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Intracellular and Intramitochondrial Distribution of Vitamin K:[1] Biochemical and Electron Microscopic Radioautographic Study

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The intracellular distribution of vitamin K_1, $K_{2(20)}$ and K_3 in the rat liver and heart was studied by sucrose linear density gradient centrifugation and it was revealed that vitamin K_1 and $K_{2(20)}$ which have long alkyl side chain at 3-position of their common frame other than K_3 were incorporated in the mitochondria. This result was well consistent with the previous one which was obtained by differential centrifugation method.

Further study of their intramitochondrial distribution revealed that they localized in the inner membrane of the mitochondria and some of them in the soluble fraction.

Electron microscopic radioautography of ^{3}H-vitamin $K_{2(20)}$ was also studied and it was again proved that this homolog had higher affinity to the membranous structure in the liver cell especially to the mitochondrial inner membrane.

This high affinity of these homologs to the mitochondria especially to the inner membrane suggests the participation of them in some regulatory process in the mitochondrial function other than blood coagulation protein synthesis.

Vitamins K function in the process of blood coagulation protein synthesis has been under study without clear resolution. The study of intracellular distribution of vitamins K may give precise information about their site of action in the cell, since only a few reports about K_1 distribution in rat liver cell have been published.[3]

Our previous work using the differential centrifugation method on the intracellular distribution of typical three homologs of vitamin K, vitamin K_1, $K_{2(20)}$, and K_3, in rat liver and heart muscle showed that K_1 and $K_{2(20)}$ which are more lipophilic than K_3 were highly incorporated into the mitochondrial fraction of both tissues.[1] Additional studies in this work with use of sucrose density gradient centrifugation method confirmed the above findings and the study of intramitochondrial distribution of these vitamin K homologs by stepwise sucrose density gradient centrifugation revealed that almost all the vitamin K_1 and $K_{2(20)}$ incorporated into the mitochondria were localized in the inner membrane fraction.

Furthermore, the electron microscopic radioautography using vitamin $K_{2(20)}$-(6-^{3}H) demonstrated that the administered $K_{2(20)}$ was incorporated into the mitochondrial membrane and other membranous structures in the liver cell, consisting with the findings of the biochemical tracer experiments.

From these results, vitamin K_1 and $K_{2(20)}$ with lipophilic alkyl side chain at 3-position of their basal structure of 1,4-naphthoquinone were deduced to have higher affinity to the membranous structure in the cell especially to the inner membrane of mitochondria. Such higher affinity to the mitochondria of lipophilic homologs of vitamin K other than K_3 suggests their role in the mitochondrial function in addition to blood coagulation protein synthesis.

Material and Method

Preparation of Labeled Compound——Vitamin K_1-(2-methyl-^{14}C) (4.1 mCi/mmole) and vitamin $K_{2(20)}$-(2-methyl-^{14}C) (4.3 mCi/mmole) were synthesized from vitamin K_3-(2-methyl-^{14}C) (5.8 mCi/mmole) which

1) Previous report: T. Konishi and S. Baba, *Chem. Pharm. Bull.* (Tokyo), **21**, 1906 (1973).
2) Location: *3-20-1, Kitashinjuku, Shinjuku-ku, Tokyo.*
3) *a*) W.D. Taggart and J.T. Matschiner, *Biochemistry*, **8**, 1141 (1969); R.G. Bell and J.T. Matschiner, *Biochem. Biophys. Acta*, **184**, 597 (1969); M.J. Thierry, H.A. Hermodson, and J.W. Suttie, *Am. J. Physiol.*, **219**, 854 (1970); *b*) M.J. Thierry and J.W. Suttie, *Arch. Biochem. Biophys.*, **147**, 430 (1971).

was purchased from Radiochemical Center (England) by the method of previous report.[4] Vitamin $K_{2(20)}$-(6-^{3}H) (42.1 mCi/mmole) was also synthesized by the method previously reported.[4]

Chemicals——Cytochrome c was purchased from Sigma Co., (U.S.A.) and all other chemicals used here were purchased from Wako Junyaku Co., (Tokyo) and have analytical purity.

Animals——Male Wistar strain rats weighing 160 to 170 g were used and three rats were grouped for a experiment.

Solubilization of Labeled Compound——Each labeled compound was dissolved in water with nonionic detergent, HCO-60, and the detergent concentration was not over 5%.

Sucrose Density Gradient Centrifugation——Rats were injected intravenously with 0.5 μmole of each labeled vitamin K and decapitated at indicated time after the administration. The liver and heart muscle were washed with saline to free from blood contamination and homogenized in 0.25M sucrose with Potter-Elvehjem homogenizer with a Teflon pestle to make 10% homogenate of each tissue and then cell debris and nuclear fraction were removed by slow centrifugation at $900 \times g$ for 10 min. The resulting supernatant was placed on the sucrose linear gradient (specific gravity 1.03 to 1.29) prepared by Hitachi model DGK-U gradient producer and centrifuged with RSP-3A rotor for 3 hr at 50000 rpm with Hitachi model 65P ultracentrifuger. After centrifugation, the fractionation was carried out by cutting every 0.5 mm from top of the gradient to bottom with tube slicer. The fractions obtained here were diluted with 1 ml of Tris-HCl(pH 7.4) containing 0.25M sucrose and every 0.2 ml of each fraction was used for radioactivity measurement, protein determination and enzyme assay.

Subfractionation of the Mitochondria——Purification and the following osmotic puncture treatment of the mitochondria was performed by the method of Parson, *et al.*[5] The mitochondria was treated with hypotonic phosphate buffer (pH 7.4) for 2 hr to separate outer, inner membranes and matrix space. Resulting osmotic shocked mitochondrial suspension was centrifuged at $2000 \times g$ for 5 min to obtain crude inner membrane fraction as pellet and crude outer membrane fraction as supernatant. Purification of each fraction was carried out by the way shown in Chart 1. Each crude fraction was placed separately on the stepwise sucrose gradient,[6] (1.03, 1.09, 1.14, and 1.19) and centrifuged with RSP-25A roter at 22000 rpm for 3 hr with Hitachi model 65P ultracentrifuger to equilibration. The outer membrane was found to be concentrated in 4th fraction as white cloud and the inner membrane was obtained in 7th fraction as pellet. The fractionation was carried out by cutting off with tube slicer. The radioactivity measurement of each fraction was carried out by the same way mentioned above.

Electron Microscopic Radioautography——A rat (body weight 100 g) was injected with 0.7 mCi of ^{3}H labeled vitamin $K_{2(20)}$ into the tail vein and decapitated after 1 hr of the administration. The small pieces of liver were fixed at 0° for 2 hr in 5% glutaraldehyde in 0.15M phosphate buffer (pH 7.4) containing 0.25M sucrose and postfixed in 2% OsO_4 in 0.25M sucrose for 2 hr. All samples were dehydrated in graded ethanols and propylene oxide and embeded in Epon. The sections showing pale gold to silver interference colors were prepared with a LKB ultratome and coated with the Sakura NR-H2 emulsion (Konishiroku Co.,), developed in Copinal (Fuji Photo Film Co.) or Elon-ascorbate,[7] fixed and stained with lead acetate.

The radioautograms were examined with the JEN-1 electron microscope at 60 kV and photographed at an instrument magnification of 6000 to 34000.

Protein determination was carried out by the method of Lowry, *et al.*[8] using bovine serum albumin as standard.

Succinate-Cytochrome c reductase activity was measured by the method of Tisdale, *et al.*[9] in which the absorbancy change at 550 nm was traced with a spectrophotometer, Hitachi model SR-1.

Radioactivity was determined with a liquid scintillation spectrometer, Aloka model LSC-502, and the resulting counts were corrected by external standard method.

Result and Discussion

Our previous experiment revealed that vitamin K_1 and K_2 which have a long alkyl side chain at 3-position of their basal frame were incorporated selectively into the mitochondrial fraction of liver and heart muscle cell after the intravenous administration. K_3 which is less lipophilic than K_1 or K_2 was not incorporated into the fraction and retained in the super-

4) T. Konishi, S. Baba, and T. Matsuura, *Radioisotopes.*, **20**, 31 (1971).
5) D.F. Parson and G.R. Williams, in "Methods Enzymol," Vol. , 10, Academic Press, N.Y., p. 443.
6) L. Sato, in "Seitaimaku no Seikagaku," ed. by T. Oda, L. Sato, and M. Nakao, Asakura, Japan, 1969, p. 12.
7) T. Mizuhira and K. Uchida, *Kagaku to Seibutsu*, **5**, 235 (1967).
8) O.H. Lowry, N.J. Rosenbrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
9) H.D. Tisdale, in "Method Enzymol.," Vol. 10, Academic Press, N.Y., p. 213.

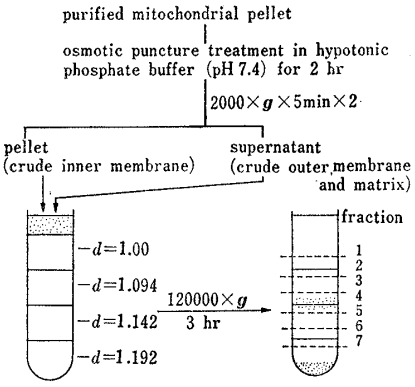

Chart 1. Stepwise Sucrose Gradient Purification of The Inner and Outer Membrane of Mitochondria

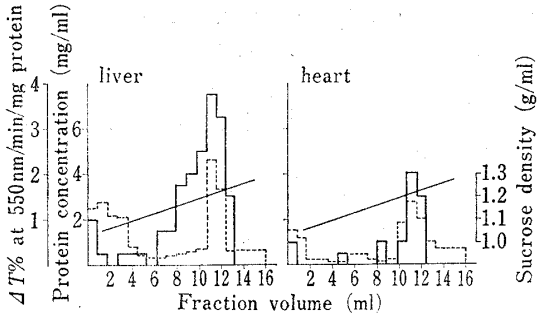

Fig. 1. Distribution of Succinate-Cytochrome c Reductase on Sucrose Linear Density Gradient

The solid line indicates the succinate-cytochrome c reductase activity and the dotted line indicates the protein concentration in each fraction.

natant fraction through the experimental period. But the conventional differential centrifugation method is not the best tool for complete separation of the intracellular structures, so a further study by sucrose linear gradient centrifugation method was needed to confirm the affinity of vitamin K to the mitochondria.

Fig. 1 shows the distribution of succinate-cytochrome c reductase activity, which was measured as mitochondrial marker. Almost all the activity was proved to be concentrated in the portion of density 1.20 and so the protein peak in this portion was identified as the mitochondrial fraction. This density is reasonable from the literature value.[10]

The density gradient centrifugation pattern of $900\times g$ supernatant of the liver and heart muscle of rats administered with vitamin K are shown in Fig. 2 to 4. The previous observation in which vitamin K seemed to have a tendency of incorporation into the mitochondria was more clearly recognized in this case. The vitamin K_1 or $K_{2(20)}$ recovered in $900\times g$ supernatant fraction were found to be highly concentrated in the portion of mitochondrial protein and some of them was also found in the portion of much lower density which may correspond to the soluble fraction or microsomal fraction.

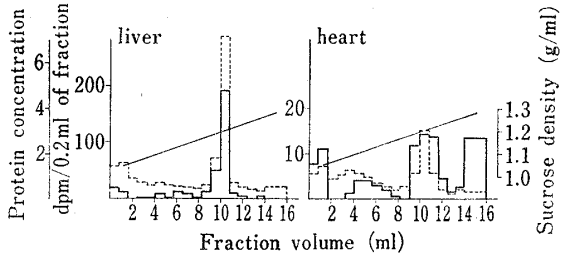

Fig. 2. Distribution of Vitamin K_1-(2-methyl-^{14}C) on Sucrose Linear Density Gradient at 6 hr after Intravenous Administration

The solid line indicates the succinate-cytochrome c reductase activity and the dotted line indicates the protein concentration in each fraction.

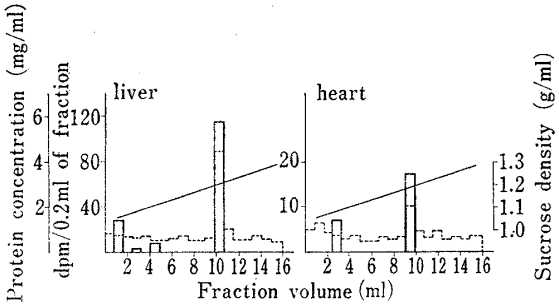

Fig. 3. Distribution of Vitamin $K_{2(20)}$-(2-methyl-^{14}C) on Sucrose Linear Density Gradient at 6 hr after Intravenous Administration

The solid line indicates the radioactivity and the dotted line indicates the protein concentration in each fraction.

The K_3 distribution was studied at 1 hr after the administration because this homolog was proved to be incorporated less than the other two lipophilic homologs into the tissues and excreted faster in urine.[1] Fig. 4 revealed that almost all the radioactivity retained in

10) H. Schuel, S.R. Tipton, and N.G. Anderson, *J. Cell. Biol.*, 22, 317 (1964).

the portion of lower density. The lower incorporation of this homolog in the particular fraction and the higher distribution in the soluble fraction are well consistent with the result obtained by differential centrifugation study.

The observations obtained here again confirmed that vitamin K homologs which have long isoprenoid side chain have higher affinity to the mitochondria. This suggests that they must have some intimate relationship with mitochondrial function in addition to blood coagulation protein synthesis.

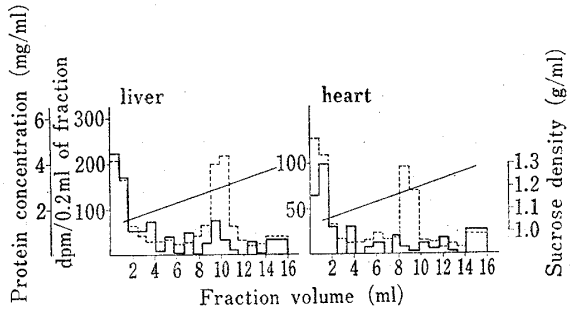

Fig. 4. Distribution of Vitamin K_2-(2-methyl-^{14}C) on Sucrose Linear Density Gradient at 1 hr after intravenous Administratin

The solid line indicates the radioactivity and the dotted line indicates the protein concentration in each fraction.

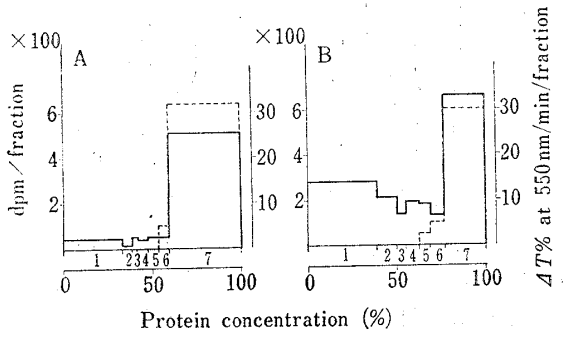

Fig. 5. Intramitochondrial Distribution of Vitamin K_1-(2-methyl-^{14}C) at 1 hr after Intravenous Administration

A: 2000×*g* pellet (crude inner membrane fraction)
B: 2000×*g* supernatant (crude outer membrane fraction)
The solid line indicates the radioactivity and dotted line indicates the succinate-cytochrome c reductase activity.

Further to examine the function of vitamins K in mitochondria, intramitochondrial distribution of them was studied.

The crude outer and inner mitochondrial membrane fractions obtained by osmotic puncture method were further purified by stepwise sucrose density gradient and the results were shown in Fig. 5 to 7. As vitamin K_1 was previously found to be incorporated more slowly into the tissues than K_2, the distribution at 2 hr after the administration was also studied in the case of K_1. Succinate-Cytochrome c reductase activity which was found to be localized in the fraction 7 in which purified inner membrane was concentrated. The purity of the

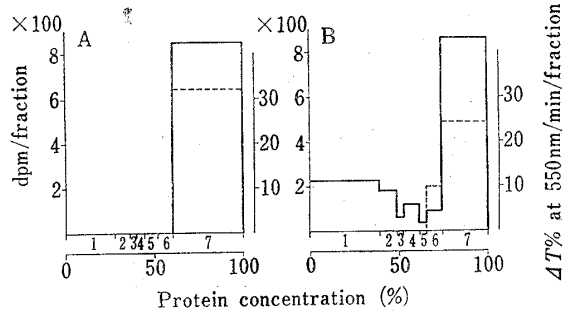

Fig. 6. Intramitochondrial Distribution of Vitamin K_1-(2-methyl-^{14}C) at 2 hr after Intravenous Administration

A: 2000×*g* pellet (crude inner membrane fraction)
B: 2000×*g* supernatant (crude outer membrane fraction)
The solid line indicates the radioactivity and dotted line indicates the succinate-cytochrome c reductase activity.

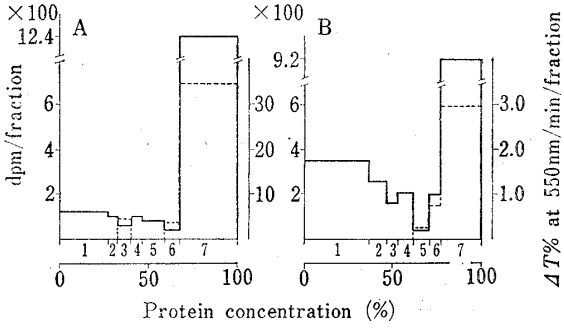

Fig. 7. Intramitochondrial Distribution of Vitamin $K_{2(20)}$-(2-methyl-^{14}C) at 1 hr after Intravenous Administration

A: 2000×*g* pellet (crude inner membrane fraction)
B: 2000×*g* supernatant (crude outer membrane fraction)
The solid line indicates the radioactivity and the dotted line indicates the succinate-cytochrome c reductase activity.

inner and outer mitochondrial membrane was also proved electron microscopically by negative stainning method.

The intramitochondrial distribution of K_1 at 1 hr after the administration shown in Fig. 5 revealed that the radioactivity in the crude inner membrane fraction was apparently localized in the fraction 7. Even in the case of crude outher membrane fraction, rather higher radioactivity was observed in the fraction 7 than the other fractions and considerable amount of the radioactivity was found in the 1 and 2 fractions, which might be attributed to the soluble proteins in the matrix space of the mitochondria. Any notable amount of radioactivity was not detected in the fraction 4 which contains outer membrane. The incorporation of K_1 into the inner mitochondrial membrane was markedly emphasized after 2 hr of the administration as shown in Fig. 6 because the ratio of the radioactivity to the enzyme activity was increased at 2 hr while no incorporation into the fraction 4 was observed in this case also.

Much higher affinity of vitamin $K_{2(20)}$ to the inner mitochondrial membrane was apparent from Fig. 7. Remarkably higher radioactivity was found to be incorporated into the inner mitochondrial fraction already at 1 hr. No appreciable amount of radioactivity was observed in the outer membrane fraction also in this case like the case of K_1. The faster incorporation of $K_{2(20)}$ into the inner mitochondrial fraction than K_1 may be reasonably explained from our previous experiment[1] in which the faster incorporation of $K_{2(20)}$ into the liver and heart

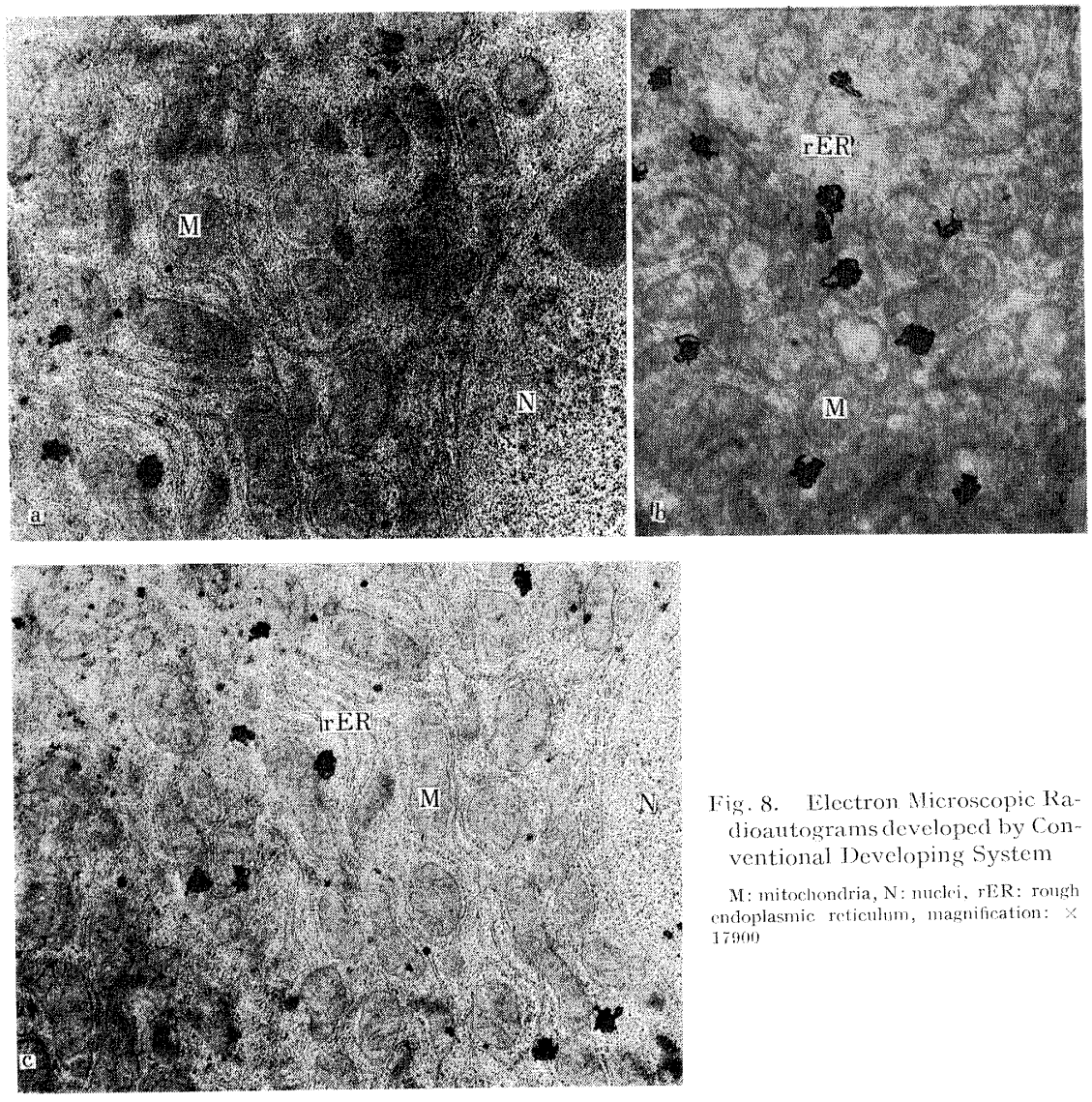

Fig. 8. Electron Microscopic Radioautograms developed by Conventional Developing System

M: mitochondria, N: nuclei, rER: rough endoplasmic reticulum, magnification: × 17900

muscle tissues and also into the mitochondrial fraction of both tissues than K_1 was clarified. These observations proved that vitamin K homologs which have a long isoprenoid side chain were well incorporated into the mitochondria and they were mostly concentrated in the inner membrane but not in the outer one, though only a small portion was contained in the matrix space of the mitochondria.

Fig. 8 to 10 shows the electron microscopic radioautograms of the liver preparation of the rat administered with vitamin $K_{2(20)}$-^3H intravenously. The developed silver grains show different size and figures owing to the developing conditions such as developing reagent, temperature and developing time. Here we tried two ways of development, one was followed by a conventional developing system at low temperature and another by ultra micrograin

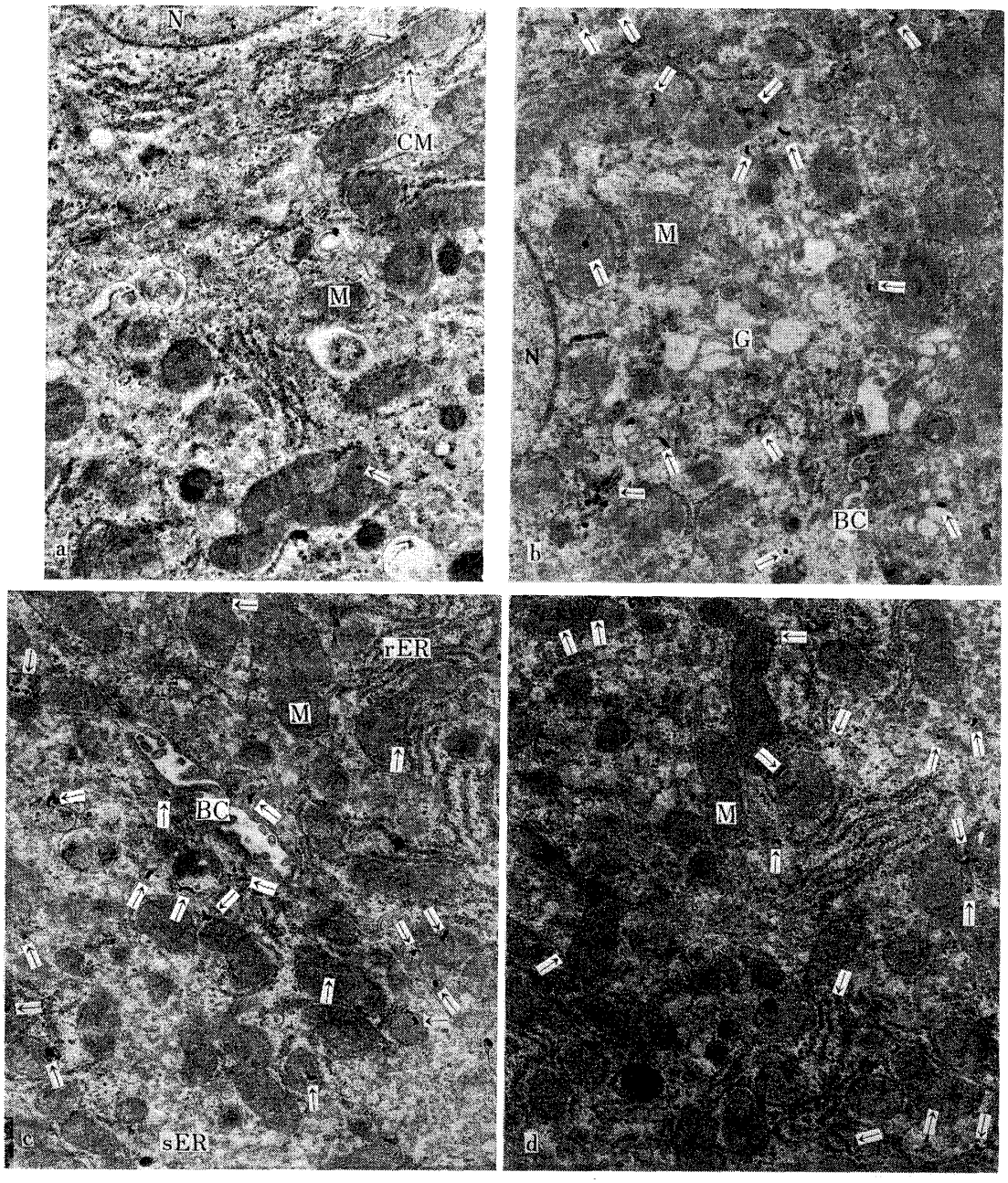

Fig. 9. Electron Microscopic Radioautograms developed by Micro grain Developing System[7]

M: mitochondria, N: nuclei, L: lysosome, G: golgi, CM: cytoplasmic membrane, BC: bile capillary, magnification: ×17900

developing system of Mizuhira, *et al.*[7] The development was carried out at several developing time periods at 15°. Former gives rather larger filamentary developed silver grains, which is easily counted up on the photographs, although it is difficult to detect the origin of the β-ray emitted from tritium. The radioautograms developed by this system are shown in Fig. 8. The filamentary developed silver grains are mainly found in the region of the mitochondria and some are also found in the portion of the endoplasmic reticulum.

The electron microscopic radioautograms developed by ultra micro grain developing system, in which the silver grains are inhibited growing to larger filament and found as a small particles, are shown in Fig. 9 and 10. The higher resolution of the β-ray origin are obtained in this case. From the observation of lower magnified radioautograms in Fig. 9, the silver grains were found on the mitochondria and endoplasmic membranous structures such as s-ER, r-ER or vacuoles. Some were also found in the cytoplasma. They however could not be

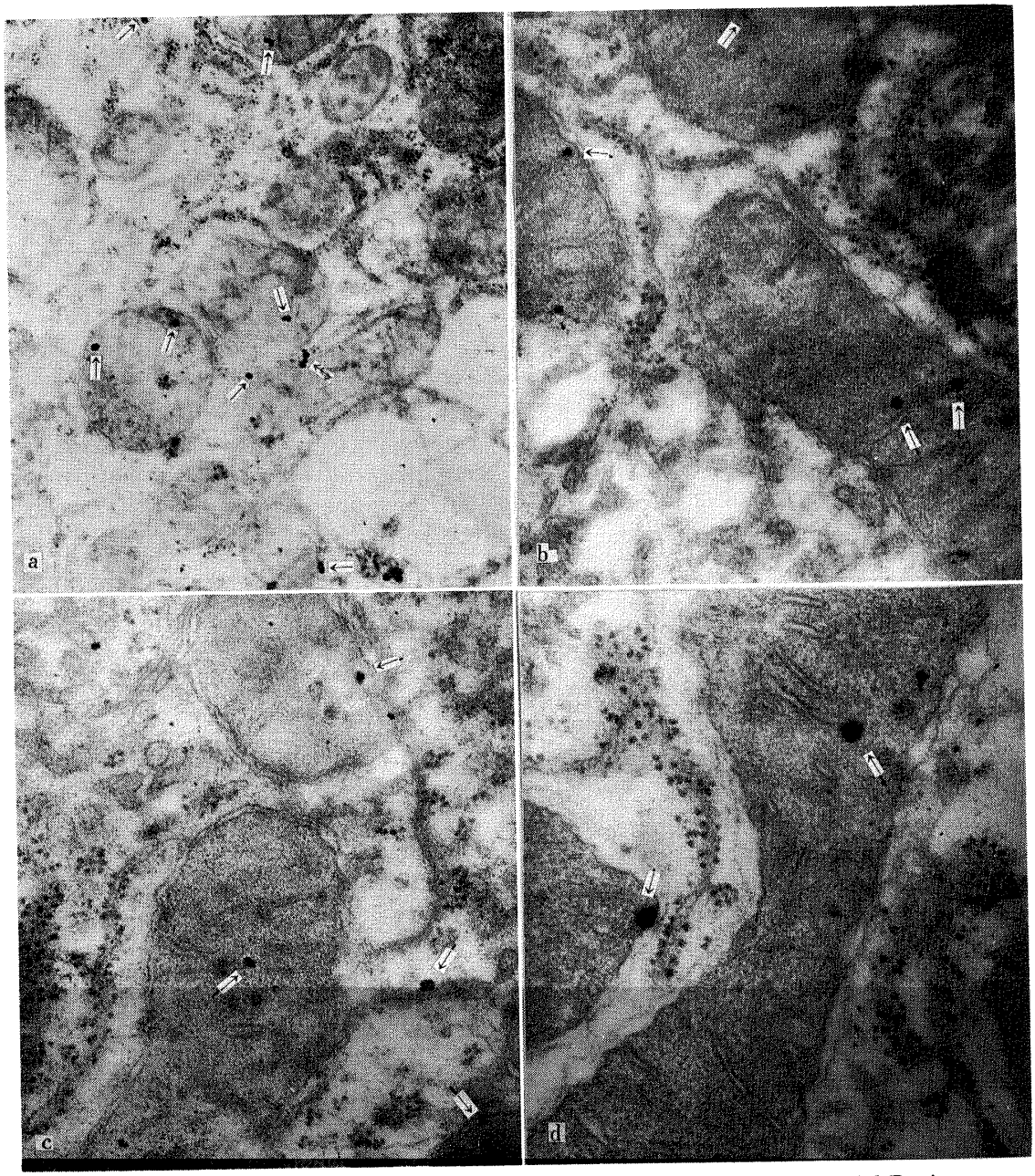

Fig. 10. Enlarged Electron Microscopic Radioautograms of Mitochondrial Region
magnification: a; ×20100, b,c; ×45100, d; ×51600

observed on other detectable structures such as lysosomes (Fig. 9-a), Golgi apparatus (Fig. 9-b), nuclear regions and nucleoli (Fig. 8-a). The larger magnification of the mitochondrial region revealed that the grains adhered on the cristae or inner membrane of the mitochondria and some were also found in the matrix space or the boundary region between inner and outer membrane (intermembrane or cristal space) (Fig. 10). And it was also confirmed that some other grains observed in the region other than mitochondria were adherent only on the membranous structure such as endoplasmic reticulum (Fig. 10-c).

Table I. Silver Grain Distribution on the Intracellulare Organellas

	No. of silver grains	% of total grains counted
Nuclei	3	1.5
Mitochondria	74	36.5
Lysosomes	6	3.0
Endoplasmic reticulum	53	26.1
r-ER	18	
s-ER	35	
Lyposome membrane	9	4.4
Plasma membrane	9	4.4
Cytoplasma (not detectable as reticulum membrane)	49	24.1

The grains found in the photographs which were indiscriminately obtained by medium instrument magnification, were contrasted with several distinctive structures and the result are shown in Table I. The number of grains observed on the mitochondria was correlated to the results obtained previously by differential centrifugation study, in which intravenously administered $K_{2(20)}$ was incorporated about 18% in the nuclear fraction, 27% in the mitochondrial, 18% in the microsomal and 37% in the supernatant fractions at 1 hr after the administration. Rather higher distribution in the nuclear fraction in the previous study may be attributable to the contamination of the mitochondria and cytoplasmic membrane fragments. This estimation is well consistent with our present observation in which the higher number of grains were observed on the mitochondria in the electron microscopic radioautograms and few on the nuclear region.

The inner mitochondrial membrane has a function of the control barrier of the substrates and ions transfer and is the site of oxidative phosphorylation. So the high affinity of vitamin K to the mitochondrial inner membrane suggests us some regulatory function of vitamin K in the mitochondria other than its role in the blood coagulation protein synthesis.

Recently Matschiner, *et al.*[11] studied the intracellular distribution of K_1 in rat liver and found that K_1 was markedly incorporated into the microsomal fraction. Especially, the Golgi apparatus was found to concentrate large amount of K_1 and so they estimated that K_1 may function in the regulation of the glycosylation of pre-prothrombin in the Golgi apparatus. But both our previous and present studies revealed rather higher affinity of K_1 and K_2 to the mitochondria than to the microsomes. High resolution electron microscopic radioautography also did not find any relationship between vitamin K and Golgi apparatus. Such mitochondrial affinity of vitamin K_1 was observed by Thierry and Suttie[3b] in the case of intravenously administered K_1. Further study should be needed for the confirmation what is the cause of these differences in the results.

11) S.E. Nyquist, J.T. Matschiner, and D.J. Morre, *Biochem. Biophys. Acta.*, **244**, 645 (1971).

However, Maryin, *et al.*[12] recently reported that the mitochondria can synthesize their own glycoproteins and the glycoprotein synthesizing system is localized in the inner mitochondrial membrane. In fact, it was revealed by Bernacke, *et al.*[13] that K_1 accelerates the glycoprotein synthesis in the mitochondria.

Otherwise, it was also clarified that inner membrane of the mitochondria contains the polyprenyl pyrophosphate chain synthesizing system and these compounds synthesized there, are estimated to have a role in the transfer of sugar into the glycoproteins.[14] This is interesting in relation to our present finding that vitamin K which has a long alkyl side chain shows higher affinity to the inner mitochondrial membrane and that possible physiological active type of vitamin K, $K_{2(20)}$,[15] shows much higher affinity to the inner membrane than the other two homologs.

Acknowledgement The authors wish to express their thanks to Mr. H. Ueda for technical assistance.

12) S.S. Maryin and H.B. Bosmann, *Biochem. Biophys. Acta*, **230**, 411 (1971).
13) R.J. Bernacki and H.B. Bosmann, *Biochem. Biophys. Res. Commun.*, **41**, 498 (1970).
14) K. Momose and H. Rudney, *J. Biol. Chem.*, **247**, 3930 (1972).
15) C. Martius and H.O. Esser, *Biochem. Z.*, **331**, 1 (1958); C. Martius, *Am. J. Clin. Nutr.*, **9**, 97 (1961); M. Billeter, W. Bolliger, and C. Martius, *Biochem. Z.*, **340**, 290 (1964).