Effects of pH, Albumin and Urate on the Inactivation Profile of Rabbit Muscle Creatine Phosphokinase[1]

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The effects of pH, albumin and urate on the inactivation behavior of rabbit muscle creatine phosphokinase (CPK) have been investigated at 39°C.

The conditions under which the inactivation of CPK shows apparent first order kinetics and biphasic behavior have been explored and regression equations are presented along with half-lives.

A circular dichroism (CD) study showed no evidence of spectral change of CPK in the range of pH 6.00—8.00, and a kinetic study showed that CPK is most stable at near neutral pH.

Both rabbit serum albumin (RSA) and urate significantly enhance the stability of the CPK activity and retard CPK coagulation during incubation. No evidence for any intermolecular interactions between RSA and CPK in pH 7.40, 50 mM phosphate buffer solution was obtained in the CD study. CPK is presumably just dispersed in the matrices of RSA and stabilized by protein colloid. On the other hand, urate is likely to form a polymeric structure in the buffer at around the physiological urate level in circulatory blood (2.5—3.5×10^{-4} M). CD spectra of CPK can be observed with a maximum around 287 nm, induced by urate. The molar ellipticity coefficient depends on the concentration of urate present in the solution.

Keywords——rabbit muscle creatine phosphokinase; pH; albumin; urate; CD; first order inactivation; biphasic inactivation; irregular inactivation

Many factors significantly affect the stability of creatine phosphokinase. The effect of serum pH on the storage stability of human creatine phosphokinase (CK) has been pointed out recently by Nealon *et al.*,[2] and we also reported the inactivation profile of rabbit muscle creatine phosphokinase (CPK) in Tris–acetate buffer solution in relation to pH and temperature.[3] Cho and Meltzer[4] found that plasma factors with molecular weights of less than 1000 are potent inhibitors of CK activity. Warren[5] reported that urate is a natural CK inhibitor and that the inactivation can be completely reversed by the addition of thiol compounds. The effect of albumin has been noted in the report of Nealon and Henderson[6] that human brain CK is extremely unstable in the presence of albumin (BSA); cardiac and muscle CK are very little affected. On the other hand, Morin[7] found that both albumin and urate enhance or stabilize human brain, cardiac and muscle CK activity. There are similarities among creatine phosphokinases of the same type from different species.[8] However, the effects of pH, albumin and urate on the stability of CPK and the mechanisms by which they are involved in the inactivation profile of CPK are still obscure.

This report presents the results of an *in vitro* study on the inactivation profile of CPK in 50 mM phosphate buffer solution and the effects of pH, albumin and urate on CPK inactivation. Some possible mechanisms by which the factors modify the stability of CPK are also discussed.

Materials and Methods

Materials——Purified rabbit muscle creatine phosphokinase (128.5 U/mg protein, mol. wt., $M_{S.D.}$: 81000[9]) and rabbit serum albumin (RSA, assumed to have mol. wt. 68000) were obtained from Sigma Co., U.S.A.

Uric acid, potassium phosphate monobasic, sodium hydroxide, sodium chloride and hydrochloric acid were supplied by Wako Co., Japan. All reagents were of biochemical or analytical grade, and were used without further purification. Phosphate buffer solution, 50 mM, pH 7.40 (39°C), was prepared from potassium phosphate monobasic and sodium hydroxide solution. RSA solution, 5%, pH 7.40 (39°C), was prepared by dissolving RSA in pH 7.40, 50 mM phosphate buffer solution, and the pH was readjusted by adding sodium hydroxide solution. Other RSA solutions (1.5%, pH 6.86, 7.00, 7.40, 8.30 and 5%, pH 7.00) were prepared by dissolving RSA in distilled water and adjusting the pH with the least possible amount of 5% NaOH or 10% HCl at 39°C.

The Initial Activity of CPK——The initial activity of CPK in the 1.5% RSA system or in the pH 7.00, 5% RSA system was in the range of 10500—13800 U/l (Figs. 1 and 2) which is comparable to the level in biological fluids (to be presented in the next report). However, due to the concentration requirement in the circular dichroism study, initial activity of CPK in the range of 143000—161000 U/l was used in pH 7.40, 50 mM phosphate buffer solution (Fig. 3). To assess the effect of urate in the presence of RSA, initial activity in the range of 91700—97000 U/l was utilized in the 5% RSA system at pH 7.40 (Fig. 4).

Incubation Conditions——CPK was dissolved in various systems (cited in each figure and table), and the resultant solutions were divided into small glass tubes with tight-fitting polyethylene caps. The tubes were incubated in a water bath at 39 ± 0.1°C (similar to the normal body temperature of rabbits). The initial 15 min of incubation was taken as the time 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 were carried out within two days after removing coagulated precipitates by means of centrifugation.

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[10] using a spectrophotometer (model UV-210, Shimadzu, Japan). The incubation was carried out twice, first to obtain the preliminary profile and secondly with carefully designed time-interval incubation to obtain the precise profile, and the similar profiles were confirmed. 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——Five parts of 5% RSA was added to one part of sample solution and well mixed, then sufficient physiological salt solution was added. The whole was well mixed and incubated in an ice bath for exactly 5 min. Subsequently, the activity of the CPK was determined. This dilution method was found to be the best. Table I shows that there will be an average of 17—19% of the CPK activity lost if physiological salt solution is used for the dilution of the CPK sample in phosphate buffer solution. It is probable that sudden dilution of CPK with salt solution in the absence of substrates or protective colloids to stabilize the intact conformation will cause spontaneous inactivation that lead to low apparent activity. The maximum value of the coefficient of variation was 7.3% through the sequential activity determinations (RSA dilution method) of all the samples studied. Though this value is slightly larger than the coefficient of variation usually observed in the case of fresh samples (less than 5%), this method is the best of several modifications of the activity determination procedure for incubated samples.

Circular Dichroism——Circular dichroism (CD) measurements were carried out on an automatic recording spectropolarimeter (model J-40A, JASCO, Japan) and a 1 cm cell was used.

Urate Determination——Urate assay was carried out in 50 mM phosphate buffer solution (pH 7.40) at 292 nm by the spectrophotometric method.[5]

Results

The Effect of Dilution Method on the Detected CPK Activity

Table I gives the detected CPK activity in 50 mM phosphate buffer solutions (pH 7.40) in the presence or absence of urate as determined by the 5% RSA dilution method and by the physiological salt solution method. It is clear that the former method retains higher activity than the latter method.

The Inactivation Profiles of CPK in RSA Solutions

The inactivation profiles of CPK in 1.5% RSA at pH 6.86, 7.40 and 8.30 at 39°C are presented in Fig. 1. Figure 2 shows the inactivation profiles of CPK in 5% RSA at pH 7.00 and 7.40 as well as in 1.5% RSA at pH 7.00, which reveals the effect of RSA concentration at the same pH.

The Inactivation Profiles of CPK in pH 7.40, 50 mM Phosphate Buffer Solution with or without RSA and Urate

The inactivation profiles of CPK in pH 7.40, 50 mM phosphate buffer solution with

TABLE I.   CPK Activity (U/1) detected in pH 7.40, 50 mM Phosphate Buffer Solution
without (A) or with (B) Urate $(3.1 \times 10^{-4} \text{M})$ during Incubation at 39°C
as determined by the 5% RSA Dilution Method (R) and
by the Physiological Salt Solution Method (S)

Incubation time (h)	0	1	3	11	32
AR	161000[a]	152000	140000	106000	91400
	9230[b]	6060	8010	2190	1400
AS	120000[a]	116000	101000	98500	80800
	11800[b]	6890	1260	1640	2210
AS/AR (%)	74.5	76.3	72.1	92.9	88.4
Average AS/AR :	81.0%				
BR	143000[a]	131000	128000	117000	78000
	1360[b]	500	2600	5580	3420
BS	119000[a]	113000	95500	89600	73500
	3290[b]	1670	1520	1760	2810
BS/BR (%)	83.2	86.3	74.6	76.6	94.2
Average BS/BR :	83.0%				

Triplicate determinations. a) Mean. b) S. D.
 AR: without urate, by 5% RSA dilution. AS: without urate, by physiological salt solution. BR: with
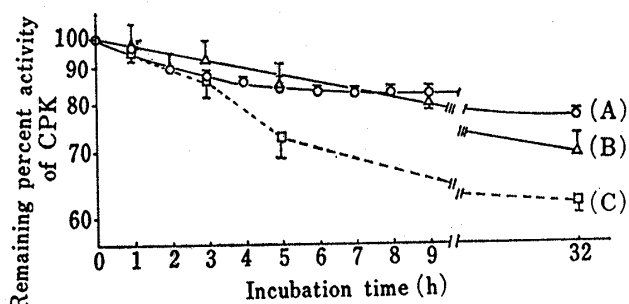urate, by 5% RSA dilution. BS: with urate, by physiological salt solution.

Fig. 1.   The Inactivation Profile of Rabbit Muscle
Creatine Phosphokinase in 1.5% RSA Solution
at 39°C

(A) pH 6.86 (initial activity: 10500 ± 451 U/l);
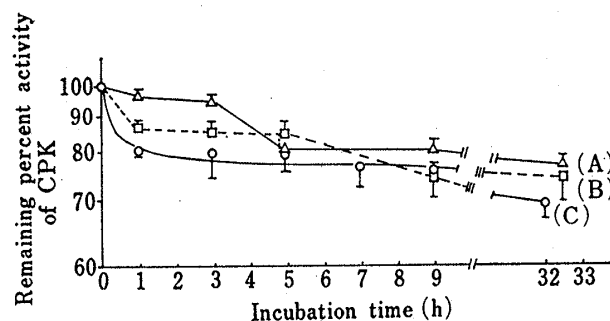(B) pH 7.40 (13200 ± 520 U/l);
(C) pH 8.30 (13800 ± 609 U/l).

Fig. 2.   The Inactivation Profile of Rabbit Muscle
Creatine Phosphokinase in 5% RSA and 1.5%
RSA Solutions at 39°C

(A) pH 7.00, 5% RSA (initial activity: 10800 ± 161 U/l);
(B) pH 7.00, 1.5% RSA (12700 ± 162 U/l);
(C) pH 7.40, 5% RSA in 50 mM phosphate buffer solution
(97000 ± 315 U/l).

or without urate are shown in Fig. 3; those in 5% RSA solution with or without
urate are illustrated in Fig. 4.

Apparent First-Order Inactivation Profile of CPK

We have reported that the inactivation of CPK is an apparent first-order reaction at pH
6.00 and 7.40 in 50 mM Tris–acetate buffer solution at 39°C.[3]   Similar apparent first-order
inactivation of CPK at 39°C is also observed in other systems such as pH 7.40, 1.5% RSA
(Fig. 1, B) and pH 7.40, 50 mM phosphate buffer solution for certain periods of incubation (Fig. 3,
A and B).   The regression equations and the half-lives are listed in Table II.

Biphasic Inactivation Profile of CPK

The inactivation profile of CPK shows a biphasic pattern in pH 6.86, 1.5% RSA and in
pH 7.40, 5% RSA solution (Figs. 1, A and 2, C or 4, B).   We have postulated that the ano-
malous fluctuation of activity observed in 50 mM Tris–acetate buffer solutions might be due
to the disintegration of microaggregates.[3]   During this study, we noticed that RSA had a
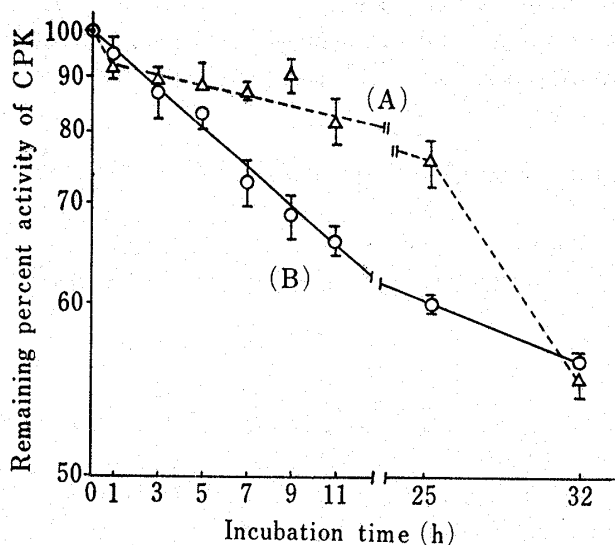
Fig. 3. The Inactivation Profile of Rabbit Muscle Creatine Phosphokinase in pH 7.40, 50 mM Phosphate Buffer Solutions with or without Urate, at 39°C

(A) with urate (3.1×10^{-4} M), initial activity: 161000 ± 9230 U/l;

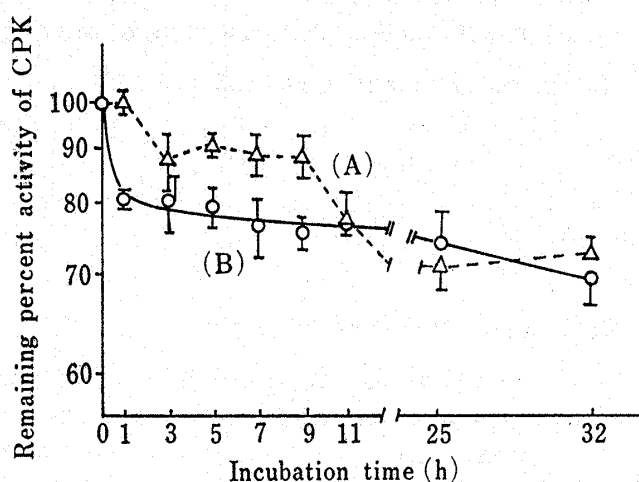(B) without urate, initial activity: 143000 ± 1360 U/l.

Fig. 4. The Inactivation Profile of Rabbit Muscle Creatine Phosphokinase in 5% RSA, 50 mM Phosphate Buffer Solution of pH 7.40 with or without Urate, at 39°C

(A) with urate (3.1×10^{-4} M), initial activity: 91700 ± 4190 U/l;

(B) without urate, initial activity; 97000 ± 3150 U/l.

TABLE II. The Regression Equations and Half-lives of CPK in Apparent First Order Inactivation at 39°C, pH 7.40

System	Initial activity	Regression equation	Time interval (h)	Half-life (h)
1.5% RSA	$13200^{a)}$ U/l $520^{b)}$	$A = 100e^{-0.028t}$	$0 \leqslant t \leqslant 9$	25.1
50 mM Phosphate buffer Without urate	$143000^{a)}$ U/l $1360^{b)}$	$A = 100e^{-0.039t}$	$0 \leqslant t \leqslant 11$	17.7
With urate	$161000^{a)}$ U/l $9230^{b)}$	$A = 91.5e^{-0.008t}$	$1 \leqslant t \leqslant 25$	86.6

A: percent activity. a) Mean. b) S.D.

solubilizing effect on CPK and at the same time retarded CPK protein coagulation during incubation. There were only traces of precipitates visible after incubation for 9 h at 39°C in RSA solutions, whereas some white coagulated precipitates could be seen after 1 h in phosphate buffer solution. Activity determinations carried out after centrifugation for 20

TABLE III. The Regression Equations and Half-lives of CPK in Biphasic Inactivation at 39°C

System	pH	Initial activity	Regression equation	Time interval (h)	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)
1.5% RSA	6.86	$10500^{a)}$ U/l $451^{b)}$	$A = 41.6e^{-0.194t} + 58.4e^{-0.028t}$	$0 \leqslant t \leqslant 9$	3.6	24.8
5% RSA	7.40	$97000^{a)}$ U/l $3150^{b)}$	$A = 20.5e^{-2.69t} + 79.5e^{-0.004t}$	$0 \leqslant t \leqslant 32$	0.3	173.3

A: percent activity. a) Mean. b) S.D

min at 4000 rpm showed little fluctuation of activity (within the 5% error range). 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 III along with the half-lives.

The Irregular Inactivation Profile of CPK

The CPK shows irregular inactivation at various pHs in the presence of RSA, *e.g.* in 1.5% RSA, pH 7.00 (Fig. 2, B), pH 8.30 (Fig. 1, C) and in 5% RSA, pH 7.00 (Fig. 2, A), pH 7.40 Fig. 4, A). The irregularity observed in these systems may be due to the combined effects of pH, RSA and urate on the stability of CPK. Further studies are required to elucidate the details.

The Circular Dichroism Spectra

The results of a CD study on CPK in 50 mM phosphate buffer solution (pH 7.40) with or without urate are depicted in Fig. 5. The CD spectra of CPK induced at around 287 nm by the presence of urate are related to the concentration of urate. The molar ellipticity coefficient at 287 nm is 13×10^4 deg cm^2/dmol (urate 2.0×10^{-4} M, Fig. 5, B) or 75×10^4 deg cm^2/dmol (urate 3.1×10^{-4} M, Fig. 5, C). In the far-ultraviolet region (250—200 nm), a 2 nm shift toward the longer wavelength side is also observed for 2×10^{-6} M CPK in the presence of 3.1×10^{-5} M urate (Fig. 5, E).

Figure 6 depicts the CD spectrum of RSA (2×10^{-6} M) and urate (3.1×10^{-4} M). Figure 7 depicts a typical CD spectrum of urate at 3.1×10^{-4} M in 50 mM phosphate buffer solution, pH 7.40.

The Stability Profile of Urate

Figure 8 depicts the stability of urate (initial concentration: 3.0×10^{-3} M) in pH 7.40, 50 mM phosphate buffer solution at 39°C. The profile was obtained from the change of absorption at 292 nm.
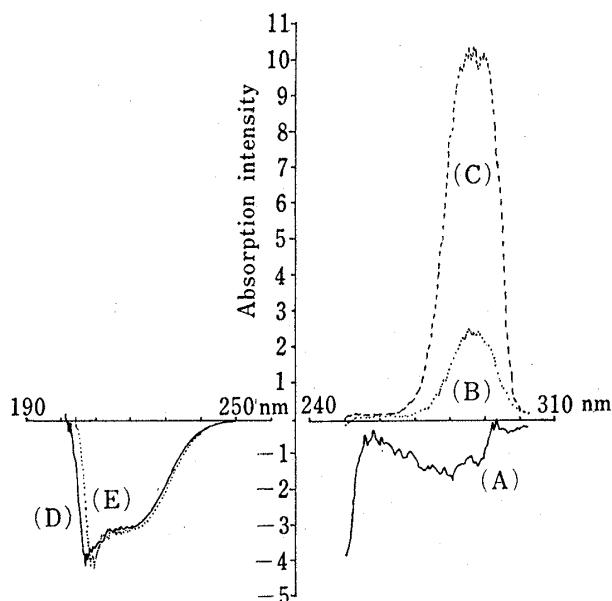

Fig. 5. The CD and the Urate-induced CD Spectra of Rabbit Muscle Creatine Phosphokinase (CPK) in pH 7.40, 50 mM Phosphate Buffer Solution at Room Temperature (15—18°C)

(A) CPK: 4×10^{-5} M; (B) CPK (4×10^{-5} M) in the presence of urate (2×10^{-4} M); (C) CPK (4×10^{-5} M) in the presence of urate (3.1×10^{-4} M); (D) CPK: 2×10^{-6} M; (E) CPK: (2×10^{-6} M) in the presence of urate (3.1×10^{-5} M).
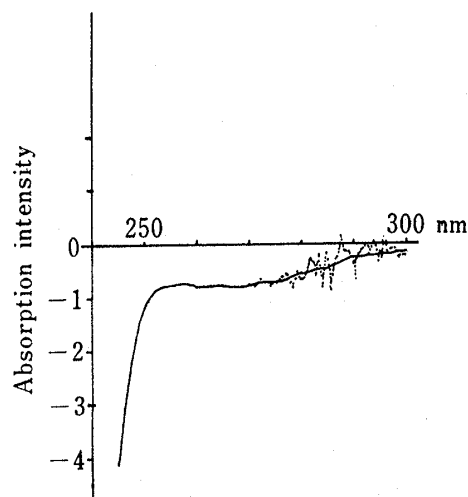

Fig. 6. The CD and the Urate-induced CD Spectra of Rabbit Serum Albumin (RSA) in pH 7.40, 50 mM Phosphate Buffer Solution at Room Temperature (15—18°C)

Continuous line: RSA (2×10^{-6} M); stippled line: RSA (2×10^{-6} M) in the presence of urate (3.1×10^{-4} M).
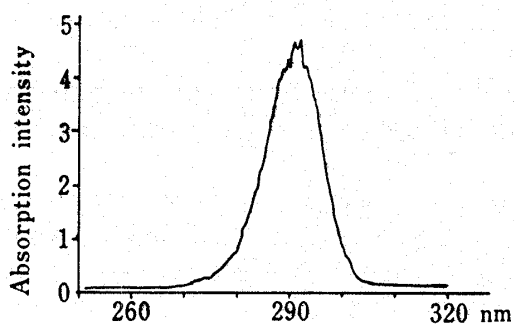
Fig. 7. The CD Spectrum of Urate (3.1 × 10^{-4} M) in pH 7.40, 50 mM Phosphate Buffer Solution at Room Temperature (15—18°C)

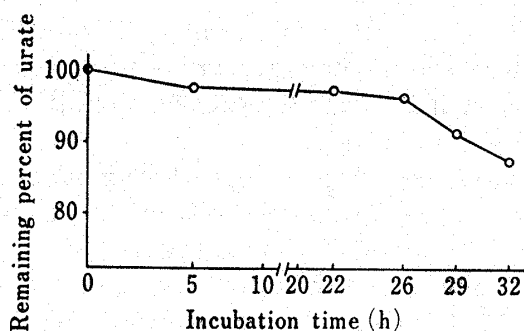Molar ellipticity coefficient: $[\theta]_{293} = 30.7 \times 10^2$ deg $cm^2/dmol$.

Fig. 8. The Stability of Urate in pH 7.40, 50 mM Phosphate Buffer Solution at 39°C

Initial concentration: 3×10^{-3} M.

Discussion

The Effect of pH on the Inactivation of CPK

Generally, CPK is most stable at near neutral pH (6.8—7.0) in the RSA system, as shown in Figs. 1 and 2. This is consistent with the result described in our preceding report on the Tris–acetate system.[3] Any pH outside this region will result in accelerated inactivation. However, it should be pointed out that CPK appears to be equally stable at pH 7.40 and at pH 6.86 in 1.5% RSA solutions (Fig. 1, B and A), at least for the first 9 h of incubation. This may be a result of rather slow inactivation during this period so that the discrepancies are masked by the S.D. (approx. 6%). An intriguing finding is that alkaline pH could retard CPK coagulation during the thermal incubation but did not improve the stability (Fig. 1, C). On the other hand, RSA and urate significantly suppress CPK protein coagulation and stabilize the activity (Figs. 3, A and 4, A). It is possible that alkaline pH causes weakening of the conformational integrity of CPK and accelerates the denaturation of CPK protein by heat. Nevertheless, there is no evidence of conformational change in the CD spectra in the range of pH 6.00—8.00 for CPK 1.5×10^{-5} M (300—250 nm) or 1.0×10^{-6} M (250—200 nm).

The Effect of RSA on the Inactivation of CPK

Figure 2 shows that under pH 7.00, 5% RSA is more effective than 1.5% RSA in stabilizing the CPK activity during incubation. However, the difference was significant only for the initial 3 h incubation, but not in the later period.

Figures 4, B and 3, B demonstrate that in the pH 7.40, 50 mM phosphate buffer solution system, RSA has a stabilizing effect on CPK at incubation periods of 7 to 32 h, but not for the initial 5 h. It is intriguing that the CPK inactivation profile in pH 7.40, 50 mM phosphate buffer solution appears to be linear, at least for the first 11 h (Fig. 3, B), but the pattern changes into a biphasic one when 5% RSA is contained in the same buffer solution (Fig. 4, B). The mechanism involved in the initial rapid inactivation of CPK observed in the presence of RSA (Fig. 4, B) is not clear. Perhaps CPK is in the form of a molecular dispersion in RSA solution and exposes the more labile CPK with heterogeneity[11]; the CPK may be in the form of micro-aggregates in 50 mM phosphate buffer solution. The initial rapid inactivation of CPK is usually observed at alkaline pH and in the presence of RSA or urate. It is possible that the solubilization effect of these factors may contribute to the increase of exposure of labile CPK with heterogeneity.[11] Further studies are required to elucidate the detail.

The CD study over 400 to 200 nm with CPK and RSA at concentrations of 2×10^{-5} M and 1×10^{-6} M shows no evidence of molecular interaction between CPK and RSA. This implies that CPK is just dispersed in the RSA matrices and the much more thermo-resistant RSA

protein colloid helps to stabilize the native conformation of the CPK and to suppress denaturation.

The stabilizing effect of albumin on CPK may be influenced by the pH (Figs. 1 and 2). Therefore, it is important to define the pH in discussing the effect of albumin on CPK.

The Effect of Urate on the Inactivation of CPK

Urate stabilizes the activity of CPK during incubation (Figs. 3 and 4). The effect is very significant in the initial 9 h of incubation in pH 7.40, 5% RSA (Fig. 4) and during 7 to 25 h in pH 7.40, 50 mM phosphate buffer solution (Fig. 3). As for the results shown in Fig. 3, during the initial 5 h of incubation in the phosphate buffer system, the effect of urate on CPK is presumably overshadowed by the slow inactivation rate and the S.D. (approx. 4%). Albumin is known to bind urate.[12] The binding seems to be rather weak, and only a very weak induced CD spectrum can be seen, as shown in Fig. 6 for RSA and urate. There was no change in the CD spectrum in the far-ultraviolet region for RSA in the presence of urate. However, considering that a very much higher concentration of RSA than CPK is present in the test system, it is possible that the binding of urate by RSA may diminish the urate effect on CPK considerably (Figs. 4, A and 3, A). The function of urate as a CPK stabilizer during 9 h incubation in 5% RSA at 39°C is consistent with the finding of Morin[7] in an incubation study on CK in serum for 80 min at 37°C.

The amount of urate decreased somewhat during the incubation and the remaining urate profile seems likely to be related to the stability of CPK (Fig. 8). However, quantitative interpretation is difficult. Curiously, at certain concentrations (2.5—3.5×10^{-4} M, a physiological urate level in human circulatory blood), urate shows a typical CD spectrum at around 292 nm as can be seen in Fig. 7. The spectrum is not evident at 1×10^{-4} M or at 6.1×10^{-4} M. In addition, urate has no asymmetric carbon and is optically inactive. Therefore, it seems that a certain polymeric structure of urate is constructed in the solution and is responsible for the phenomenon observed in Fig. 7. Warren[5] suggested that the mechanism by which urate interacts with CK must involve the sulfhydryl groups of the enzyme. However, it seems more likely that CPK is protected in the polymeric structure of urate in the present case. Nevertheless, the stabilizing effect of urate in the thermal inactivation of CPK can not be overlooked.

References and Notes

1) This paper constitutes Part II of the series entitled "Biopharmaceutical Studies on Muscle Creatine Phosphokinase."
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