[Chem. Pharm Bull. 21(9)1906—1913(1973)]

UDC 615.356.033

Vitamin K Distribution in Rat Liver and Heart Muscle and Some Metabolic Properties[1)]

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(Received November 17, 1972)

Intracellular distribution and some additional studies of metabolic fate of vitamin K homologs were studied by isotopic tracer with tritium or carbon-14 labeled vitamin K homologs, vitamin K_1, $K_{2(20)}$ and K_3, to obtain the information about their site of action in the animal tissue.

There are several reports on the intracellular distribution of K_1 but no clear result has been obtained about the incorporation site of vitamin K_1 in the animal cell and no report has been found in the case of $K_{2(20)}$, which is estimated to be a physiologically active type of K homologs in the animal.

In our present experiment, K_1 was found to be concentrated in the mitochondrial fraction after a long period of time and $K_{2(20)}$ was faster incorporated in the liver and heart muscle than K_1 and showed remarkably higher affinity to the mitochondrial fraction. K_3 which is less lipophilic than the other two homologs, was less incorporated into the both tissues and stayed in the supernatant fraction.

From these observations, it was revealed that K_1 and K_2 which have a long isoprenoid side chain have a higher mitochondrial affinity like other isoprenoid vitamins and the higher affinity of vitamin K to the mitochondrial fraction suggest the possibility of their function in some regulation process in mitochondria other than blood coagulation protein synthesis.

It is known that Vitamins K (K) are responsible for the synthesis of the coagulation proteins in animal liver parenchymal cells, but the mechanism of their function and the site of action of these K homologs in this process are not yet clarified completely. Moreover, there is few information of their physiologically active form in the animal body. Although there are the following three typical homologs in this vitamin, it is still obscure what their structural differences contribute to: Vitamin K_1 (K_1), which is the best known of this vitamin, has a phytyl side chain, and occurs naturally in green plants. Vitamin K_2 group, which has an isoprenyl side chain of various lengths, is found in some bacteria. Vitamin K_3, which has no side chain at 3-position in their common skeleton, is medically used as a synthetic vitamin.

Of the K_2 group, vitamin $K_{2(20)}$ (K_2), which has a C-20 isoprenyl side chain, is most important because of its ubiquitous distribution in animal tissues. Martius, *et al.*[3)] suggested that this vitamin K_2 is possibly a physiological active form of K in the animal body from the finding that the K homologs administered are converted into K_2 in the animal body.

Recently, several workers[4)] also reported the conversion of K_1 into K_2, but further experiments are needed for its confirmation because the identification of K_2 produced is only based on the *Rf* value in thin-layer chromatography (TLC).

Another interesting point is that K has a structure similar to such lipophilic quinones as ubiquinone and tocopherylquinone. Ubiquinone also has a long isoprenyl side chain similar to that of K_2, and tocopherylquinone has a saturated isoprenoid side chain like K_1. Such

1) Presented at the 90th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, July, 1970.
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3) C. Martius and H.O. Esser, *Biochem. Z.*, **331**, 1 (1958); M. Billeter and C. Martius, *ibid.*, **333**, 430 (1960); M. Billeter, W. Bolliger and C. Martius, *ibid.*, **340**, 290 (1964).
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structural similarity of these lipophilic quinones suggests their common physiological activity.

To estimate these possibilities, it would be interesting to study the intracellular distribution of these K homologs in the two tissues of a rat, liver and heart muscle, which are the site of coagulation protein synthesis and the site of respiration metabolism, respectively.

There are several reports on the intracellular distribution of K_1,[4a,5,6] but no clear result has been obtained about the incorporation site of K in the animal cell and no report has been found in the case of K_2. We synthesized K_2 labeled with tritium and carbon-14[7] and compared the distribution and some metabolic fate of this homolog in a rat and some other animals with that of K_1[8] and revealed that K_2 is absorbed faster and retained better in the tissues than K_1.

We studied the intracellular distribution of K_2 and other two homologs, K_1 and K_3, and obtained several informations. Some additional studies such as clearance in liver and heart muscle, urinary and fecal excretion, and the chemical characteristics of metabolites were made.

Material and Method

Vitamin $K_{2(20)}$-(6-^{3}H) (42.1 mCi/mmole) was synthesized from 2-methylnaphthalene-6-^{3}H, which was obtained by tritiation of 2-methyl-6-bromonaphthalene,[7] Vitamin K_1-(2-methyl-^{14}C) (4.3 mCi/mmole) was synthesized from vitamin K_3-(2-methyl-^{14}C) (5.8 mCi/mmole) which was purchased from Radiochemical Center (England).

Cytochrome c was purchased from Sigma Chem. Co. (U.S.A.) and all other chemicals used were those of analytical grade and purchased from Wako Junyaku Co. (Tokyo).

Male Wistar strain rats weighing 150 to 160 g were used. Each labeled vitamin K was dissolved in water with nonionic detergent, HCO-60, and a volume corresponding to 0.5 μmole of each sample (about 0.5 ml) was injected into the tail vein. The rats were decapitated and the removed tissues were well washed with saline to free from blood. The cell fractionation was carried out by means of differential centrifugation.[9] Liver and heart muscles were homogenized in 0.25 M sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle to make 10% homogenate of the tissues, and then unbroken cells and cell debris were removed off by slow centrifugation of $500\times g$ for 5 min. The nuclear and mitochondrial fractions were obtained by centrifugation at $900\times g$ for 10 min and at $12500\times g$ for 20 min, respectively. The nuclear and mitochondrial pellets were resuspended in fresh 0.25 M sucrose and recentrifuged for purification. The supernatant of $12500\times g$ was centrifuged for 60 min at $105000\times g$ using Hitachi ultracentrifuger model 65P, to obtain the microsomal fraction as a pellet and a supernatant fraction. Each of the fractions obtained here was resuspended and diluted into an aliquot with 0.25 M sucrose.

Radioactivity measurement, protein determination, and enzyme assay were carried out by using 0.2 ml of each fraction.

Succinate-cytochrome c reductase activity was measured by the method of Tisdale, *et al.*[10] in which the absorbancy change at 550 nm was traced with a spectrophotometer, Hitachi model ESP-3. The specific activity of the enzyme was shown as ΔT% at 550 nm per mg of protein of each fraction per min. Protein concentration was measured by the method of Lowry, *et al.*[11] using boving serum albumin as standard. The radioactivity was determined with a liquid scintillation spectrometer, Aloka model LSC-501, and the resulting count were corrected by external standard method.

The radioactivity in the tissue was extracted with 20 volumes of acetone–chloroform mixture (1: 1) and the resulting extract was concentrated and developed by the thin-layer chromatographic systems as follows: I) Wakogel B-5F: benzene–chloroform (1: 1), II) Wakogel B-5F inpregnated $AgNO_3$: *n*-heptane–benzene (4: 1) and III) Wakogel B-5F inpregnated 2% Paraffin oil: acetone–water (95: 5). The resulting chromatogram was scanned by a thin-layer radiochromatoscanner, Aloka model TLC-1.

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10) H.D. Tisdale, "Methods in Enzymology," Vol. 10, Academic Press, N.Y., p.213.
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Result and Discussion

Previous studies on the metabolic fate of K_2 in some animals[8] revealed that this homolog was more easily absorbed from gastrointestimal tract and was more effectively incorporated into the tissues than K_1. A little difference in the number of unsaturated bonds in the side chain made a considerable difference in their behavior in the animal body. Therefore, it was of interest to compare the metabolic fate of these three typical K homologs under the same experimental conditions. Studies were made at 1 and 24 hr after the administration to examine the difference in the distribution pattern of K after a short and long period of time after their administration.

Table I. Incorporation of Vitamin K into Rat Liver and Heart Muscle after Intravenous Administration

	Vit. K_1		Vit. $K_{2(20)}$		Vit. K_3	
	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr
Liver	323.7[a]	222.4	730.5	120.2	59.4	12.3
Heart muscle	169.1	357.5	203.8	119.4	46.7	6.0

a) Expressed as equivalent nmole per g wet tissue calibrated from the specific activity mentioned in "Material and Method".

Table I shows the incorporation of each K homolog in the liver and heart muscle of a rat after intravenous injection. In the liver, the highest incorporation at 1 hr after the administration was found in K_2 and nextly higher incorporation in K_1 but, after 24 hr, the concentration of K_1 was higher than those of K_2 and K_3. The same results were obtained in the case of heart muscle. Incorporation of K_3 was very low in both tissues in comparison with the other two homologs. The turnover factor, which is expressed by the ratio of the radioactivity incorporated at 1 hr after the administration against that at 24 hr, gives more detailed informations. The turnover factor of 1.73 and 5.92 were obtained for K_1 and K_2 in the case of the liver respectively, and this suggests rather higher decreasing rate of K_2 than K_1 in the liver. In the heart muscle, the values of each homolog were lower than those in the liver, being 0.38 for K_1 and 3.00 for K_2. This indicates slower turnover of K in the heart muscle than in the liver, although the value for K_2 was remarkably higher than that of K_1 in this case. The fact that the incorporation of K_1 in the heart muscle increased at 24 hr after the administration is characteristic to this homolog. The lowest incorporation and the larger turnover factor of K_3 which was 4.25 and 7.00 for liver and heart muscle, respectively, reveals much lower affinity of K_3 to the tissues than the other two more lipophilic K homologs.

Table II. Urinary and Fecal Excretion of Vitamin K in Rat during First 24 hr after Intravenous Administration

Sample	Urinary excretion	Fecal excretion	
		Acetone–chloroform extractable fraction	Acetone–chloroform unextractable fraction
Vitamin K_1	15.42[a]	13.33	19.60
Vitamin $K_{2(20)}$	3.13	6.04	0.17
Vitamin K_3[b]	40.80	1.43	0.20

a) Expressed as percent for administered dose; mean of three experiments.
b) result of one experiment

On the other hand, comparison of urinary and fecal excretion of these homologs shows the least excretion of K_2 in urine and feces during the first 24 hr after the administration. In contrast to the previous results, in which K_2 was excreted more than K_1 in feces after intraperitoneal injection,[8a] greater fecal excretion of K_1 was observed in the present case. The equal level of the incorporation of K_2 in the liver and heart muscle at 24 hr after the administration shown in Table I and the lower excretion in urine and feces suggest the tendency for homogeneous distribution of this homolog in animal tissues and this observation is consistent with the previous experiment in which the K distribution in the tissues was studied.[8]

Table III. Succinate–Cytochrome c Reductase Activity in Subfractions of Rat Liver and Heart Muscle

Fraction	Liver	Heart muscle
Nuclear	2.51±0.2[a]	20.77±0.35
Mitochondria	6.13±0.12	36.78±9.02
Microsome	1.13±0.30	3.13±0.76
Supernatant	0.96±0.18	1.12±0.04

a) Expressed as $\Delta T\% \times 10^{-1}$ at 550nm/mg protein/min and mean±SD of three experiments.

Since ubiquinone[12] and tocopherol[13] are known to be incorporated into the mitochondrial fraction, K was estimated to be incorporated in the fraction so the succinate-cytochrome c reductase activity of each fraction was measured as a mitochondrial marker to examine the contamination of the mitochondria in other fractions, and the result was shown in Table III. Although complete separation of each fraction cannot be expected by the differential centrifugation, considarable migration of mitochondria into the nuclear fraction was observed in both tissue preparations, especially in heart muscle. Therefore, it would be necessary to take account of the mitochondrial migration in evaluating the results obtained from the nuclear fraction.

Table IV. Intracellular Incorporation of Vitamin K_1 in Rat Liver and Heart Muscle after Intravenous Administration

	1 hr		24 hr	
	Relative % distribution[a]	Concentration (nmole/mg protein)	Relative % distribution	Concentration (nmole/mg protein)
Liver				
nuclear	22.3±16.6[b]	24.4[c]	15.7±12.3	8.5
mitochondria	10.2± 7.0	5.4	35.6± 3.7	11.1
microsome	28.5±10.3	17.8	19.8± 7.2	7.3
supernatant	40.0±14.4	11.2	29.0± 6.8	4.5
Heart muscle				
nuclear	10.6± 3.4	4.7	29.8± 6.2	11.2
mitochondria	4.0± 2.9	5.4	32.3± 7.6	26.2
microsome	6.8± 4.4	12.1	21.1± 5.9	36.4
supernatant	78.6± 9.9	31.8	16.8± 7.6	6.7

a) Expressed as percent for the total radioactivity incorporated among the fractions.
b) expressed as mean ±SD of three experiments
c) Expressed as Vit. K_1 equivalent calibrated from the specific activity mentioned in "Material and Method" and expressed as $\times 10^2$.

12) F.L. Crane, "Biochemistry of Quinones," ed. by R.E. Morton, Academic Press, N.Y., 1965, p. 199.
13) M.M. Oliveira, W.B. Weglichi, A. Nason and P.P. Nair, *Biochem. Biophys. Acta*, **180**, 98 (1969).

Table IV shows the intracellular distribution of K_1. The highest distribution of K_1 at 1 hr after the administration was found in the supernatant fraction of the liver preparation, followed by the microsomal and nuclear fractions. Almost all the radioacticity was also found in the supernatant fraction of the heart muscle preparation. The distribution in the mitochondrial fraction at 24 hr after the administration increased markedly in the liver preparation, while that of nuclear, microsomal and supernatant fractions decreased. On the other hand, the radioactivity in the supernatant fraction of the heart muscle preparation at 1 hr after the administration tended to migrate into the particular fractions of the cells after 24 hr, and also in this case, the highest distribution was found in the mitochondrial fraction. On the other hand, the comparison of the concentration of K_1 in each fraction revealed the highest value in the nuclear fraction of the liver at 1 hr after the administration and next the microsomal and supernatant fractions. K_1 seems to have a tendency to be incorporated and accumulated in the mitochondrial fraction with passage of time because the mitochondrial fraction contained a larger amount of K_1 at 24 hr after the administration than at 1 hr, while the concentration in other fractions decreased compared to that at 1 hr. In the heart muscle preparation, the largest amount of K_1 was found in the supernatant fraction and the secondary largest amount in the microsomal fraction shortly after the administration. It is observed that fractions other than the supernatant concentrated much higher amount of K_1 at 24 hr than at 1 hr after the administration. The ratio of concentration at 24 hr against that at 1 hr was larger in the mitochondrial fraction than in the microsomal fraction, but the highest concentration was found in the microsomal fraction.

Table V. Intracelluar Incorporation of Vitamin $K_{2(20)}$ in Rat Liver and Heart Muscle after Intravenous Administration

	1 hr		24 hr	
	Relative % distribution[a]	Concentration (nmole/mg protein)	Relative % distribution	Concentration (nmole/mg protein)
Liver				
nuclear	18.5±0.8[b]	60.0[c]	7.1± 2.2	1.9
mitochondria	26.9±3.6	31.2	45.9± 2.8	7.4
microsome	17.5±6.3	35.0	9.1± 4.6	2.2
supernatant	37.1±5.9	32.8	38.1± 3.9	4.2
Heart muscle				
nuclear	29.0±3.8	31.7	15.3±10.4	2.4
mitochondria	22.0±1.5	22.5	36.6±12.5	18.5
microsome	10.5±3.9	16.8	11.2± 1.2	10.2
supernatant	38.6±5.5	22.4	36.9±14.4	7.7

a) Expressed as percent for the total radioactivity incorporated among the fractions.
b) expressed as mean±SD of four experiments
c) Expressed as Vit. $K_{2(20)}$ equivalent calibrated from the specific activity mentioned in "Material and Method" and expressed as $\times 10^2$.

K_2 showed much higher distribution in the mitochondrial fraction of the liver preparation than K_1 even at 1 hr after the administration as shown in Table V. However, the highest distribution was shown in the supernatant fraction as in the case of K_1. After 24 hr, the affinity of K_2 to the mitochondria was markedly emphasized and almost all the radioactivity was divided into two fractions of the liver preparation, mitochondrial and supernatant fractions. On the other hand, in the heart muscle preparation a rather higher distribution was found in the fractions other than supernatant fraction at 1 hr after the administration. The distribution pattern of K_2 in the heart at 24 hr after was similar to that of the liver preparation in which a higher distribution of the radioactivity was observed in the mitochondrial and supernatant fractions. The concentration of K_2 in each fraction of the liver was not so largely

different at 1 hr after the administration, but the nuclear fraction showed the highest value. After 24 hr, the highest concentration of K_2 was observed in the mitochondrial fraction but the concentration in each fraction apparently decreased. A little difference in the behavior of K_2 from that of K_1 was observed in the heart muscle. The highest concentration of K_2 was found in the nuclear fraction, then in the mitochondrial and the supernatant fractions at 1 hr after the administration in contrast with that K_1 was highly concentrated in the supernatant fraction. After 24 hr, the highest concentration of K_2 was found in the mitochondrial fraction and then in the microsomal fraction. Although the concentration in each fraction at 24 hr was lower than that at 1 hr, the decreasing rate in the microsomal and mitochondrial fractions was apparently lower than those of other two fractions.

Table VI. Intracellular Incorporation of Vitamin K_3 in Rat Liver and Heart Muscle after Intravenous Administration

	1 hr		24 hr[b]	
	Relative % distribution[a]	Concentration (nmole/mg protein)	Relative % distribution	Concentration (nmole/mg protein)
Liver				
nuclear	7.7±0.4[c]	0.97[d]	13.3	0.36
mitochondria	11.3±0.3	1.15	29.3	0.60
microsome	13.7±7.7	2.67	24.2	0.62
supernatant	66.4±6.6	3.46	33.2	0.39
Heart muscle				
nuclear	11.6±2.5	0.58	11.7	0.01
mitochondria	8.5±4.5	0.65	33.8	0.69
microsome	9.2±0.8	2.58	54.5	2.17
supernatant	69.8±8.8	11.32	0.0	—

a) Expressed as percent for the total radioactivity incorporated among the fractions.
b) result of one experiment
c) expressed as mean±SD of three experiments
d) Expressed as Vit.K_3 equivalent calibrated from the specific activity mentioned in "Material and Method" and expressed as $\times 10^2$.

The distribution of K_3 is shown in Table VI, which indicates that almost all the radioactivity was retained in the supernatant fraction of both tissues at 1 hr after the administration and a very low distribution in other fractions. After 24 hr, no specificity was found in the distribution pattern of K_3 in both tissues except that the supernatant fraction of the heart muscle preparation lost the radioactivity. A relatively high concentration of K_3 was found in the supernatant fraction of both tissues shortly after its administration, although the concentration was not so high as other two homologs in both tissues and at both time periods. It is interesting that the decreasing rate of concentration in the microsomal fraction of heart muscle preparation was rather low.

An appreciable amount of K_1 and K_2 was shown to be incorporated in the nuclear fraction in both tissues shortly after the administration and the migration of mitochondria into the nuclear fraction is not enough to explain these high values. Some of the radioactivity in the nuclear fraction may depend partly on the adsorption of K on the cytoplasmic membrane fragments contaminated because the radioactivity in the nuclear fraction decreased after 24 hr, but more detailed studies should be needed for confirmation.

Bell and Matschiner[6] studied the intracellular distribution of intracardially administered K_1 in the rat liver 0.5, 8, and 26 hr after the administration and reported that the specifically high relative distribution was observed in the microsomal fraction throughout each experimental period and that about one-half of the extractable radioactivity in the liver was incorporated into the microsomal fraction. But in our present experiment, neither specific incor-

poration of K_1 nor of K_2 and K_3 into the microsomal fraction was observed. The higher distribution in the mitochondria than the microsomes at 24 hr was rather apparent, although the microsomal fraction showed a relatively high value at 1 hr. And also, K_2 showed a characteristic affinity to the mitochondrial fraction and this was remarkable at 24 hr in both tissues, liver and heart muscle. However, it is not sufficient to discuss the affinity to each K homolog to each fraction only by relative distribution because in our observation the concentration of homologs in each tissues at 24 hr markedly decreased with the exception of K_1 in the heart muscle.

K_1 was again proved to be concentrated in the mitochondrial fraction at 24 hr by comparison of concentration. This observation is consistent with the result obtained by Thierry, *et al.*[5] in which K_1 was incorporated into the mitochondrial fraction after intravenous administration and the microsomal incorporation was not so high. At 24 hr after the administration of 10 μg of K_1, 9.6 pmole per mg of mitochondrial protein was retained yet and this was still the highest amoung the fractions. A little difference was revealed from our present experiment, that K_1 was more concentrated in the mitochondrial fraction at 24 hr than at 1 hr after the administration in both tissues. K_1 seems to show lower turnover in the liver mitochondrial fraction than in other fraction. In the case of K_2 also, the concentration in the mitochondrial fraction of the liver was the highest at 24 hr although it was lower than that of K_1, indicating that the turnover of K_2 in the liver mitochondria seemed also to be the lowest. It is estimated from the obervations mentioned below that K_2 was incorporated faster and more concentrated in the mitochondria than K_1, although K_1 seemed to turnover slower than K_2. The concentration of K_2 in the mitochondrial fraction shortly after the administration was higher than that of K_1, while K_1 increased markedly in it at 24 hr. A considerable amount of K_2 was still retained in the mitochondrial fraction at 24 hr after the administration but the concentration was lower than that of K_1.

The highest concentration of K_3 in the supernatant fraction of both tissues shortly after the administration and its rapid disappearance at 24 hr suggest its lower affinity to the particular fractions in the cell.

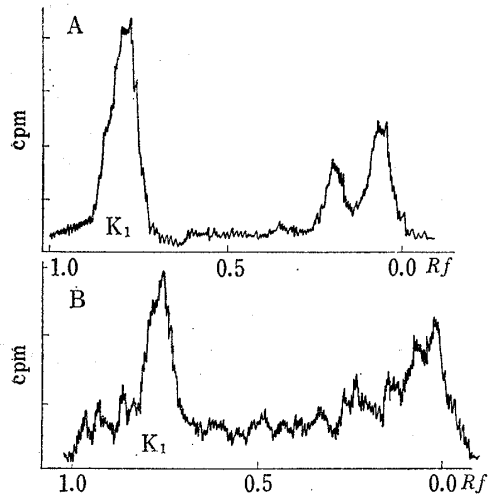

Fig. 1. Scannogram of Liver Extract of ^{14}C-Vitamin K_1 Administered Rat

A: developing system:
Wakogel B-5F benzene–chloroform (1:1)
B: developing system:
Wakogel B-5F inpregnated 2% paraffine acetone–water (95:5)

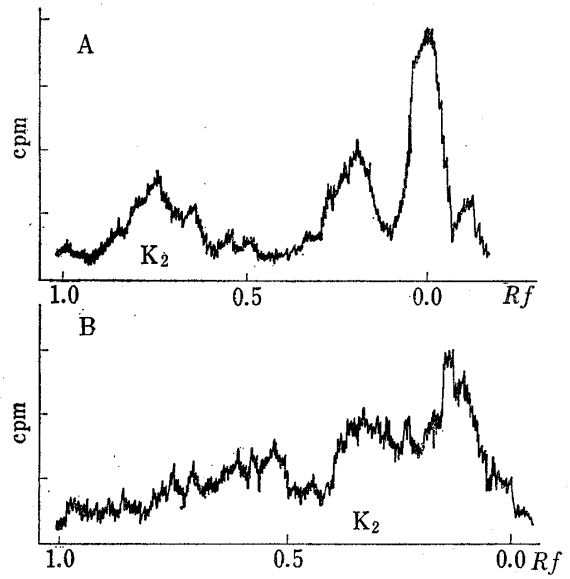

Fig. 2. Scannogram of Liver Extract of ^{3}H-Vitamin $K_{2(20)}$ Administered Rat

A: developing system:
Wakogel B-5F benzene–chloroform (1:1)
B: developing system:
Wakogel B-5F inpregnated $AgNO_3$ *n*-heptane–benzene (4:1)

In the case of K_2, the distribution tendency in each fraction was very similar in both tissues, where almost all the radioactivity was found in the mitochondrial and supernatant fractions and the highest concentration was also found in the mitochondrial fraction.

On the other hand, there was little difference in the distribution pattern between in the liver and heart muscle in the case of K_1 and K_3. Rather higher distribution of K_1 and K_3 in the supernatant fraction shortly after the administration in the heart muscle was characteristic and the radioactivity in it seemed to migrate into the particular fractions with passage of time. The amount of K_1 and K_3 in the supernatant fraction was not so high at 24 hr, while much higher distribution of K_2 was observed in the fraction even at 24 hr.

Fig. 1 and 2 show the TLC scannograms of acetone–chloroform extract of the rat liver after intravenous administration of K_1 or K_2. It is obvious that a considarable amount of both K_1 and K_2 was changed to more polar metabolites within 1 hr after the administration. Rather faster change of K_2 than K_1 in the liver was observed by comparison of their peak range on the TLC scannogram and there could not be found any conversion of K_1 to K_2 on this scannogram. Further study of metabolites in the liver at 24 hr is needed to clarify which form of K metabolites shows affinity to the mitochondria, although such study could not be done in this case because of smaller radioactivity.

Although it is difficult to find direct relationship between the intracellular distirbution pattern of K and their role in the coagulation protein synthesis, rather higher distribution of K in the microsomal fraction shortly after the administration is interesting in connection with the active site of K in prothrombin synthesis in which K acts at or after the translational level on ribosome.[14] However characteristically higher affinity of K which has alkyl side chain to the mitochondria become apparent from the observations that K_1 tended to be concentrated in the miochondria after a long period of time. A considarable amount of K_2 was incorporated in the mitochondria shortly after the administration and this tendency of K_2 to be concentrated was strengthened at a later period.

K_3 which is less lipophilic than other two homologs did not show any characteristic affinity to the mitochondria.

As the other structurally similar lipophilic vitamins such as ubiquinone and tocopherol are known to be highly incorporated into the liver mitochondria,[12,13] the lipophilic side chain may be responsible for their affinity to the mitochondria. A higher affinity of K_2 to the mitochondria than K_1 would be reasonable from the observations that the mitochondrial lipids are highly unsaturated[15] and the side chain of K_2 is similar to that of ubiquinone.

The higher affinity of K to the mitochondria suggest the possibility of the participation of K in some regulation process in the mitochondria other than blood coagulation protein synthesis, such as oxidative phosphorylation, transport regulation, or membrane preservation. However, little difference in the distribution pattern of K in both liver and heart muscle may suggest the possibility of their function in the ion or substrate transport regulation or membrane preservation in the mitochondria rather than in oxidative phosphorylation.

To clarify these possibilities, some additional studies, such as intramitochondrial distribution, by biochemical tracer and electron microscopic autoradiography are now under way.

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