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Mode of Action of Menaquinone-4 on Blood Coagulation

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The prothrombin activity in plasma was highly correlated with that in liver in normal rats, bishydroxycoumarin (dicumarol)-treated rats and CCl_4-treated rats. Menaquinone-4($K_{2(20)}$) significantly increased the prothrombin activities in plasma and in liver of rats which had hypoprothrombinemia induced by administration of dicumarol or CCl_4. $K_{2(20)}$ significantly promoted the prothrombin synthesis in liver homogenate in the rats treated with dicumarol or CCl_4. Furthermore, it was observed that $K_{2(20)}$ exerted a promoting effect on prothrombin synthesis in the microsomal fraction of liver. However, when plasma was incubated with $K_{2(20)}$, the prothrombin time of the plasma was not shortened. The effects of $K_{2(20)}$ on the prothrombin activity and on the prothrombin synthesis of liver were more rapid and stronger than those of vitamin K_1.

It is concluded that $K_{2(20)}$ promotes prothrombin synthesis in liver parenchymal cells and improves hypoprothrombinemia caused by dicumarol or CCl_4 in rats.

It has been demonstrated that vitamin K_1 (abbr.: K_1) orally given to animals is converted into menaquinone-4 (abbr.: $K_{2(20)}$)[2] and such is the case with vitamin K_3.[3] Our previous study of the potency of $K_{2(20)}$ and K_1 on the coagulation of blood revealed that $K_{2(20)}$ was more potent than K_1 in protecting the bleeding death caused by bishydroxycoumarin (dicumarol) in mice.[4] In addition, when $K_{2(20)}$ or K_1 was orally given to rabbits with hypoprothrombinemia caused by warfarin, the prothrombin time in the group given $K_{2(20)}$ was more rapidly improved as compared with that in the group given K_1 of the same dose as $K_{2(20)}$.[5] $K_{2(20)}$ was more potent than K_1 in preventing hypoprothrombinemia caused by daily administration of dicumarol and neomycin in rabbits.[6] Furthermore, in the study of hemostatic effect on hemorrhage caused by various hemorrhagenic agents in rats, $K_{2(20)}$ significantly shortened the bleeding time in the rats given dicumarol or carbon tetrachloride (CCl_4).[7] On the other hand, K_1 had significant shortening effect on bleeding time in rats given dicumarol but had no effect in rats given CCl_4.[7]

In the present study, the effect of $K_{2(20)}$ on liver prothrombin synthesis and blood coagulability was investigated to clarify the mode of action of $K_{2(20)}$ on blood coagulation in rats which were treated with dicumarol or CCl_4.

Materials and Methods

Materials——The materials used were bishydroxycoumarin (dicumarol, Nakarai Chemicals Co.); CCl_4 (Iwaki Kagaku Co.); $K_{2(20)}$ and K_1 (Eisai Co.); thromboplastin (Thrombokinase "Geigy," Fujisawa Pharmaceutical Co.); non-ionic surface active agent HCO-60 (Nikkol HCO-60, Nikko Chemicals Co.); olive oil (JP., Sanko Seiyaku Co.). Other chemicals were of guaranteed reagent.

$K_{2(20)}$ and K_1 were made soluble with a non-ionic surface active agent, HCO-60 of 7 times their quantities. After they were diluted with distilled water, 10 mg/ml and 100 μM of $K_{2(20)}$ or K_1 were used for *in vivo* and

1) Location: *Koishikawa, Bunkyo-ku, Tokyo, 112, Japan.*
2) C. Martius and C. Alviono, *Biochem. Z.*, **340**, 316 (1964); M. Billeter, W. Bolliger, and C. Martius, *ibid.*, **340**, 290 (1964); M. Billeter and C. Martius, *ibid.*, **333**, 430 (1960).
3) W.V. Taggart and J.T. Matschiner, *Biochemistry*, **8**, 1141 (1969).
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6) T. Tajima, H. Kawashima, Y. Kato, T. Ohgoh, and K. Miyao, *Folia Pharmacol. Japon.*, **67**, 419 (1971).
7) T. Tajima, T. Ohgoh, and K. Miyao, *Folia Pharmacol. Japon.*, **67**, 478 (1971).

in vitro experiments, respectively. Thromboplastin suspension was prepared from Thrombokinase "Geigy" tablet with 2.5 ml of distilled water per a tablet within 3 hr prior to use.

Animals——Male Wistar strain rats (ranging in age from 12 to 16 weeks) from the Eisai colony were used. They were caged in a room with constant temperature of 22±2°. A standard stock diet (AC-1, Nihonkurea Co.) and drinking water were freely given.

Preporation of $BaSO_4$-adsorbed Plasma——A volume of 8 ml of blood from the abdominal aorta was obtained under pentobarbital anesthesia by a syringe, in which 2 ml of 3.13% sodium citrate was previously filled. After blood and sodium citrate were mixed thoroughly, the mixture was centrifuged at 3000 rpm ($1050 \times g$) for 10 min to separate plasma. Then, prothrombin, factor VII, factor IX, factor X and factor XI were removed from the plasma by shaking with 1 g of barium sulfate ($BaSO_4$). It was confirmed that the $BaSO_4$-adsorbed plasma did not clot for 400 sec by Quick's one-stage procedure. The $BaSO_4$-adsorbed plasma was kept at −20° and was used within a week after preparation.

Measurement of Prothrombin Activity in Plasma——Eight ml of blood was obtained from test animal by the above mentioned procedure and the blood plasma was separated from the blood by centrifuging at 3000 rpm for 10 min. Prothrombin activity in the plasma was measured by Quick's one-stage procedure. A Fibrometer (Baltimore Biological Lab. Inc.) was used to measure the prothrombin time of plasma. A volume of 0.2 ml of thromboplastin suspension was put in a Fibrotube and warmed at 37° for 10—15 min. Then, 0.1 ml of plasma previously warmed at 37° for 5—10 min, was added to the thromboplastin suspension and prothrombin time was measured. Observations were repeated at least three times on the same plasma and their average value was used as a prothrombin time for the plasma.

Measurement of Prothrombin Activity in Liver——After bleeding the rat to death, perfusion cannula was inserted into the portal vein and the liver with the portal vein cannulated was removed. A volume of 100 ml of physiological saline previously cooled at 4° was infused into the portal vein at a rate of 20 ml/min to wash out blood of the liver. Then, 10 g of the liver was homogenized with 50 ml of 0.25 M sucrose by a VirTis "45" Hi-Speed homogenizer (VirTis Inc.). The homogenate was centrifuged at 15000 rpm ($27000 \times g$) for 20 min by a refrigerated high speed centrifuge (model KP-200A, Kubota Co.) at 4° and the supernatant was obtained. A volume of 0.1 ml of thromboplastin suspension was put in a Fibrotube and warmed at 37° for 10—15 min. Then, 0.1 ml of the supernatant and 0.1 ml of $BaSO_4$-adsorbed plasma which were warmed at 37° for 5—10 min separately, were added to the thromboplastin suspension and then clotting time was measured by a Fibrometer.

Hypoprothrombinemia——Nine rats were orally given 50 mg/kg of dicumarol. Then, a dose of 10 mg/kg of $K_{2(20)}$, 10 mg/kg of K_1 or 1 ml/kg of vehicle (7% HCO-60 solution) was intramuscularly administered to the rats 20—21 hr after the treatment of dicumarol. The other nine rats were subcutaneously injected 0.2 ml/kg of CCl_4 which was diluted to 10 times its volume with olive oil. A dose of 10 mg/kg of $K_{2(20)}$, 10 mg/kg of K_1 or 1 ml/kg of vehicle was intramuscularly administered to the rats 20—21 hr after treatment of CCl_4. Prothrombin activities of plasma and of liver were determined by Quick's one-stage procedure 3—4 hr after the administration of $K_{2(20)}$, K_1 and vehicle. As a control, five normal rats were used to determine prothrombin activities of plasma and of liver.

Prothrombin Synthesis in Liver——Livers were removed 24 hr after oral administration of 50 mg/kg of dicumarol or subcutaneous administration of 0.2 ml/kg of CCl_4 to rats. Livers of normal rats and of rats treated with dicumarol or CCl_4 were used for the observation of prothrombin synthesis. Five ml of liver homogenate or 5 ml of the microsomal fraction (the supernatant of centrifuged liver homogenate at $27000 \times g$ for 20 min), 4 ml of 0.1 M phosphate buffer with pH 7.4 and 1 ml of 100 μM $K_{2(20)}$ or 1 ml of 100 μM K_1 were mixed. Each mixture was incubated at 37° for 0.5, 1, 2, or 3 hr. Clotting time of the mixture was measured with Quick's one-stage procedure.

Interaction between $K_{2(20)}$ or K_1 and Plasma——Plasma was obtained 24 hr after oral administration of 50 mg/kg of dicumarol or subcutaneous administration of 0.2 ml/kg of CCl_4 to rats. A volume of 0.1 ml of 100 μM $K_{2(20)}$ or 100 μM K_1 was added to 1 ml of normal rat plasma, and to plasma of the rat treated with dicumarol or CCl_4. Each mixture was incubated at 37° for 0.5, 1 and 2 hr, respectively. Each prothrombin time was measured by Quick's one-stage procedure.

Results

Effect on Hypoprothrombinemia caused by Dicumarol or CCl_4

a) **Prothrombin Activity in Plasma**——As shown in Table I and Table II, the prothrombin time of plasma in normal rats was 12.1±0.1 sec. When 50 mg/kg of dicumarol was orally administered to rats, the prothrombin time of plasma was significantly prolonged and was 41.8±2.0 sec 24 hr after dosing. When 10 mg/kg of $K_{2(20)}$ or 10 mg/kg of K_1 was intramuscularly given to the rats 20—21 hr after the administration of dicumarol, the prothrombin

time of plasma 3—4 hr after dosing was significantly shortened; that of $K_{2(20)}$ group was 12.6±0.6 sec and that of K_1 group was 15.5±1.7 sec.

When 0.2 ml/kg of CCl_4 was subcutaneously injected to rats, the prothrombin time of plasma was also significantly prolonged and was 23.7±1.8 sec. In contrast, when 10 mg/kg of $K_{2(20)}$ was intramuscularly given to the rats 20—21 hr after the injection of CCl_4, the prothrombin time of plasma was significantly shortened and was 17.8±0.9 sec. However, no remarkable shortening of the prothrombin time was found in rats administered with K_1.

b) **Prothrombin Activity in Liver**——As shown in Table I and Table III, prothrombin time of liver in normal rats was 209±2 sec. The prothrombin time of liver 24 hr after oral administration of 50 mg/kg of dicumarol was significantly prolonged and was 272±12 sec. When 10 mg/kg of $K_{2(20)}$ or 10 mg/kg of K_1 was intramuscularly given to the rats 20—21 hr after dicumarol-treatment, the prothrombin time of the liver was significantly shortened and was 211±12 sec and 215±5 sec, respectively. As shown in Fig. 1, prothrombin time of plasma was highly correlated with that of liver. In the case of dicumarol-pretreatment, the coefficient of correlation was 0.87 and the line of regression was $y=1.96x\pm186$.

The prothrombin time of liver 24 hr after the subcutaneous injection of 0.2 ml/kg of CCl_4 was also significantly prolonged and was 273±8 sec. Intramuscularly administered $K_{2(20)}$ had significant shortening effect on prothrombin time of liver in the rats given CCl_4 and it was 243±6 sec. On the other hand, when K_1 was administered to the rats, the prothrombin time

TABLE I. Effect of $K_{2(20)}$ on Hypoprothrombinemia Induced by Dicumarol and by CCl_4 in Rats

Treatment	Drugs	(A) Prothrombin time of plasma	(B) Prothrombin time of liver
1) Non.	non.	11.8 sec	213 sec
		12.3	203
		10.9	205
		12.8	208
		12.5	215
	mean ± SE	12.1±0.1	209± 2
2) Dicumarol 50 mg/kg (*p.o.*)	control (vehicle)	42.3	274
		38.2	250
		44.9	291
	mean ± SE	41.8±2.0	272±12
3) Dicumarol 50 mg/kg (*p.o.*)	$K_{2(20)}$ 10 mg/kg (*i.m.*)	13.3	220
		13.0	204
		11.5	210
	mean ± SE	12.6±0.6	211± 5
4) Dicumarol 50 mg/kg (*p.o.*)	K_1 10 mg/kg (*i.m.*)	13.8	206
		14.6	218
		19.2	220
	mean ± SE	15.9±1.7	215± 5
5) CCl_4 0.2 ml/kg (*s.c.*)	Control (vehicle)	20.3	256
		24.8	279
		26.1	283
	mean ± SE	23.7±1.8	273± 8
6) CCl_4 0.2 ml/kg (*s.c.*)	$K_{2(20)}$ 10 mg/kg (*i.m.*)	16.2	231
		19.3	250
		17.9	248
	mean ± SE	17.8±0.9	243± 6
7) CCl_4 0.2 ml/kg (*s.c.*)	K_1 10 mg/kg (*i.m.*)	19.7	250
		23.1	276
		22.1	281
	mean ± SE	21.6±1.0	269±10

(A) 1), 2); 1), 5); 2), 3); 2), 4); 5), 6): significant at $p=0.01$. 6), 7): significant at $p=0.05$. 3), 4); 5), 7): not significant.
(B) has the same result on the statistic analysis as (A) has.

of liver was little shortened. In the case of CCl_4-pretreatment, prothrombin time of plasma was also highly correlated with that of liver and the coefficient of correlation was 0.93 and the line of regression was $y=5.59x+143$.

Table II. Analysis of Variance
Prothrombin Time of Plasma in Rats (sec)

Source of variation	Sum of squares	d.f.	Mean square	Variance ratio F	Probability p
Inter-group	2020.7922	6	236.7987	73.48	<0.01
Error	73.3326	16	4.5833		
Total	2094.1243	22			

Significant limits of the difference between inter-groups were calculated with following equation:

$$d=t_{(\phi e,\ \alpha)} X \sqrt{Ve\ (\frac{1}{n1}+\frac{1}{n2})}$$

Significant limit of the difference between normal group and dicumarol or CCl_4 treated group was 4.6 sec at $p=0.01$ and 3.4 sec at $p=0.05$.

Significant limit of the difference between control group and $K_{2\ (20)}$ or K_1 administered group was 5.2 sec at $p=0.01$ and 3.8 sec at $p=0.05$.

Table III. Analysis of Variance
Prothrombin Time of Liver in Rats (sec)

Source of variation	Sum of squares	d.f.	Mean square	Variance ratio F	Probability p
Inter-group	17933.1129	6	2988.8522	19.86	<0.01
Error	2406.8001	16	150.4250		
Total	20339.9130	22			

Significant limits of the difference between inter-groups were calculated with following equation:

$$d = t_{(\phi e,\ \alpha)} X \sqrt{Ve\ (\frac{1}{n1}+\frac{1}{n2})}$$

Significant limit of the difference between normal group and dicumarol or CCl_4 treated group was 27 sec at $p=0.01$ and 19 sec at $p=0.05$.

Significant limit of the difference between control group and $K_{2\ (20)}$ or K_1 administered group was 30 sec at $p=0.01$ and 22 sec at $p=0.05$.

Effect on Prothrombin Synthesis in Liver

a) Normal Rat Liver——Prothrombin time of the liver homogenate and of the liver microsomal fraction in normal rats was shortened within 1 hr incubation, but thereafter no change was found. The prothrombin time in both of liver homogenate and liver microsomal fraction was not affected by addition of $K_{2(20)}$ or K_1 (Fig. 2-A and Fig. 2-B).

b) Dicumarol-pretreated Rat Liver——In liver homogenate of dicumarol-pretreated rats, addition of $K_{2(20)}$ significantly shortened the prothrombin time at 1-hr incubation time and also thereafter, whereas addition of K_1 significantly shortened the prothrombin time after 2 hr-incubation (Fig. 2-C).

In the microsomal fraction, the prothrombin time was also significantly shortened by addition of $K_{2(20)}$ or K_1. The effect of $K_{2(20)}$ was more rapid and stronger than that of K_1 (Fig. 2-D). However, these effects of $K_{2(20)}$ and K_1 on the prothrombin time in microsomal fraction were not remarkable compared with those in liver homogenate.

c) CCl_4-pretreated Rat Liver——In liver homogenate of CCl_4-pretreated rats, the prothrombin time was significantly shortened 1 and 2 hr after incubation by addition of $K_{2(20)}$. However, addition of K_1 showed no significant effect (Fig. 2-E). In the liver microsomal fraction, the effect of $K_{2(20)}$ or K_1 was indefinite (Fig. 2-F).

Interaction between $K_{2(20)}$ or K_1 and Plasma

When 1 ml of normal rat plasma, of dicumarol-treated rat plasma and of CCl_4-treated rat plasma were respectively incubated with 0.1 ml of 7% HCO-60 solution at 37°, prothrombin time of each plasma was shortened after 30 min of incubation time, and thereafter was

prolonged. The prothrombin time of each plasma was not shortened by addition of $K_{2(20)}$ or K_1 (Fig. 3).

Discussion

This study was performed in order to investigate the mode of action of $K_{2(20)}$ on blood coagulation. For this purpose, the prothrombin activity in liver and blood coagulability were examined by the measurement of prothrombin time in liver and plasma based on Quick's one-stage procedure.

It was recognized that the prothrombin activity in liver was highly correlated with that in plasma (Fig. 1). $K_{2(20)}$ and K_1 markedly improved the prothrombin activities in liver and in plasma which were decreased by oral administration of dicumarol to rats. $K_{2(20)}$ had a significant restorative effect on prothrombin activities in liver and in plasma which were decreased by CCl_4-pretreatment, but K_1 had no significant effect.

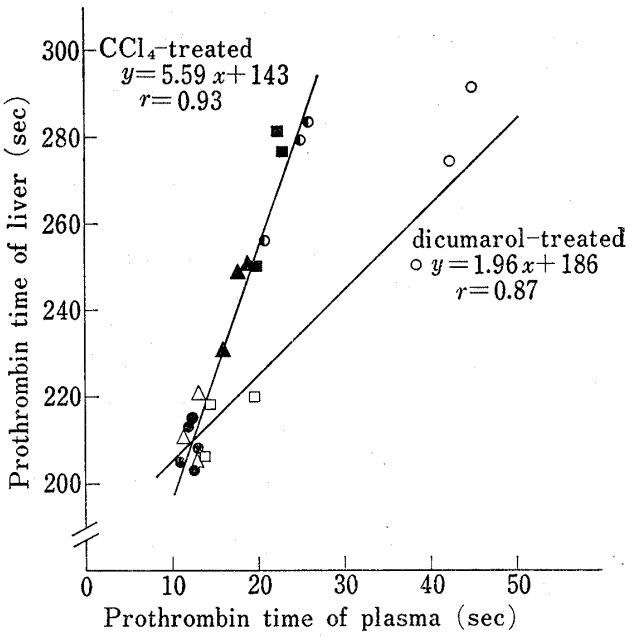

Fig. 1. Relationship between Prothrombin Time of Plasma and That of Liver

A dose of 10 mg/kg of $K_{2(20)}$, 10 mg/kg of K_1 or 1 ml/kg of 7% HCO-60 solution was intramuscularly administered to the rats 20—21 hr after the treatment of dicumarol (50 mg/kg, *p.o.*) or CCl_4 (0.2 ml/kg, *s.c.*). Prothrombin activities of plasma and of liver were determined by Quick's one-stage procedure 3—4 hr after the administration of $K_{2(20)}$, K_1 and HCO-60.
●: normal rat, ○: dicumarol-treated rat, △: dicumarol-treated and $K_{2(20)}$-administered rat, □: dicumarol-treated and K_1-administered rat, ◐: CCl_4-treated rat, ▲: CCl_4-treated and $K_{2(20)}$-administered rat, ■: CCl_4-treated and K_1-administered rat.

The regression coefficient between prothrombin activity in plasma and that in liver of rats in the case which was pretreated with dicumarol, was 1.96, whereas in the case which was pretreated with CCl_4 it was 5.59. A difference in the regression coefficient between these two cases suggests that the action of dicumarol and CCl_4 on blood coagulation system is different in mechanism.

Prothrombin synthesis in liver homogenate to dicumarol-pretreated rats was markedly promoted by adding 10 μM of $K_{2(20)}$ or K_1. In this condition, $K_{2(20)}$ was found to be more rapid than K_1 in the onset of action. This result agrees with that in the previous study with the therapeutic effect of K_1 and $K_{2(20)}$ on hypoprothrombinemia caused by warfarin in rabbits.[5] Prothrombin synthesis in liver microsomal fraction of dicumarol-treated rats was significantly promoted by $K_{2(20)}$. In the liver homogenate of CCl_4-pretreated rats, $K_{2(20)}$ significantly increased prothrombin formation but K_1 did not. It is interesting that this result agrees with that of bleeding time, *i.e.* $K_{2(20)}$ has a significant shortening effect on the bleeding time in rats given CCl_4 but K_1 had no effect.[7]

Matsuoka, *et al.*[8] reported that hypoprothrombinemia and decrease of mitochondria in liver were found when the liver function was damaged by CCl_4 in rabbits. Stoffel, *et al.*[9] reported that $K_{2(20)}$ was synthesized from vitamin K_3 and geranyl-geranyl pyrophosphate in chicken liver homogenate. Fujita, *et al.*[10] recognized that $K_{2(20)}$ and K_1 restored the coupled phosphorylation of membrane fragments with oxidative ability damaged by near-ultraviolet irradiation in *Micrococcus lysodekticus*, and that $K_{2(20)}$ was more than three times as effective

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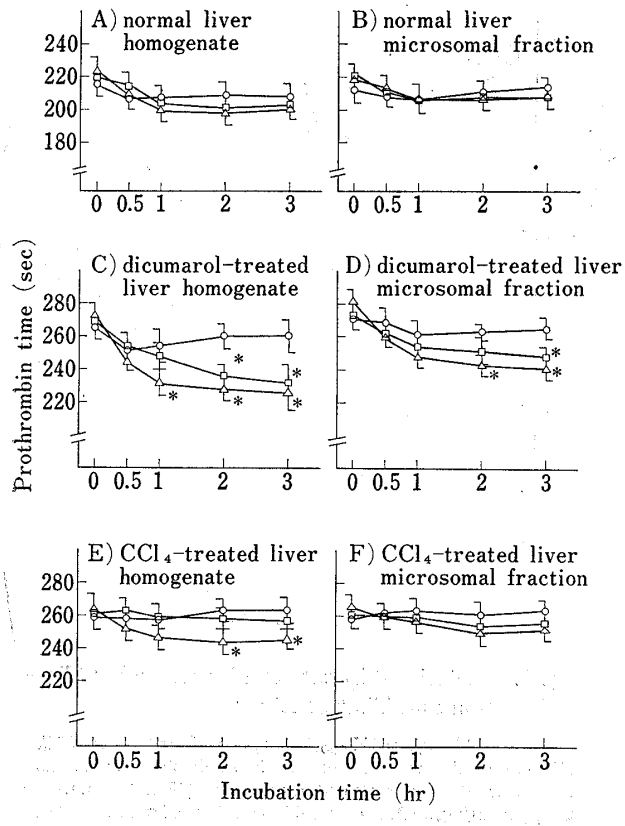

Fig. 2. Effect of $K_{2(20)}$ on Prothrombin Synthesis in Rat Liver

Livers were removed 24 hr after administration of dicumarol (50 mg/kg, *p.o.*) or CCl_4 (0.2 ml/kg, *s.c.*) to rats. Five ml of liver homogenate or the microsomal fraction (the supernatant of centrifuged liver homogenate at $27000 \times g$ for 20 min), 4 ml of 0.1M phosphate buffer with pH 7.4 and 1 ml of 100 μM $K_{2(20)}$, 100 μM K_1 or 0.7% HCO-60 were mixed and incubated at 37°. A) and B) show the change in the prothrombin time of liver homogenate and liver microsomal fraction in normal rats, respectively. C) and D) show those in dicumarol-treated rats, E) and F) show those in CCl_4-treated rats, respectively. Each group was performed five observations. —○—: Control, —△—: $K_{2(20)}$, —□—: K_1.
*: significant difference to control group, with a probability of $p=0.05$. ⊥: standard error.

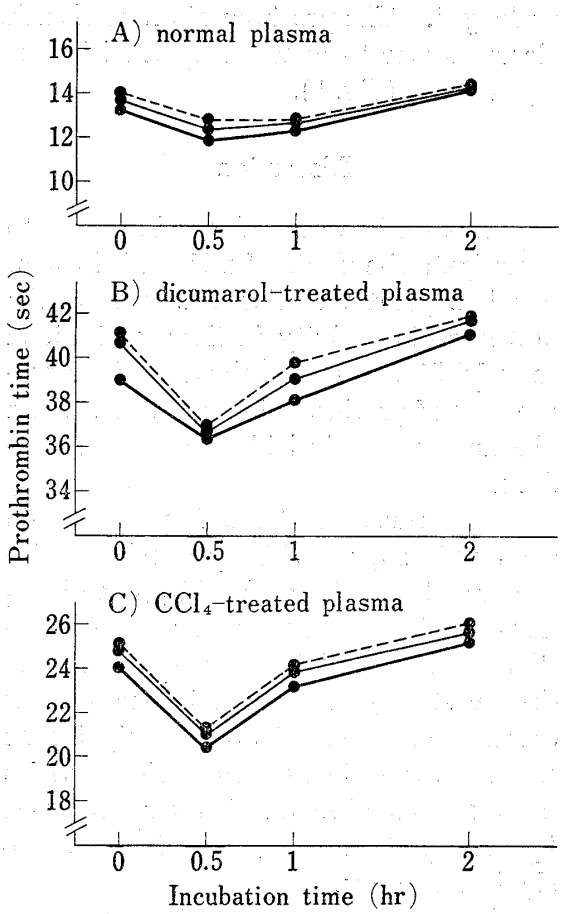

Fig. 3. The Change in the Prothrombin Time of Plasma Incubated with $K_{2(20)}$

A volume of 1ml of citrated plasma, obtained from normal rats, dicumarol (50 mg/kg, *p.o.*)-pretreated rats or CCl_4 (0.2 ml/kg, *s.c.*)-pretreated rats, was mixed with 0.1 ml of 0.7% HCO-60, 100 μM $K_{2(20)}$ and K_1, respectively, and incubated at 37°. A), B) and C) show the change in the prothrombin time in normal plasma, dicumarol-treated plasma and CCl_4-treated plasma, respectively. Each group was performed three observations and their average value was in this figure.
—●—: control (HCO-60 solution), ---●---: $K_{2(20)}$, —●—: K_1.

as K_1. From above-mentioned reports and results of the present experiments, it could be considered that energy of oxidative phosphorylation in mitochondria contributes to the conversion of K_1 to $K_{2(20)}$ and to the prothrombin synthesis.

As to the site of prothrombin synthesis, Lash, *et al.*[11] reported that prothrombin was synthesized in liver mitochondria. However, the present study revealed that prothrombin synthesis in liver microsomal fraction was significantly promoted by $K_{2(20)}$ in dicumarol-treated rats. This result suggests that prothrombin is synthesized in liver microsome.

When $K_{2(20)}$ or K_1 was incubated with citrated plasma obtained from normal rats, dicumarol-pretreated rats and CCl_4-pretreated rats, respectively, the prothrombin time of each plasma was not shortened and rather prolonged.

From the results of the present study concerning the mode of action of $K_{2(20)}$ on the blood coagulation, it was found that $K_{2(20)}$ promotes prothrombin synthesis in liver parenchymal cells and improves hypoprothrombinemia caused by dicumarol or CCl_4 in rats.

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