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ENZYMATIC ALKYLATION OF MENAQUINONE-0 TO MENAQUINONES BY MICROSOMES FROM CHICK LIVER

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SUMMARY

1. The alkylation of menaquinone-0 to menaquinone-4 occurs in chick liver. Studies have been carried out to localize the enzyme in a cell particulate and demonstrate its activity *in vitro*. Injection of [6,7-$^3H_2$]menaquinone-0 into vitamin K-deficient chicks resulted in the biosynthesis of menaquinone-4 within 15 min. After 3 h, the distribution among mitochondria, microsomes, and cytosol were 24, 76 and 0%, respectively. Liver cell fractions were incubated in phosphate buffer with [6,7-$^3H_2$]menaquinone-0 or [6,7-$^3H_2$]menaquinol-0 with or without geranyl pyrophosphate, farnesyl pyrophosphate, or geranylgeranyl pyrophosphate for 1 h. Menaquinones formed were identified by thin-layer chromatography and gas–liquid chromatography. With geranylgeranyl pyrophosphate, the incorporation into cell particulates were 0.14, 0.68, and 0.09% for mitochondria, microsomes, and cytosol. The addition of farnesyl pyrophosphate, geranyl pyrophosphate, and geranylgeranyl pyrophosphate to microsomes increased the overall synthesis of menaquinones from 0.03 to 0.55, 0.24 and 0.10 nmoles, respectively. It appears that the homologue synthesized is controlled by isoprenyl pyrophosphate available for condensation.

INTRODUCTION

The alkylation of menaquinone-0 to menaquinone-4 occurs in chick and rat liver[1–3] and accounts for the biological activity of menadione in the mammal and bird. Menaquinone-0 thus functions as a provitamin in these species. Although MARTIUS AND ESSER[1] tentatively identified the product of menaquinone-0 alkylation as menaquinone-4 from countercurrent distribution, DIALAMEH *et al.*[2] and TAGGART[4] have unequivocally identified the product by mass spectrometry. The rate of conversion of administered menaquinone-0 to menaquinone-4 in chick liver is of the order of 1% of the dose administered in 6 h. The other major metabolites are the glucuronide and sulfate of reduced menaquinone-0.

This report deals with the study of the distribution of menaquinone-4, synthesized from menaquinone-0, among cell particulates in vitamin K-deficient chicks and the study of the *in vitro* alkylation of menaquinone-0 by microsomes employing isoprenyl pyrophosphates as a source of lipophilic side chain.

MATERIALS AND METHODS

Chemicals

[6,7-$^3H_2$]Menaquinone-0 was prepared from sodium menadiol phosphate by the method of TAGGART AND MATSCHINER[3]. Geraniol and farnesol were obtained commercially and purified by column chromatography. Geranylgeraniol was synthesized from nerolidol by the method of UPPER AND WEST[6]. The pyrophosphate derivatives of these alcohols were prepared by the method of CHILDS AND BLOCH[7].

Studies in whole chicks

1-day old chicks were fed a vitamin K-deficient diet[8] for two weeks at which time they weighed approx. 150 g and showed plasma prothrombin values of less than 5% of normal. It has previously been demonstrated that 10 μg of menaquinone-0 administered intramuscularly to these deficient chicks resulted in a prothrombin content of 90 $\pm$ 10% of normal in 6 h. It had also been observed that the incorporation of menaquinone-0 into menaquinone-4 in the liver reached a maximum of 1% of the administered dose in 1 h and remained essentially constant thereafter. [6,7-$^3H_2$]-Menaquinone-0 with specific activity of 74 mC/mmole in doses of 10 μg per chick were injected into the pectoralis muscle of vitamin K-deficient chicks. They were killed at the end of 3 h. Livers from 3 chicks were quickly removed, perfused for a few sec with ice cold 0.1 M phosphate buffer (pH 7.4), containing 0.03 M nicotinamide, 0.004 M $MgCl_2$ and 0.125 M sucrose, pooled and then homogenized in 9 vol. of the same medium. Homogenates were centrifuged for 10 min at 600 $\times$ *g* to eliminate tissue fragments, cells and nuclei. The supernatants were collected and centrifuged at 10000 $\times$ *g* in order to collect mitochondria. The mitochondria pellets were washed and resuspended with the same buffer system and recentrifuged at 10000 $\times$ *g*. Supernatant fractions containing microsomes were combined, and centrifuged at 106000 $\times$ *g* in an L-2 ultracentrifuge for 1 h and the pellet washed and respun. Total microsomal, mitochondrial, and cytoplasmic fractions were combined and lyophilized. The lyophilized residues were extracted with ethyl ether and menaquinone-4 isolated by use of the combined procedures of MATSCHINER AND TAGGART[9] and DIALAMEH AND OLSON[10]. The radioactivity of each fraction associated with menaquinones was determined by counting in a Packard Scintillation Spectrometer.

Studies of liver particulate suspensions

Liver particulates from vitamin K-deficient and normal chicks were prepared as described above. The mitochondria microsomes and cytosol from 1.5 g of vitamin K-deficient chick liver were incubated in 10 ml of 0.1 M phosphate buffer (pH 7.4) for varying periods at 30°. In one series of experiments, the microsomes from rat liver were compared with those from chick liver. At the end of the experiment, the cell particles were dried, extracted with diethyl ether and menaquinones isolated and counted as previously described.

RESULTS

When 10 μg of [6,7-$^3H_2$]menaquinone-0 was injected intramuscularly into vitamin K-deficient chicks, and cell particulates isolated, menaquinone-4 was found

in all cell fractions as shown in Table I. Microsomes contained 66% of the total menaquinone-4 isolated, mitochondria contained 32% and the cytosol contained a negligible 2%. This result pointed to the possibility that the reticulum was the site of alkylation of menadione in the chick and experiments were planned to investigate this problem *in vitro*.

Homogenates of chick liver in bicarbonate and phosphate buffers were prepared and [6,7-$^3H_2$]menaquinone-0 in various amounts were incubated for 1 h at 30°. The results are shown in Table II. Phosphate buffer provided the best medium for the conversion of menaquinone-0 to menaquinones. It was also observed that 1.0 μg of substrate per 15 ml of buffer gave the maximum conversion of substrate to product.

In order to identify the particulate fraction responsible for the alkylation activity of chick liver homogenate, cell particulate fractions were prepared from vitamin K-deficient chick liver. Various fractions were incubated with or without added farnesyl pyrophosphate. The results are presented in Table III. It may be seen that the microsomal fraction had the highest activity per g of liver but also per g of protein. Mitochondria had one sixth the activity of microsomes and the cytosol, again, had negligible activity. The presence of farnesyl pyrophosphate augmented

TABLE I

DISTRIBUTION OF MENAQUINONE-4 IN CELL PARTICLES OF VITAMIN K-DEFICIENT CHICK LIVER AFTER ADMINISTRATION OF [6,7-$^3H_2$]MENAQUINONE-0

Each value represents the average of 3 experiments. The experiments were done with pooled livers from three chicks. Each chick was injected with 10 μg ($5.8 \cdot 10^{-2}$ μmoles) of [6,7$^3H_2$]menaquinone-0 (specific activity 74 mC/mmole) were sacrificed after 3 h.

Particles	*Distribution of menaquinone-4*	
	pmoles/mg protein	%
Microsomes	0.350	66
Mitochondria	0.105	32
Cytosol	0.008	2

TABLE II

EFFECT OF CONCENTRATION OF MENAQUINONE-0 ON ENZYMATIC ACTIVITY OF HOMOGENATE

Each value represents the average of 3 experiments. Each experiment consists of two incubations. Incubation mixtures contain: Homogenate of 1.5 g liver in 15 ml buffer (pH 7.4), 1.6 μC radioactivity was added to each homogenate, the total amount of substrate was determined by adding non radioactive menaquinone-0. The mixture was incubated 1 h at 30°.

[6,7-$^3H_2$]*Menadione added* (*nmoles*)	*Menaquinone-4 synthesized* (*pmoles*)	
	Bicarbonate buffer	*Phosphate buffer*
1.1	21	68
2.9	40	122
5.8	43	185
11.6	32	186
58.0	1.4	24
464	0	0

TABLE III

ENZYMATIC ACTIVITY OF LIVER CELL PARTICLES

Each value is the average of 3 experiments. Each experiment consists of two incubations each containing particles from 1.5 g liver in 10 ml phosphate buffer (pH 7.4), $3 \cdot 10^{-7}$ M [6,7-$^3H_2$]-menaquinone-0 (specific activity 1.1 C/mmole and $6 \cdot 10^{-5}$ M farnesyl pyrophosphate. The control consisted of the full medium less particulates. The mixture was incubated 1 h at 30°.

Particles	*Incorporation into menaquinone-0 (pmoles/mg protein)*	
	+ Farnesyl-P-P	*− Farnesyl-P-P*
Microsomes	5.80	0.95
Mitochondria	1.00	0.12
Cytosol	0.04	0.03
Control	0.00	0.00

TABLE IV

REQUIREMENTS OF THE ALKYLATION SYSTEM FOR MENAQUINONE IN CHICK LIVER MICROSOMES

Full system contained microsomes from 3.0 g chick liver in 20 ml of phosphate buffer (pH 7.4), 1.0 μg of [6,7-$^3H_2$]menaquinone-0 (specific activity 1.1 C/mmole), 60 μM farnesyl pyrophosphate in two beakers each containing 10 ml of medium. The beakers were incubated for 2 h at 30° in 100% O_2.

Experimental condition	*Menaquinone synthesized (pmoles)*
Full system	246
− O_2	319
− active microsomes (boiled)	2
− farnesyl-*P-P*	30
− farnesyl-*P-P* + farnesol	31

the synthesis of menaquinones 6–8 fold in microsomes and mitochondria, but not in the cytosol. It was decided to concentrate experimentation of the microsomal fraction because of its greater activity.

The requirements of the microsomal system for alkylation of menadione are presented in Table IV. Using the microsomes from 3.0 g of chick liver suspended aerobically in phosphate buffer, and farnesyl pyrophosphate as a source of the lipophilic side chain, 246 pmoles of menaquinone was synthesized. It was identified by gas–liquid chromatography[10] and mass spectrometry as menaquinone-3, corresponding to the added farnesyl-*P-P* in length of side chain. In the absence of oxygen (N_2 atmosphere) synthesis of menaquinone was increased 30%. If the microsomes were inactivated by boiling the biosynthesis was nil. If farnesyl pyrophosphate were omitted or farnesol substituted for the farnesyl pyrophosphate, the yield dropped to about 12% of that obtained with the full system.

Since menaquinone-4 is the physiological product of menadione alkylation, it was of interest to study the relative efficacy of homologous isoprenyl pyrophosphates in the microsomal system. The results of comparisons made among geranyl pyrophosphate, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate and

TABLE V

EFFECT OF FREE ISOPRENYL ALCOHOLS AND THEIR PYROPHOSPHATE DERIVATIVES ON THE RATE OF ALKYLATION OF MENAQUINONE-0 BY CHICK MICROSOMES

Each value represents the average of three experiments. Each experiment consists of two incubations each containing microsomes of 1.5 g liver in 10 ml phosphate buffer (pH 7.40); 0.50 μg [6,7-$^3H_2$]menaquinone-0 (specific activity 1.1/C mmole) and 200 μg isoprenyl alcohols or their pyrophosphates. Incubation period 3 h at 30°.

Conditions	*% incorporation into menaquinones*
Geranyl pyrophosphate	4.92
Geraniol	0.40
Farnesyl pyrophosphate	9.45
Farnesol	0.43
Geranylgeranyl pyrophosphate	0.85
Geranylgeraniol	0.38
Control	0.42

their respective free alcohols are shown in Table V. It may be seen that farnesyl pyrophosphate was the best isoprenyl donor, geranyl-*P-P* was second, and the physiologic donor geranylgeranyl-*P-P*, least effective. In fact, the blank value of 0.42% conversion which represented menaquinone-4 biosynthesis, was only doubled by adding more geranylgeranyl-*P-P*. In contrast, the yield from farnesyl-*P-P* was 20 times the blank value. It was established by gas–liquid chromatography, furthermore, that the expected menaquinone, *i.e.* menaquinone-2 from geranyl pyrophosphate, menaquinone-3 from farnesyl pyrophosphate, and menaquinone-4 from geranylgeranyl pyrophosphate were obtained.

Finally, comparisons between chick microsomes and rat microsomes were carried out to determine the specie differences, if any, which might account for the

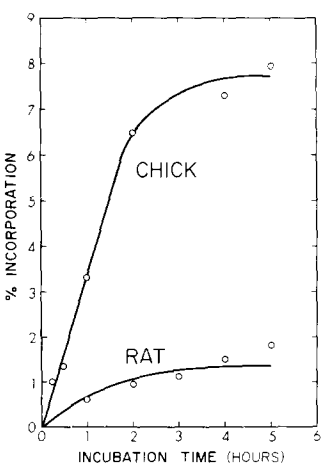

Fig. 1. Relative rates of alkylation of menaquinone-0 microsomes from chick and rat liver. Each point represents the results of incubation of 2 beakers each containing the microsomes from 1.5 g of chick or rat liver, 0.5 μg of [6,7-$^3H_2$]menaquinone-0 (74 mC/mmole), and 200 μg of farnesyl pyrophosphate. Samples were incubated in phosphate buffer with an N_2 atmosphere for varying times up to 5 h at 30°.

relatively rapid rate of alkylation of menaquinone-0 in the chicken and the relatively slow rate in the rat. Farnesyl pyrophosphate was used as the side chain donor. The data are presented in Fig. 1. It was found that chick microsomes were 6–7 times as effective in converting menaquinone-0 to menaquinone-3 as rat microsomes, in a 5-h incubation period.

DISCUSSION

MARTIUS AND ESSER[1] were the first to demonstrate conversion of menadione to menaquinone-4 in vitamin K-deficient chicks. They administered 1-4-[2-*Me*-^{14}C]-naphthoquinone with the specific activity of 14 mC per mM, given by mouth to vitamin K-deficient chicks over an 8 day period. Menaquinone-4 was not isolated but lipid extracts of tissue were subjected to countercurrent distribution in a Craig apparatus using *n*-heptane and methylglycol. The radioactivity partitioned with authentic markers of menaquinone-4. This is interpreted as evidence for the conversion of the precursor menaquinone-0 to menaquinone-4.

These observations were confirmed and extended by DIALAMEH *et al.*[2] through the use of [6,7-$^{3}H_2$]menaquinone-0 with specific activities of the order of 70 mC per mM. The product was unequivocally isolated by sequential chromatography on various systems followed by mass spectrometry.

In the early report by MARTIUS AND ESSER[1], it was claimed that the alkylation had been demonstrated *in vitro* using unlabeled menadione and radioactive mevalonic acid. Again the produce was tentatively identified by countercurrent distribution.

In a subsequent report, MARTIUS[11] showed that $1 \cdot 10^{-4}$ M [^{14}C]menaquinone-0 could be converted into menaquinone-4 by incubation with geranylgeranyl pyrophosphate. Again, the identification was made by countercurrent distribution. The activity was claimed to have been localized in the mitochondrial fraction of the homogenate but no further information was supplied.

The experiments reported here clearly demonstrate the *in vitro* alkylation of menaquinone-0 by homogenates of liver from vitamin K-deficient chicks. The highest activity appears to reside in the microsome which is at variance with the above report by MARTIUS[11]. The microsomal fraction was 4–5 times as active as the mitochondrial fraction both in the presence and the absence of added isoprenyl pyrophosphate. It was of interest that the physiological product, menaquinone-4 was not produced in highest yield by cell particulate fractions *in vitro*. It was surprising that farnesyl pyrophosphate was the best isoprenyl donor, geranyl pyrophosphate was next, and the physiological donor, geranylgeranyl pyrophosphate, was least effective. The corresponding products menaquinone-3, menaquinone-2, and menaquinone-4 were identified by gas phase chromatography. The alkylation can proceed anaerobically as would be predicted from the mechanism first suggested by LYNEN *et al.*[12] which involves a classic electrophilic attack of the carbonium ion (generated by the leaving of the pyrophosphate group) upon the reduced naphthoquinone.

It would appear from these observations that the control of the homologue synthesized in any tissue is the result of mechanisms which control the availability of isoprenyl pyrophosphate. It would appear that although the capacity for alkylation is highest with farnesyl pyrophosphate, farnesyl pyrophosphate concentrations are not permitted to rise to the point where they compete with geranylgeranyl pyrophos-

phate as an alkyl donor for menaquinone-0. This is probably due to the rapid condensation of farnesyl pyrophosphates to squalene.

It has been demonstrated repeatedly that menaquinone-0 is not as effective a provitamin in the rat as it is in the chick. TAGGART AND MATSCHINER[3] have shown that the appearance of prothrombin in corresponding with menaquinone-4 is delayed approx. 4 h in the rat whereas in the chick, OLSON *et al.*[13] has shown an essentially linear increase in prothrombin by the administration of menaquinone-0 to chicks. This response has been correlated with extremely rapid alkylation of menaquinone-0 to menaquinone-4 *in vivo*[2]. The observations reported here that the alkylating ability of microsomes from chicks is approx. 6–7 times as effective as those from rats in converting menaquinone-0 to menaquinone-3 would explain the delayed response of the rat to the administration of menaquinone-0. It provides further evidence for the view that the active vitamin capable of stimulating prothrombin biosynthesis must be an alkylated derivative.

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