delta A/\text{min}$  (the increased absorbance at 340 nm per minute)  $\times$  dilution factor  $\times 4127$ . The  $\Delta A/\text{min}$  was obtained from the maximum value at the reaction steady phase (at least 4 min) during 30 min reaction. The upper limit for the total CPK activity in terms of the reagent capacity of the kit is about 200 U/1 and the method of dilution described in the leaflet using normal saline alone does not work well for the incubated samples; further, it has been pointed out that normal saline dilution of a serum sample leads to erroneous results in activity determination.<sup>7)</sup> Thus, a modified dilution method was used in this study. Each sample was subjected to triplicate determinations, and the activity is represented as mean  $\pm$  standard deviation (S.D.).

**Method of Dilution in Activity Determination**—An equal volume of Tris-acetate buffer solution containing 2 mM ADP and 10 mM magnesium acetate (pH 7.40) was added to the sample, and then sufficient normal saline was added. The whole was mixed well and incubated in an ice bath for exactly 20 min. Subsequently, the activity of the remaining CPK was determined with the diluted sample. Since the substrate added at the dilution stage does not include the reaction starter, phosphocreatine, the dilution does not interfere with the activity determination of the fresh sample but does improve the sensitivity of detection. The dilution method also results in a smaller standard deviation than simple dilution with normal saline triplicate determinations.

## Results

### The Apparent First-Order Inactivation Profile of CPK

The isoelectric point of rabbit muscle creatine phosphokinase is reported to be pH 6.0—6.1.<sup>8)</sup> At very close to this pH, the inactivation profile of CPK in 50 mM Tris-acetate buffer solution follows apparent first-order kinetics, as shown in Fig. 1. The activation energy of the inactivation at pH 6.0 was estimated from the Arrhenius equation, and is 31.8 kcal/mol. The inactivation rate constant increases 1.7 times when the temperature is increased by 5°C from 30°C to 35°C, but increases 4.6 times when the temperature is raised by 9°C from 30°C to 39°C. An apparent first-order inactivation pattern can also be observed at 39°C, pH 7.40 as demonstrated in Fig. 2. At 39°C, the inactivation rate constant of CPK at pH

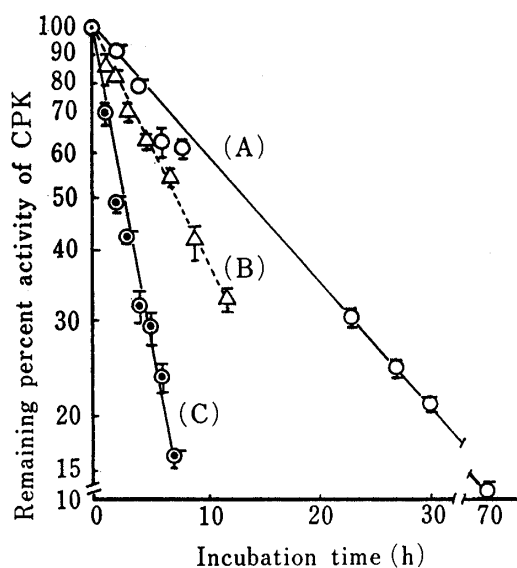


Fig. 1. The Inactivation Profile of Rabbit Creatine Phosphokinase (CPK) in 50 mM Tris-acetate Buffer Solutions

(A) 30°C, pH 5.95 (initial activity:  $1572.5 \pm 48.8$  U/l);  
(B) 35°C, pH 5.93 ( $1157.2 \pm 71.1$  U/l); (C) 39°C, pH 5.90 ( $1400.3 \pm 35.2$  U/l).

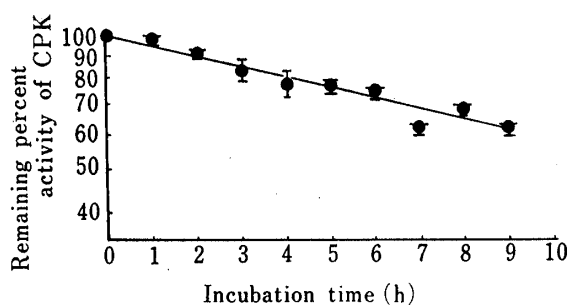


Fig. 2. The Inactivation Profile of Rabbit Creatine Phosphokinase (CPK) in pH 7.40, 50 mM Tris-acetate Buffer Solution at 39°C

Initial activity:  $2823.0 \pm 141.7$  U/l.

TABLE I. The Regression Equations and Half-lives of CPK in Apparent First Order Inactivation

pH	Temp.(°C)	Regression Equation	Half-life (h)
5.95	30	$A = 100 \times e^{-0.0509t}$	13.6
5.93	35	$A = 100 \times e^{-0.0873t}$	7.9
5.90	39	$A = 100 \times e^{-0.2344t}$	3.0
7.40	39	$A = 100 \times e^{-0.0583t}$	11.9

A: percent activity.

5.90 is 1.5 times that at pH 7.40. The first-order regression equations and the half-lives are shown in Table I.

### The Biphasic Inactivation Profile of CPK

Fig. 3 illustrates that CPK in pH 6.70 Tris-acetate buffer solution at both 30°C and 39°C undergoes two types of inactivation: an initial rapid inactivation ( $\alpha$ -phase) and a gradual inactivation ( $\beta$ -phase). The best fitting curves can be represented as biexponential equations by the least-squares method and they are given in Table II together with their half-lives.

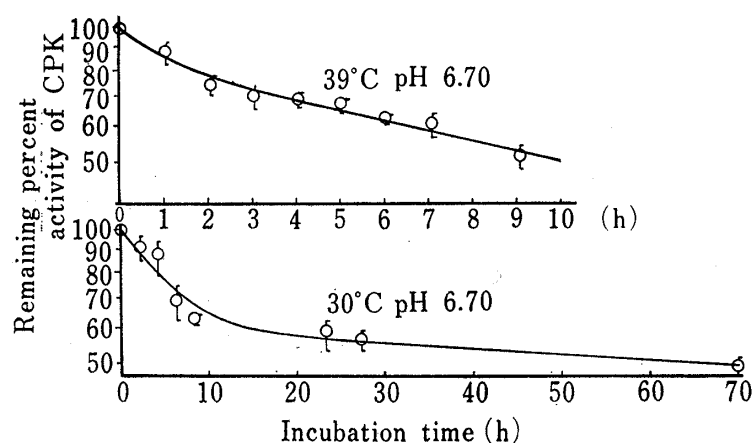


Fig. 3. The Inactivation Profile of Rabbit Creatine Phosphokinase (CPK) in 50 mM Tris-acetate Buffer Solution pH 6.70 at 30°C and at 39°C  
Initial activity: 30°C ( $3140.0 \pm 52.9$  U/l),  
39°C ( $2673.6 \pm 100.6$  U/l).

TABLE II. The Regression Equations and Half-lives of CPK in pH 6.70 Tris-acetate Buffer Solution at 30°C and at 39°C

pH	Tempe.(°C)	Regression Equations	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)
6.70	30	$A = 44.46 e^{-0.2067t} + 60.62 e^{-0.00317t}$	3.4	218.7
6.70	39	$A = 16.95 e^{-1.043t} + 83.87 e^{-0.0512t}$	0.7	13.5

A: percent activity.

### The Irregular Inactivation Profile of CPK

The time courses of the inactivation of CPK at pH 7.00, 7.40 and 8.00 at 30°C as well as that at pH 8.00 at 39°C form zigzag patterns and anomalous activity fluctuations are seen as illustrated in Figs. 4 and 5. Namely, in spite of a low remaining activity determined at a certain time, the same sample will show a higher activity at a later incubation time. Similar anomalous fluctuation has also been described in the literature<sup>5b,9)</sup> on serum CPK activity in

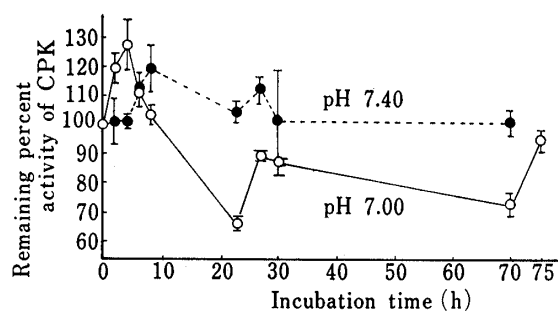


Fig. 4. The Inactivation Profile of Rabbit Creatine Phosphokinase (CPK) in 50 mM Tris-acetate Buffer Solution pH 7.00 and pH 7.40 at 30°C

Initial activity: pH 7.00 ( $2341.4 \pm 61.8$  U/l),  
pH 7.40 ( $3075.0 \pm 51.4$  U/l).

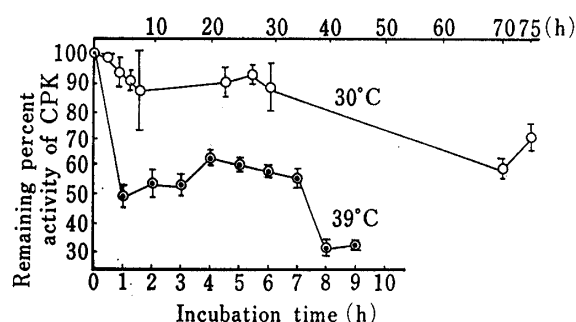


Fig. 5. The Inactivation Profile of Rabbit Creatine Phosphokinase (CPK) in 50 mM Tris-acetate Buffer Solution pH 8.00 at 30°C and at 39°C

Initial activity: 30°C ( $3989.4 \pm 157.6$  U/l),  
39°C ( $2515.4 \pm 82.2$  U/l).

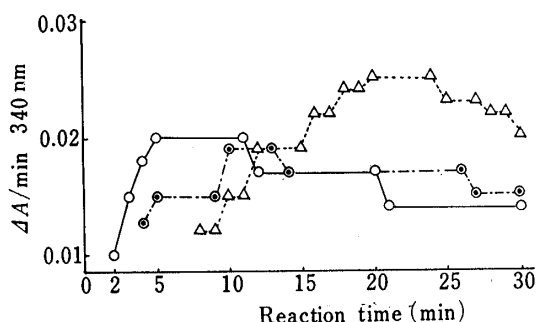


Fig. 6. The Reaction Patterns of Fresh CPK and Incubated CPK in Tris-acetate Buffer Solution with the Optimized Oliver-Rosalki Method at 25°C

(○) fresh CPK in pH 7.40, 50 mM Tris-acetate;  
(●) incubated CPK in pH 6.70, 50 mM Tris-acetate at 39°C for 8 h; (△) incubated CPK in pH 8.00, 50 mM Tris-acetate at 39°C for 1 h.

storage studies, but the phenomenon has not been explained. Different reaction patterns during activity determination reaction have been observed between a fresh CPK sample (without incubation) and an incubated CPK sample, as demonstrated in Fig. 6.

### Discussion

The irreversible inactivation of CPK is considered to be primarily thermal and the reversible inactivation appears to be an oxidation-reduction phenomenon.<sup>10)</sup> The CK-NAC activated optimized Oliver-Rosalki method<sup>6)</sup> provides a reactivation process so that the loss of activity detected is considered to be mainly due to irreversible inactivation. The rates

and the patterns of the irreversible inactivation of CPK seem to depend on both temperature and pH (Figs. 1–5). Although less work has been reported on the influence of pH than on that of temperature on the stability of CPK, it is clear that pH is also critically important to the stability of CPK.

The strength of hydrophobic interactions in proteins increases with rising temperature<sup>11)</sup> and acetate ions have been reported to reduce the hydrophilic properties of proteins.<sup>12)</sup> The conditions that allow the inactivation profiles of CPK to appear as linear (Figs. 1 and 2) are those under which CPK molecules show rather strong hydrophobic interactions, for instance, at pH close to the isoelectric point. Therefore, it seems reasonable to assume that CPK molecules are dispersed in Tris-acetate buffer solution in the form of microaggregates. Thus, the thermal inactivation proceeds from the outer layer of the aggregates toward the inner layer by diffusion of heat to give an apparent first-order kinetic inactivation. At 39°C, pH 7.40, it is obvious that the temperature effect on the hydrophobic interaction predominates over the ionic repelling effect due to the pH. As a result, apparent first-order kinetic inactivation is observed.

The optimum pH for the storage of human serum creatine phosphokinase has been suggested by Nealon *et al.*<sup>13)</sup> to be approximately 6.50, whereas the optimum pH to obtain the maximum activity of human creatine phosphokinase in the Oliver-Rosalki coupled enzyme reaction is 6.70.<sup>6)</sup> It seems probable from the experimental findings that purified rabbit

muscle CPK is similarly most stable at near neutral pH. The purified CPK has been resolved into six bands of monomer by starch gel electrophoresis at pH 6.3 in a Tris-citrate buffer system, and heterogeneity of the isoenzyme is postulated.<sup>14)</sup> Presumably, a part of isoenzyme with conformational differences resulting from alternations in primary structure or ligand heterogeneity might behave different thermostability. Thus, the  $\alpha$ -phase inactivation might result from the inactivation of the most thermolabile isoenzyme in the aggregates under the mildest incubation conditions and optimum pH (Fig. 3, a distinct  $\alpha$ -phase at pH 6.70, 30°C).

When the pH value of the solution is increased from the isoelectric point, CPK will be ionized. It is obvious that a counter force relationship exists in the aggregates. Namely, there are the repelling ionic interactions of CPK molecules (influenced by the solution pH) and the attracting hydrophobic interactions (influenced by the incubation temperature). When the ionic repelling force is predominant, disintegration of the aggregates will occur together with the denaturation and inactivation of CPK. The disintegration of aggregates results in the release of the intact CPK which is bound inside the aggregates. This might be the cause of the fluctuation of CPK activity observed in Figs. 4 and 5. It is evident that the size of CPK aggregates formed in the buffer systems depends on the concentration of CPK protein, temperature, pH and the incubation time. A colloidal suspension is formed and is visible when dissolving CPK with activity higher than 3000 U/l in pH 6.00 Tris-acetate buffer solution at room temperature. At pH higher than 7.00, CPK with activity higher than 5000 U/l will form a suspension of coagulated white precipitates after incubation for a few hours at 39°C. Although no appreciable precipitate was observed over the activity range studied, it is likely that microaggregates with a size of less than 50  $\mu$  (the visible limit size) are formed in the solution along with the inactivation process in view of the different reaction patterns observed for fresh CPK sample and the incubated samples. Fig. 6 shows data for a fresh sample prepared by dissolving CPK in Tris-acetate buffer solution; the maximum initial velocity was reached quickly with a short lag time. In contrast, the incubated CPK samples show several pseudo-steady phases before they reach maximum velocity. Further, the coefficient of variation in triplicate determinations increases from 2–4% in fresh samples to 10–18% in incubated samples. Serum alkaline phosphatase has also been noted to increase its apparent activity on standing at room temperature, and disintegration of aggregates was proposed to explain the phenomenon.<sup>15)</sup> It is possible a similar explanation can be applied to the fluctuation of CPK activity.

The inactivation profile of CPK in biological fluids is under investigation in this laboratory.

**Acknowledgement** This work has been supported by a grant to Hsingchu Hsu from the National Science Council, Republic of China.

#### References and Notes

- 1) This paper constitutes Part I of a series entitled "Biopharmaceutical Studies on Muscle Creatine Phosphokinase."
- 2) J. Eschar and H.J. Zimmerman, *Am. J. Med. Sci.*, **253**, 272 (1967); S. Ebashi, Y. Toyokura, H. Momoi, and H. Sugita, *J. Biochem. (Tokyo)*, **46**, 103 (1959); J.C. Dreyfus, G. Schapira, J. Resnais, and L. Secbat, *Rev. Eur. Etud. Clin. Biol.*, **5**, 386 (1960); J.P. Colombo, R. Richterich and E. Rossi, *Klin. Wochenschr.*, **40**, 37 (1962); J.W. Hess, R.P. MacDonald, R.I. Frederick, R.N. Jones, J. Neely, and D. Gross, *Ann. Intern. Med.*, **61**, 1015 (1964).
- 3) J.E. Gray, R. Weaver, M. Prestrud, and R.M. DeHaan, *Toxicol. Appl. Pharmacol.*, **17**, 297 (1970); J.E. Gray, R. Weaver, J. Moran, and E.S. Feenstra, *Toxicol. Appl. Pharmacol.*, **27**, 308 (1974); F.R. Sidell, D.L. Culver, and A. Kaminskis, *J. Am. Med. Assoc.*, **229**, 1894 (1974); H.O. Gloor, C. Vorbürger, and J. Schadelin, *Schweiz. Med. Wschr.*, **107**, 948 (1977); O. Svendsen, F. Rasmussen, P. Nielsen, and E. Steiness, *Acta Pharmacol. et Toxicol.*, **44**, 324 (1979).
- 4) H.M. Eppenberger, D.M. Dawson, and N.O. Kaplan, *J. Biol. Chem.*, **242**, 204 (1967); D.A. Nealon and A.R. Henderson, *Clin. Chem.*, **21**, 392 (1975); N. Hall, P. Addis, and M. DeLuca, *Biochem. Biophys. Res. Commun.*, **76**, 950 (1977); A.M. Magro, *Immunology*, **39**, 323 (1980).

- 5) a) E.J. Milner-White and D.C. Watts, *Biochem. J.*, **122**, 727 (1971); b) H.W. Cho and H.Y. Meltzer, *Am. J. Clin. Path.*, **71**, 75 (1979); c) L.G. Morin, *Clin. Chem.*, **23**, 1569 (1977).
- 6) H.U. Bergmeyer, H. Breuer, H. Buttner, A. Delbruck, D. Laue, W. Pilz, E. Schmidt, F.W. Schmidt, D. Stamm, and G. Szasz, *J. Clin. Chem. Clin. Biochem.*, **15**, 255 (1977).
- 7) F.A. Graig, J.C. Smith, and F.F. Foldes, *Clin. Chim. Acta*, **15**, 107 (1967); I. Dobosz, *Clin. Chim. Acta*, **50**, 301 (1974); E. Laurella, F. DeCarolis, S. Moretti, and B. Petrone, *Rass. Int. Clin. Ter.*, **60**, 470 (1980).
- 8) L. Noda, S.A. Kuby, and H.A. Lardy, *J. Biol. Chem.*, **209**, 203 (1954).
- 9) W.H.S. Thomson, *Clin. Chim. Acta*, **23**, 105 (1969).
- 10) L.G. Morin, *Clin. Chem.*, **23**, 646 (1977).
- 11) G. Nemethy and H.A. Scheraga, *J. Phys. Chem.*, **66**, 1773 (1962).
- 12) V.M. Rosenoer, M. Oratz, and M.A. Rothschild, "Albumin Structure, Function and Uses," Pergamon Press, Inc., New York, 1976, p. 13.
- 13) D.A. Nealon, S.M. Pettit, and A.R. Henderson, *Clin. Chem.*, **26**, 1165 (1980).
- 14) C. Traugott and E.J. Massaro, *Biochim. Biophys. Acta*, **295**, 549 (1973).
- 15) R.J. Henry, "Clinical Chemistry," Harper and Row, Inc., New York, 1964, p. 482.