Chem. Pharm. Bull. 30(3)1009—1013(1982)

The Inactivation Profile of Rabbit Muscle Creatine Phosphokinase in Biological Fluids[1]

Hsingchu Hsu[a] and Jun Watanabe*,[b]

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

(Received August 11, 1981)

The inactivation profiles of rabbit muscle creatine phosphokinase (CPK) in heparinized whole blood and plasma under various pH conditions at 39°C have been investigated. Under pH 7.40, at 39°C, the inactivation profile of CPK in heparinized whole blood follows apparent first-order kinetics. The inactivation rate constant is 0.054 h^{-1} and is very close to that observed in patients with acute myocardial infarction (reported by Steele and Cohn) but much less than that resulting from exercise (reported by King *et al.*). The nature of the inactivation of CPK *in vitro* is presumably thermal denaturation, and there is no evidence to show that blood cells or dissolved oxygen are involved. The inactivation profiles of CPK in heparinized whole blood at pH other than 7.40 appear to be biphasic. The regression equations and the half-lives are presented.

Keywords——rabbit muscle creatine phosphokinase; heparinized whole blood; plasma; pH; first-order inactivation; biphasic inactivation

The nature of the inactivation of creatine phosphokinase (CK) *in vitro* or *in vivo* is still uncertain. The view that sulfhydryl groups of the CK enzyme are involved in the inactivation is challenged by several experimental findings. Smith *et al.*[2] demonstrated an anomalous sulfhydryl group in CK and concluded that the iodoacetamide-sensitive active-site sulfhydryl group in CK is not essential for the enzyme activity. Warren[3] also reported that the oxidative inactivation of sulfhydryl groups in CK can be readily reversed by the addition of thiol compounds and he also postulated that the irreversible inactivation of CK is primarily thermal on the basis of an *in vitro* incubation study. In the preceding report, we demonstrated that pH, albumin and urate also play important roles in the inactivation of rabbit muscle CK *in vitro*.[4] The fate of enzymes in the circulatory system is usually uncertain and the available experimental evidence, as pointed out by Henley *et al.*,[5] is scanty and contradictory. Thus, the inactivation rates of human CK determined during exercise[6] or in the progress of delivery[7] and in patients[8] appear to be paradoxical.

This report presents the results of an *in vitro* study on the inactivation profile of rabbit muscle creatine phosphokinase (CPK) in biological fluids at 39°C (normal body temperature of the rabbit) under various pH conditions. The nature of the inactivation of CPK is also discussed.

Materials and Methods

Materials——Purified rabbit muscle creatine phosphokinase (CPK, 128.5 U/mg protein) and rabbit serum albumin (RSA) were obtained from Sigma Co., U.S.A. Sodium hydroxide, sodium chloride, acetic acid, sodium azide and sodium heparin were supplied by Wako Co., Japan. All reagents were of biochemical or analytical grade, and were used without further purification. Blood specimens were obtained from male white rabbits weighing 2.7—3.0 kg. Heparinized whole blood and plasma contained 0.05% (w/v) each of sodium heparin and sodium azide. The pH values were adjusted to 6.75; 7.40; and 8.03 at 39±0.1°C with the least possible amount of 5% NaOH or 10% acetic acid. The heparinized whole blood and plasma of pH 7.72 and the denatured plasma of pH 8.30 were used without adjusting the pH with alkali or acid after

adding sodium heparin and sodium azide. Denatured plasma was obtained by incubating the fresh plasma at 56°C for 30 min. A comparative study between arterial and venous blood collected from the ear vessel was carried out at 35°C with blood specimens from the same rabbit and without adjusting the pH.

The Initial Activity——The initial activity was set in the range of 10000—20000 U/l (Figs. 3, 4, and 5) in whole blood and in plasma so that the *in vitro* study would provide data comparable with the results of an *in vivo* study which is in progress in this laboratory. Since the serum CPK level of conscious rabbits fluctuated by hundreds U/l due to emotional distress and other factors in our preliminary experiment, the initial activity of 10000—20000 U/l might be sufficient for the effect of endogeneous CPK fluctuation during *in vivo* study to be neglected. A comparative study between arterial and venous blood was carried out at an initial activity of 521—561 U/l so as to avoid the possibility of overlooking small differences when high initial activity was employed (Fig. 2). Because denatured plasma is widely used for the dilution of high activity specimens during activity determination without consideration of the pH effect, and activity reaching thousands U/l in sera can occur during muscular lesions or acute muscular diseases, initial activity of 3080 U/l was used in the denatured plasma system to elucidate the stability of CPK. The initial activity of endogeneous CPK was 374±11 U/l, which was obtained by elevating the CPK level of the rabbit by exercise and emotional agitation for a few minutes.

Incubation Conditions——CPK was dissolved in physiological salt solution and was added to the biological fluids; it increased the volume by approximately 2%. The resultant fluids were divided into small glass tubes with tight-fitting polyethylene caps. The tubes were incubated in a water bath at 39±0.1°C, except for the comparative study for the arterial and venous blood which was incubated in a water bath at 35±0.1°C. The initial 15 min of incubation was taken as the time required for temperature equilibration so that the zero time count was started after the end of the 15th minute. After certain periods of incubation, the samples were serially removed from the water bath and submerged in an ice bath. The activity determinations of the separated plasma fraction were carried out within two days. The incubation was carried out twice, first to obtain a preliminary profile and secondly with carefully designed time-interval incubation to obtain the detailed profile, and the similar profiles were confirmed.

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[9] using a spectrophotometer (model UV-210, Shimadzu, Japan). Each sample was subjected to triplicate determinations and the activity is represented as mean±standard deviation (S.D.).

Method of Dilution in Activity Determination——Five parts of 5% RSA solution was added to one part of sample solution and well mixed, and then sufficient physiological salt solution was added. The whole was well mixed and incubated in an ice bath for exactly 5 min, then the activity was determined.

Results

The Effect of Initial Activity, Endogenous and Purified CPK on the Inactivation Profile

There was evidence that neither the initial activity level nor the source of CPK (endogenous or purified CPK) would affect the inactivation profile in whole blood if the

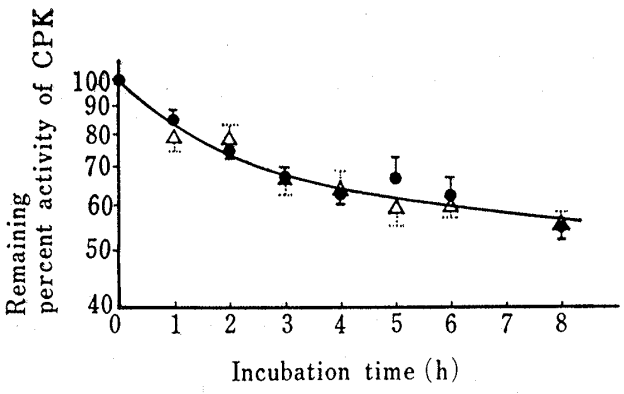

Fig. 1. The Inactivation Profile of Endogenous CPK and Purified CPK in Heparinized Whole Blood at pH 7.72 at 39°C

△: endogeneous CPK (initial activity: 374±11 U/l); ●: purified CPK (17200±518 U/l).

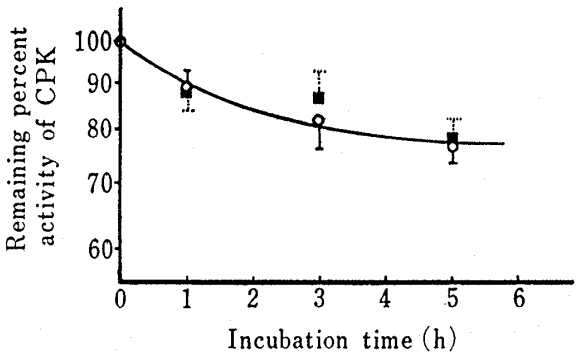

Fig. 2. The Inactivation Profile of CPK in Heparinized Arterial and Venous Whole Blood Samples at pH 7.53 at 35°C

■: arterial blood (initial activity: 521±27 U/l); ○: venous blood (561±32 U/l).

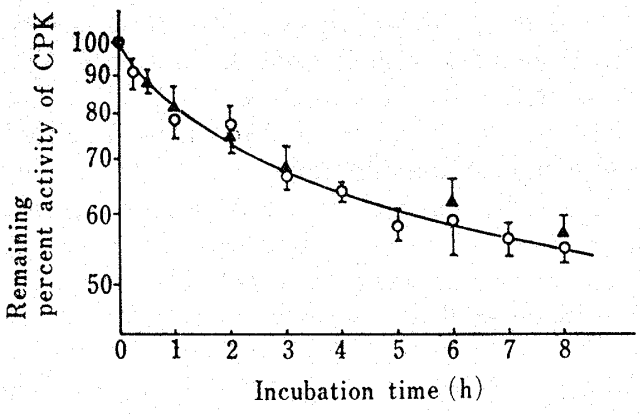

Fig. 3. The Inactivation Profile of CPK in Heparinized Whole Blood and Plasma, at pH 7.72 at 39°C

○: whole blood (initial activity: 17200±518 U/l); ▲: plasma (17900±527 U/l).

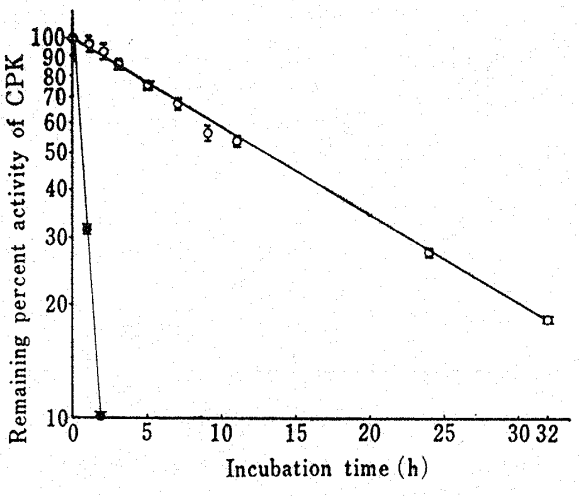

Fig. 4. The Apparent First Order Inactivation Profile of CPK at 39°C

○: pH 7.40, heparinized whole blood (initial activity: 9300±282 U/l); ●: pH 8.30, denatured plasma (3080±381 U/l). Solid lines represent the regression lines obtained by the least-squares method.

pH and temperature were defined, as shown in Fig. 1. Thus, endogenous CPK with an activity of 374 U/l showed essentially the same inactivation profile as 17200 U/l of purified CPK in heparinized whole blood under pH 7.72 at 39°C.

The Effect of Arterial or Venous Blood and Blood Cells on the Inactivation Profile of CPK

Figure 2 shows that the inactivation profiles of CPK in heparinized arterial and venous whole blood samples (pH 7.53, 35°C) with similar initial activity were the same. This implies that the amount of dissolved oxygen is unlikely to affect the irreversible denaturation of CPK. It is also clear that blood cells are irrelevant to CPK inactivation. As can be seen in Fig. 3, there is no difference between the inactivation profile of CPK in plasma and in whole blood at the same pH and temperature with similar initial activity.

Apparent First-Order Inactivation Profile of CPK

We have reported that the inactivation of CPK occurs in an apparent first-order fashion at pH 7.40, 39°C in many systems. Thus, CPK in 50 mM Tris–acetate buffer[4b] or in 1.5% RSA and in 50 mM phosphate buffer[4c] showed apparently linear inactivation, at least during the first 9—11 h of incubation. Under pH 7.40, 39°C, CPK showed a significant apparent first-order inactivation in heparinized whole blood for the whole time course studied (32 h) with a relatively small S.D. (less than 4%). Unexpectedly, CPK in pH 8.30, denatured plasma also showed a linear inactivation pattern. The rate of inactivation was extremely rapid, so that only 0.5% of the initial activity remained after 4 h at 39°C. These results are illustrated in Fig. 4 and the regression equations along with the half-lives are listed in Table I.

TABLE I. The Regression Equations and Half-lives of CPK in Apparent First Order Inactivation at 39°C

System	pH	Initial activity (U/l)	Regression equation	Time interval	Half-life (h)
Heparinized whole blood	7.40	9300±282	$A=100e^{-0.054t}$	$0\leq t\leq 32$(h)	12.8
Denatured plasma	8.30	3080±381	$A=100e^{-1.3t}$	$0\leq t\leq 4$(h)	0.5

A: Percent activity. Initial activity: mean±S.D. Triplicate determinations.

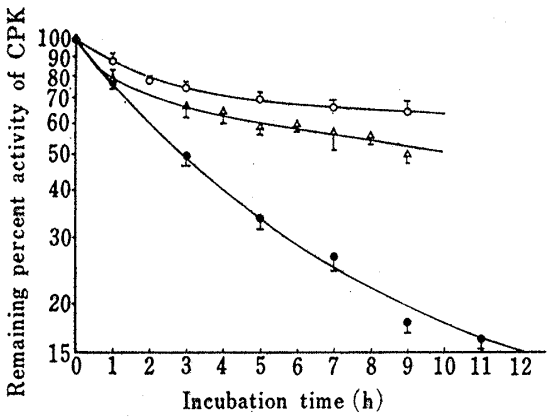

Fig. 5. The Biphasic Inactivation Profile of CPK in Heparinized Whole Blood at 39°C

○: pH 6.75 (initial activity: 8500±152 U/l); △: pH 7.72 (17200±518 U/l); ●: pH 8.03 (9360±573 U/l). Solid lines represent the regression lines obtained by the least-squares method.

Biphasic Inactivations Profile of CPK

The inactivation profile of CPK appeared to be biphasic in boilogical fluids at pH other than 7.40 at 39°C, as is shown in Fig. 5. The biphasic inactivation profiles can be described as biexponential functions and the best-fitting equations obtained by the least-squares method are listed in Table II along with the half-lives. There was no irregular inactivation of CPK in biological fluids under the conditions studied. This might be due to the high content of proteins in the biological fluids, which may have contributed to the homogeneous dispersion of CPK in the system. Further studies are required to clarify the details.

TABLE II. The Regression Equations and Half-lives of CPK in Biphasic Inactivation at 39°C at Various pH Values in Heparinized Whole Blood

pH	Initial activity (U/l)	Regression equation	Time interval (h)	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)
6.75	8500[a]	$A=28.6e^{-0.549t}+71.7e^{-0.013t}$	$0\leq t\leq 9$	1.3	53.3
	152[b]				
7.72	17200[a]				
	518[b]	$A=19.2e^{-1.76t}+80.3e^{-0.055t}$	$0\leq t\leq 9$	0.4	12.6
8.03	9360[a]				
	537[b]	$A=73.1e^{-0.326t}+26.5e^{-0.061t}$	$0\leq t\leq 24$	2.1	11.4

A: Percent activity. *a*) Mean. *b*) S.D. Triplicate determinations.

Discussion

The Effect of pH on the Inactivation of CPK in Biological Fluids

It is clear that the stability behavior and the inactivation profile of CPK in blood and in plasma also depend on the pH, as expected (Figs. 4 and 5). CPK appears to be most stable at near neutral pH (6.8—7.0) in biological fluids, as has been observed in buffers and in RSA solutions.[4b,c] It is important to note that due to the different pattern of inactivation, CPK appears to be less stable at pH 6.75 than at 7.40 during the initial stage of incubation at 39°C. However, the situation is reversed after 9 h incubation time. This is particularly important in considering the pH dependency of CPK stability.

The Inactivation Rate of CPK in Blood

The results of an *in vitro* study on the inactivation profile of CPK in whole blood at pH 7.40, 39°C showed an apparent linear pattern (Fig. 4). This is consistent with the findings of Shell and Sobel[10] that the disappearance of total CK and cardiac CK from the ciculation in patients with acute myocardial infarction conforms closely to first-order kinetics. It is interesting to note that the *in vitro* CPK inactivation rate constant in whole blood at normal rabbit body temperature (39±0.1°C) obtained in this study is 0.054 h^{-1} (eq. to 9.0×10^{-4} min^{-1}, Table I) and is very close to the *in vivo* average disappearance rate constant of total CK,

$(8.6\pm1.8)\times10^{-4}$ min^{-1}, obtained from twenty patients with acute myocardial infarction by Steele and Cohn.[8] Morin[11] also reported the first-order decay constant of human muscle creatine phosphokinase in serum matrix at 37°C to be 8.3×10^{-4} min^{-1} from an *in vitro* incubation study. The consistency between *in vivo* and *in vitro* studies suggests that the *in vitro* inactivation profile of creatine phosphokinase under artificial physiological conditions may reflect the inactivation profile *in vivo* reasonably well. Considering that no evidence was obtained that CPK is subjected to liver microsomal oxidative inactivation in our preliminary experiment, as well as the above findings that blood cells or dissolved oxygen are probably not involved in the irreversible inactivation of CPK *in vitro*, it seems likely that the nature of the inactivation of CPK both *in vitro* and *in vivo* is primarily thermal rather than directly physiological, as postulated by Warren[3] and Morin.[11] Nevertheless, there is another possibility, *i.e.*, that the rate of change of serum CK level in acute myocardial infarction patients may be related to the release of CK from the heart and the disappearance of CK from the circulation. In fact, a much larger inactivation rate constant of human CK *in vivo* (k_d) has been reported by King *et al.*[12] to result from exercise: $k_d=0.0022$ to 0.0028 min^{-1}. In addition, there is evidence that marked cardial CK inactivation occurs in cardial lymph *in vitro* and *in situ*,[10] and it is well known that reticuloendothelium is important for the transport of macromolecules. The disappearance of serum CPK *in vivo* might occur *via* more complicated pathways than simple inactivation by body temperature. If this is the case, a much faster inactivation rate would be seen *in vivo* than that *in vitro*. An *in vivo* kinetic study is in progress in this laboratory.

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

References and Notes

1) This paper constitutes Part III of the series entitled "Biopharmaceutical Studies on Muscle Creatine Phosphokinase."
2) D.J. Smith and G.L. Kenyon, *J. Biol. Chem.*, **249**, 3317 (1974); D.J. Smith, E.T. Maggio, and G.L. Kenyon, *Biochemistry*, **14**, 766 (1975).
3) W.A. Warren, *Clin. Chem.*, **18**, 473 (1972).
4) *a*) D.A. Nealon, S.M. Pettit, and A.R. Henderson, *Clin. Chem.*, **26**, 1165 (1980); *b*) H. Hsu and J. Watanabe, *Chem. Pharm. Bull.*, **29**, 3350 (1981); *c*) H. Hsu, S. Ozeki, and J. Watanabe, *Chem. Pharm. Bull.*, **30**, 1002 (1982).
5) K.S. Henley and F.W. Schmidt, *J. Am. Med. Assoc.*, **174**, 977 (1960).
6) P. Schnor, P. Grande, and C. Christiansen, *Acta Med. Scan.*, **208**, 229 (1980); A.J. Siegel, L.M. Silverman, and R.E. Lopez, *Yale J. Biol. Med.*, **53**, 275 (1980).
7) G. Papa, G. Magri, M. DeSieena, P. Tarzia, and S. Lombardi, *Boll. Soc. Ital. Biol. Sper.*, **56**, 1474 (1980).
8) B.W. Steele and J.N. Cohn, *Clin. Chem.*, **22**, 1202 (1976).
9) 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).
10) W.E. Shell and B.E. Sobel, *Circulation*, **53**, Supp. I, 98 (1976).
11) L.G. Morin, *Clin. Chem.*, **23**, 646 (1977).
12) S.W. King, B.E. Statland, and J. Savory, *Clin. Chem.*, **22**, 1203 (1976).