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Total body phylloquinone and its turnover in human subjects at two levels of vitamin K intake

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The aims of this study were to determine the total body phylloquinone and its metabolic turnover in human subjects using a tracer dose of [5-H^3]phylloquinone containing $55{\cdot}5 \times 10^4$ MBq/mmol. Seven subjects aged 22 to 49 years were given 0·3 μg isotopic phylloquinone intravenously on a control diet (75 μg phylloquinone/d) and blood, urine and faeces were sampled periodically for 6 d. Five of these subjects were studied a second time after 3–8 weeks on a low-vitamin K diet (8 μg/d). The changes in the radioactivity of plasma phylloquinone with time were analysed by the method of residuals and fitted to a curve composed of two exponential components. The size of the exchangeable body pool was calculated by isotope dilution. Plasma phylloquinone levels fell during vitamin K restriction but the vitamin K-dependent coagulation factors did not change. After injection the first exponential decay curve $t_{1/2}$ was 1·0 (SD 0·47) h in the subjects on the control diet and 0·49 (SD 0·27) h after vitamin K restriction. On the control diet, the second exponential $t_{1/2}$ was 27·6 (SD 124) h that did not change on the low-vitamin K diet ($t_{1/2} = 25{\cdot}1$ (SD 13·5) h). These results indicate that the turnover time for phylloquinone in human subjects is about 1·5 d. Urinary excretion of ^{3}H-metabolites ranged from 30 % of the administered dose on the control diet to 38 % on the restricted diet and had the same turnover rate as the second component of the plasma decay curves. The exchangeable body pool of phylloquinone declined from about 1·0 μg/kg before restriction to lower values after vitamin K restriction. The faecal excretion of phylloquinone and its metabolites fell from 32 % of the administered dose on the control diet to 13 % on the restricted diet.

Vitamin K: Radioactive phylloquinone: Exchangeable body pool: Metabolic turnover

Great advances have been made in the understanding of the distribution, function and metabolism of vitamin K since its discovery by Henrik Dam. (Dam, 1935; Olson, 1999) Less well documented is the content of vitamin K in the human body and the extent of its metabolic turnover. The first attempt to measure phylloquinone turnover in human subjects was made by Shearer *et al.* (1972), who administered 1 mg [1′, 2′-$^3H_2$]phylloquinone (200 MBq/mmol) intravenously to each of three young male volunteers whose plasma was sampled at intervals for 96 h. They observed that after 2 h only 10 % of the initial radioactivity remained in the plasma space, and that the decay curves over 6 h could be resolved into two exponential functions with half-times of 20–24 min and 121–150 min. Later Shearer *et al.* (1974) repeated their study using 45 μg-phylloquinone of higher specific activity (3515 MBq/mmol) and obtained similar results. Bjornsson *et al.* (1979) carried out similar turnover studies in four healthy male volunteers using 300 μg [1′, 2′-$^3H_2$]phylloquinone (3237 MBq/mmol). They also observed two exponential components to the decay curves of 26 (SD 8) min and 166 (SD 10) min over 10 h.

None of these investigators could calculate the body pool of phylloquinone because at that time there was no suitable method for measuring phylloquinone in plasma. The first method for measuring plasma vitamin K utilizing HPLC and u.v. absorption as a method of detection was published by Shearer *et al.* (1982) and showed that the

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concentration of plasma phylloquinone was in the pM range. The procedure was subsequently improved with respect to methods of detection by employing electrochemical (Ueno & Suttie, 1983) and fluorescence (Haroon *et al.* 1986) techniques. As more results on the phylloquinone content of animal foods become available (Booth *et al.* 1996*b*; Shearer *et al.* 1996), it became clear that the vitamin K content of animal organs was very low (1–10 μg/kg) suggesting that the body pool of vitamin K in man was of the order of 100 μg. Thus, the doses of phylloquinone given in the previous studies either exceeded or approached the size of the body pool. As a result, the turnover values were in the pharmacologic rather than the physiologic range.

The only solution to this problem was to obtain labelled phylloquinone of a much higher specific activity than that used in earlier studies. Some years ago, Joseph Würsch at the Hoffmann-LaRoche Company in Basel, Switzerland, devised a method to synthesize [5-H^3]phylloquinone with a specific activity of $55{\cdot}5 \times 10^4$ MBq/mmol. The repetition of his synthesis enabled us to inject a fraction of a μg of isotopic phylloquinone with a total radioactivity of about $37{\cdot}0 \times 10^4$ MBq in human subjects and measure its turnover under more physiological conditions. In addition, we have compared the turnover of phylloquinone in human subjects at two levels of vitamin K intake (75 *v.* 8 μg/d). A preliminary report of this work has been published (Olson *et al.* 1984).

Subjects and methods

Subjects

Seven healthy adults were chosen from a number of volunteers that were obtained through newspaper advertising and notices posted in the University of Pittsburgh (Pittsburgh, PA, USA). Each volunteer was interviewed by a staff physician and dietitian. A medical and dietary history and a physical examination were completed on each subject. In addition, laboratory tests were conducted that included a complete blood count, urine analysis, and the measurement of plasma glucose, uric acid, Ca, total protein, albumin, alkaline phosphatase, total and direct bilirubin, lactic dehydrogenase, serum glutamic oxalacetic transaminase, blood urea-N, inorganic phosphate, cholesterol, triacylglycerol, creatinine, and the thyroid hormones triiodothryronine and tetraiodothryonine. Coagulation studies included a partial thromboplastin time and prothrombin time. Two laboratory workers, a 49-year-old premenopausal female and a 31-year-old male and five young unemployed male steel workers ranging in age from 22 to 35 years of age were accepted as participants in the study. They were free of cardiovascular, endocrine, hepatic, gastrointestinal and haematologic disease and were non-smokers.

The two laboratory workers (subjects 1 and 2) were studied as outpatients at St Louis University Medical Center, St Louis, MO, USA, in 1982 and the five steel workers (subjects 3–7) were studied as in-patients on the metabolic unit of the University of Pittsburgh Clinical Research Center, Presbyterian University Hospital, Pittsburgh, PA, USA from 1982–3. Three of them completed the full study, but two of them left before completion of the period of vitamin K restriction. Each study was approved by the Human Investigation (IRB) Committees of St Louis University School of Medicine and the University of Pittsburgh School of Medicine. A signed informed consent form was obtained from each subject. The mean value of the BMI of these subjects was 25·3 kg/m^2 and packed cell volume 45·6 %. The plasma volumes were calculated from blood volumes derived from body weight (Weenesland *et al.* 1959) and total body water estimated from the BMI (Edelman & Liebman, 1959; Ritz, 2000).

Protocol

The protocol consisted of a control period of 1–2 weeks, followed by the feeding of a low-vitamin K diet for 3–8 weeks. [5-H^3]Phylloquinone ($55{\cdot}5 \times 10^4$ MBq/mmol) in amounts varying from 216–408 ng (0·27–0·50 MBq)/ml human serum albumin (50 ml/l) was injected intravenously into each subject at the end of the control period, and again at the end of the low-vitamin K diet period. The low-dose

Table 1. Composition of control and low-vitamin K diets*

Diet	Energy (MJ/d)	% Energy from: Protein	Carbohydrate	Fat	Phylloquinone (μg/d) Mean	SD
Control	8·03–12·80	14	50	36	75	10
Low-vitamin K	7·95–12·55	14	66	20	8	2

* Vivonex High Nitrogen formula (Norwich-Easton Pharmaceuticals, Norwich, NY, USA) (12·6 MJ/kg) supplied 52·8 g essential L-amino acids/kg (g/kg): isoleucine 5·76, leucine 9·12, lysine 6·61, methionine 6·36, phenylalanine and tyrosine 11·02, threonine 5·77, tryptophan 1·77, valine 6·36, non-essential amino acids 85·8 (including (g): alanine 7·19, arginine 5·65, aspartic acid 15·35, glutamine 25·29, glycine 13·65, histidine 3·28, proline 9·60, serine 5·76). Vitamins and minerals except for vitamin K were supplied in amounts to meet or exceed the recommended dietary allowance (National Research Council, 1980) including 'safe and adequate amounts of other vitamins and minerals'. Carbohydrates were supplied as glucose and glucose oligosaccharides and fat as safflower oil plus linoleic acid. Energy in the low-vitamin K diet was supplied by the Vivonex High Nitrogen formula (85 %, energy) and by a food supplement containing ginger ale, Jell-O, crackers, popcorn, cornflakes, tuna, spaghetti, yogurt, oranges, bananas, cornmeal, margarine and strawberries (15 % energy).

vitamin K diet was fed to the two laboratory workers for 3 weeks. The diets are described in Table 1 (Greenstein *et al.* 1957; Winitz *et al.* 1965) The control diet contained 75 (SD 5) μg phylloquinone as listed in food tables (Booth *et al.* 1996*a*; Shearer *et al.* 1996) and the low-vitamin K diet contained 8 (SD 2) μg phylloquinone as listed in the above food tables and determined by a bioassay in warfarin-resistant rats (Hermondson *et al.* 1969). Samples of blood, urine and faeces were collected for 6 d after each injection. Heparinized blood (5 ml) was collected at 10 min, 30 min and 1·0, 2·0, 4·0 and 10·0 h after the injection on the first day and then daily for 7 d to measure the ^{3}H content of the plasma. The two laboratory workers received all of their meals in the diet-kitchen of the St Louis University Medical School. The others were studied in a metabolic unit of the University of Pittsburgh. All five steel workers received the control diet and the first injection of [5-H^3]phylloquinone. Three of the five steel workers (subjects 3–5) consumed the low-vitamin K diet for 8 weeks and during the last 4 weeks, each of these subjects also received one of three supplements designed to enhance the vitamin K deficiency, i.e. 3 g. neomycin/d (subject 3) (Frick *et al.* 1967), 1 g D-α-tocopherol/d (subject 4) (Corrigan & Marcus, 1974) and 15 mg retinol/d (subject 5) (Matschiner *et al.* 1967). Each received the second injection of [5-H^3] phylloquinone at the end of the 8 weeks. The energy intake, estimated from dietary histories of the subjects varied from 8·03–12·8 MJ/d. During the control period the energy intake was adjusted (within 5 %) to maintain entry weights on each subject.

Fasting plasma phylloquinone and the vitamin K-dependent factors II, VII, IX, X and the II:Ag ratio were measured weekly. Serum cholesterol, triacylglycerol, carotene, retinol and α-tocophenol were measured at 2-week intervals.

Twenty-four hour urine samples were collected daily for 6 d in plastic containers using thymol as a preservative following the isotopic phylloquinone injections. Aliquots were frozen and stored briefly for measurements of radioactivity. Faeces were collected in pre-weighed paint cans and weighed daily. Distilled water was added to the paint can to obtain a combined weight of water and faeces of 1000 g and these stool samples were shaken with beads for 15 min. Duplicate aliquots of the stool were transferred to centrifuge tubes and two volumes of Folch reagent ($CHCl_3$-methanol (2:1, v/v) added. Each tube was shaken for 30 min, spun and aliquots taken from both the methanol–water and $CHCl_3$ layers to determine total radioactivity.

Methods

Synthesis of [5-^{3}H]phylloquinone

The synthesis of the radioactive phylloquinone was accomplished according to the method of Josef Würsch of the Research Laboratories of Hoffmann-LaRoche, Basel, Switzerland. In order to label phylloquinone with ^{3}H specifically in the 5-position, 2-napthoic acid was brominated to form the 5-bromo-2-napthoic acid. The product was methylated with diazomethane, and then reduced with lithium aluminum hydride to yield 5-bromo-2-methyl naphthalene. Next, 5-bromo-2-chloromethylnapthalene was formed with sulfonyl chloride and reduced with lithium aluminum hydride to form 5-bromo-2-methylnapthalene. This derivative was then oxidized with chromic oxide to 5-bromo-4-naphthoquinone and the diacetate formed by catalytic reduction with H_2 and esterification with acetic anhydride. The 5-bromo-2-methyl-1,4-naphthoquinone diacetate was then reduced catalytically with nascent tritium ($107{\cdot}2 \times 10^4$ MBq) to displace the bromine and form the [5-^{3}H]-2-methyl-1,4-naphthohydroquinone diacetate. The labelled product was then hydrolysed with potassium hydroxide and the [5-^{3}H]-2-methyl-1,4-naphthohydroquinone condensed with phytol in the presence of boron trifluoride etherate to yield [5-^{3}H] phylloquinone. The final product was purified by HPLC to a constant specific activity of $55{\cdot}5 \times 10^4$ MBq mmol. It showed a single peak on TLC and had a classical u.v. spectrum.

To prepare a solution for injection, an aliquot of the isotopic phylloquinone in acetonitrile was evaporated under N_2 and taken up in 0·2 ml US Pharmacopeia ethanol and again taken to dryness. This was repeated several times to insure complete removal of the acetonitrile. The radioactive phylloquinone was then transferred to a tube containing human serum albumin (50 ml/l) with three 100 μl aliquots ethanol and filtered through a sterile 20 μm nalgene filter. This solution (1 ml) was injected intravenously into each subject.

To test for sterility, aliquots of the solution of [^{3}H]phylloquinone were placed in thioglycol tubes and incubated at 37°C for 1 week. The tubes showed no signs of bacterial growth. The sterile human serum albumin solution (1 ml) was injected into a 2 kg rabbit whose temperature showed no elevation for 48°C. No rash or other signs of toxicity were noted.

Measurement of plasma phylloquinone

The phylloquinone in plasma was measured by the method of Ueno & Suttie (1983), which depends upon the extraction with hexane–propanol (2:1, v/v) of 3–5 ml plasma spiked with [5-^{3}H-]phylloquinone (0·27 ng containing 333 Bq). Preliminary separation of phylloquinone from other lipids in the hexane extract was accomplished on a silicic acid semi-preparative HPLC column with tetrahydrofuran (750 ml/l) and hexane as the mobile column. A portion of the eluate, identified by the presence of [^{3}H] phylloquinone, was dried, redissolved in methanol–ethanol (40:60, v/v) and injected into a μ-Bondapak C_{18} analytical column (Waters Corporation, Milford, MA, USA) using buffered methanol–ethanol–water (27:57:8, by vol) containing sodium perchlorate (0·4 ml/l) and perchoric acid (0·02 ml/l) at a flow rate 1·5 m1/min. The concentration of the vitamin was determined by reductive electrochemical detection using a glassy C electrode and an Ag–AgCl reference electrode. The potential was maintained at −400 mV *v.* the reference electrode and the system was flushed with N_2 to exclude O_2. The amount of phylloquinone was determined from the peak height of the analytical HPLC chromatogram and corrected for

recovery of the added radioactive 5-^{3}H-phylloquinone that ranged from 50–80 %.

Measurement of radioactivity

Levels of ^{3}H in the plasma and urine samples were determined by adding aliquots of 1 or 2 ml plasma, urine or faecal extracts to 20 ml aqueous counting solubiliser (Amersham, Bucks., England) solubiliser and counting in a Beckman liquid scintillation spectrometer (Beckman Co., Palo Alto, CA, USA). The observed counts were converted to d.p.m. using efficiencies determined by the channels ratio method and then to Bq. Selected plasma samples were subjected to extraction with hexane–ispropanol and chromatographed on silica gel. Greater than 90 % of the radioactivity was present in the phylloquinone fraction for the first 2 h of the study.

Measurement of clotting factors

Prothrombin time was measured on automated equipment obtained from the Instrumentation Laboratory, Lexington, MA, USA. Assays for factors II (prothrombin), VII, IX and X were determined in one-stage clotting assays employing plasmas deficient in each factor. Factor II antigen was determined by the rocket electrophoresis method

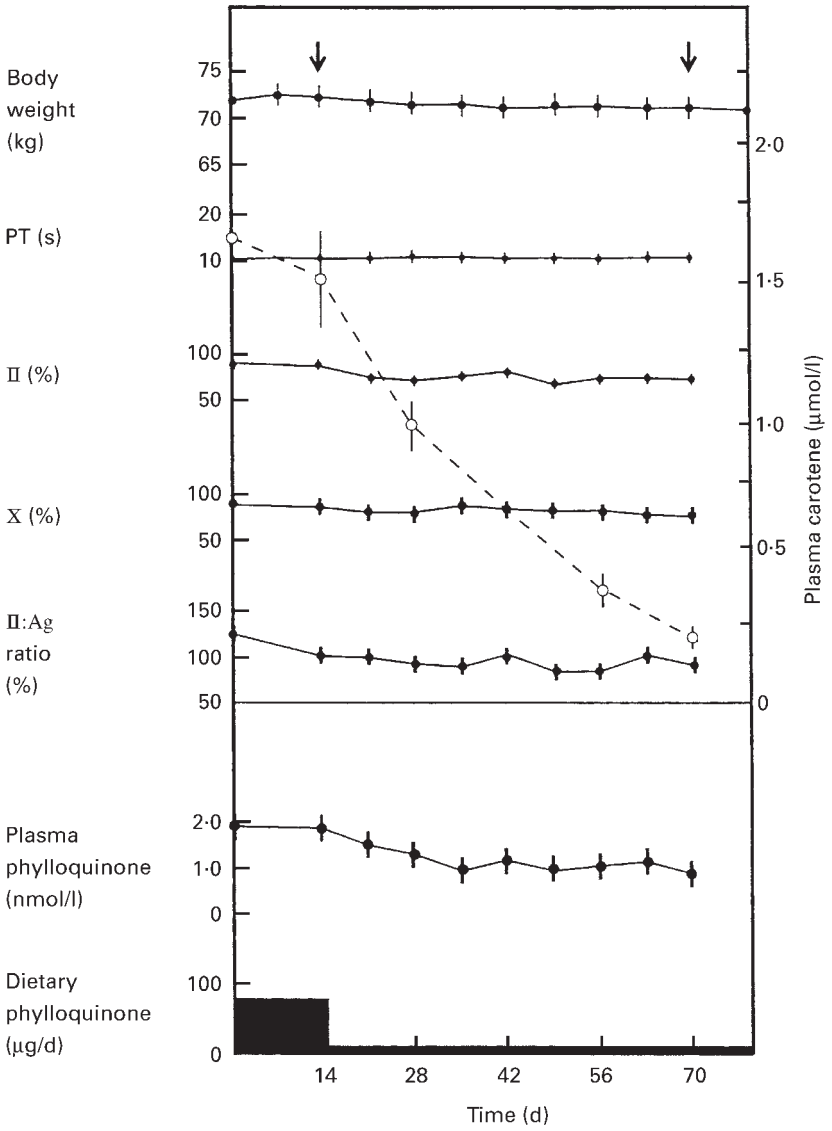

Fig. 1. Weekly changes in body weight, prothrombin time, coagulation factor activity (II, X, II:Ag ratio), plasma phylloquinone and dietary intake of phylloquinone for subjects (3, 4 and 5) who completed the 8-week period of vitamin K restriction. For details of subjects, diets and procedures, see Table 1 and p. 544. – – ○ – – Plasma carotene; ↓, time of injections of isotopic phylloquinone; PT, prothombin time. Factors VII and IX were normal and are not shown. Values are means for three subjects with standard errors shown by vertical bars.

Table 2. [5-^{3}H]Phylloquinone metabolism in human subjects*

	Control diet								Low-vitamin K diet							
	Total [^{3}H]phyllo-quinone		Intercept 1			Intercept 2	$t_{1/2}$		Total [^{3}H]phyllo-quinone		Intercept 1			Intercept 2	$t_{1/2}$	
Subject	MBq	Bq/ml	(% Bq)	$t_{1/2}$ (h)	k_1 (h)	(% Bq)	(h)	k_2 (h)	MBq	Bq/ml	(% Bq)	$t_{1/2}$ (h)	k_1 (h)	(% Bq)	(h)	k_2 (h)
1	0·30	138	17·8	1·14	0·61	4·95	54·2	0·0128	0·32	138	37·6	0·210	3·30	17·5	6·56	0·1056
2	0·32	115	14·3	0·67	1·03	4·09	32·1	0·0216	0·32	115	11·9	0·910	0·76	5·52	23·62	0·0293
3	0·27	107	16·8	2·09	0·33	4·88	28·7	0·0241	0·38	152	18·5	0·741	0·94	3·62	16·96	0·0409
4	0·32	95	39·6	0·70	0·99	1·96	27·2	0·0255	0·47	138	17·7	0·299	2·32	1·72	46·58	0·0149
5	0·30	125	16·8	0·68	1·03	1·76	18·9	0·0366	0·50	210	10·6	0·306	2·27	1·09	31·56	0·0220
6	0·27	79	29·1	0·68	1·02	6·77	16·8	0·0412								
7	0·27	100	34·8	0·97	0·71	4·87	15·4	0·0450								
Mean			24·2	0·99	0·82	4·18	27·6	0·0296			19·3	0·493	1·92	5·9	25·06	0·0425
SEM			3·6	0·18	0·10	0·63	4·7	0·004			4·3	0·124	0·41	2·7	6·04	0·015
Statistical significance of effect: P†			NS	<0·05	<0·05	NS	NS	NS			NS	<0·05	<0·05	NS	NS	NS

*For details of subjects, diets and procedures see Table 1 and p. 544.
†Control diet *v.* low-vitamin K diet.

of Laurell (1972) using a commercial rabbit antibody to human prothrombin.

Measurement of plasma lipids

Serum cholesterol was measured by the method of Abell *et al.* (1952) and serum triacylglycerol by the method of Van Handel & Zilversmit (1957). Plasma rentinol and carotene were determined by the method of Neeld & Pearson (1963) which employs the Carr–Price reaction using fluoroacetic acid. Plasma α-tocopherol levels were determined by the method of Quaife *et al.* (1949) which is based upon the Emmerie–Engel reaction.

Analysis of data

The isotopic decay curves were analysed by the method of residuals from 10 min onward (Gibaldi & Perrier, 1982). These calculations ignore the very fast initial rate of decay due to dilution in the plasma space ($t_{1/2}$ 3–4 min) and represents an error of less than 5 %. The data were fitted to a curve with two exponential components describing a two-compartment open model:

$$P = A_1 e_1^{-k_1 \cdot t} + A_2 e_2^{-k_2 \cdot t}$$

The initial radioactivity was calculated by dividing the radioactivity of the injected dose by the plasma volume to obtain Bq/ml. P is the % remaining radioactivity at any time, t is time, A_1 and A_2 are the two amplitudes and k_1 and k_2 are the two fractional turnover rates. These four variables was determined for each data set to obtain the best fit to the above equation according to a least squares algorithm using the Microsoft EXCEL program (Richmond, WA, USA).

The exchangeable body pool of phylloquinone (A) in the organism was calculated by the equation:

$$A = i(\alpha/\alpha_0 - 1),$$

where i is the amount of isotopic substance given, α is the specific activity of the [5–^{3}H]phylloquinone injected and α_0 is the specific activity of the body pool at t 0 calculated from the intercept of the second exponential curve.

The fractional turnover constant $k = 0{\cdot}693/t_{1/2}$ where $t_{1/2}$ is the time required to half the relevant radioactivity. The absolute turnover rate is obtained by multiplying the pool size by k ($a = Ak$). The turnover time $t_t = 1/k = 1{\cdot}44\, t_{1/2}$.

The results are presented as the mean values and standard deviations unless specified otherwise. The significance of differences between subjects on the control diet and on the vitamin K restricted diet was examined by unpaired t tests (two-tailed). Differences with $P < 0{\cdot}05$ were considered significant. These calculations were performed with SPSS for Windows, version 10·1 (SPSS Inc., Chicago, IL, USA).

Results

Clinical and laboratory findings

All seven of the subjects remained healthy during the study. As noted earlier, a medical and dietary history was obtained from each subject and a physical examination and various laboratory tests were done before beginning the study. Besides clotting assays, various tests to measure haematologic, renal, liver and endocrine function were performed at the beginning and at the end of the study. Except for subject 1, all results remained within normal limits. The plasma phylloquinone values decreased from 1·94 (SD 0·76) to 1·05 (SD 0·54) nmol/l during the study, a change of 46 % as shown in Fig. 1, although it is likely that the true values were lower as noted on p. 547. There was, nevertheless, no evidence of bleeding as indicated by the lack of petechiae, ecchymoses, anaemia, haematuria or occult blood in the stool. There were no significant changes in body weight or in the level of coagulation factors in any of the subjects on the control or even those on the low-vitamin K diet for 8 weeks (Fig. 1) as also noted by others (Allison *et al.* 1987; Suttie *et al.* 1988; and Ferland *et al.* 1993). Evidence of adherence to the diet by these subjects was obtained by noting changes in their plasma lipids before and after 8 weeks on the low-vitamin K diet that was also low in fat, high in polyunsaturated fat and devoid of cholesterol and carotene. In the three subjects who completed the 8-week study, serum cholesterol fell from 4·60 (SD 0·49) mmol/l (1·78 (SD 0·19) μg/l) to 2·84 (SD 0·21) mmol/l (1·10 (SD 0·08) μg/l); and triacylglycerol fell from 1·36 (SD 0·0·06) mmol/l (1·23 (SD 0·06) μg/l) to 0·61 (SD 0·12) mmol/l (0·55 (SD 11) μg/l. There was a good correlation between phylloquinone levels and total serum cholesterol.(r 0·97, $P<0·005$), but a poor correlation between phylloquinone and triacylglycerol levels (r 0·63, not significant at the $P<0·05$ level). This suggests that the principal carrier for phylloquinone during restriction of phylloquinone was LDL. Plasma carotene fell from 1·56 (SD 0·31) μmol/l (840 (SD 170) μg/l) to 0·24 (SD 0·05) μmol/l (130 (SD 30) μg/l) as shown in Fig. 1.

The 49-year-old female subject (subject 1) was the only subject to demonstrate a significant change in coagulation factor levels. After 3 weeks on the low-vitamin K diet, her prothombin time increased from 13·1 to 22·9 s, her factor II (prothrombin) declined from 102 to 50 % and her factor X decreased from 78 to 29 %. Her plasma phylloquinone declined from 0·82 to 0·51 nmol/l and her body phylloquinone shrank from 17·1 to 2·8 μg. She, like the others, did not demonstrate any evidence of bleeding despite these changes. She received an intramuscular dose of 1 mg phylloquinone after completing the study.

The use of daily 'stressor' supplements to attempt to increase the vitamin K requirement, i.e. 2·0 g neomycin (subject 3), 1·0 g D-α-tocopherol (subject 4) and 15 mg retinol (subject 5) for the last 4 weeks of the low-vitamin K diet appeared to have little or no effect on plasma phylloquinone. The plasma α-tocopherol level of subject 4 who received the α-tocopherol supplement rose from 16·9 μmol/l (7·10 mg/l) to 26·0 μmol/l (11·20 mg/l) over the 4-week period. Subject 5, who received the retinol supplement, also showed an increase in plasma retinol from 1·46 μmol/l (410 μg/l) to 1·9 μmol/l (550 μg/l). Retinyl esters were not determined.

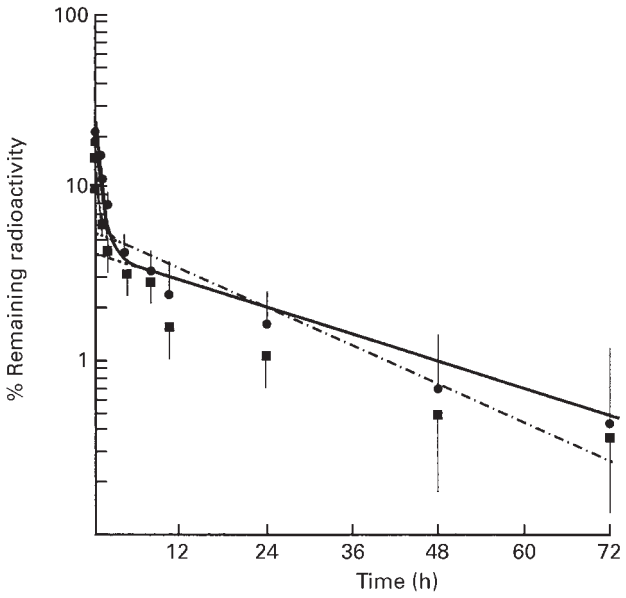

Fig. 2. Plots of the phylloquinone radioactivity *v.* time after intravenous injection of [5-^{3}H]phylloquinone in seven subjects on the control diet and five of the same subjects after dietary vitamin K restriction for 8 weeks. For details of subjects, diets and procedures, see Table 1 and p. 544. ●, Mean values before the low-vitamin K diet, ■, mean values following the low-vitamin K diet; ——, before the low-vitamin K diet, – · – · –, following the low-vitamin K diet. The lines represent the best fit of all the data in each case and the long linear portion represents the second exponential of each curve with extension to show the intercept at zero time. Values are mean with standard errors shown by vertical bars.

Phylloquinone kinetics

Of the subjects who entered the study, plasma kinetics of [5-^{3}H] phylloquinone were obtained for all subjects after the control diet and for five subjects after taking the low-vitamin K diet for 8 weeks (three subjects) or three weeks (two subjects).

The results on phylloquinone turnover derived from these studies are shown in Table 2 and Fig. 2. The initial values of plasma radioactivity were calculated from the dose injected and the plasma volume and the decline in plasma radioactivity with time was computed as a percentage of the initial radioactivity. Data on zero time intercepts, $t_{1/2}$ and k (fractional turnover rates) for the two exponential curves obtained before and after ingestion of the low-vitamin K diet are shown in Table 2. Approximately 75 % of the injected radioactivity disappeared from the plasma after 10 min and 90 % by 2 h.

The half-time $t_{1/2}$ for the first exponential component was 1·0 (SD 0·47) h for subjects on the control diet and 0·49 (SD 0·28) h for subjects on the low-vitamin K diet, ($P<0·05$) . This was also reflected by reciprocal changes in the k values. The half-times for the second exponential component were 27·6 (SD 12·4) h for the subjects on the control diet and 25·1 (SD 13·5) for the subjects on the low-vitamin K diet. The mean uptake of phylloquinone

Table 3. Plasma and body phylloquinone content in human subjects before and after feeding the low-vitamin-K diet*

Before/after	Subject	Dose injected † MBq	Dose injected † Amount (ng)	Plasma phylloquinone (nmol/l)‡	Specific activity phylloquinone (μBq/ng)	Pool size (μg)	Pool size (μg/kg)
B	1	0·32	261	0·82	18·37	17·11	0·281
A		0·32	261	0·51	105·58	2·76	0·046
B	2	0·32	261	2·91	3·58	88·76	1·18
A		0·32	261	2·00	7·05	44·98	0·60
B	3	0·26	216	2·04	5·67	46·20	0·72
A		0·37	306	1·24	9·82	37·70	0·58
B	4	0·32	261	2·53	1·63	194·7	2·17
A		0·46	378	0·89	5·92	77·75	0·86
B	5	0·30	243	1·40	3·48	85·02	1·34
A		0·50	408	0·62	8·18	60·52	0·95
B	6	0·26	216	3·33	1·97	132·6	1·28
B	7	0·27	222	1·87	5·48	49·26	0·76

B, before receiving the low-vitamin K diet; A, after receiving the low-vitamin K diet.
* For details of subjects, diets and procedures, see Table 1 and p. 544.
† [5-^{3}H]phylloquinone was injected intravenously at the end of the control period and again at the end of the low-vitamin K diet period.
‡ Plasma phylloquinone in ng/ml may be obtained by dividing the values in nmol/l by 2·22.

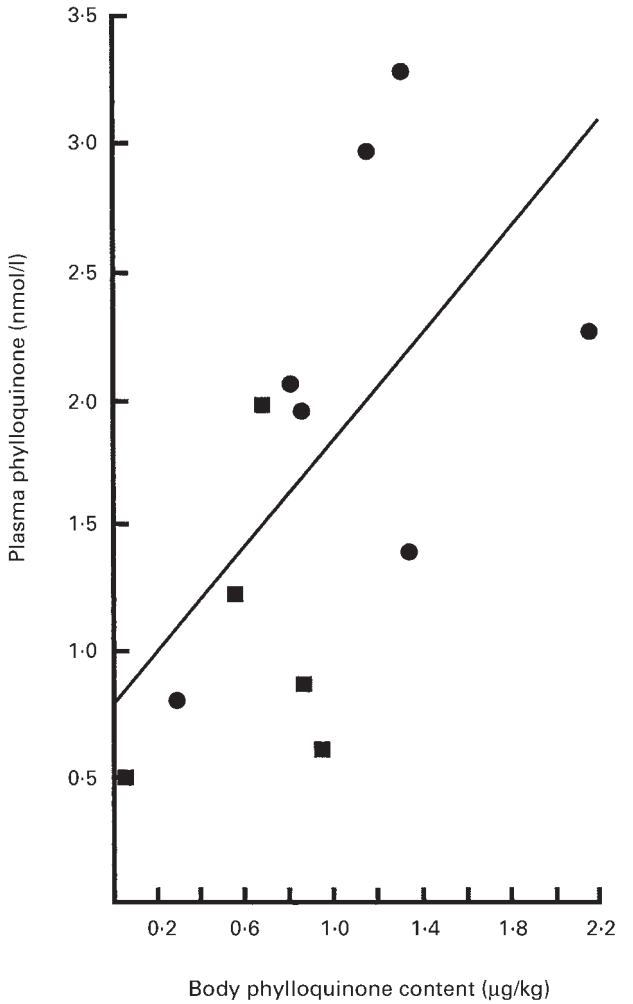

Fig. 3. A plot of the values for plasma phylloquinone (nmol/l) *v.* body phylloquinone (μg/kg) for subjects before and after dietary vitamin K restriction for 8 weeks. For details of subjects, diets and procedures, see Table 1 and p. 544. ●, Values of seven subjects before vitamin K restriction; ■, values for seven subjects after vitamin K restriction. $y = 0{\cdot}80 + 0{\cdot}99x$, where y is plasma phylloquinone and x is the body pool $r + 0{\cdot}60$, ($P < 0{\cdot}05$).

by the tissues appeared to be more rapid after vitamin restriction, but the ultimate metabolic disposition occurred at the same rate. The corresponding turnover times were 39·7 (SD 17·9) and 36·1 (SD 19·4) h respectively. These insignificant differences are also shown in Fig. 2. This presents decay curves that best fit the data calculated by the method of least squares before and after vitamin K restriction.

The phylloquinone pool size determined by isotope dilution is shown before and after vitamin K restriction in Table 3. These values are calculated from the zero

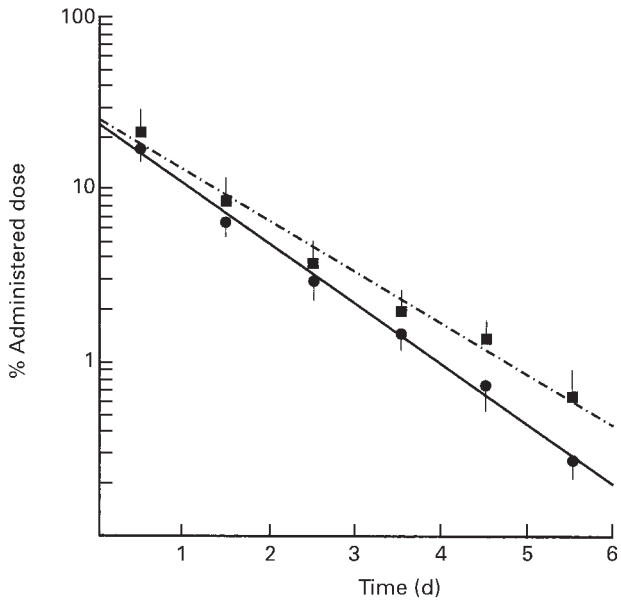

Fig. 4. Plots of total urinary ^{3}H *v.* time for six subjects on the control diet and for five subjects after dietary restriction of vitamin K. For details of subjects diets and procedures, see Table 1 and p. 544. ●, mean values before the low-vitamin K diet; and ■, mean values following the low-vitamin K diet; ——, regression line for subjects on the control diet; – · – · –, regression line for subjects after vitamin K restriction. Values are means with standard errors shown by vertical bars.

time intercept of the second exponential curve that represents the metabolic turnover of the body pool. The computed body pools of phylloquinone varied from 17–194 μg phylloquinone with mean value of 87·6 (SD 55·6) μg phylloquinone. These values shrank to a mean value of 44·7 (SD 25·1) after vitamin K restriction. When expressed on a body-weight basis, the values before vitamin K restriction were 1·14 (SD 0·64) μg/kg and after restriction 0·61 (SD 0·32) μg/kg. The difference of 0·53 μg/kg was a change of 47 % ($P<0·05$) . When the values for plasma concentration were plotted against the body pool for each case, the plot shown in Fig. 3 was obtained. The formula for the line is $y = 0·80 + 0·99x$ where y is plasma phylloquinone (nmol/l) and x is body pool (μg/kg body weight), n 0·60, $P<0·05$. The regression line shows that the depletion of the body pool exceeds the reduction in plasma phylloquinone at any given time.

Excretion of ^3H in the urine and stool

The excretion of metabolic products of [^{3}H] phylloquinone in the urine was 30·0 (SD 4·0) % of the dose injected in subjects on the control diet and 38·3 (SD 21·9) after 3–8 weeks on vitamin K restricted diets. Significant radioactivity was detected in the urine for 6 d after the injection. The $t_{1/2}$ for the decline in excretion of radioactive products was 20·1 (SD 0·7) h before ingestion of the low-vitamin K diet and 24·8 (SD 1·8) h afterwards, an insignificant difference. As shown in Fig. 4, the slopes of the lines for the decay of urinary metabolites were essentially identical to those of the terminal exponential curves of the plasma radioactivity, indicating that both curves represent the true turnover rate of the vitamin. The excretion products in the stool, representing hepatic biliary secretion (Shearer *et al.* 1974) were significantly reduced from 32·8 (SD 1·8) % on the control diet to 13·3 (SD 1·0) % after vitamin K restriction. The total radioactivity recovered in the urine and stool accounted for less than 70 % of the total radioactivity injected as shown in Table 4. Since the plasma pool showed no detectable radioactivity after 6 d, the radioactivity that is unaccounted for remains in some other pool in the body, possibly in adipose tissue.

Discussion

The previous and present work on the turnover of phylloquinone in human subjects is presented in Table 5. Data on the tracer used, its specific activity, dose administered and the computed $t_{1/2}$ for the two components of the biexponential curves are presented in each case. It is clear that the doses used by previous investigators are large in comparison with the one used by us and were 0·5–10-fold the size of the apparent body pool. It is also true that the period of observation of the plasma radioactivity was shorter (10 *v.* 72 h). The lack of time or significant plasma radioactivity in the terminal phases of the previous studies may have prevented these investigators from observing the slope of the true terminal decay curve of plasma radioactivity. It seems likely that the apparent 10-fold difference in the terminal decay rates is due to the higher doses and shorter times used by previous investigators.

Such pharmacological doses have been shown to give shorter turnover times in other studies. In the case of glucose, using a tracer dose of [^{14}C] glucose, Zilversmit (1960) observed that the fractional turnover rate for blood glucose in humans was 0·1/h and the pool size 5 g. When 50 g glucose was administered intravenously the turnover rate was 1·0/h, a 10-fold increase (Karam *et al.* 1986) and when the absolute turnover rate was calculated the difference in the rate of glucose disposal between the physiologic and pharmacologic doses was 100-fold. Similar effects have been noted with the turnover of 1,25-dihydroxycholecalciferol before and after loading with the hormone (Frolik & DeLuca, 1973).

We observed that half-times for phylloquinone turnover estimated from both plasma and urinary values were approximately 1 d for subjects on both the control and vitamin K-restricted diets. Bjornsson *et al.* (1979) concluded from the short half-times of plasma phylloquinone observed in their study that the body pool turned over 30–50 %/hour. Shearer & Barkhan, (1979) argued, however, that the results available from their studies did not provide the evidence needed to calculate the turnover rate. It is likely that Shearer *et al.* (1972, 1974) took into account the fact that although their plasma values gave $t_{1/2}$ 1·7–2·5 h, the rate of excretion of labelled metabolites in the urine gave values of 18–24 h, similar to what we have observed. They also cited four conditions that should prevail before an accurate estimate of turnover could be calculated. These include specificity of measurement, size of the tracer dose, requirement for a steady state, and rapid mixing with the exchangeable pool.

As regards specificity of measurement, we are aware that the method of Ueno & Suttie (1983) and Mummah-Schendel & Suttie (1986) gave high values (2-fold) when compared with the method of Sadowski *et al.* (1989) in studies of healthy populations. In the method of Ueno & Suttie (1983) this effect appears to be due to a stable impurity in the HPLC eluate that adjoins the phylloquinone peak and is read as a part of the phylloquinone peak. If we use the lower, and presumably more accurate values for plasma phylloquinone, the exchangeable pool size in subjects on the control diet would drop from 1·14 (SD 0·64) μg/kg to 0·57 (SD 0·32) μg/kg. This lower value deviates even more from the body pool calculated independently from the product of the daily phylloquinone intake (1 μg/kg per d) (Booth *et al.* 1996*a*) times the turnover time (1·5 d) to

Table 4. Excretion of radioactive products of [5-^{3}H]phylloquinone during 6 d following administration of isotopic phylloquinone†

(Mean values with their standard errors for seven subjects)

	% Dose given					
	Urine		Stool		Total	
Diet	Mean	SEM	Mean	SEM	Mean	SEM
Control	30·0	1·8	31·8	0·81	61·8	2·0
Low-vitamin K	38·3	9·8	13·3***	0·51	51·6	9·8

† For details of subjects, diets and procedures, see Table 1 and p. 544.
Mean value was significantly different from that of the control diet
***$P<0·001$.

Table 5. Reports of phylloquinone turnover in human subjects

Reference	Tracer	Specific activity (Mbq/mmol)	Dose		Kinetic data*	
			Mass (μg)	Activity (MBq)	$t_{1/2}\alpha$ (h)	$t_{1/2}\beta$ (h)
Shearer *et al.* (1972)	[1′, 2′-^{3}H]Phylloquinone	203	1000	0·44	0·37	2·3
Shearer *et al.* (1974)	[1′, 2′-^{3}H] Phylloquinone	3255	45	0·33	0·21	1·7
Shepherd, *et al.* (1977)	[1′, 2′-^{3}H] Phylloquinone	203	600	0·27	0·20	3·4
Bjornsson *et al.* (1979)	[1′, 2′-^{3}H] Phylloquinone	3255	300	2·22	0·43	2·8
Present study	[5-^{3}H] Phylloquinone	550 000	0·3	0·37	1·00	27·6

* $t_{1/2}\alpha$ and $t_{1/2}\beta$ are respectively half-times for the first and second exponential decay curves of plasma radioactivity.

yield a body pool of 1·5 μg/kg. Further, Shearer *et al.* (1972) noted that in their short-term study that water-soluble metabolites of isotopic phylloquinone accumulated in the plasma over time, reducing the relative amount of phylloquinone present. Since we measured total radioactivity in our subjects, we recalculated the slopes of our terminal curves for subjects on the control and low-vitamin K diets using Shearer's data prorated over 6 h. Under these conditions we found that the zero time intercepts decreased by 20 % and the slopes increased by 10 %, but the corrected values were not significantly different from the uncorrected ones. Within the error of our method, the body pool of phylloquinone in healthy subjects (0·6–1·2 μg/kg) is much smaller than that observed for other fat-soluble vitamins. In human subjects, the body pool for vitamin D (all forms), is 5 μg/kg, for vitamin A it is 5000 μg/kg and for vitamin E it is 40 000 μg/kg (Olson and Munson, 1995).

As regards the requirement for a tracer dose in turnover studies (Shearer & Barkhan, 1979), it is clear from Table 5 that tracer doses were not employed in the previous studies. As regards the quality of the tracer used, it is possible that impurities in a tracer could alter the uptake and excretion of its total radioactivity. The [5-^{3}H]phylloquinone we used was 99 % pure by TLC and showed a classic u.v. spectrum. Two hours after injection, greater than 90 % of the isotope was associated with authentic phylloquinone. Shearer *et al.* (1972,1974) reported that of the material they received from Hoffmann-LaRoche (Basel, Switzerland) labelled in the 1′–2′ position, only 90 % of the original compound travelled with pure phylloquione on TLC. Further, they reported that 1 h after injection only 80 % was associated with carrier phylloquinone and after 7 h this had decreased to 40 %.

The third criterion cited by Shearer & Barkhan (1979) for estimation of the body pool size is a steady state. It can be assumed that the subjects on the control diet were in a steady state because they had consumed a diet with a constant intake of vitamin K for 1–2 weeks. As regards these subjects after 3–8 weeks on a low-vitamin K diet, it is not clear whether any of them had reached a final steady state. Furthermore, the average plasma phylloquinone in our depleted subjects was determined to be 1·05 (SD 0·54) nmol/l. As with the subjects on a normal diet, we suspect the value is high, but may be relatively much higher than that of normal subjects because of the aforementioned impurity in the HPLC eluate. Ferland *et al.* (1993) reported average values of 0·18 nmol/l in thirty-two subjects given a diet containing 10 ug/d of phylloquinone/d for 13 d. If we assume that we overestimated the plasma phylloquinone in our subjects on the low-vitamin K diet by 5-fold, the calculated plasma value would have been 0·27 (SD 0·14) nmole/l and the computed body pool would have been 0·12 (SD 0·6) μg/kg, not different from the 0·15 μg/kg predicted from the daily intake (0·1 μg/kg per d) × turnover time (1·5 d). It is of some interest that these assumptions about the error of the Ueno & Suttie (1983) method predict a body pool that is too low by 50 % for the subjects on the control diet and about right for subjects on the low-vitamin K diet. Nonetheless, discussion of the errors in our plasma phylloquinone result have illuminated the limits that can be set for the accuracy of the estimates of body pool size that are ± 50 % for persons on a normal diet. For persons on a vitamin K-low diet, the values are lower, but the variance is greater.

The signs of adaptation to the low vitamin K intake in all subjects were: (1) a more rapid entry of phylloquinone into cells, as indicated by the reduction of the $t_{1/2}$ of the first exponential of the plasma decay curve; (2) a reduction in the biliary excretion of metabolites into the gut as indicated by the reduction in faecal H_3 from 32–13 % of the dose given. The result of Hodges *et al.* (1993) suggest that most of the vitamin K in the body is in the bones and that includes both phylloquinone and the menaquinones. However, it is unclear, whether in response to a low-vitamin K diet there is a shift of phylloquinone from bones to liver, or a shift within the liver to dependence on menaquinones for γ-carboxyglutamate synthesis. Taggart & Matschiner (1969) found in the rat that the minimum concentration in liver for the synthesis of prothrombin is 10 nM (4·5 ng/g). The value for human subjects may be lower, or the dependence on menaquinones greater, since two studies (Shearer *et al.* 1988; Usui *et al.* 1990) have reported that phylloquinone in adult human liver ranges from 2·2–48·8 nM (1–22 ng/g).

The fourth characteristic of a satisfactory turnover study mentioned by Shearer & Barkhan (1979) is rapid mixing of the isotope administered with the total body pool. The fact that 35–40 % of the radioactive phylloquinone given was not accounted for after 6 d suggests that there may be a pool of phylloquinone in the human body with a very slow turnover rate that was not determined in our present study and would expand the above estimates of the total body pools by 30 %. We speculate that it may be adipose tissue. Wiss & Gloor (1966) reported in the rat that the injection of a large dose (2 mg) of isotopic phylloquinone

resulted in the retention of 15 % of this dose in the skin and fat after 24 h. Rosenstreich *et al.* (1971) observed that unaltered vitamin D in adipose tissue was the principal storage form of vitamin D after administration of [4-^{14}C] cholecalciferol to rats.

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References

Abell LL, Levy BB, Brodie DB & Kendall FE (1952) A simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *Journal of Biological Chemistry* **195**, 357–366.

Allison PM, Mummah-Schendel LL, Kindberg CG, Harmes CS, Bang NU & Suttie JW (1987) Effects of vitamin K deficient diet and antibiotics in normal human volunteers. *Journal of Laboratory and Clinical Medicine* **110**, 180–188.

Bjornsson TD, Meffin PJ, Swezey SE & Blaschke TF (1979) Effects of clofibrate and warfarin alone and in combination on the disposition of vitamin K_1. *Journal of Pharmacology and Experimental Therapeutics* **210**, 322–326.

Booth SL, Pennington JAT & Sadowski JA (1996*a*) Food sources and dietary intakes of vitamin K_1 (phylloquinone) in the American diet data from the FDA Total Diet Study. *Journal of the American Dietetic Association* **96**, 149–154.

Booth SL, Sadowski JA & Pennington JAT (1996*b*) Phylloquinone (vitamin K_1) content of foods in the US Food and Drug Administrations Total Diet Study. *Journal of Agriculture and Food Chemistry* **43**, 1574–1579.

Corrigan JJ & Marcus FI (1974) Coagulopathy associated with vitamin E ingestion. *Journal of American Medical Association* **230**, 1300–1302.

Dam H (1935) Antihaemorrhagic vitamin of the chick. *Biochemical Journal* **29**, 1273–1285.

Edelman IS & Liebman J (1959) Anatomy of body water and electrolytes. *American Journal of Medicine* **271**, 256–277.

Ferland G, Sadowski JA & O'Brien ME (1993) Dietary-induced subclinical vitamin K deficiency in normal human subjects. *Journal of Clinical Investigation* **91**, 1761–1768.

Frick G, Riedler G & Brögli H (1967) Dose response and minimum daily requirement for vitamin K in man. *Journal of Applied Physiology* **23**, 387–389.

Frolik CA & DeLuca HF (1973) The stimulation of 1,25-dihydroxycholecalciferol metabolism in vitamin D-deficient rats by 1,25-dihydroxycholecalciferol treatment. *Journal of Clinical Investigation* **52**, 543–547.

Gibaldi M & Perrier D (1982) Appendix C, Method of Residuals. In *Pharmacokinetics*, 2nd ed., pp. 433–444. New York, NY: Marcel Dekker.

Greenstein JP, Birnbaum SM, Winitz M & Otey MC (1957) Quantitative nutritional studies with water-soluble chemically defined diets in growth, reproduction and lactation in rats. *Archives of Biochemistry and Biophysics* **72**, 396–416.

Haroon Y, Bacon DS & Sadowski JA (1986) Liquid-chromatographic determination of vitamin K_1 in plasma with fluorometric detection. *Clinical Chemistry* **32**, 1925–1929.

Hermondson MA, Suttle JW & Link KP (1969) Warfarin metabolism and vitamin K requirement in the warfarin resistant rat. *American Journal of Physiology* **217**, 1316–1319.

Hodges SJ, Bejui J, Leclercq CP & Delmas PD (1993) Detection and measurement of vitamins K_1 and K_2 in human cortical and trabecular bone. *Journal of Bone and Mineral Research* **8**, 1005–1008.

Huff RL & Feller DO (1956) Relation of circulating red cell volume to body density and obesity. *Journal of Clinical Investigation* **35**, 1–10.

Karam JH, Salber PR & Forsham PH (1986) Pancreatic hormones and diabetes mellitus. In *Basic and Clinical Endocrinology*, 2nd ed., pp. 523–574 [FS Greenspan and PH Forsham, editors]. Los Altos, CA: Lange Medical Publications.

Laurell C-B (1972) Electroimmunoassay. *Scandinavian Journal of Clinical Laboratory Investigation* **29**, Suppl. 124, 21–37.

Matschiner JT, Amelotti JM & Doisy EA (1967) Mechanism of the effect of retinoic acid and squalene on vitamin K deficiency in the rat. *Journal of Nutrition* **91**, 303–306.

Mummah-Schendel L & Suttie JW (1986) Serum phylloquinone concentration in a normal adult population. *American Journal of Clinical Nutrition* **44**, 686–689.

National Research Council (1980) *Recommended Dietary Allowances*, 9th ed., Washington, DC: National Academy Press.

Neeld JB Jr & Pearson WN (1963) Macro and micromethods for the determination of vitamin A using trifluoroacetic acid. *Journal of Nutrition* **79**, 454–462.

Olson RE (1999) Vitamin K. In *Modern Nutrition in Health and Disease*, pp. 363–380 [M Shils, JA Olson, M Shike and A Ross, editors]. Baltimore, MD: Williams and Wilkins.

Olson RE, Meyer RG, Chao J & Lewis JH (1984) The vitamin K requirement of man. *Circulation* **70**, II-97, Abstr.

Olson RE & Munson PL (1995) Fat-soluble vitamins. In *Principles of Pharmacology Basic Concepts and Clinical Applications*, pp. 927–947 [PL Munson, RA Mueller and GR Breese, editors]. New York, NY: Chapman & Hall.

Quaife ML, Scrimshaw NS & Lowry OH (1949) A micromethod for the assay of total tocopherols in blood serum. *Journal of Biological Chemistry* **180**, 1229–1235.

Ritz P (2000) Body water spaces and cellular hydration during healthy aging. *Annals New York Academy of Sciences* **904**, 474–483.

Rosenstreich SJ, Rich C & Volwiler W (1971) Deposition in and release of vitamin D_3 from body fat: evidence for a storage site in the rat. *Journal of Clinical Investigation* **50**, 579–687.

Sadowski JA, Hood SJ, Dallal GE & Garry PJ (1989) Phylloquinone in plasma from elderly and young adults: factor influencing its concentration. *American Journal of Clinical Nutrition* **50**, 100–108.

Shearer MJ, Bach A & Kohlmeier M (1996) Chemistry, nutritional sources, tissue distribution and metabolism of vitamin K with special reference to bone health. *Journal of Nutrition* **126**, 1181S–1186S.

Shearer MJ & Barkhan P (1979) Vitamin K_1 and the therapy of massive warfarin overdosage. *Lancet* **ii**, 266–267.

Shearer MJ, McBurney A & Barkhan P (1974) Studies in the absorption and metabolism of phylloquinone (vitamin K_1) in man. *Vitamins and Hormones* **32**, 513–542.

Shearer MJ, McCarthy PT, Crampton OE & Mattock MB (1988) The assessment of human vitamin K studies from tissue measurements. In *Current Advances in Vitamin K Research*, pp. 437–452 [J Suttie, editor]. New York, NY: Elsevier Science Publishing Co.

Shearer MJ, Mallinson CN, Webster GR & Barkhan P (1972) Clearance from plasma and excretion in urine, faeces and bile of an intravenous dose of tritiated vitamin K_1 in man. *British Journal of Haematology* **22**, 579–588.

Shearer MJ, Rahim S, Barkhan P & Stimmler L (1982) Plasma vitamin K in mothers and their newborn babies. *Lancet* **ii**, 460–463.

Shepherd AMM, Wilson NM & Stevenson IH (1977) Vitamin K pharmacokinetics: Response in young and elderly patients. *Clinical Pharmacology Therapy* **21**, 117, Abstr.

Suttie JW, Mummah-Schendel BS, Shah DV, Lyle BJ & Greger JL (1988) Vitamin K deficiency from dietary vitamin K restriction in humans. *American Journal of Clinical Nutrition* **47**, 475–480.

Taggart WV & Matschiner JT (1969) Metabolism of menadione 6,7-^{3}H in the rat. *Biochemistry* **8**, 1141–1146.

Ueno T & Suttie JW (1983) High pressure liquid chromatographic-reductive electrochemical detection analyses of serum trans-phylloquinone. *Analytical Biochemistry* **133**, 62–67.

Usui Y, Tanimura H, Nishimura N, Kobayashi N, Okanove T & Ozawa K (1990) Vitamin K concentrations in the plasma and liver of surgical patients. *American Journal of Clinical Nutrition* **51**, 846–852.

Van Handel E & Zilversmit DB (1957) Micromethod for the direct determination of serum triglycerides. *Journal of Laboratory and Clinical Medicine* **50**, 152–157.

Weenesland R, Brown E, Hopper J JR, Hodges JL Jr, Guttentag OE, Scott KG, Tucker IN & Bradley B (1959) Red cell, plasma and blood volume in healthy men measured by radiochromium (^{51}Cr) cell tagging and hematocrit: Influence of age, somatotype, and habits of physical activity on the variance after regression of volumes to height and weight combined. *Journal of Clinical Investigation* **38**, 1065–1077.

Winitz M, Graft J, Gallagher N, Narkin A & Seedman DA (1965) Evaluation of chemical diets as nutrition for man-in-space. *Nature* **205**, 741–743.

Wiss O & Gloor U (1966) Absorption, distribution, storage and metabolism of vitamin K and related quinones. *Vitamins and Hormones* **24**, 575–586.

Zilversmit DB (1960) The design and analysis of isotopic experiments. *American Journal of Medicine* **29**, 832–848.