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The Implication of Thoracic Duct Lymph in the Distribution and Elimination of Rabbit Muscle Creatine Phosphokinase[1)]

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The implication of thoracic duct lymph in the distribution and the elimination of rabbit muscle creatine phosphokinase (CPK) was investigated *in situ* and *in vitro* with 30 rabbits. The rate of thoracic duct lymph flow per kg body weight in anesthetized rabbits was 0.97 ± 0.21 $ml \cdot h^{-1} \cdot kg^{-1}$ (mean $\pm$ S.D., $n=9$). The CPK activity (U) that appeared in thoracic duct lymph after laparotomy (15 cm dissection along the mid-line) was 0.129 ± 0.038 U/h ($n=4$) in untreated rabbits and 4.55 ± 2.23 U/h ($n=5$) in rabbits given intramuscular administration of CPK (1000 U/kg body weight). The rate of transfer of CPK from the circulation to the thoracic duct lymph in rabbits after intravenous administration of CPK (1000 U/kg) was 30.0 ± 11.1 U/h ($n=5$).

The inactivation rate constant of CPK in thoracic duct lymph (pH 7.40, 39 °C, $0.098 \pm 0.023\ h^{-1}$, $n=5$) was larger than that in heparinized whole blood. The result implies that CPK may be partly inactivated in the lymph *in vivo*. The inactivation rate constant of CPK in thoracic duct lymph fluid without lymphocytes (pH 7.40, $0.159 \pm 0.013\ h^{-1}$, $n=3$) was much larger than that in the thoracic duct lymph. The calcium concentration (14.4 ± 0.810 mg/dl, $n=7$) and the magnesium concentration (3.93 ± 0.530 mg/dl, $n=7$) in thoracic duct lymph were determined. The possible mechanism involved in the inactivation of CPK in thoracic duct lymph is discussed.

Keywords——rabbit muscle creatine phosphokinase; thoracic duct lymph; lymph flow; transfer of CPK (plasma to lymph and muscle to lymph); first order inactivation; calcium concentration; magnesium concentration

In the preceding report,[2)] the pharmacokinetic parameters for the disposition of rabbit muscle creatine phosphokinase (CPK) after intravenous administration were presented. The *in vivo* study revealed that the disposition of CPK could be described by a two-compartment body model, and the elimination rate constant of CPK (k_{10}) was much larger than the inactivation rate constant observed *in vitro*.[3)] Further, the distribution volume of CPK (Vd_{ss}) was larger than the plasma volume. Therefore, there must be some mechanism involved in the elimination of CPK *in vivo* other than simple inactivation in the circulation by heat (body temperature), and the extravascular system might be involved in the distribution of CPK. Robison *et al.*[4)] reported that cardiac creatine phosphokinase can be inactivated in cardiac lymph even faster than in the blood after myocardial infraction in dogs. It is known that direct elimination from the plasma by filtration in the kidney can only be assumed for enzymes with a molecular weight of less than 60000.[5)] CPK has a molecular weight of 81000,[6)] and has not been detected in urine in our study. Besides, it was impossible in our laboratories to demonstrate the inactivation of CPK in liver microsomal fraction in a preliminary study. Wakim and Fleisher[7)] demonstrated that the transfer of intravenously administered glutamate oxaloacetate transaminase (GOT) and glulamate pyruvate transaminase (GPT) from serum to lymph is quite fast in dogs and the transport of macromolecules from blood to lymph was also

reported by Crone.[8] It is possible that the lymphatic system may be involved in the distribution and elimination of CPK *in vivo*.

This report presents the results of an *in situ* and *in vitro* study on the implication of thoracic duct lymph in the distribution and elimination of CPK. The possible mechanism of inactivation of CPK in thoracic duct lymph is discussed.

Materials and Methods

Materials——Rabbit muscle creatine phosphokinase (CPK: 85.4±0.5 and 72.2±0.2 U/mg protein), rabbit serum albumin (RSA), sodium heparin, sodium azide and Tris(hydroxymethyl)aminomethane were obtained from Sigma Co., U.S.A. Sodium chloride, EDTA disodium, ether, glacial acetic acid and sodium pentobarbital were supplied by E. Merck Co., West Germany.

Rabbits——Thirty male white rabbits weighing 2.26—3.57 kg were used. The plasma activity of CPK in these rabbits before the experiments was 35.9±24.7 U/l (mean±S.D., $n=30$). Rabbits were fed as usual until experiments. Four rabbits were used in blank tests (collection of thoracic duct lymph without any treatment). Each group of five rabbits was used for the intramuscular or intravenous administration experiment and another seven rabbits were used for the determination of calcium or magnesium concentration in thoracic duct lymph or in the plasma under anesthesia. Seven rabbits were used as control rabbits, from which "intact" plasma was obtained for comparison with the plasma obtained under anesthesia. The remaining two rabbits were used for the collection of thoracic duct lymph which was used in the incubation study on the inactivation of CPK in lymph.

Intravenous or Intramuscular Administration of CPK——CPK was dissolved in 2% RSA solution and filtered through a 0.22 micrometer Millipore filter (Millipore Corp., U.S.A.). The activity of CPK in the filtrate was determined and 1000 U/kg body weight in 1.5 ml was administered into the marginal ear vein within 20 s or into the vastus lateralis muscle on both sides (each 0.75 ml) at exactly 1 cm depth with a 23 G needle (Top Surgical Manufacturing Co., Japan).

Time Schedule in Experiments——Rabbits were anesthetized 0.1 h and laparotomized 0.25 h after the aministration of CPK. Collection of thoracic duct lymph was started 0.5 h after the administration. The time schedule in a blank test was in accordance with the above schedule.

Blood Samples——Venous blood samples were serially collected into heparinized tubes (containing dried sodium heparin to form 0.75% sodium heparin in blood) from the marginal ear vein on the opposite side from that of administration (before anesthesia) and from the posterior caval vein (after laparotomy for thoracic duct cannulation). The collected blood was centrifuged at 4 °C, 3500 rpm for 3 min within 10 min after each collection. The plasma was pipetted into small glass tubes with tight-fitting polyethylene caps and stored at 4 °C. The determination of CPK activity was carried out within 24 h after the blood collection.

Lymph Samples——Thoracic duct lymph was collected under anesthesia. Rabbits were anesthetized with sodium pentobarbital (30 mg/kg body weight) and if necessary, ether was used to maintain the anesthetized condition. Rabbits were laparotomized carefully along the mid-line of the abdomen for exactly 15 cm to expose the thoracic duct; the thoracic duct was canulated with polyethylene tubing No. 20 (Igarashi Ika Kogyo Co., Japan) and fixed with cyanoacrylate adhesive (Alteco-Ace, Type EE, Alpha Techno Co., Japan). Since thoracic duct lymph emerged continuously after the cannulation, the lymph was collected in a series of heparinized small glass tubes (containing dried sodium heparin to form 2—2.5% sodium heparin in lymph). The mid-point time of collection was taken to represent the time of the sample collection. The volume of lymph was estimated from its weight and weight per unit volume. The activity of CPK was determined within 30 min after collection.

Incubation Study of CPK in Thoracic Duct Lymph——Thoracic duct lymph collected from two untreated rabbits in heparinized tubes was mixed with sodium azide (to form 0.5% sodium azide in lymph) and CPK (initial activity as given in the legends to figures and tables). The incubation study was carried out under four different conditions as follows.

(1) Fresh thoracic duct lymph (containing 2% sodium heparin and 0.5% sodium azide) without any adjustment of pH, was used (pH 7.82).

(2) The pH of fresh thoracic duct lymph (1) was adjusted to 7.40 with the least possible amount of 100 mM Tris-acetate buffer solution (pH 6.70) which increased the total volume by 2.5%. The resultant lymph was used.

(3) The fresh thoracic duct lymph with pH 7.40 (2) was filtered through a 0.45 micrometer Millipore filter to remove lymphocytes and the lymph filtrate, pH 7.40, was used.

(4) Ethylenediaminetetraacetic acid (EDTA) disodium was added to fresh thoracic duct lymph (1) to form 25 mM EDTA in lymph and then the pH was adjusted to 7.40 with the least possible amount of 100 mM Tris-acetate buffer solution (pH 8.50) which increased the total volume by 3.5%. The resultant lymph was filtered through a 0.45 micrometer Millipore filter and the filtrate, pH 7.40, was used.

The thoracic duct lymphs thus prepared (1 to 4) were each pipetted into a series of small glass tubes with tight-fitting polyethylene caps and incubated in a water bath at 39±0.2 °C (normal body temperature of rabbits). The

initial 20 min of incubation was taken as the time required for temperature equilibration, so that at the end of the 20th min the time count was started (zero time). 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 5 h after withdrawal of each sample from the water bath. The study on the incubation of CPK in thoracic duct lymph was carried out with 3 to 5 different initial activities of CPK for each specified condition, using the thoracic duct lymph obtained at different times from two untreated rabbits.

Activity Determinations——If necessary, the plasma and the lymph were diluted with 5% RSA solution and isotonic saline solution. The activity of CPK was determined at 25 °C with a Merckotest kit (E. Merck Co., West Germany), which is based on the optimized Oliver–Rosalki method,[9] using a spectrophotometer (Hitachi model 320, Japan). Duplicate determinations were performed on each sample and the range was less than 6% in this study.

Determination of Calcium and Magnesium——The concentrations of calcium and magnesium in rabbit plasma or in thoracic duct lymph obtained from seven untreated rabbits under anesthesia and those in rabbit plasma obtained from the intact control rabbits were determined by using an atomic absorption spectrophotometer (Hitachi model 170-30, Japan). The plasma from anesthetized rabbits was separated from a blood sample which was obtained from the posterior caval vein after the collection of 4—5 ml of thoracic duct lymph in each rabbit. The plasma obtained from the control rabbits was collected from the marginal ear vein. Five replicate determinations were performed on each sample and the mean value was used in estimating the concentration of calcium or magnesium in the sample by use of the calibration equation obtained by the least-squares method from the results for standard solutions.

Results

Rate of Thoracic Duct Lymph Flow

Figure 1 depicts the regression line of the cumulative volume of collected thoracic duct lymph *versus* the time of lymph collection in nine rabbits. The average rate of thoracic duct lymph flow in anesthetized rabbits was estimated to be 2.58 ± 0.52 ml/h (k_0, mean $\pm$ S.D., $n=9$) and the rate of thoracic duct lymph flow per kg body weight was 0.97 ± 0.21 ml·h^{-1}·kg^{-1} (k_0/BW).

Amount of Activity of CPK (U) Appeared in Thoracic Duct Lymph of Untreated, and Intramuscularly or Intravenously CPK-Injected Rabbits

Figures 2 and 3 depict the profiles of the amount of activity of CPK (U) appeared in thoracic duct lymph in a blank test and following intramuscular or intravenous administration of CPK 1000 U/kg body weight). The profile in the blank test shows a zero order appearance with an average rate of 0.129 ± 0.038 U/h (0.58—4.1 h, $n=4$); whereas the profile in the intramuscular administration experiment shows a biphasic pattern. A slow initial phase with a zero order appearance rate of 1.60 ± 0.55 U/h (0.5—2.0 h, $n=5$) was followed by a fast phase with an appearance rate of 4.55 ± 2.23 U/h (2.2—5.0 h).

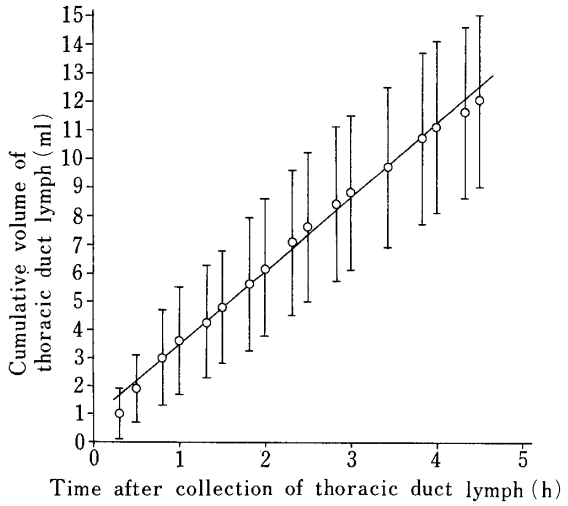

Fig. 1. Profile of Cumulative Volume of Thoracic Duct Lymph after Lymph Drainage in Anesthetized Rabbits

Mean $\pm$ S.D., $n=9$.

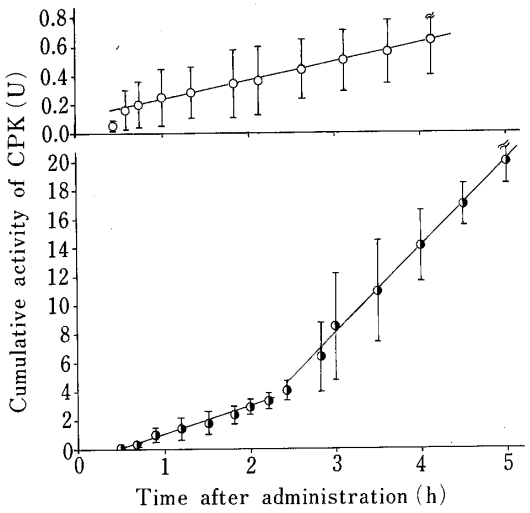

Fig. 2. Profiles of Cumulative Activity of CPK in Thoracic Duct Lymph in Blank Test or after Intramuscular Administration (1000 U/kg Body Weight)

○—○, blank test, mean±S.D., $n=4$; ◑—◑, intramuscular administration experiment, mean±S.D., $n=5$.

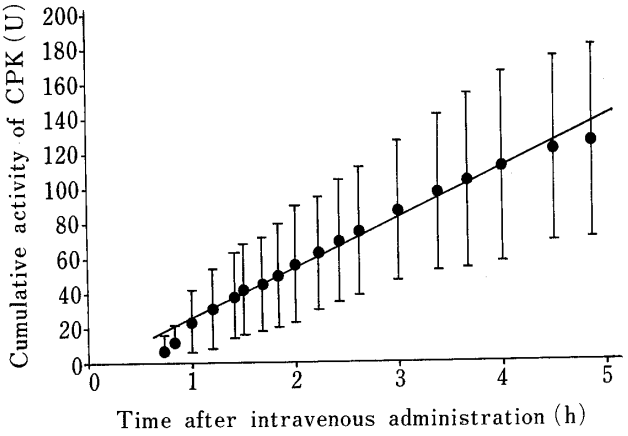

Fig. 3. Profile of Cumulative Activity of CPK in Thoracic Duct Lymph after Intravenous Administration (1000 U/kg Body Weight)

Mean±S.D., $n=5$.

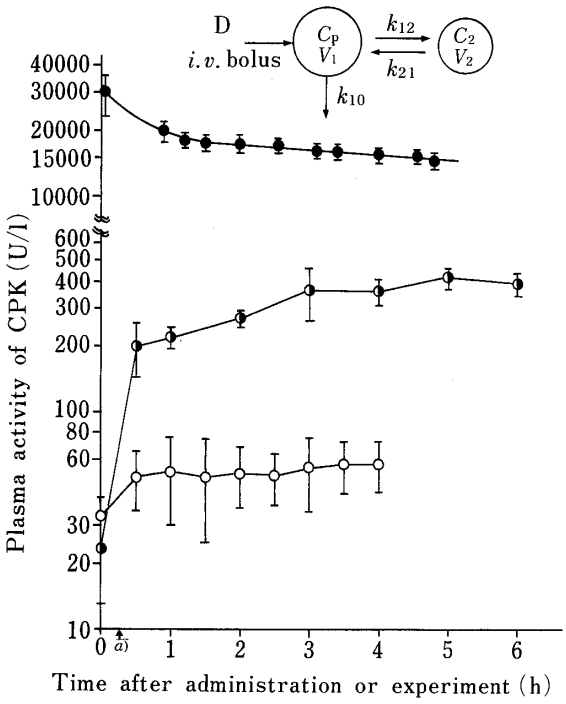

Fig. 4. Profiles of Plasma Activity of CPK in Anesthetized Rabbits during Blank Test, and Intramuscular or Intravenous Administration Experiments

○—○, blank test, mean±S.D., $n=4$; ◑—◑, intramuscular administration experiment (1000 U/kg), mean±S.D., $n=5$; ●—●, intravenous administration experiment (1000 U/kg), mean±S.D., $n=5$. The solid line represents the regression curve obtained by the least-squares method.

a) Laparotomy was carried out.

The rate of transport of CPK from the circulation to the thoracic duct lymph after intravenous administration of CPK (1000 U/kg) was much faster than the rate of appearance of CPK after intramuscular administration. A zero order appearance rate of 30.0±11.1 U/h (0.72—4.8 h, $n=5$) was observed.

Plasma Activity Level of CPK during the Collection of Thoracic Duct Lymph

Figure 4 depicts the profiles of plasma activity of CPK during the blank test, intramuscular and intravenous administration experiments. After the laparotomy for thoracic duct lymph collection, a moderate elevation of plasma activity of CPK was observed in both the untreated and intramuscularly administered rabbits.

The profile in the intravenous administration experiment showed a biphasic pattern similar to those observed in the preceding report.[2] The mean plasma activity of the profile could be fitted to a two-compartment body model and expressed by the equation $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ using the least-squares method. The pharmacokinetic parameters of CPK after intravenous administration in anesthetized rabbits during thoracic duct lymph drainage are

TABLE I. Pharmacokinetic Parameters of CPK after Intravenous Administration (1000 U/kg Body Weight) in Anesthetized Rabbits during Thoracic Duct Lymph Drainage (Value for Parameter ± Standard Error)[a]

Parameter		Parameter	
A, U/l	13000 ± 732	k_{12}, h^{-1}	0.915 ± 0.157
B, U/l	18700 ± 616	k_{21}, h^{-1}	1.41 ± 0.295
α, h^{-1}	2.36 ± 0.444	Vd_{ss}, ml[b]	135 ± 16.0
β, h^{-1}	0.047 ± 0.009	Vd_{ss}/BW, %[c]	5.19 ± 0.775
k_{10}, h^{-1}	0.079 ± 0.013	$t_{1/2\beta}$, h	14.8 ± 2.83

a) According to the method in the textbook "Statistical Adjustment of Data," ed. by W. E. Deming, John Wiley and Sons, Inc., New York, 1946.
b) $Vd_{ss} = V_1 + V_2$.
c) BW: body weight, 2.60 ± 0.08 kg (mean ± S.D., $n = 5$).

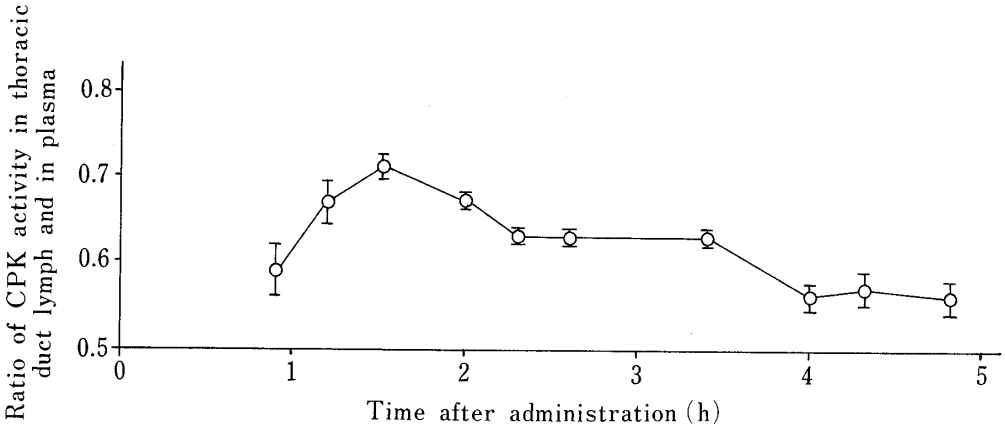

Fig. 5. Profile of the Ratio of CPK Activity in Thoracic Duct Lymph and in the Plasma after Intravenous Administration (1000 U/kg Body Weight, $n = 5$)

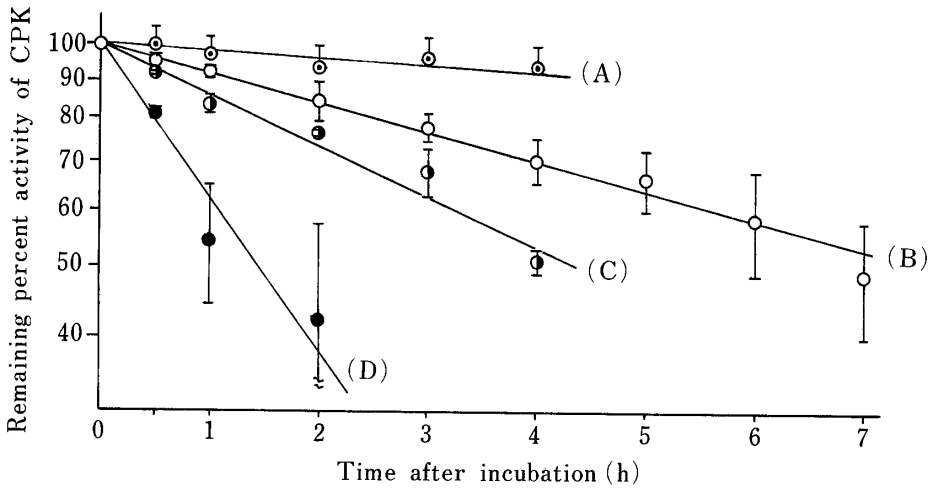

Fig. 6. Inactivation Profiles of CPK in Thoracic Duct Lymph under Various Conditions at 39 °C

(A), lymph filtrate with 25 mM EDTA, pH 7.40, initial activity 6950 ± 901 U/l, $n = 3$; (B), fresh thoracic duct lymph, pH 7.40, initial activity 9940 ± 2570 U/l, $n = 5$; (C), lymph filtrate, pH 7.40, initial activity 6770 ± 1940 U/l, $n = 3$; (D), fresh thoracic duct lymph, pH 7.82, initial activity 5630 ± 307 U/l, $n = 3$.

Table II. Apparent First Order Inactivation Rate Constant of CPK (K_1) in Thoracic Duct Lymph, 39 °C

Conditions	pH	Initial activity (n)	K_1, h^{-1} (mean ± S.D.)
Fresh thoracic duct lymph	7.40	9940 ± 2570 U/l (5)	0.098 ± 0.023
Lymph filtrate	7.40	6770 ± 1940 U/l (3)	0.159 ± 0.013
Fresh thoracic duct lymph	7.82	5630 ± 307 U/l (3)	0.671 ± 0.026
Lymph filtrate-EDTA	7.40	6950 ± 901 U/l (3)	0.014 ± 0.006

n: number of replicate determinations.

Table III. Calcium and Magnesium Concentrations in Fresh Thoracic Duct Lymph and in the Plasma

Samples	Calcium concn., mg/dl Mean ± S.D., $n=7$	Magnesium concn., mg/dl Mean ± S.D., $n=7$
Thoracic duct lymph	14.4 ± 0.810[b]	3.93 ± 0.530
Plasma (anesthetized rabbits)	12.7 ± 1.90	4.97 ± 0.732[a]
Plasma (intact control rabbits)	10.5 ± 0.768[b]	3.76 ± 0.453[a]

a) $0.05 < p < 0.10$. b) $p < 0.001$.

listed in Table I.

It should be noted that under the condition of thoracic duct lymph drainage and anesthesia (the body temperature measured at the liver site was about 33 °C), the values of α, Vd_{ss}/BW, k_{12} and $t_{1/2\beta}$ were much larger and β was much smaller than those of the intact rabbits reported in the preceding study.[2]

Ratio of CPK Activity in Thoracic Duct Lymph and in the Plasma after Intravenous Administration

Figure 5 depicts the profile of the ratio of CPK activity in thoracic duct lymph and in the plasma after intravenous administration of CPK. The average ratio (lymph/plasma) was 0.62 ± 0.05 (0.9—4.8 h).

Inactivation Profile of CPK in Thoracic Duct Lymph

Figure 6 depicts the inactivation profile of CPK in thoracic duct lymph at pH 7.40 or 7.82, 39 °C. The inactivation of CPK in thoracic duct lymph followed apparent first order kinetics. The stability of CPK was enhanced by the presence of EDTA and decreased in the lymph filtrate and at alkaline pH. Table II depicts the apparent first order rate constants of inactivation of CPK obtained by the least-squares method.

Concentrations of Calcium and Magnesium in Rabbit Plasma and Thoracic Duct Lymph

Table III lists the results of determination of calcium and magnesium in thoracic duct lymph and in the plasma by atomic absorption spectrophotometry. It should be noted that the calcium concentration in thoracic duct lymph was significantly higher than that in the plasma of intact control rabbits. However, the difference between the calcium concentration in thoracic duct lymph and in the plasma of anesthetized rabbits was not significant ($p > 0.20$). The concentration of magnesium in the plasma was increased by the effect of hypothermia (during anesthesia), and this is similar to the phenomenon reported for hamster or cat.[10] The magnesium concentration in intact control rabbit plasma determined in this study was higher

than the reported value for rabbits (2.52 ± 0.24 mg/dl).[11] The difference between the reported value and the value in this study may be due to the different feed used and the different method of determination.

Discussion

Transfer of CPK from Muscle Site to Lymph

After the laparotomy for the collection of thoracic duct lymph in untreated rabbits, the CPK liberated from injured muscle cells was gradually transported to the lymph. The average rate for the appearance of the amount of CPK activity (U) in thoracic duct lymph was 0.129 ± 0.038 U/h (Fig. 2). The plasma activity of CPK was elevated moderately during the experiment (Fig. 4). This is probably due to phenomena similar to those observed in humans. It is well known that there is about 16.7% lymph flow into the circulation through channels other than the thoracic duct in humans,[12] and that the lymph flow increases during exercise.[13]

However, at 2.2 h after intramuscular administration of CPK (1000 U/kg), the rate of appearance of CPK in thoracic duct lymph was 4.55 ± 2.33 U/h (Fig. 2). This result indicates that injected CPK was transported to thoracic duct lymph from the muscle site with an average rate of 4.42 ± 2.37 U/h (corrected for the rate in the blank test) in anesthetized rabbits. If we accept the average figure for the total lymph flow (83.3% *via* the thoracic duct and the remainder *via* other channels),[12] the rate of appearance of injected CPK in total lymph can be roughly estimated to be 5.30 ± 2.84 U/h in anesthetized rabbits. Although the rate of appearance of CPK in intact rabbits may be larger than this value, the transfer of injected CPK from the muscle site to the circulation *via* the lymphatic system appears to be a rather slow process.

Transfer of CPK from Blood to Thoracic Duct Lymph

The rate of transfer of CPK from blood to thoracic duct lymph was about 6 times the rate of appearance of CPK from the muscle site in thoracic duct lymph (Figs. 2 and 3). The result suggests that part of the intravenously administered CPK is rapidly transferred to the lymphatic system. Therefore, it is clear that the lymphatic system is not only involved in the absorption of CPK from the muscle site but is also involved in the distribution of CPK.

Possible Mechanism Involved in the Inactivation of CPK in Thoracic Duct Lymph

The inactivation rate constant of CPK observed in thoracic lymph, pH 7.40 ($0.098 \pm 0.023\ h^{-1}$, Table II), was larger than that reported in heparinized whole blood ($0.054\ h^{-1}$),[3] under the same incubation conditions. It is also interesting that under the same incubation conditions, CPK was inactivated significantly faster in lymph filtrate ($0.159 \pm 0.013\ h^{-1}$) than in the lymph. Since the size of lymphocytes is about 7—20 micrometers,[14] they can be removed with a 0.45 micrometer Millipore filter. The result implies that CPK inhibitors may be present in the lymph and the inhibitory action on CPK might be interfered with in the presence of lymphocytes. It is intriguing that the presence of 25 mM EDTA in lymph filtrate greatly enhanced the stability of CPK (Fig. 6).

It was demonstrated that the addition of cation chelators to serum caused a 14 to 16% increase in the CPK activity of fresh samples.[15] Calcium ion is known to be CPK inhibitor *in vitro*.[15] Nealon and Henderson[16] reported that calcium ion competes with magnesium ion to inhibit the activity of CPK *in vitro*. It has been established that only 50—60% of total calcium ion in the plasma is in the active free form and the remainder is bound to plasma protein (mainly to albumin).[17] The albumin concentration in human thoracic duct lymph (average 1.5—3.0 g/dl) is significantly lower than that in the plasma (average 4.1 g/dl).[18] It is probable

that a similar situation exists in rabbits. Although the concentration of total calcium ion in thoracic duct lymph was parallel to that in the plasma of anesthetized rabbits, increases of the active free form of calcium ion in thoracic duct lymph due to the low albumin concentration cannot be ruled out. A change of the conformation of CPK protein has been reported when CPK reacts with divalent cations in the presence of adenosine diphosphate (ADP).[19] Also, it was suggested that CPK can be protected by matrices of albumin against thermal denaturation.[20] Presumably, the low albumin concentration in thoracic duct lymph might facilitate the binding of active free form of calcium ion with CPK and accelerate the thermal denaturation. Further studies are required to elucidate the nature of the inactivation of CPK in lymph and the mechanism in the presence of EDTA.

It is clear that after liberation by cell breakdown, CPK is transported into the intravasal compartment *via* the lymphatic system and CPK in the circulation can also be distributed to the lymphatic system. Since CPK can be inactivated by heat (body temperature) in the interstitium, lymph and blood, and also might be filtered at lymph nodes, the activity of CPK detected in the circulation after muscle lesion may be only a limited proportion of the total liberated CPK. It is not clear what proportion of the liberated CPK at the injured muscle site can gain ingress into the circulation. An absolute bioavailability study on the intramuscular administration of CPK may provide useful insight, and is in progress in our laboratories.

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