The Formation of Menaquinone-4 (Vitamin K) and its Oxide in some Marine Invertebrates

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Three echinoderms, a crustacean, a mollusc and a coelenterate were injected with either [2-¹⁴C]mevalonate and unlabelled menadione (2-methyl-1,4-naphthaquinone) or 2-[¹⁴C]methyl-1,4-naphthaquinone. In each case the label was incorporated into menaquinone-4. [2-¹⁴C]Mevalonate was incorporated into menaquinone-4 in the crab (*Carcinus maenas*) only in the presence of menadione, the organism presumably being unable to synthesize the naphthaquinone nucleus. The starfish *Asterias rubens* and *C. maenas* incorporated the label into a compound which was identified chromatographically as 2,3-epoxymenaquinone-4. The significance of menaquinone-4 and its epoxy derivative in these animals is discussed.

It has been recognized for many years that vitamin K is required by higher animals for the formation of four blood-clotting proteins, prothrombin and factors VII, IX and X (Naeye, 1956). Until recently the precise area of function of the vitamin was not understood. In the first place it was thought to be a cofactor for the blood-clotting proteins, then participation in oxidative phosphorylation appeared likely and later it seemed that it played a part in mRNA formation (see Pennock, 1966). It is now clear, however, that vitamin K is involved in the carboxylation of glutamate residues in precursor proteins, e.g. prothrombin, to yield the functional blood-clotting factors (Esmon *et al.*, 1975; Fernlund & Stenflo, 1975). The blood-clotting factors contain several γ -carboxyglutamate residues at the *N*-terminal part of the protein and these residues are involved in Ca²⁺ binding. In the absence of vitamin K, carboxylation does not occur and prothrombin and the other factors are not formed.

As yet it is not clear how the vitamin fulfils its role in γ -carboxylation, but it appears to function during the carboxylation reaction. When microsomal fractions from vitamin K-deficient rats (which have present the precursor protein awaiting carboxylation) are incubated with phyloquinone (vitamin K₁), NaH¹⁴CO₃ and a little cytoplasm, the label appears in the prothrombin in 15 min, and it can be shown that the radioactivity is located in the carboxyl groups of γ -carboxyglutamate residues (Esmon *et al.*, 1975). Whether vitamin K plays a coenzymic role remains to be seen.

Little or nothing is known of metabolites of vita-

min K in animals except phyloquinone 2,3-oxide. This compound was isolated as a metabolite of phyloquinone in rats (Matschiner *et al.*, 1970) and was then shown to have vitamin K activity in the vitamin K-deficient rat (Bell & Matschiner, 1970). The two compounds appeared to be interconvertible, but the anticoagulant Warfarin blocked the formation of phyloquinone from its oxide (Bell & Matschiner, 1970). The metabolic significance of the oxide at present remains undecided.

The requirement for vitamin K by higher animals can be satisfied by menadione, and it is reasonable to suppose that it is functional after addition of a polyprenyl side chain to form a menaquinone. Chickens and rats have been shown to form menaquinone-4 after administration of menadione, and in particular the microsomal fraction of vitamin K-deficient chicken liver was able to condense menadione with geranylgeranyl pyrophosphate to yield menaquinone-4 (Martius & Esser, 1958; Taggart & Matschiner, 1969; Dialameh *et al.*, 1970).

The problem arises as to how widespread the role of vitamin K in carboxylation is in the animal kingdom. Is carboxylation of glutamate residues to form a Ca²⁺-binding protein specific only for blood-clotting proteins, or are other such proteins vitamin K-dependent? The purpose of this paper is to examine the status of vitamin K in organisms that do not have the four blood-clotting proteins mentioned above and in fact do not possess a similar type of coagulation system. Such animals are to be found in invertebrate phyla. Martius *et al.* (1965) found that the earthworm (*Lumbricus terrestris*) and snail (*Helix pomatia*) were

capable of producing menaquinone-4 from injected menadione, and the work described here extends these studies to marine invertebrates.

Experimental

Materials

Animals were obtained from the Marine Biological Station, Millport, Isle of Cumbrae, Scotland, U.K. They were maintained in aerated sea-water aquaria at 8°C. The carnivorous animals were fed on *Mytilus edulis* and the herbivorous animals were given *Fucus vesiculosus*.

Menaquinones-3 and -4 were synthesized by condensation of menadiol with either DL-nerolidol (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) or geranyl-linalool, a generous gift of Messrs. F. Hoffmann-La Roche, Basle, Switzerland, by courtesy of Dr. O. Isler (Rüegg *et al.*, 1960). The product was eluted from a column of acid-washed alumina (Brockmann Grade III; M. Woelm, Eschwege, Germany) in 2% (v/v) diethyl ether in light petroleum (b.p. 40–60°C).

2,3-Epoxymenaquinones-3 and -4 were formed by treatment of the relevant menaquinone with H₂O₂ in aqueous ethanol containing Na₂CO₃ (Tishler *et al.*, 1940). The epoxides were purified by preparative t.l.c., utilizing adsorption and reversed-phase systems (see Table 1).

Methods

Incubation. The radioactive precursors used in these experiments were 2-[¹⁴C]methyl-1,4-naphthoquinone ([¹⁴C]menadione) and DL-[2-¹⁴C]mevalonate (The Radiochemical Centre, Amersham, Bucks., U.K.). The [¹⁴C]menadione was dissolved in equal amounts of ethanol and aq. 10% (v/v) Tween 80 and as a routine 12.5 μCi in 0.2 ml was injected into the animals. In experiments with [¹⁴C]mevalonate, unlabelled menadione (usually 1.4 mg) in 0.1 ml of ethanol/aq. Tween 80 was injected 1 h before the administration of the [2-¹⁴C]mevalonate (10 μCi) in 0.2 ml of 0.1 M-sodium phosphate buffer, pH 7.4.

Injection was accomplished by a variety of methods; through the mouth for the sea-urchins (*Psammechinus miliaris* and *Echinus esculentis*) and sea-anemone (*Actinia equina*), through the leg joint for the crab (*Carcinus maenas*), into the coelomic cavity for the starfish (*Asterias rubens*), and into the foot muscle for the whelk (*Buccinum undatum*). The animals were maintained in small aquaria for 24 h before being killed.

Extraction of lipids. After removal of shell (where present) the organism or tissue was homogenized in chloroform/methanol (2:1, v/v), the extract was filtered, methanol was removed by washing with water and finally the chloroform extract was dried over Na₂SO₄. At this stage carrier amounts of menaquinone-4 (210 nmol) were added, because in initial

Table 1. *Thin-layer-chromatographic properties of menaquinones-3 and -4 and 2,3-epoxymenaquinone-4*

Thin layers of silica gel G were 0.25 mm thick for analytical work and 0.5 mm for preparative work (system 1). The di-isopropyl ether used in system 2 was passed through a column of acid-washed alumina (Brockmann grade O) just before use to remove the quinol (hydroquinone) normally present to prevent peroxide formation. The reversed-phase system 3 is described by Walton & Pennock (1972). R_F values are given in the Table.

Compound	System 1	System 2	System 3
	Silica gel G/6% (v/v) diethyl ether in light petroleum	10% (w/w) AgNO ₃ /silica gel G/60% (v/v) di-isopropyl ether in light petroleum	Paraffin-impregnated keiselsüßer/aq. 90% (v/v) acetone
Menaquinone-3	0.39	0.40	0.72
Menaquinone-4	0.41	0.27	0.55
2,3-Epoxymenaquinone-4	0.41	0.21	0.72

experiments menaquinone-4 could be detected radioactively but not chemically. The extract was then evaporated to dryness under N₂.

Column chromatography. The neutral lipids were separated from the phospholipids on a column of silicic acid. (100 mesh; Mallinckrodt Chemical Works, St. Louis, MO, U.S.A.)/Celite 503 (Koch-Light) (2:1, w/w). The vitamin K fraction was eluted by chloroform together with the neutral-lipid fraction, or preferably by 1% diethyl ether in light petroleum. When vitamin K was eluted with the neutral-lipid fraction it was further purified on acid-washed alumina as in the preparation of menaquinone-3.

Thin-layer chromatography. This is shown in Table 1. The best method for separating menaquinone-4 and 2,3-epoxymenaquinone-4 is clearly the reversed-phase system (3). However, with this system 2,3-epoxymenaquinone-4 and menaquinone-3 have identical R_F values. Ag⁺-impregnated silica gel G (argentation t.l.c.; system 2) resolves all three compounds, but the separations are only small and reproducibility is difficult to achieve. At best the system worked with freshly purified solvent, plates used not more than 1 h after preparation, and small amounts of menaquinones chromatographed.

Detection of menaquinones on thin-layer chromatograms. Menaquinones-3 and -4 have strong absorption in the u.v. region ($\lambda_{\max}$, 248 nm, ϵ 19000) and can be detected quite easily on chromatograms with fluorescein. Epoxymenaquinones absorb u.v. light ($\lambda_{\max}$, 259 nm, shoulder 305 nm; ϵ_{259} 6166, ϵ_{305} 2042), but are much more difficult to detect because of the smaller ϵ value. The compounds could be detected equally well by spraying with 2,4-dinitrophenylhydrazine, both giving orange colours.

Reversed-phase high-pressure liquid chromatography. This was carried out by using a μ Bondapak C₁₈ column with acetonitrile/water (17:3, v/v) as solvent in a ALC 100 analytical liquid chromatograph (Waters Assoc., Stockport, U.K.). With a flow rate of 2.5 ml/min, retention times were: menadione,

5.0 min, menaquinone-3, 17.0 min, 2,3-epoxymenaquinone-4, 20.0 min, and menaquinone-4, 27.5 min.

Radioactivity. This was detected as described by Walton & Pennock (1972).

Results

Incorporation of 2-[¹⁴C]methyl-1,4-naphthaquinone ([¹⁴C]menadione) into menaquinone-4

[¹⁴C]Menadione (12.5 μ Ci, 244 μ g) was injected into two shore crabs (*C. maenas*) and a similar amount into six small sea-urchins (*Psammechinus miliaris*). After a 24 h incubation the animals were killed and the lipids extracted and fractionated as described in the Experimental section. The vitamin K fraction was found to be radioactively labelled and in each organism radioactivity co-chromatographed with menaquinone-4 in all three t.l.c. systems. Menaquinone-4 contained a total of 4838 d.p.m. in *C. maenas* and 13630 d.p.m. in *P. miliaris*, and in *C. maenas* a related compound (which was later shown to be 2,3-epoxymenaquinone-4) contained a total of 2842 d.p.m. No other menaquinone homologue was found to be labelled and in each case only radioactivity was detected, no menaquinone-4 was found chemically.

Identification of 2,3-epoxymenaquinone-4

In the experiments with [¹⁴C]menadione described above and in later experiments with [2-¹⁴C]mevalonate, radioactivity was detected in a compound from *C. maenas* lipids which was similar to menaquinone-4 in chromatographic properties but distinguishable from it on some systems. The unknown metabolite migrated like menaquinone-4 or -3 on adsorption t.l.c., was identical with menaquinone-3 on reversed-phase t.l.c., but ran slower than either menaquinone-3 or -4 on argentation t.l.c. This compound, being labelled with either [¹⁴C]menadione or [2-¹⁴C]mevalonic acid as precursor, was clearly related to men-

Table 2. *Effect of menadione on the incorporation of [2-¹⁴C]mevalonate into menaquinone-4 and its epoxide in C. maenas*

C. maenas (two crabs in each case) were given 0.25 mg, 2.75 mg or no menadione, followed by 10 μ Ci of [2-¹⁴C]mevalonate, and the lipids were examined after 24 h incubation. —, Not detected.

Menadione administered (mg)	Incorporation (d.p.m.)	
	Menaquinone-4	Epoxymenaquinone-4
0	—	—
0.25	20560	10498
2.75	72250	15970

quinone-4, but also from its chromatographic properties could not be a related homologue, e.g. menaquinone-3 or menaquinone-5.

In the rat, 2,3-epoxyphyloquinone has been found to be a metabolite of exogenous phyloquinone (Matschiner *et al.*, 1970) and such an epoxide related to menaquinone-4 might well account for the properties of the unknown metabolite. Accordingly 2,3-epoxymenaquinone-4 was formed by oxidation of menaquinone-4 with H₂O₂ and its identity confirmed by mass spectrometry in an A.E.I. M.S.12 mass spectrometer.

The mass spectrum showed a molecular ion at *m/e* 460 as expected and major fragment peaks at (1) *m/e* 391 (*M*–69), (2) *m/e* 323 (*M*–137) and (3) *m/e* 255 (*M*–205), corresponding to the loss of one, two and three isoprene units respectively.

Another peak (*m/e* 213) could be the 2,3-epoxyquinone with a vinyl group at position 2. No major fragment peak was observed to correspond to the pyrylium ion proposed by Di Mari *et al.* (1966) in the mass spectrum of phyloquinone. This is to be expected, as the stable pyrylium ion requires the presence of a quinoid nucleus and in the 2,3-epoxymenaquinone-4 this structure has been lost by epoxidation.

The chromatographic properties of the epoxymenaquinone-4 are shown in Table 1 with those of menaquinone-4, which illustrates that the former migrates to exactly the same position as the unknown metabolite described above, i.e. migrating with menaquinones-3 and -4 on adsorption t.l.c., with menaquinone-3 on reversed-phase t.l.c. and just behind menaquinone-4 on argentation t.l.c. Carrier 2,3-epoxymenaquinone was added to the radioactive metabolite and radioactivity accompanied the carrier on all three t.l.c. systems and also was inseparable from carrier on high-pressure liquid chromatography.

Incorporation of DL-[2-³H]mevalonate and [¹⁴C]menadione into menaquinone-4 and its epoxide in C. maenas

To form menaquinone-4 from menadione the organism must attach a geranylgeranyl side chain on

to the naphthaquinone nucleus, and presumably this side chain is formed from mevalonate. Consequently a double-labelling experiment was carried out with *C. maenas*, two animals being injected with [¹⁴C]menadione (12.5 μ Ci) and [2-³H]mevalonate (25 μ Ci, sp. radioactivity 200 μ Ci/mmol). After a 24 h incubation the animals were killed and the lipids extracted and fractionated. Both menaquinone-4 and its epoxide were labelled with ¹⁴C and ³H (menaquinone-4 contained 3703 d.p.m. of ¹⁴C radioactivity and 25780 d.p.m. of ³H radioactivity and the 2,3-oxide 4333 d.p.m. and 27900 d.p.m. of ¹⁴C and ³H radioactivity respectively).

Incorporation of DL-[2-¹⁴C]mevalonate into menaquinone-4 and its epoxide in C. maenas in the presence and absence of menadione

One pair of crabs (*C. maenas*) was injected with a total of 2.75 mg of unlabelled menadione. Another pair received a total of 0.25 mg, and a third pair received no menadione. Each pair of animals was then injected with 10 μ Ci of [2-¹⁴C]mevalonate and after 24 h the animals were killed and treated as above.

The results shown in Table 2 indicate clearly that *C. maenas* requires a nuclear precursor (in this case menadione) for menaquinone biosynthesis. Increasing amounts of menadione gave higher incorporations of mevalonate into menaquinone-4. Menadione is insoluble in water and only slightly soluble in ethanol and Tween 80 solution, and it was not possible to inject more than about 1.4 mg of menadione into each animal. With a higher dosage of menadione perhaps higher rates of incorporation could be achieved. The lower dose of menadione administered (0.25 mg) was about the same as the [¹⁴C]menadione given in earlier experiments, i.e. 12.5 μ Ci, 0.244 mg, and gave a good but not maximal incorporation of mevalonate.

Incorporation of [2-¹⁴C]mevalonic acid into menaquinone-4 by marine invertebrates

The experiments were extended to include other marine invertebrates utilizing [2-¹⁴C]mevalonate as radioactive precursor ([¹⁴C]menadione was no longer available from The Radiochemical Centre). Menadione (1.4 mg) and [2-¹⁴C]mevalonate (10 μ Ci) were injected into each animal or, if more than one animal was used, the dose was equally divided between animals in that group and the incubation carried on for 24 h. The animals were killed and treated as above and the results are shown in Table 3.

All animals synthesized menaquinone-4 to some extent. The relatively low incorporation of radioactivity into menaquinone-4 in the whelk (*Buccinum undatum*) may well reflect the difficulties encountered in injecting both menadione and mevalonate into the extremely hard foot muscle. In the starfish

Table 3. Incorporation of [2-¹⁴C]mevalonate into menaquinone-4 and its oxide by some marine invertebrates. Each group of animals (or animal where only one was used) were injected with menadione (1.4 mg) and [2-¹⁴C]mevalonate (10 μ Ci). After 24 h the animals were killed and lipids extracted and separated as described in the Experimental section. —, Not detected.

Organism	No. of animals	Incorporation (d.p.m.)	
		Menaquinone-4	Epoxymenaquinone-4
<i>Carcinus maenas</i>	1	42910	38300
<i>Asterias rubens</i>	2	5649	4438
<i>Echinus esculentus</i>	1	25160	—
<i>Actinia equina</i>	6	4462	—
<i>Buccinum undatum</i>	2	622	266

(*Asterias rubens*) and whelk, radioactivity migrated with 2,3-epoxymenaquinone-4. No such activity was detected in the sea-anemone (*Actinia equina*) or sea-urchin (*Echinus esculentus*). The incorporation of label into menaquinone-4 and its oxide was much higher in a specimen of *C. maenas* (also included in this series of experiments) than into similar compounds in the other invertebrates.

Location of menaquinone-4 and oxide in the hepatopancreas

A single specimen of *C. maenas* was injected with menadione and [2-¹⁴C]mevalonate as described previously. After 24 h incubation the animal was killed and dissected to yield hepatopancreas, muscle fraction and the remainder of the carcass. The three fractions were extracted and menaquinone-4 and oxide examined for radioactivity.

Of the radioactivity recovered from the whole animal in the vitamin K fraction (i.e. menaquinone-4 and epoxymenaquinone-4), 83.4% was in the hepatopancreas and the remaining 16.6% was equally divided between the other two fractions. The ratio of radioactivity detected in menaquinone-4 and epoxymenaquinone-4 was approx. 1:1 in each tissue examined, i.e. hepatopancreas, muscle and remainder of carcass.

Discussion

The data presented in this paper show that members of four marine invertebrate phyla are able to synthesize menaquinone-4 from menadione and mevalonate, and are in agreement with the results of Martius *et al.* (1965) on members of two terrestrial phyla.

What is the significance of menaquinone-4 formation in these animals? Is this quinone formed as a result of non-specific enzymes condensing a prenyl pyrophosphate with the exogenous menadione? In other words, might this menaquinone be formed by the enzymes normally used for ubiquinone formation? This seems unlikely, as most of the animals studied

have been shown to form ubiquinone-10 from mevalonic acid and not ubiquinone-4; further, *C. maenas* and *A. equina* incorporate quite large amounts of label from [2-¹⁴C]mevalonic acid into farnesol (and almost certainly farnesyl pyrophosphate) (Walton & Pennock, 1972), and yet no menaquinone-3 was detected in the experiments described here. Therefore the formation of menaquinone-4 suggests a quite specific enzymic reaction.

If menaquinone-4 is made as a routine in invertebrates, what is the source of the naphthoquinone nucleus? Without added menadione no menaquinone-4 was synthesized by *C. maenas*, and as far as is known menadione is not a naturally occurring compound. Billeter & Martius (1960) reported that pigeons and chickens were capable of removing the phytyl side chain of vitamin K₁ (phyloquinone) and utilizing the nucleus for menaquinone-4 synthesis, and this seems to be a possibility, particularly in herbivorous animals, who are likely to obtain plenty of phyloquinone in the diet. Alternatively there may be a trace of a naphthalenic compound in the diet which can be converted by the invertebrate into naphthoquinone or a similar nuclear precursor of menaquinone.

What is the most likely function, if any, of menaquinone-4 in these invertebrates? In some bacteria, ubiquinone is not present in the electron-transport chain and its place is essentially taken by a menaquinone (see Pennock, 1966). It is possible that in invertebrates menaquinone-4 functions in the mitochondrial electron-transport chain in place of ubiquinone. However, although little is known about electron transport in invertebrates, ubiquinone has been found to be present and to function in mitochondrial electron transport in *C. maenas* (Burrin & Beechey, 1966); all the animals used in the present study have been shown to contain and synthesize ubiquinone, and therefore a role for menaquinone-4 in electron transport seems unlikely.

In mammals vitamin K is needed for γ -carboxylation of glutamate residues in the formation of four blood-clotting zymogens, but although invertebrate blood does 'clot', the process is different from the

mammalian system, and the blood-clotting zymogens are not involved (Fuller & Doolittle, 1972). The four blood-clotting factors are zymogens of proteolytic enzymes, and presumably during evolution of the mammalian clotting system, zymogens passing in the bloodstream from the liver, where they are made to the gut, where they would function in digestion, have been adapted to a blood-clotting role. Therefore in invertebrates these or similar zymogens may contain γ -carboxyglutamate residues and may function in digestion, and of course would need menaquinone for their formation.

Alternatively the role of vitamin K in γ -carboxylation of glutamate residues in proteins may not be confined to the four blood-clotting factors. γ -Carboxyglutamates may occur in other Ca^{2+} -binding proteins, all requiring vitamin K for their synthesis, and indeed there is evidence of at least one other protein of unknown function purified from bovine plasma (Stenflo, 1976).

The finding that as well as menaquinone-4 a derivative, 2,3-epoxymenaquinone-4, is also formed in some of the animals is of great interest. As described in the introduction, 2,3-epoxyphyloquinone is formed from phyloquinone in rats, especially in the presence of anticoagulants (Bell & Matschiner, 1970). The hypothesis of Bell & Matschiner (1970) is that warfarin inhibits the conversion of the epoxy derivative into phyloquinone and so a phyloquinone (or vitamin K) deficiency occurs and 2,3-epoxyphyloquinone might in fact act as a competitive inhibitor. The formation of 2,3-epoxymenaquinone-4 in some of these marine invertebrates in proportions as high as those of menaquinone-4 suggests a possible role for the oxide. The formation of menaquinone-4 in rats and invertebrates and the occurrence of 2,3-epoxyphyloquinone or 2,3-epoxymenaquinone in rats and invertebrates might be coincidence, but it is tempting to suggest that menaquinone-4 plays a role in γ -carb-

oxylation of glutamate residues in rats and invertebrates and that 2,3-epoxyphyloquinone or 2,3-epoxymenaquinone-4 is involved in this reaction.

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