Dietary modification of potential vitamin K supply from enteric bacterial menaquinones in rats

BY J. C. MATHERS

Department of Agricultural Biochemistry and Nutrition, University of Newcastle, Newcastle upon Tyne NE1 7RU

AND FRESIA FERNANDEZ AND M. J. HILL

Bacterial Metabolism Research Laboratory, PHLS Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire SP4 0JG

AND P. T. McCARTHY AND M. J. SHEARER

Haematology Research Laboratory, Guy's Hospital, London SE1 9RT

AND A. OXLEY

Department of Haematology, Royal Victoria Infirmary, Newcastle upon Tyne

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Rats given a low-fibre diet based on boiled white rice developed symptoms of severe vitamin K deficiency within 23 d. Inclusion of autoclaved black-eye beans (*Vigna unguiculata*) in the diet prevented the bleeding syndrome. To test the hypothesis that deficiency resulted from low phylloquinone intake exacerbated by inadequate production of menaquinones by the enteric bacteria, a follow-up experiment was carried out in which groups of rats were given an all-rice diet, a rice + beans diet or a stock diet. Rats on the all-rice diet had significantly lower faecal concentrations of the main menaquinone-producing bacterial species (*Bacteroides fragilis* and *Bacteroides vulgatus*) than animals on either of the other two diets. This coupled with the much lower faecal output on this diet suggests that total menaquinone production was low for the all-rice diet. The alterations in faecal flora were associated with several significant changes in caecal metabolism. Rats given the stock diet had much shorter caecal transit times and a considerably greater proportion of butyric acid in volatile fatty acid end-products than did rats on either of the other two diets.

Caecal fermentation: Menaquinones: Vitamin K: Rat

Phylloquinone (vitamin K_1) from green leafy vegetables is the main dietary source of vitamin K required for the post-translational carboxylation of glutamate residues to produce γ-carboxyglutamic acid (Gla) in at least seven plasma proteins, four of which are involved in blood clotting. Vitamin K-dependent Gla-containing proteins are also present in bone (osteocalcin), kidney, atherosclerotic plaque and several other tissues (Suttie, 1985). Adult man appears to be very resistant to the development of a primary deficiency of the vitamin (Suttie, 1985), but haemorrhagic disease of neonates is occasionally reported and seems to be more common in breast-fed than bottle-fed infants (von Kries *et al.* 1988). Some species of enteric bacteria produce menaquinones (vitamin K_2; Collins & Jones, 1981) having activity as vitamin K (Suttie, 1985) and such menaquinones are frequently assumed to provide about half the human requirement (Passmore & Eastwood, 1986). In vitro studies using everted rat colonic sacs have shown that menaquinones may be absorbed by a passive non-saturable process (Hollander *et al.* 1976), but whilst menaquinones have been detected in liver from many species including man (Suttie, 1985) there is no

Table 1. *Expt A. Composition of experimental diets (g/kg)*

Experimental diets...	2A	3A	4A	5A
Rice	750	637·5	525	412·5
Beans*	0	112·5	225	337·5
Casein + methionine†	120	100	80	60
Sucrose	30	50	70	90
Maize oil	50	50	50	50
Vitamin + mineral premix‡	50	50	50	50

* Black-eye beans (*Vigna unguiculata*) cooked and ground.

† Casein–methionine (100:1, w/w).

‡ Contained (g/kg premix): $CaHPO_4$ 350, $MgSO_4.7H_2O$ 60, NaCl 15, KCl 95, $FeSO_4.7H_2O$ 3, $MnSO_4.4H_2O$ 4, $ZnSO_4.7H_2O$ 4·5, $CuCl_2.2H_2O$ 0·27, KIO_3 0·005; and (mg/kg premix): Rovimix AD_3 500/100 (Roche) 200, Rovimix E50 Adsorbate (Roche) 1200, folic acid 15, riboflavin 60, thiamin hydrochloride 90, pyridoxine hydrochloride 75, choline chloride 27000, calcium pantothenate 50, biotin 20, cyanocobalamin 1 in a sucrose base.

information on rates of menaquinone production or absorption in vivo. Vitamin K deficiency in breast-fed babies may result from limited transfer of the vitamin across the placenta (Shearer *et al.* 1982), relatively low concentrations of phylloquinone in breast milk (Haroon *et al.* 1982) and the development of a gut flora in which bacteria devoid of menaquinones, e.g. bifidobacteria and lactobacilli, predominate (Bullen *et al.* 1977).

Administration of antibiotics to vitamin K-deprived human subjects (O'Reilly, 1971; Allison *et al.* 1987) and rats (Black *et al.* 1942) has been used to produce vitamin K deficiency but the effects of dietary modification on the metabolic activities of the large intestinal flora are poorly understood and there is no quantitative information on the ways in which such modification may influence the contribution made by enterically produced menaquinones in any species. In the first experiment reported here, vitamin K deficiency occurred in rats within 23 d of giving a diet based on boiled white rice. The follow-up experiment was designed to investigate the potential for dietary manipulation of the large intestinal population of menaquinone-producing bacteria. Findings on the fermentation characteristics accompanying such changes are also presented.

EXPERIMENTAL

Expt A

Animals and housing. Male Wistar rats, initial weight approximately 150 g, were purchased from A. Tuck & Son, Battlebridge, Essex and housed initially in plastic cages with wire-mesh floors (three per cage) and fed on a stock diet (41B, Oxoid Ltd, Basingstoke, Hampshire) *ad lib.* until they weighed 234 (SD 9·5)g. Twenty rats were then housed individually in Perspex and stainless-steel metabolism cages (Thompson, 1970) with expanded metal floors (experimental diet-fed animals). The animal room was maintained at a temperature of $25 \pm 1°$ and a relative humidity of $53 \pm 0·5\%$.

Diets. Four experimental diets (2A–5A) containing various proportions of polished rice (*Oryza sativa*) and black-eye beans (*Vigna unguiculata*) were formulated (Table 1). The protein contents of the diets were kept approximately constant (Matschiner & Doisy, 1965) at about 185 g/kg dry matter by varying the proportions of casein + methionine and of sucrose. Before inclusion in the diets, the rice was cooked by boiling in open pans for 10 min and the beans soaked in hot water for 40 min and autoclaved at 115°, 69 kPa (10 psi) for 15 min. The cooked rice and beans were dried at 60° and ground through a 1 mm screen. The experimental diets were offered at 15 g air dry weight/d. For comparative

purposes, samples of liver and blood were also obtained from rats of the same strain and from the same supplier and given access *ad lib.* to a commercially prepared pelleted chow (41B, Oxoid Ltd). Water was available *ad lib.* to all animals.

Experimental procedures. Groups of five rats were given each of the experimental diets for 25 d after which time survivors were killed by diethyl ether inhalation. Blood was collected by cardiac puncture and 2-ml portions were transferred into tubes containing 0·2 ml 0·15 M-sodium citrate solution for prothrombin clotting time and other assays. Animal carcasses were stored at −20° until required for analyses. Stock-fed rats were killed at intervals either by chloroform inhalation (when livers only were required) or diethyl ether inhalation (when blood samples were required); the caecums were removed for other purposes and the carcasses stored at −20° until required for analysis.

Analytical methods. Platelet-poor plasma was prepared by centrifuging citrated blood at 2000 ***g*** for 5 min and assayed for prothrombin clotting time and blood clotting factors II, V, VII, IX and X using methods described by Paul *et al.* (1987). Values for clotting factors are expressed as percentages of a control pooled human plasma with a prothrombin time of 12 s. For liver analyses, frozen carcasses were allowed to thaw sufficiently to permit removal of the livers which were washed in ice-cold saline (9 g sodium chloride/l), blotted dry, weighed and transported packed on dry ice. Lipids of rat diets and livers were extracted with acetone and partitioned into hexane as previously described (Shearer, 1986*a*). Phylloquinone (vitamin K_1) was measured in lipid extracts by a multi-stage purification procedure with final analysis by high-performance liquid chromatography and dual-electrode electrochemical detection in the redox mode (Hart *et al.* 1985; Shearer, 1986*b*). Quantification of phylloquinone was made by the method of internal standardization using menaquinone-6 as the internal standard for diet analyses and 2′,3′-dihydrophylloquinone for liver analyses. Post-mortem investigation including histology was carried out by standard procedures on three rats given the all-rice diet (diet 2A).

Expt B

Eighteen male Wistar rats (mean initial weight 211 g) were placed in individual steel and Perspex metabolism cages (Thompson, 1970) fitted with glass separators to permit collection of urine and faeces.

Diets and feeding. Three diets were used. Diet 1B was a stock diet (Oxoid 41B) milled to pass a 1 mm screen and containing 2 g chromic oxide/kg as an indigestible marker. Diets 2B and 5B were formulated as for diets 2A and 5A in Expt A but with the addition of 0·5 mg menadione (a synthetic source of vitamin K) and 2 g Cr_2O_3 (an unabsorbed marker)/kg at the expense of sucrose (National Research Council, 1978). Six animals were allocated to each diet and each was offered 15 g diet once daily at 10.00 hours. Water was available *ad lib.*

Experimental procedures. Rats were weighed on days 1, 15, 24, 25 and 26. After 14 d adaptation to diets, complete collections of urine and faeces were made for 7 d. Food residues over the latter period were collected, dried and weighed. On each of days 24, 25 and 26, freshly voided faeces were collected by picking up each rat by the tail which provoked defaecation. Approximately 0·5 g freshly voided faeces was immediately transferred into a pre-weighed bottle containing 4·5 ml Glycerol Transport Broth (100 g glycerol/l, Lab-Lemco, Oxoid Ltd). It was suspended in this fluid by breaking up the pellet with a mounted steel needle followed by 'whirli' mixing, and stored at −80°. Between 13.00 and 17.00 hours on day 26, rats were anaesthetized by diethyl ether inhalation, laparotomy was performed and samples of blood withdrawn from the portal vein and heart. The liver was excised, rinsed in ice-cold saline, blotted dry, weighed and stored at −80°. The gastrointestinal tract was removed and the caecum excised and weighed. The pH of the

caecal contents was measured and duplicate samples (approximately 0·5 g) were mixed 2:1 (w/v) with deproteinizing solution (metaphosphoric acid solution (200 g/l) containing 50 mM-3-methyl-valeric acid). Further samples of caecal contents were transferred into pre-weighed tubes for determination of dry matter (DM) and Cr_2O_3 contents. The caecal tissue was washed, blotted dry and weighed.

Chemical analyses. Volatile fatty acids (VFA) in caecal contents were measured by gas–liquid chromatography using a 2 mm internal diameter column packed with 10% SP-1200–1% phosphoric acid on 80/100 Chromosorb (Supelco Inc., Belliforte, Pa 16823, USA) in a PU 4550 gas–liquid chromatograph (Pye Unicam, Cambridge). Samples (100 mg) of foods, caecal contents and faeces were weighed into graduated Pyrex tubes and ashed at 450° for 16 h. The residue was digested using 1·2 ml acid mixture (300 ml $MnSO_4.4H_2O$ solution (100 g/l) diluted to 1 litre with orthophosphoric acid) and 1·6 ml potassium bromate solution (45 g $KBrO_3$/l) and made to 10 ml with distilled water. To portions of the digest, 1 ml calcium chloride solution (5·47 g $CaCl_2.6H_2O$/l) and 0·1 ml sodium silicate solution (7·55 g $Na_2SiO_3.5H_2O$/l) were added and, after appropriate dilution, the chromium concentration was measured by atomic absorption spectrophotometry (SP9; Pye Unicam, Cambridge).

Bacteriology. Bacteriological analyses of the faecal samples were performed by the method of Borriello *et al.* (1978). In this method the media are pre-reduced and stored anaerobically before use. All dilutions and plating techniques were carried out in an anaerobic chamber. Qualitative and quantitative analysis were performed using a range of selective and non-selective media which enabled enumeration of aerobic, microaerophilic, obligate and facultative anaerobic organisms. Identification of the organisms was confirmed by the method of Holdeman *et al.* (1977), whilst facultative organisms were identified by the method of Cowan & Steel (1965). The counts of colony-forming units per g of faeces were expressed as $\log_{10}$ counts ($\log_{10}$cfu/g).

Statistical analysis

Values from both experiments were examined by one-way analysis of variance. In Table 2, data for stock-fed rats were not included in the analysis of body masses. For faecal bacteriological values in Expt B (see Table 7, p. 647), where measurements were made for each animal on each of the 3 d, means for each animal were computed and used in the analysis of variance. Variation between days within animals will be discussed elsewhere. For Expt A, linear regression was used to describe the relationship between liver phylloquinone concentration and concentration of phylloquinone in the diet. For Expt B, differences between treatment means were tested using the following orthogonal contrasts:

contrast 1: diet 1B *v.* (diet 2B + diet 5B),
contrast 2: diet 2B *v.* diet 5B.

RESULTS

Expt A

Animal health. All the rats on the experimental diets ate normally and grew well (Table 2) over the first 15 d of the study. Thereafter, whilst rats on diets containing beans appeared normal, those given the all-rice diet (diet 2A) began to show signs of ill-health. Reduced food intake was the earliest symptom and was first observed 16–23 d after introduction to the experimental diets. Faeces became malformed with some scouring. The rats became moribund and the first (rat no. 20) died on day 18. Rat no. 14 haemorrhaged from the right paw on day 20 and was killed by chloroform inhalation. Rats on the all-rice diet showed

Table 2. *Expt A. Body mass, growth rate, faecal dry matter (DM) output, liver mass, liver lipid concentration and concentrations of vitamin K_1 (phylloquinone) in the food and livers of rats given the stock diet and experimental diets containing various proportions of black-eye beans* (Vigna unguiculata) *and rice*

(Values are means with pooled standard error for five rats per group)

		Experimental				
Diet*...	Stock 1A	2A	3A	4A	5A	SE (*n* 5)
Body mass (g)	698	293†‡	320	316	304	4·7
Growth rate§ (g/7 d)	—	25·3‡	28·4	26·2	25·0	1·27
Faecal DM output§ (g/7 d)	—	4·83‡	4·95	6·28	7·29	0·149
Liver mass (g/kg body mass)	44·5	40·4‖	38·7	41·2	40·2	1·15
Liver lipid (g/kg liver mass)	24·9	26·9‖	22·3	21·2	20·6	1·40
Dietary vitamin K_1 (μg phylloquinone/kg)	52	16	28	36	49	—
Liver vitamin K_1 (μg phylloquinone/kg)	2·58	0·14‖	0·38	0·40	1·29	0·583

* For details, see Table 1 and p. 640.
† Measured on day 18 (rat nos. 5 and 20); day 25 for other rats on experimental diets.
‡ Three rats only (values for two rats which were unwell were excluded).
§ Measured over days 11–18.
‖ Two rats only.

a loss of the normal pink colour from the ears and other extremities and the remaining three rats on this diet were killed on days 18 (rat no. 5), 22 (rat no. 30) and 24 (rat no. 36). Post-mortem investigations were carried out on rat nos. 5, 20 and 30. Rat no. 20 had subcutaneous bruising and haemorrhages, the liver was very pale and the kidneys were congested. The urine was heavily blood-stained and contained a fibrin clot. The intestinal contents were mucoid and the large intestine was full of dark brown digesta. Cultures from a range of tissues showed a scanty growth of non-haemolytic coliforms only. Pathological findings in rat no. 5 were similar but less marked. The post-mortem appearance was similar to that in animals which had received an anticoagulant. Rat no. 30 did not show any of these symptoms at post-mortem.

Vitamin K status

The measured vitamin K_1 concentration in white rice was very low (2 μg phylloquinone/kg) compared with that in the black-eye beans (108 μg phylloquinone/kg). Measured phylloquinone concentrations in the experimental diets (2A–5A) increased with increasing proportion of beans in the diet (Table 3), and were higher by 8–12 (mean 10) μg/kg than those calculated from the proportions of rice, beans and maize oil in the diets (assuming a value of 30 μg phylloquinone/kg oil obtained for a different batch of this ingredient). There was a significant ($P < 0{\cdot}05$) positive relationship between the concentration of phylloquinone in the diet (x) and liver phylloquinone concentration (y):

$$y = -0{\cdot}68\,(\text{SE}\,0{\cdot}53) + 0{\cdot}037\,(\text{SE}\,0{\cdot}014)x,\ (r\,0{\cdot}56, n\,17).$$

Whilst the stock diet contained a concentration of phylloquinone similar to that of the diet containing the highest level of beans (diet 5A), the liver phylloquinone concentration from animals fed on the stock diet was much higher than that of those given the experimental diets. The mean hepatic vitamin K_1 concentration of animals on the diet with the highest

Table 3. *Expt A. Prothrombin clotting times and concentration of some coagulation factors in plasma of rats given the stock diet and experimental diets containing various proportions of black-eye beans* (Vigna unguiculata) *and rice*

Diet*...	Stock 1A (*n* 4)	Experimental 2A Rat no. 36	3A Rat no. 21	5A Rat no. 3
Prothrombin clotting time (s)	15 (0·9)†	> 60	24	15
Concentration of coagulation factors in plasma‡				
Factor II	45 (9·1)	5	8	52
Factor V	110 (11·7)	120	105	120
Factor VII	124 (11·0)	4	13	160
Factor IX	40 (9·9)	< 1	7	40
Factor X	30 (2·4)	1	4	33

* For details, see Table 1 and p. 640.
† Standard deviation of a single observation is given in parentheses.
‡ Expressed as a percentage of a control pooled human plasma with a prothrombin time of 12 s.

level of beans (diet 5A) was only half that of the animals given the stock diet. Some of the differences in liver phylloquinone concentrations between stock-fed and experimental animals may be due to age or body-weight and further information in this area is required. The mean plasma prothrombin clotting times of stock-fed rats was 15 s, and was the same as that observed for rats given diet 4A (*n*2) and diet 5A (*n*3). Rats given diet 3A had prothrombin times of 16, 17 and 24 s, whilst the only animal (rat no. 36) tested from the all-rice-diet (diet 2A) group had a prothrombin time in excess of 60 s. The plasma concentrations of coagulation factors for some rats are given in Table 3. Concentrations of factor V (non-vitamin K-dependent) were similar in all animals tested, but concentrations of the vitamin K-dependent factors II, VII, IX and X were severely reduced in the rats with the longest prothrombin clotting times (and given diets 2A and 3A) compared with stock-fed rats or rat no. 3 which had been given the diet with the highest level of beans (diet 5A). Liver weights (as a proportion of body mass) were similar for rats given the experimental diets but significantly greater for those consuming the stock diet. Diet did not affect the concentration of lipid in the liver (Table 2).

Expt B

All animals remained healthy throughout the study. Food consumption was almost total with the difference in DM intake between diet 1B and the other diets being due to the lower DM content of the stock diet (Table 4). The rats grew well on both rice-containing diets at nearly 6 g/d. The 27% lower growth for rats on the stock diet was as expected, since the DM intake was lower and DM digestibility considerably reduced (Table 4) on this diet. Faecal DM output (g/kg DM intake) was 5·9 and 3·5 times greater with diet 1B than with diets 2B and 5B respectively. Liver weights (per unit body mass) were 10% greater for rats given the rice-containing diets than for those fed on the stock diet (Table 4).

Caecal metabolism

Rats given the very-low-fibre all-rice diet (diet 2B) had caecums weighing only half that of rats given either of the other two diets (Table 5). These between-diet differences were also found in the mass of caecal tissue which on diet 2B was only 0·72 of that on the stock and rice + beans diets. The proportion of DM in caecal contents was highest on diet 1B and

Table 4. *Expt B. Food intakes and faecal outputs, growth rates and liver weights of rats given a stock diet and two experimental diets based on white rice with and without black-eye beans* (Vigna unguiculata)

(Values are means with pooled standard errors for six rats per group)

Diet†...	Stock 1B	All rice 2B	Rice + beans 5B	SE of mean	Statistical significance of contrasts: Diet 1B *v.* (diet 2B + diet 5B)	Statistical significance of contrasts: Diet 2B *v.* diet 5B
DM content of diet (g/kg)	887	948	948	—	—	—
Growth rate (g/d)‡	4·2	5·9	5·8	0·24	***	NS
DM intake‡ (g/d)	13·2	14·0	14·1	0·09	***	NS
Faecal DM output‡ (g/kg DM intake)	266	45	76	2·1	***	***
Liver wt (g/kg body wt)	30·6	33·3	34·2	0·92	*	NS

NS, not significant; DM, dry matter.
* $P < 0·05$, *** $P < 0·001$.
† For details, see Table 1 and p. 641.
‡ Measured over days 15–26 of study.

Table 5. *Expt B. Total caecal weight, caecal contents and tissue weights, pH of caecal contents and transit time through the caecum of rats given a stock diet and two experimental diets based on white rice with and without black-eye beans* (Vigna unguiculata)

(Values are means with pooled standard errors for six rats per group)

Diet†...	Stock 1B	All rice 2B	Rice + beans 5B	SE of mean	Statistical significance of contrasts: Diet 1B *v.* (diet 2B + diet 5B)	Statistical significance of contrasts: Diet 2B *v.* diet 5B
Caecal wt (g)	6·15	3·14	6·56	0·337	**	***
Caecal contents dry wt (g)	1·27	0·48	0·97	0·060	***	***
Caecal contents DM (g/kg)	253	202	178	3·9	***	***
Caecal tissue wet wt (g)	1·11	0·80	1·11	0·054	*	***
Caecal pH	5·8	6·3	5·7	0·07	*	***
Caecal transit time (h)	7·1	19·7	20·1	1·64	***	NS

NS, not significant; DM, dry matter.
* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† For details, see Table 1 and p. 641.

lowest for the bean-containing diet (diet 5B), so that the dry weight of caecal contents was 2·6 and 1·3 times greater with diet 1B than with diets 2B and 5B respectively. The pH of caecal contents was considerably higher in animals fed on the all-rice diet than in those fed on the other two diets, which both produced similar values (Table 5).

The proportion of ingested Cr_2O_3 recovered in faeces was high, with means of 1·00, 1·09 and 1·06 (SE 0·021) for diets 1B, 2B and 5B respectively. The mean transit time of Cr_2O_3

Table 6. *Expt B. Total volatile fatty acid (VFA) concentrations and molar proportions of individual VFA in caecal contents from rats given a stock diet and two experimental diets based on white rice with and without black-eye beans* (Vigna unguiculata)

(Values are means with pooled standard errors for six rats per group)

Diet†...	Stock 1B	All rice 2B	Rice + beans 5B	SE of mean	Statistical significance of contrasts: Diet 1B *v.* (diet 2B + diet 5B)	Statistical significance of contrasts: Diet 2B *v.* diet 5B
Total VFA (mmol/kg caecal contents)	114	140	154	6·7	**	NS
Molar proportions of VFA (mmol/mol)						
Acetate	652	781	770	10·9	***	NS
Propionate	107	135	111	4·9	*	**
Isobutyrate	6	8	1	1·0	NS	***
Butyrate	214	55	99	11·0	***	*
Isovalerate	9	10	6	0·8	NS	**
Valerate	12	12	13	0·9	NS	NS

NS, not significant.
* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† For details, see Table 1 and p. 641.

in the caecum (estimated as total Cr_2O_3 in the caecum divided by the rate of Cr_2O_3 intake; see Faichney, 1975 and Goodlad & Mathers, 1987) was similar for diets 2B and 5B, but was very much shorter for animals given the stock diet (Table 5).

There was no significant ($P > 0·05$) difference in the total VFA concentration in the caecum for the all-rice and rice + beans diets but, because of the greater caecal contents mass, the VFA pool size was 2·6 times greater for the bean-containing diet. Total VFA concentration in caecal contents was significantly ($P < 0·01$) lower for the stock diet. The proportions of individual VFA were considerably influenced by diet, with a much lower proportion of acetate on diet 1B than on diets 2B and 5B. The proportion of propionate was highest for the all-rice diet and similar for the stock and rice + beans diets. Butyrate proportions were almost four times higher for diet 1B than for diet 2B, with an intermediate value for diet 5B. After taking the caecal masses into account, the caecal butyrate pool sizes were 18, 83 and 123 μmol/rat for the all-rice, rice + beans and stock diets respectively. For the minor VFA, there were no significant differences between diets for valerate, but the rice + beans diet resulted in lower proportions of both *iso*-acids than either the stock or all-rice diets which were similar (Table 6).

Faecal bacteriology

Diet had no detectable effect on faecal concentrations of total anaerobes, whether facultative or obligate, but there were considerable between-diet differences for individual bacterial species (Table 7). Faecal concentrations of total *Bacteroides*, *Bacteroides fragilis* and *Bacteroides vulgatus* were similar for the stock and rice + beans diets but significantly lower for the all-rice diet. *Fusobacterium* sp. concentrations were significantly lower on the rice + beans diets compared with the all-rice diet. *Veillonella* sp. were also significantly lower on the all-rice diet. The latter diet was associated with higher concentrations of anaerobic Gram + ve rods and both rice-based diets (diets 2B and 5B) resulted in higher

Table 7. *Bacterial concentrations in freshly voided faeces from rats given a stock diet and two experimental diets based on white rice with and without black-eye beans* (Vigna unguiculata)

(Values are means with pooled standard errors for six rats per group)

Diet† ... Bacterial species	Mean $\log_{10}$ colony-forming units/g: Stock 1B	All rice 2B	Rice + beans 5B	SE of mean	Statistical significance of contrasts: Diet 1B *v.* (diet 2B + diet 5B)	Diet 2B *v.* diet 5B
Total anaerobes: facultative	9·2	9·4	9·6	0·11	NS	NS
obligate	8·9	9·0	9·3	0·10	NS	NS
Total *Bacteroides*	8·7	7·7	8·6	0·14	**	***
Bacteroides fragilis	8·1	7·0	8·2	013	**	***
Bacteroides vulgatus	8·5	7·3	8·4	0·25	NS	**
Fusobacterium sp.	6·8	7·1	5·0	0·40	NS	**
Veillonella sp.	2·5	2·0	2·8	0·23	NS	*
Bifidobacterium sp.	7·6	8·3	8·4	0·19	**	NS
Eubacterium sp.	6·9	6·2	7·5	0·64	NS	NS
Anaerobic Gram + ve rods	6·2	8·1	4·5	0·84	NS	**
Clostridium sp. Lec + ve	2·7	1·9	1·6	0·23	**	NS
Clostridium sp. Lec − ve	4·4	3·2	2·3	0·44	**	NS
Lactobacillus sp.	8·3	8·3	8·4	0·13	NS	NS
Total aerobes	7·8	8·9	9·2	0·18	***	NS
Enterobacteria	6·5	7·7	7·9	0·29	**	NS
Enterococci	5·8	7·0	6·7	0·32	*	NS

Lec + ve, lecithinase-positive; Lec − ve, lecithinase-negative; NS, not significant.
* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† For details, see Table 1 and p. 640.

faecal concentrations of *Bifidobacterium* sp. and aerobic species. Both lecithinase-positive and lecithinase-negative clostridia were present in higher concentrations in faeces from stock-fed rats than in those given the rice-based diets. Counts of *Lactobacillus* sp. were similar for all three diets (Table 7).

DISCUSSION

The occurrence of vitamin K deficiency symptoms in Expt A was unexpected since the rat is usually considered to be fairly resistant to the development of vitamin K deficiency. Deficiency has occurred in animals given diets low in phylloquinone when coprophagy is prevented (Barnes & Fiala, 1959; Mameesh & Johnson, 1959) or when rats are reared germ-free (Gustafsson, 1959). Administration of antibiotics to rats (Kornberg *et al.* 1944; Matschiner & Doisy, 1965) or man (O'Reilly, 1971; Allison *et al.* 1987) has been used to reduce enteric menaquinone production in studies of vitamin K depletion, and the clinical appearance of vitamin K deficiency is sometimes associated with antibiotic use (Pineo *et al.* 1973; Colvin & Lloyd, 1977; Hooper *et al.* 1980; Krasinski *et al.* 1985). Such findings together with the presence of menaquinones in some species of enteric anaerobes (Collins & Jones, 1981; see Table 8) and the possibility that menaquinones could be absorbed across the large-intestinal mucosa (Hollander *et al.* 1976) suggest, but do not prove, that enterically produced menaquinones are an important source of vitamin K activity. Among the factors which could have contributed to the appearance of vitamin K deficiency symptoms in rats given diet 2A are (1) low dietary phylloquinone concentration (Table 2); (2) low production of menaquinones by the large-intestinal bacteria because of limited

Table 8. *Menaquinones (MK) produced by the main species of the enteric flora**

Species	MK					
	Major components			Minor components		
Bacteroides fragilis	MK-11	MK-10†	MK-12	MK-9	MK-8	MK-7
Bacteroides vulgatus	MK-11	MK-10†	MK-12	MK-9	MK-8	MK-7
Fusobacterium sp.	‡			‡		
Veillonella sp.	MK-7		MK-6			
Eubacterium sp.§	‡			‡		
Eubacterium lentum	MK-6	MMK-6	DMMK-6			
Clostridium sp. Lec+ve	‡			‡		
Clostridium sp. Lec−ve	‡			‡		
Lactobacillus sp.	‡			‡		
Enterobacteria sp.	Q8	MK-8	DMK-8			
Enterococcus sp.	DMK-9		DMK-8	DMK-7	DMK-6	

Q-*n*, ubiquinone; MK-*n*, menaquinone; DMK-*n*, demethylmenaquinone; MMK-*n*, methylmenaquinone; DMMK-*n*, dimethylmenaquinone, where *n* indicates the number of isoprene units in the side-chain; Lec+ve, lecithinase positive, Lec−ve, lecithinase-negative.

* Data from Collins & Jones (1981), Collins *et al.* (1985) and Fernandez & Collins (1987).

† Present in comparable amounts.

‡ Lacks MK.

§ Except *Eubacterium lentum*.

supply of fermentable substrate to the large intestine from this very-low-fibre diet, coupled with the proliferation of a flora in which menaquinone-producers were in a minority; and (3) failure to absorb sufficient menaquinones directly from the large bowel or indirectly from the small intestine after coprophagy. The rats in the present study were housed in metabolism cages with expanded metal floors, which although not completely preventing consumption of faeces reduces this to a relatively insignificant amount (J. C. Mathers, unpublished results; see also Uchida & Komeno, 1988).

Measurements on freshly voided faeces showed that quite different floras were established in animals given the stock diet (1B), the all-rice diet (2B) and the rice + beans diet (5B) (see Table 7). The menaquinone profiles of the main enteric species found in the present study are listed in Table 8. Kindberg *et al.* (1987) inoculated different groups of germ-free rats with groups of organisms which were known to produce menaquinones (*Bacteroides vulgatus* and *Escherichia coli*) or did not produce menaquinones (*Bifidobacterium longum* and *Clostridium ramosum*). They did not detect menaquinones in the faeces or livers from animals given the latter two inoculants, but rats colonized with *Bacteroides vulgatus* had high concentrations of menaquinone MK-10 and significant amounts of MK-9 and MK-11 in faeces, whilst the *E. coli*-inoculated rats had MK-8 and MK-7 as major faecal menaquinones. Liver concentrations of menaquinones mirrored faecal concentrations and were higher in animals housed in wire-bottomed cages than in those in cages designed to prevent coprophagy. These findings demonstrate (1) that the predominant menaquinones of enteric bacteria grown in vivo are the same as those reported from pure-culture studies (Collins & Jones, 1981; Ramotar *et al.* 1984) and (2) that these menaquinones may be absorbed and appear in the liver (and, therefore, are a potential source of vitamin K activity). The results do not clarify to what extent coprophagy is necessary for efficient absorption. Faeces from animals given diet 2B had significantly reduced concentrations of the main menaquinone producers, i.e. *Bacteroides fragilis* and *Bacteroides vulgatus* (Table 7) and this coupled with the very low faecal output on this diet (Table 4) suggests that total enteric menaquinone production was low for animals given the all-rice diets (2A and 2B).

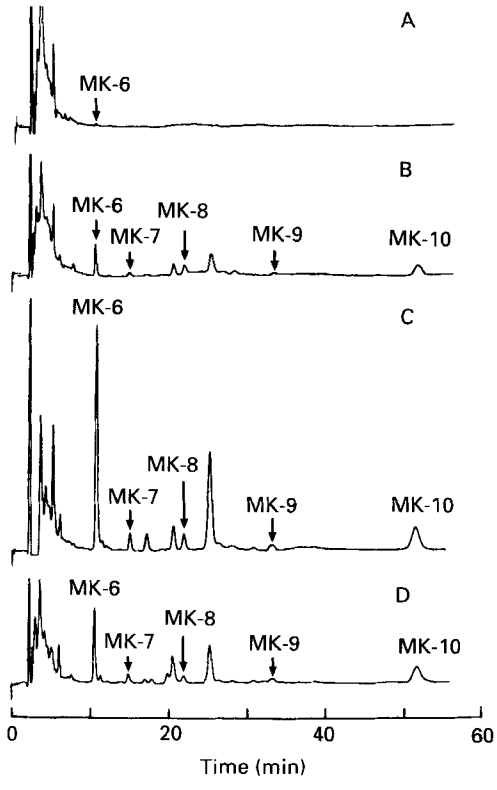

Fig. 1. Expt 1. Chromatograms illustrating the menaquinone (MK) profiles in the livers of four representative rats (A to D) given experimental diets 2A to 5A respectively. The rat numbers, liver phylloquinone (K_1) concentrations and prothrombin times (PT) are given below in parenthesis.

A. Diet 2A (rat 36, liver K_1 0·13 ng/g, PT > 60 s).
B. Diet 3A (rat 21, liver K_1 0·36 ng/g, PT 24 s).
C. Diet 4A (rat 19, liver K_1 0·52 ng/g, PT 15 s).
D. Diet 5A (rat 18, liver K_1 0·33 ng/g, PT 15 s).

Initial results of menaquinone measurements on the livers from rats from Expt A indicate very much lower concentrations of menaquinone when the all-rice diet (2A) was given than when bean-containing diets were eaten.

Chromatograms of hepatic menaquinones in four representative rats each given one of the four experimental diets (2A–5A) are shown in Fig. 1. Two of these rats (nos. 36 and 21) were vitamin K-deficient as evidenced by the prolonged prothrombin times and low levels of individual vitamin K-dependent clotting factors (Table 3). The most severely vitamin K-deficient rat (no. 36) with a prothrombin time of > 60 s had been given the all-rice diet and had both the lowest liver phylloquinone concentration (0·13 ng/g) and no detectable menaquinones apart from a trace of MK-6 (Fig. 1A). Rat no. 21 with a prothrombin time prolonged to 24 s also showed evidence of lower hepatic MK-6 and MK-10 concentrations (Fig. 1B) compared with rats fed on diets containing a higher proportion of beans (Fig. 1C, D).

The reasons for the altered faecal flora observed here are uncertain. Classical studies in man (see Moore & Holdeman, 1975; for review, see Borriello, 1986) have indicated that whilst there is considerable inter-person variability, the faecal flora of an individual is stable and not readily changed by dietary means. However, the metabolic activity of the large-

bowel flora is influenced by diet. For example, the polysaccharide-degrading systems of *Bacteroides* appear to be inducible in vitro (Salyers, *et al.* 1985), and Wyatt *et al.* (1986) have reported a rapid increase in the numbers of faecal flora able to degrade gum arabic after this polymer was included in the diet of a human volunteer. Several studies have shown that dietary manipulation changes the pattern of VFA which are the major fermentation end-products of enteric bacteria (Horn *et al.* 1986; Cheng *et al.* 1987; Key & Mathers, 1987). Marked changes in VFA pattern were also observed in Expt B (see Table 6). Inclusion in the diets of rats of fibre sources such as pectin, or of starches which are resistant to small-intestinal digestion may alter the activities of enzymes such as nitrate reductase (*EC* 1.7.99.4) in faecal samples (Mallett *et al.* 1987), and β-glucuronidase (*EC* 3.2.1.31) and nitroreductase in caecal contents (Mallett *et al.* 1988). Oral administration of antibiotics such as bacitracin and erythromycin alter the pattern of VFA in human faeces (Hoverstad *et al.* 1986), whilst the growth-promoting antibiotic Avoparcin alters caecal VFA in rats (Mathers & Finlayson, 1989). Dietary manipulation may provoke changes in VFA end-products because of alterations in substrates flowing to the large bowel (Goodlad & Mathers, 1988) and alterations in the environmental conditions of the large intestine such as pH or transit time. The results in Table 5 show that those diets which would be expected to supply the greater amounts of substrate to the large bowel (diets 1B and 5B) resulted in larger caecal masses and lower pH. Diet 1B which contained the greatest proportion of indigestible material was associated with very short caecal transit times, which is likely to have increased the selective pressure on the caecal flora. It is likely that it was the differences in amounts and types of dietary carbohydrate which were responsible for the changes in both flora and fermentation end-products observed in the present study. Addition of wheat bran to a maize-based diet resulted in significant changes in the faecal flora of pigs, whilst addition of maize oil had no effect (Moore *et al.* 1987). Similarly replacement of white by wholemeal bread in the diet of rats provoked large changes in caecal VFA patterns (Key & Mathers, 1987) which were unaffected by dietary oil concentration (Key & Mathers, 1988).

Since the present study was completed, a report has appeared by Uchida & Komeno (1988) who compared menaquinone concentrations in intestinal contents and liver and who concluded that 'menaquinones do not seem to play a significant role in clotting factor synthesis in rats'. In contrast, Ramotar *et al.* (1988) reported that rats fed on cooked rice-based diets (O'Reilly, 1971) had lower concentrations of phylloquinone in blood and liver when compared with chow-fed animals, but prothrombin clotting time was increased and caecal menaquinone concentrations reduced only when animals received subcutaneous injections of the antibiotic moxolactam. Ramotar *et al.* (1988) concluded that 'diets have a major effect upon intestinal menaquinone profiles, and secondarily (*sic*) may alter intrahepatic stores of vitamin (K)'. The effectiveness of intestinally-derived menaquinones as a source of vitamin K remains controversial. However, our study has shown that dietary modification using normal foods may alter the potential vitamin K supply from enteric bacterial menaquinones by altering the density of large-bowel menaquinone-producing bacteria. Further work is needed to explain the reasons for such changes.

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