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SHORT COMMUNICATIONS

Epoxidation of several vitamins K by rat liver microsomes

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Gamma-carboxylation of specific glutamic acid residues, the vitamin K-dependent post-translational modification of a number of proteins [1–4], occurs in microsomes, and carboxylating systems *in vitro* have been studied in microsomes prepared from liver, kidney, bone and placenta [5, 6,*†]. Epoxidation of phylloquinone (vitamin K_1), in which the vitamin is converted to its stable 2, 3-epoxide, also occurs in microsomes derived from these organs [7, 8]. A number of criteria suggest that carboxylation and epoxidation are closely associated: both require oxygen and a reducing agent such as NADH, both activities are proportional to the amount of prothrombin precursor present in liver microsomes, and agents such as tetrachloro-4-pyridinol which inhibit carboxylation also block epoxidation.

Vitamins K other than K_1 support carboxylation. To investitigate further the relationship of epoxidation and carboxylation, we have synthesized a series of radiolabeled vitamins K and studied their rates and extents of epoxidation by rat liver microsomes.

A vitamin K deficiency was induced in 250 g male CD rats, and microsomes were prepared from their livers, as has been described [9].

Tritiated menadione was prepared from tritiated menadiol sodium diphosphate (Amersham-Searle; sp. act. 96 Ci/mmole) by acid hydrolysis [10]; tritiated phylloquinone (K_1), menaquinone-2 (MK-2) and menaquinone-3 (MK-3) were synthesized by coupling phytol, geraniol and farnesol, respectively, to tritiated menadiol by published procedures [10, 11]. [U-^{14}C-phytyl]-2-phytyl-1,4-naphthoquinone (demethylphylloquinone or DMK_1) was prepared by coupling [^{14}C]-phytol to 1, 4-naphthoquinol [10, 11]. Vitamin K epoxides were prepared by oxidation of their respective vitamins K [11]. After synthesis the parent vitamins and their epoxides were chromatographed in benzene on preparative 500μ thin-layer chromatography (t.l.c.) plates. After elution of the compounds with acetone, they were dried with N_2, and their structures were confirmed using N.M.R. spectroscopy. The compounds were stored in the dark at −20°. On the day of the assay the radioactive vitamins were purified away from impurities, including epoxide generated during storage, using either the reversed-phase thin-layer chromatography or high-pressure liquid chromatography techniques described below. Vitamins were eluted from thin-layer plates with acetone; all were dried with N_2 and then dissolved in ethanol for use in subsequent epoxidase assays. At this point at least 93 per cent of radioactivity could be identified as vitamin; the per cent of epoxide present before incubation (zero time) is indicated in Table 1.

Epoxidation reactions (total volume 0.5 ml) were carried out as reported [8]. In incubations with K_1, DMK_1, MK-2 and MK-3, 1 ml H_2O was added at the times indicated, and the vitamins and their respective epoxides were extracted immediately into 6 ml of hexane–isopropanol. It was shown that 85–90 per cent of the radioactivity present after incubation could be extracted by this method. Epoxide formation was measured by reversed-phase thin-layer chromatography, using paraffin-impregnated silica gel G plates. The epoxides of K_1 and DMK_1 were separated from the parent vitamin using a solvent system of acetone–H_2O (92 : 8); for MK-2 and MK-3 the ratio of acetone to H_2O was 75 : 25. Recovery of compounds from the t.l.c. plates and scintillation counting were as described previously [8]. Menadione was separated from its epoxide on a Waters Associates 440 high-pressure liquid chromatograph utilizing an analytical C_{18} μ Bondapak column. After the addition of carrier menadione and menadione epoxide, aliquots of the reaction mixtures were chromatographed directly. The running buffer was 70% methanol in H_2O, pH 5.4, with a flow rate of 2 ml/min. The retention times for the epoxide and menadione were 4.25 and 4.90 min respectively. The peaks were collected separately for scintillation counting.

When the vitamins K were compared for their capacities to support carboxylation in intact microsomes, menadione and DMK_1 were inactive while MK-2 and MK-3 were, respectively, 10 and 80 times more potent than K_1 itself [9]. To

* J. Lian, unpublished data.

† P. Friedman *et al.*, manuscript in preparation.

Table 1. Conversion of vitamins K to their epoxides*

Vitamin	Per cent converted to epoxide: 0-min incubation	5-min incubation	10-min incubation	15-min incubation	Relative activity in carboxylation reaction† (%)
K_1	3	6.4	10.2	15.7	5
DMK_1	2	< 1	< 1	< 1	0
Menadione	2	< 1	< 1	< 1	0
MK-2	7	29	37	42	25
MK-3	7	25	36	44	100

* Incubations were in room air at 37° for the times indicated. Reaction volume was 0.5 ml and contained 0.1 ml microsomes, 25 mM imidazole–HCl (pH 7.2) 250 mM sucrose, 50 mM KCl, 2.5 mM magnesium acetate, 4 mM NADH, and 1 μM sodium warfarin. The vitamins were each at a concentration of 6.5×10^{-7} M, and the reactions had 12,000 cpm of either ^{14}C or ^{3}H. Conversion is expressed as the per cent of radioactivity converted to the epoxide (the ratio of cpm in the epoxide spot/cpm in the epoxide plus vitamin spots minus the same ratio from a zero time control). All numbers are the average of duplicate determinations that differed by less than 5 per cent. If boiled microsomes were incubated for 15 min with vitamins K_1, MK-2 or MK-3, no conversion to epoxide occurred.

† Carboxylation assays were carried out for each vitamin using a concentration of 6.5×10^{-7} M. Conditions were as have been reported [9].

compare the extents and rates of epoxidation equal concentrations of radiolabeled vitamins K were incubated in separate epoxidation reactions, and the percentage of each converted to its epoxide at the times indicated was determined (Table 1). After 15 min, menadione and DMK_1 were not converted to the epoxide in detectable quantities (i.e. 50 per cent above the zero-time epoxide level). MK-2 and MK-3 as well as K_1 were epoxidized; at 15 min the percentage conversions of MK-2 and MK-3 were about equal and greater than that of K_1. Little further epoxidation occurs with longer incubations. The percentage conversions at the earlier two time points show that the rates of epoxidation of MK-2 and MK-3 are more rapid than that of K_1.

We have demonstrated that vitamins K, other than phylloquinone (K_1), can be converted by the rat liver microsome "phylloquinone epoxidase" to their respective epoxides. In addition, of the vitamins K tested, only those which support carboxylation—K_1, MK-2 and MK-3—are epoxidized in detectable quantities. It is possible, although unlikely, that the hydroquinones of DMK_1 and menadione would be substrates for epoxidation. These were not tested in the current study but were shown previously to be no more active than their respective quinones in carboxylation. The menaquinones, which are more potent than K_1 in the carboxylation reaction, are epoxidized more rapidly and to a greater extent than K_1. Knauer *et al.* [12] have reported that the *cis* isomer of vitamin K_1 which has little biological activity [13], is also epoxidized to only a small extent. In experiments in which tritiated MK-2 and MK-3 epoxide were incubated for 15 min with the microsomes in a N_2 atmosphere, negligible conversion to MK-2 or MK-3 was detected; thus, 1 μM warfarin is sufficient to inhibit the reduction of these epoxides by the microsomal epoxide reductase, and we have not underestimated the amount of epoxide formed because of recycling of the epoxide back to the vitamin.

These data suggest strongly that carboxylation and epoxidation events are linked. The data say nothing about the nature of this linkage. While the epoxidation event may be required for carboxylation, this is not necessarily the situation. Indeed, vitamin K hydroquinone, the active form of the vitamin for carboxylation [5, 9] and an intermediate in the generation of the epoxide [14], may be oxidized to the quinone during carboxylation. If, during this reaction, there is *in situ* generation of H_2O_2, the H_2O_2 could subsequently convert the quinone to its epoxide.

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Equal inhibitory effect of dimethyl- and monomethyl-β-aminopropionylic derivatives of dibenzazepine on the uptake of [^{3}H]noradrenaline by rat brain synaptosomes

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According to the current hypothesis, the relationship of serotonin(5-HT)- and noradrenaline (NA)-positive qualities plays an important role in the action mechanism of tricyclic antidepressants providing the thymoanaleptic and psychoenergising components of antidepressive effect respectively [3,4,9]. It is supposed that the enhancement of central monoaminergic processes by trycyclic antidepressants is connected with their inhibiting effect on the neuronal uptake mechanism (membrane pumps) for monoamines [5,10]. Thus, the evaluation of the effect of drugs, manifesting in the routine screening tests the qualities of antidepressants, on the uptake of 5-HT and NA is of interest.

It was shown that some β-aminopropionylic derivatives of dibenzazepine (DBA) revealed in the routine screening tests the antidepressants activity [8]. In a previous work the effect of these drugs on 5-HT uptake was studied [11].

In the present paper data on the effect of these drugs on the NA uptake is reported.

Transmembrane transport of NA was evaluated by studying the [^{3}H]-NA uptake by a crude fraction of the synaptosomes of brain stem of female Wistar rats [13]. Drugs were introduced into the incubation medium 5 min before [^{3}H]-NA (for detailed method see [6]). The statistical significance of the results was determined by Student's t test.