The Role of Shikimic Acid in the Biosynthesis of Vitamin K₂

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1. Shikimic acid was shown to be a precursor of vitamin K₂ (MK-8) in *Escherichia coli*. 2. The benzene ring of the naphthaquinone arises from shikimic acid. 3. The methyl group of methionine is incorporated into vitamin K₂. 4. A scheme relating the biosynthesis of vitamin K₂ and ubiquinone to the general pathway of aromatic biosynthesis is proposed.

Virtually nothing is known about the route of biosynthesis of the naphthaquinone part of the molecule of vitamin K. It is thought that, in moulds and higher plants, naphthaquinone synthesis in general may take place by the acetate pathway (for references see Geissman, 1963). Gatenbeck & Bentley (1965), studying biosynthesis of the naphthaquinone, javanicin, produced by *Fusarium javanicum*, have come to the same conclusion.

Lederer and his colleagues (Lederer, 1964; Azerad, Bleiler-Hill & Lederer, 1965) have obtained incorporation of the [¹⁴C]methyl group of [*Me*-¹⁴C]-methionine into vitamin K₂ [MK-9 (2H)] (for nomenclature, see Scholes & King, 1965) with whole cells and cell-free preparations from *Mycobacterium phlei*.

The following experiments show that shikimate is probably a precursor of vitamin K₂ in *Escherichia coli* and *Aerobacter aerogenes* and an attempt has been made to relate this finding to the general scheme of aromatic biosynthesis. A preliminary account of part of this work has been published (Cox & Gibson, 1964).

EXPERIMENTAL

Organisms. The organisms used were a wild-type strain of *E. coli* (strain 518), an aromatic auxotroph of *A. aerogenes* (strain 170-44) unable to convert 3-enolpyruvoylshikimate 5-phosphate into chorismate and a methionine auxotroph (strain 5/17) derived from *E. coli* 518 after ultraviolet irradiation and penicillin selection. *A. aerogenes* strain 170-44 has been described previously (Gibson & Gibson, 1964). The position of the metabolic block in the methionine auxotroph was not determined. The organisms were kept on meat-infusion-agar slopes and subcultured monthly.

Cultivation of cells for extraction of quinones. Cells were usually grown at 37° as 11. batch cultures shaken in 21. flasks. The minimal medium used was that described by Vogel & Bonner (1956). Sterile glucose (30 ml. of 16%, w/v, soln./l.) was added before use. Supplements when required were as described in the text. Each flask was inoculated with 30 ml. of an overnight culture in the same medium.

Chemicals. Chemicals generally were the highest purity obtained commercially and not further purified except for 3,4-dihydroxybenzaldehyde, which was recrystallized before use. The [G-¹⁴C]shikimic acid was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. The light petroleum used throughout was b.p. 60–80°.

Spectroscopy. Spectra were measured in the following instruments: for ultraviolet spectra a Cary model 11 recording spectrophotometer; for infrared spectra a Perkin-Elmer Infracord; for mass spectra an A.E.I. MS9 mass spectrometer.

Extraction of quinones. For the routine examination of vitamin K₂ and ubiquinone content of cells, 31. of culture was grown for 16 hr., giving a total wet weight of cells of about 15 g. The wet cells were continuously extracted with about 100 ml. of acetone for 1½ hr., after which 100 ml. of ether was added and the extraction continued for a further 1½ hr. The volume of extract was reduced to about 20 ml. on a boiling-water bath and then extracted with 50 ml. of light petroleum. The light-petroleum extract was then reduced in volume to about 5 ml. for application to thin-layer chromatography plates. Larger quantities of cells were divided into 30 g. (wet wt.) batches and extracted as above with acetone and light petroleum for 3 hr. periods.

Chromatography and crystallization of quinones. Chromatography was carried out on Merck silica-gel G plates (0.5 mm. thick), which were not activated. About 10 mg. of each quinone was chromatographed on a 20 cm. plate. The developing solvent was light petroleum–chloroform (1:4, v/v). The quinones gave visible bands with the approximate *R_F* values: vitamin K₂ (MK-8), 0.7; ubiquinone-8, 0.4. The area of the chromatogram containing the quinone was removed into a Soxhlet thimble and continuously extracted with 50 ml. of ether for about 1 hr. The ether solution was concentrated and rechromatographed and the adsorbent extracted as above. After concentration of the ether solution to about 5 ml., 10 ml. of methanol was added and the remainder of the ether evaporated off on a hot plate. The volume of methanol was reduced to about 2 ml. and the quinone crystallized at –15°.

Degradation of [¹⁴C]vitamin K₂. (a) Vitamin K₂ to 1,4-diacetoxy-2-methylnaphthalene-3-acetic acid. The details for one such experiment were as follows; 12 mg. of vitamin K₂ (MK-8) recrystallized to constant specific activity was added to 340 mg. of vitamin K₁ and the mixture reductively

acetylated, ozonized and oxidized by using the method developed for the degradation of ubiquinones by Bentley, Ramsey, Springer, Dialameh & Olson (1965). The product was purified by the following steps: two crystallizations from ether-light petroleum; sublimation at 180° under reduced pressure; crystallization from ether-light petroleum; sublimation as above; crystallization from benzene; sublimation as above; crystallization from ether-light petroleum. The final yield was 100mg. (about 40%) of product with m.p. 209°. The m.p. agrees with that given by McCorquodale *et al.* (1939).

(b) 1,4-Diacetoxy-2-methylnaphthalene-3-acetic acid to phthalic anhydride. The method was based on that described by Binkley, McKee, Thayer & Doisy (1940) for the degradation of the diacetate of dihydrovitamin K₂ (MK-6). 1,4-Diacetoxy-2-methylnaphthalene-3-acetic acid (100mg.) recrystallized to constant specific activity was added to a solution of 3.5g. of KMnO₄ in 50ml. of acetone and refluxed for 5hr. The MnO₂ was cleared with SO₂, 30ml. of water added and the solution acidified with HCl. After evaporation of the acetone the aqueous solution was extracted five times with equal volumes of ether. The pooled ether extracts were evaporated to dryness under reduced pressure and the residue was taken up in about 50ml. of ether. The volume of the ether solution was reduced to about 2ml. and the phthalic acid precipitated with light petroleum.

The product was converted into phthalic anhydride and purified by subliming twice at 180° and atmospheric pressure. To purify to constant specific activity the phthalic anhydride was repeatedly crystallized from ether-light petroleum and resublimed. The final yield was 17mg. (about 30%). The identity of the phthalic anhydride was checked by m.p. and mixed m.p. (130°).

Identification of vitamin K₂ (MK-8) and ubiquinone-8 from E. coli. The vitamin K₂ from *E. coli* was identified by examination of the u.v. and i.r. spectra (Isler & Wiss, 1959). The presence of eight unsaturated isoprene units (Bishop, Pandya & King, 1962) was confirmed by mass spectrometry, which gave a mass of 716.

The identity of ubiquinone was checked by u.v. and i.r. spectra (Langemann & Isler, 1965) and a mass measurement gave 726, thus confirming the presence of ubiquinone-8 in *E. coli* (Lester & Crane, 1959). Mass spectrometry of the ubiquinone isolated from *E. coli* K12 showed a peak indicating a small proportion of ubiquinone-9.

Isotope counting techniques. Radioactivity on thin-layer chromatograms was measured with a Nuclear-Chicago Actigraph with an attachment for thin-layer plates. For counting of solid samples aluminium planchets of approx. 7cm.² area were used in a Nuclear-Chicago automatic gas-flow counting system.

The sample was plated by adding a solution to the planchet and 30mg. of silica gel G was added to aid plating. The resultant slurry was spread uniformly over the surface of the planchet by a finely drawn-out glass rod and then dried gently.

In all experiments, the time was taken for at least 5120 counts, which gave a reliable error of 2.3%. A background count and a standard count were included with each set.

A compound was regarded as being at constant specific activity if, after further purification, the value obtained for the activity was within 4% of the value of the starting material. Where the specific activities of two compounds

were directly compared the sample weights were sufficiently similar to make errors due to self-absorption less than 1%.

RESULTS

Demonstration of the incorporation of shikimic acid into vitamin K. Preliminary experiments on the incorporation of shikimate were carried out by growing the cells in the presence of [¹⁴C]shikimate. The cells were harvested, extracted as described above and the extracts chromatographed on thin-layer plates. Vitamin K and ubiquinone appeared as yellow bands and the plates were scanned for

Fig. 1. Incorporation of [¹⁴C]shikimate into vitamin K and ubiquinone and the effect of various compounds thereon. Cultures of wild-type *E. coli* (518) were grown in 2l. of glucose-citrate-mineral salts medium plus L-phenylalanine, L-tyrosine and L-tryptophan (each at 0.1 mM) plus 8.4 μg. of [¹⁴C]shikimic acid (specific activity 8.4 mc/mole). Cultures were incubated for about 6hr. with the following additions (each at 0.1 mM): (a) none; (b) 4-hydroxybenzoic acid; (c) 3,4-dihydroxybenzaldehyde. Cells were harvested, quinones extracted and chromatographed on silica-gel thin-layer plates that were then scanned for radioactivity. Radioactivity: full-scale deflection represents: ----, 3000 counts/min.; —, 1000 counts/min.

radioactivity. From similar plates with material obtained from cells grown without the addition of tracer the yellow bands were extracted and the quinones identified. Shikimate was incorporated into both vitamin K and ubiquinone (Fig. 1a). It was possible to test likely intermediates in the biosynthesis of vitamin K and ubiquinone by including an excess of unlabelled compound with the radioactive shikimate in the growth medium. If a compound enters the cell and competes with an endogenously produced intermediate, then the amount of labelling in the quinone will be decreased. The addition of unlabelled 4-hydroxybenzoate effectively depressed the incorporation of labelled shikimate into ubiquinone (Fig. 1b) but did not affect the incorporation into vitamin K. 3,4-Dihydroxybenzaldehyde and adrenalone were found to affect the incorporation of [^{14}C]shikimate into vitamin K. A number of compounds tested did not affect the incorporation of [^{14}C]shikimate into either vitamin K or ubiquinone and these included catechol, 4-hydroxyphenylpyruvate, 2,3-dihydroxybenzoate, phenylpyruvate and menadione.

Vitamin K formation by an aromatic auxotroph. To obtain some information about the route of biosynthesis of vitamin K from shikimate, vitamin K formation by an auxotrophic strain of *A. aerogenes* was examined. Strain 170-44 is unable to convert 3-enolpyruvoylshikimate 5-phosphate into chorismate and requires for growth the addition of tryptophan, phenylalanine, tyrosine and 4-aminobenzoic acid to the basal glucose-citrate-mineral

salts medium. Cells grown with these growth factors did not form detectable quantities of vitamin K. However, the further addition of 3,4-dihydroxybenzaldehyde (final concn. $10\mu\text{M}$) to the growth medium resulted in the formation of vitamin K. It was noted that under all conditions tested the cells of *A. aerogenes* 170-44 formed ubiquinone (see the Discussion section).

Comparison of the amount of [^{14}C]shikimic acid incorporated into vitamin K and ubiquinone. Cells of wild-type *E. coli* were grown in minimal medium plus phenylalanine, tyrosine and tryptophan (final concn. of each 0.1mM) as described in the Experimental section, with the addition of [$\text{G-}^{14}\text{C}$]shikimic acid (final concn. 0.1mM ; specific activity $750000\text{ counts/min./m-mole}$). After growth the cells were harvested, and the quinones were extracted, chromatographed twice and then crystallized twice from methanol before determination of specific activity. The compounds were then recrystallized to constant specific activity. The values at constant specific activity were: vitamin K_2 (MK-8), $80000\text{ counts/min./m-mole}$; ubiquinone-8, $95000\text{ counts/min./m-mole}$.

Incorporation of shikimic acid into the naphthaquinone of vitamin K_2 . To the vitamin K_2 obtained as in the above experiment, vitamin K_1 was added as carrier (for details see the Experimental section) and the vitamin K mixture then reductively acetylated, ozonized and oxidized to 1,4-diacetoxy-2-methylnaphthalene-3-acetic acid, which was in turn further oxidized to phthalic anhydride as

Table 1. Incorporation of [^{14}C]shikimate into the benzene ring of vitamin K_2

Cells were grown in glucose-citrate-mineral salts medium (Expt. 1, 30l.; Expt. 2, 10l.) plus L-phenylalanine, L-tyrosine and L-tryptophan (each at 0.1mM) to which had been added [$\text{G-}^{14}\text{C}$]shikimic acid (Expt. 1, final concn. 0.1mM of specific activity $750000\text{ counts/min./m-mole}$; Expt. 2, $3\mu\text{C}$ of specific activity 5mc/m-mole). Vitamin K_2 (MK-8) (Expt. 1, 12mg. ; Expt. 2, 5mg.) isolated from *E. coli* 518 was mixed with carrier vitamin K_1 and degraded through 1,4-diacetoxy-2-methylnaphthalene-3-acetic acid to phthalic anhydride. In Expt. 2, 6mg. of vitamin K_2 was also added to the vitamin K_2 from the cells before purification.

Specific activity (counts/min./m-mole)

	Vitamin K_2^*	1,4-Diacetoxy-2-methylnaphthalene-3-acetic acid	Phthalic anhydride
Expt. 1.	1300	1200	1230
Expt. 2	700	620	640

* Corrected for addition of carrier.

Table 2. *Incorporation of [1,2-¹⁴C₂]acetate or [Me-¹⁴C]methionine into vitamin K*

Cells were grown in 10l. of glucose-citrate-mineral salts medium with the labelled compound added with the inoculum. For the methionine auxotroph the medium was supplemented with L-methionine (final concn. 0.1 mM). Procedures were as described for Table 1 and in the Experimental section. Vitamin K₂ (MK-8) was added as carrier to the vitamin K₂ from the cells before purification.

Labelled compound added to medium	Strain of <i>E. coli</i>	Specific activity (counts/min./m-mole)		
		Vitamin K ₂ (MK-8)*	1,4-Diacetoxy-2-methylnaphthalene-3-acetic acid	Phthalic anhydride
Sodium [1,2- ¹⁴ C ₂]acetate (0.1 mc of specific activity 1.5 mc/m-mole)	Wild-type	1400	680	100
[Me- ¹⁴ C]Methionine (10 μC of specific activity 23 mc/m-mole)	Methionine auxotroph	1400	1470	

* Corrected for addition of carrier.

described in the Experimental section. The results obtained are summarized in Table 1. The results indicate that shikimate is incorporated into the benzene ring of the naphthaquinone. It was also shown during these experiments that the shikimate contributed only to the naphthaquinone portion of the vitamin K molecule since all the radioactivity of the original vitamin K₂ was present in the phthalic anhydride.

Incorporation of [1,2-¹⁴C₂]acetate and [Me-¹⁴C]-methionine into vitamin K. Cells of *E. coli* 518 were grown in the glucose-citrate-mineral salts medium to which [1,2-¹⁴C₂]acetate was added. Vitamin K₂ was isolated from the cells and, after the addition of vitamin K₁ as carrier, the degradation procedures as above were applied and these results are summarized in Table 2. Some radioactivity is found in the phthalic anhydride and the significance of this result is discussed below.

The methionine auxotroph of *E. coli* (strain 5/17) was grown in the glucose-citrate-mineral salts medium to which had been added [Me-¹⁴C]-methionine. The vitamin K₂ from the cells was isolated, carrier vitamin K₁ added and the degradation carried out giving the results summarized in Table 2. The radioactivity is retained in the 1,4-diacetoxy-2-methylnaphthalene-3-acetic acid.

DISCUSSION

The results presented above indicate that the benzene ring of the naphthaquinone portion of vitamin K₂ (MK-8) arises from shikimate in *E. coli*. The specific activity of the vitamin K₂ obtained after incorporation of [¹⁴C]shikimate was low compared with that of the original specific activity of the [¹⁴C]shikimate. However, the specific activity of

the vitamin K was about the same as that of the ubiquinone isolated and these low activities indicate that, even with all the aromatic amino acids present, there is some endogenous shikimate formed with which the exogenous shikimate does not compete very effectively.

It would seem that experiments on the origin of the fungal naphthaquinones might be re-examined. For example, the experiments of Gatenbeck & Bentley (1965) on the biosynthesis of the naphthaquinone javanicin do not give any direct information on the incorporation of [¹⁴C]acetate into the benzene ring of the naphthaquinone. It is possible that, with the [¹⁴C]acetate being added at the eighth day of the experiment, the glucose has been utilized and acetate would be readily utilized for the formation of shikimate. In the present experiments acetate was incorporated to some extent into the carbon atoms of the degradation products of vitamin K₂. Further experiments are needed to decide whether the acetate contributes directly to the quinone ring or to the benzene ring through shikimate, or both.

The incorporation of the methyl group of methionine into vitamin K has confirmed the results of Lederer (1964) and Azerad *et al.* (1965) but proof is still lacking that this methyl group is the source of the 2-methyl group of the naphthaquinone.

The observation that shikimic acid is incorporated into ubiquinone and vitamin K raises the question where the biosynthetic routes for these compounds diverge from the pathways of biosynthesis of aromatic compounds. Ubiquinone has previously been suggested to arise from 4-hydroxybenzoate (Parson & Rudney, 1964), which in turn is formed from chorismate (Gibson & Gibson, 1964). In *E. coli* and *A. aerogenes* it would seem that ubiquin-

Scheme 1. Outline of probable pathways of biosynthesis of aromatic compounds in *E. coli* and *A. aerogenes*. Broken lines represent pathways that have not been confirmed by experiments with cell-free extracts. The *E. coli* strain tested could not convert 4-hydroxybenzaldehyde into 4-hydroxybenzoate.

one and vitamin K may be formed from shikimate. However, *A. aerogenes* 170-44, which is unable to form chorismate from shikimate, grown on a mixture of the three aromatic amino acids plus 4-aminobenzoate forms no detectable vitamin K but normal amounts of ubiquinone, suggesting that there might be an alternative pathway to ubiquinone in this organism. An auxotroph of *E. coli* unable to carry out one of the reactions in the common pathway of aromatic biosynthesis formed no detectable vitamin K and only traces of ubiquinone. Preliminary experiments with cell-free extracts of wild-type *A. aerogenes* and *E. coli* (F. Gibson & R. C. Bayly, unpublished work) have shown that *A. aerogenes* can convert tyrosine, 4-hydroxyphenylpyruvate or 4-hydroxybenzaldehyde into 4-hydroxybenzoate, whereas *E. coli* cannot. As has been pointed out by Parson & Rudney (1965) the rapid chemical conversion of 4-hydroxyphenylpyruvate into 4-hydroxybenzaldehyde complicates the study of this pathway. A tentative outline of the pathways of biosynthesis of aromatic compounds is set out in Scheme 1.

The branch point after shikimate for the formation of vitamin K is probably at chorismate. The inability of the strain of *A. aerogenes* unable to convert 3-enolpyruvoylshikimate 5-phosphate into chorismate to form vitamin K places the branch point at chorismate or later. The addition of phenylpyruvate or 4-hydroxyphenylpyruvate to a medium containing [^{14}C]shikimate did not suppress the incorporation of the radioactivity into vitamin K. Chorismate and prephenate are not growth factors, probably cannot enter the intact cell and therefore cannot be tested by isotope-competition experiments with whole cells. Stimulation of

growth by 3,4-dihydroxybenzaldehyde has only been described for aromatic auxotrophs with metabolic lesions before chorismate, suggesting that prephenate is probably not a precursor. Thus, though the possibility of a pathway through prephenate cannot be excluded, it is tentatively suggested that the pathway is as shown in Scheme 1.

3,4-Dihydroxybenzaldehyde was tested as a possible precursor of vitamin K because of the observation by Davis (1952*a,b*) that, under certain conditions, some multiple aromatic auxotrophs required an extra growth factor (sixth factor). This sixth-factor requirement could be satisfied by 3,4-dihydroxybenzaldehyde or related compounds such as adrenaline. There is little reason to suppose that 3,4-dihydroxybenzaldehyde itself is on the direct route of biosynthesis of vitamin K. Related compounds such as 3,4-dihydroxybenzoate did not interfere with the incorporation of [^{14}C]shikimate but whether such compounds can enter the intact cell is not known. It might be noted that compounds such as 2,3-dihydroxybenzoate and 3,4-dihydroxybenzoate are excreted by various aromatic auxotrophs (Pittard, Gibson & Doy, 1961, 1962). Apart from the possible roles of 4-hydroxybenzoate and 3,4-dihydroxybenzaldehyde, nothing is known of the intermediates involved in the biosynthesis of vitamin K and ubiquinone from chorismate.

The ability to prepare cells deficient in vitamin K and ubiquinone should be of assistance in studies on the little-understood functions of these compounds.

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REFERENCES

- Azerad, R., Bleiler-Hill, R. & Lederer, E. (1965). *Biochem. biophys. Res. Commun.* **19**, 194.
- Bentley, R., Ramsey, V. G., Springer, C. M., Dialameh, G. H. & Olson, R. E. (1965). *Biochemistry*, **4**, 166.
- Binkley, S. B., McKee, R. W., Thayer, S. A. & Doisy, E. A. (1940). *J. biol. Chem.* **133**, 721.
- Bishop, D. H. L., Pandya, K. P. & King, H. K. (1962). *Biochem. J.* **83**, 606.
- Cox, G. B. & Gibson, F. (1964). *Biochim. biophys. Acta*, **93**, 204.
- Davis, B. D. (1952a). *J. Bact.* **64**, 729.
- Davis, B. D. (1952b). 2nd Congr. int. Biochem., Paris: Symp. *Metabolisme Microbien*, p. 32. Paris: Masson.
- Gatenbeck, S. & Bentley, R. (1965). *Biochem. J.* **94**, 478.
- Geissman, T. A. (1963). In *Biogenesis of Natural Compounds*, p. 590. Ed. by Bernfeld, P. Oxford: Pergamon Press Ltd.
- Gibson, M. I. & Gibson, F. (1964). *Biochem. J.* **90**, 248.
- Isler, O. & Wiss, O. (1959). *Vitam. & Horm.* **17**, 53.
- Langemann, A. & Isler, O. (1965). In *Biochemistry of Quinones*, p. 133. Ed. by Morton, R. A. London: Academic Press (Inc.) Ltd.
- Lederer, E. (1964). *I.U.B. Symp. Ser.* **33**, 63.
- Lester, R. L. & Crane, F. L. (1959). *J. biol. Chem.* **234**, 2169.
- McCorquodale, D. W., Cheney, L. C., Binkley, S. B., Holcomb, W. F., McKee, R. W., Thayer, S. A. & Doisy, E. A. (1939). *J. biol. Chem.* **131**, 357.
- Parson, W. W. & Rudney, H. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 444.
- Parson, W. W. & Rudney, H. (1965). *J. biol. Chem.* **240**, 1855.
- Pittard, A. J., Gibson, F. & Doy, C. H. (1961). *Biochim. biophys. Acta*, **49**, 485.
- Pittard, A. J., Gibson, F. & Doy, C. H. (1962). *Biochim. biophys. Acta*, **57**, 290.
- Scholes, P. B. & King, H. K. (1965). *Biochem. J.* **97**, 754.
- Vogel, H. J. & Bonner, D. M. (1956). *Microb. Genet. Bull.* **13**, 43.