since measurements of $O_2$ uptake with intact bacteria or extracts indicated approximately the same ratio of activities. Further, bacteria growing on D(-)-mandelate did not accumulate benzaldehyde, which shows that D(-)-mandelate dehydrogenase, rather than benzaldehyde dehydrogenase I (Cook et al., 1975), was now rate-limiting.

D(-)-Mandelate dehydrogenase resembles L(+) mandelate dehydrogenase in (a) its location in the membrane, (b) inhibition by oxalate, CN$^-$ and EDTA and (c) range of electron acceptors. However, the new enzyme is less stable during storage, sonication or heating. L(+) Mandelate dehydrogenase activity is not affected by D(-)-mandelate (Kennedy & Fewson, 1968a), but D(-)-mandelate dehydrogenase is inhibited by L(+) mandelate.

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An Assay Procedure for the Vitamin K$_1$ 2,3-Epoxide-Reducing System of Rat Liver involving High-Performance Liquid Chromatography

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Since the discovery in Scotland of a population of wild rats resistant to anticoagulant rodenticides (Boyle, 1960), others were reported from several European countries (Greaves, 1970) and the United States (Jackson & Kaukeinen, 1972). The resistance gene from wild warfarin-resistant rats trapped in Wales has been introduced into Wistar-derived rats in England to give the HW strain (Greaves & Ayres, 1967) and into Sprague Dawley-derived rats in the United States (Pool et al., 1968). It was shown in America by Hermodson et al. (1969), that these resistant rats did not differ from susceptible Holtzmann rats in their ability to metabolize and excrete warfarin, but they did possess an increased requirement for dietary vitamin K$_1$, possibly owing to the presence of an altered protein receptor with a lowered binding affinity for both vitamin K$_1$ and warfarin. A similar situation seemed to occur in HW rats (Martin, 1973; Townsend et al., 1975). In warfarin-treated susceptible rats, vitamin K$_1$ 2,3-epoxide accumulated in the liver (Bell et al., 1972) as the enzyme system reducing it to vitamin K$_1$ was inhibited by warfarin in vivo (Bell & Matschiner, 1970). However, Sprague-Dawley-derived resistant animals did not show this increase, but the study suggested that the altered enzyme was less effective in catalysing the vitamin K$_1$ epoxide reduction (Bell & Caldwell, 1973), and this was confirmed by studies in vitro (Matschiner et al., 1974; Zimmermann & Matschiner, 1974). They showed that the liver enzyme system was located in the liver microsomal fraction and required either the 10000g precipitate or a thiol-group reagent as a source of reducing equivalents. These workers used $^3$H-labelled vitamin K$_1$ epoxide and separated the vitamin K$_1$ produced by reverse-phase t.l.c. This is not a readily available assay, and we have evaluated an alternative method for use in screening animals for resistance and the assessment of new rodenticides.

Male Wistar rats (200-250g) were killed by cervical dislocation and their livers macerated in 0.25m-sucrose/0.025m-potassium phosphate buffer (pH7.4)/5.0m-dithiothreitol to give a 10% (w/v) homogenate. Cell fractions were obtained by centrifugation
(Bunyan et al., 1972). Vitamin K$_1$ epoxide was prepared from vitamin K$_1$ by the method of Tishler et al. (1940). The enzyme assays were carried out in 25 ml flasks open to the air and shaken in a water bath at 37°C. The postmitochondrial supernatant (2 ml) was pre-incubated with the homogenizing buffer (1 ml) or buffer containing warfarin sodium, for 5 min and the reaction started by adding vitamin K$_1$ epoxide in 50 μl of ethanol. After 20 min the contents of each flask were added to 7 ml of propan-2-ol/hexane (3:2, v/v) and 1 g of NaCl in an ice-cold stoppered glass centrifuge tube and mixed with a vortex mixer. The two phases were separated by centrifuging at 2000 g for 15 min. The upper phase was removed, dried by passing it down a short column of anhydrous Na$_2$SO$_4$ and collected in a glass vial. The lower phase was extracted with two 5 ml volumes of hexane and the upper phases were treated as previously. The combined extract was concentrated by evaporation under N$_2$ and quantitatively transferred to a tapered stoppered tube. The extract was evaporated just to dryness, immediately redissolved in 100 μl of chloroform and sealed until analysed. All the procedures after the preincubation were carried out in a darkened room.

Fig. 1. *Separation of vitamin K$_1$ and its epoxide by high-performance liquid chromatography* 

The trace was obtained from a mixture of 0.8 μg of vitamin K$_1$ epoxide (A) and 0.6 μg of vitamin K$_1$ (B) injected on to a 50 cm column of Permaphase ODS and eluted with methanol/water (90:10, v/v) at 1.5 ml/min.
Concentration of vitamin K₁ epoxide (p~)

Fig. 2. Effect of vitamin K₁ epoxide concentration on the rate of its reduction and the inhibition produced by warfarin

The effects of 3μm-warfarin sodium (▲ and ●) on the reduction of vitamin K₁ epoxide to vitamin K₁ by warfarin-susceptible (△, △) and warfarin-resistant (○, ○) rat liver preparations are shown. △, ○, No added warfarin.

Vitamin K₁ and its epoxide were separated by high-performance liquid chromatography (LC 750, ARL, Luton, U.K.) by using a 50cm Permaphase ODS column and methanol/water (90:10, v/v) at a constant pressure of 1.03MPa. The u.v. detector (254 nm) gave a response that was linear over the range 50–500ng of vitamin K₁ and 2–16μg of vitamin K₁ epoxide when standard solutions were analysed separately or as mixtures. The recovery of vitamin K₁ and its epoxide from assay mixtures where the enzyme preparation was added after the addition of the extracting solvent were reproducible, and not less than 80%. The trace of a typical assay is shown in Fig. 1.

The assay was found to be linear over the range 50–200mg fresh weight of liver, and 5–40min. A comparison of the effect of substrate concentration on the activity of extracts prepared from Wistar susceptible (TAS) and resistant (HW) rats showed that the control value for the TAS-strain rats was 250nmol of vitamin K₁ formed/h per g of liver and that for the HW strain was 40% lower (Fig. 2). In the presence of 3μm-warfarin sodium, both strains had the same activity, approx. 75nmol of vitamin K₁ formed/h per g of liver.


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