THE ROLE OF MENAQUINONES IN MAINTAINING NORMAL COAGULATION HOMEOSTASIS IN MAMMALS

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By

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1. ABSTRACT

The menaquinone profiles of intestinal bacteria were characterised. TLC and HPLC analysis of 92 strains (74 from clinical and stool specimens and 18 type strains) revealed that Bacteroides fragilis "group" organisms and facultatively anaerobic gram-negative aerobes such as Escherichia coli and Klebsiella spp. were the major producers of menaquinones. Among the gram-positive anaerobes, only members of the Propionibacterium spp. contained menaquinones.

Febrile neutropenic cancer patients who were entered into two clinical trials of empiric antimicrobial therapy were examined for the incidence of hypoprothrombinemia. Their feces were quantitatively cultured and were also analysed for vitamin K content. Hypoprothrombinemia was more common in patients receiving antimicrobial regimens that eradicated both B. fragilis group organisms and E. coli. Eradication of menaquinone producing organisms was correlated with reduction of vitamin K_1 and menaquinone concentrations in the feces of these subjects indicating that antimicrobial regimens which suppress the endogenous source of the vitamin may induce antibiotic-associated coagulopathy.

To test the hypothesis that bacterially produced menaquinones were important in human nutrition and that antibiotic-associated hypoprothrombinema occurs secondary to the removal of the endogenous source of the vitamin, an animal model using rats was developed. Animals were placed on vitamin K_1 deficient and sufficient diets alone, or in combination with antimicrobial agents capable of eradicating the menaquinone producing intestinal flora. Hypoprothrombinemia could only be produced when rats were simultaneously deprived of

dietary vitamin K_1 with elimination of menaquinone producing organisms in the intestinal lumen. In addition, hypoprothrombinemia was not observed if animals were deprived of vitamin K_1 alone, with the persistence of the endogenous menaquinone pool. Hypoprothrombinemia could be corrected in animals deprived of both dietary vitamin K_1 and the endogenous menaquinone pool, by ingestion of killed B. fragilis organisms or by allowing recolonization of the intestine with menaquinone producing microbes. As well, hypoprothrombinemia occurred with equal facility in animals given antibiotics containing the N-methylthiotetrazole (NMTT) side chain (moxalactam) or lacking it (cefoxitin).

These data support the hypothesis that menaquinone plays an important role in maintaining normal coagulation homeostasis in mammals and that antibiotic-associated hypoprothrombinemia occurs secondary to the depletion of the endogenous menaquinone pool and does not require the presence of NMTT inhibition of the microsomal carboxylase.

2. INTRODUCTION

Vitamin K exists naturally in two forms: vitamin K_1 or phylloquinone and vitamin K_2 or menaquinone (MK). Vitamin K_1 is found in the chloroplast membranes of plants where it functions as a redox cofactor in electron transport. Menaquinones are constituents of bacterial plasma membranes and also function as redox cofactors in electron transport.

Recently a number of reports have indicated that menaquinones may also serve as a useful marker in the classification of bacteria.

Vitamin K is important in mammalian coagulation since it functions as a cofactor in the carboxylation of glutamic acid residues on four protein coagulation factors, II, VII, IX and X. The reaction is mediated by a membrane bound carboxylase; post-translational carboxylation of 10 glutamic acid residues to carboxyglutamic acid on the precursor coagulation factors allows them to bind calcium and take part in the coagulation cascade. Vitamin K deficiency in mammals is therefore reflected in reduced levels of the "active" coagulation factors and is characterised by elevated prothrombin times and if the deficiency is sufficiently severe, results in bleeding.

It is generally believed that the human requirements for vitamin K are fulfilled by vitamin K_1 through the diet and that menaquinones produced by intestinal bacteria are not bioavailable and therefore unimportant (Udall, 1965). This has led to considerable controversy surrounding the relative importance of these sources. The fundamental question as to whether endogenous menaquinone plays a major role in maintaining normal coagulation homeostasis still remains unsolved.

The importance of the intestinal pool was first recognized by Greaves (1939), who found that only 12 of 77 rats fed a diet of rice developed hypoprothrombinemia, which corrected spontaneously. Gustaffson (1962), showed that only germ-free rats on a vitamin K_1 deficient diet developed vitamin K-responsive hypoprothrombinemia which could be corrected by contaminating these animals with E. coli or a Micrococcus spp.

Barnes and Fiala (1958,1959), and later Mameesh and Johnson (1960a,1960b), using a diet essentially consisting of glucose and soy protein, demonstrated that rats became severely hypoprothrombinemic only when coprophagy was prevented. Rats with normal access to their feces developed moderately severe hypoprothrombinemia. From these findings it was inferred that menaquinone could satisfy the requirements of rats but only to an extent depending on the degree of coprophagy. This implied that menaquinone produced in the intestine was not absorbed in-situ but must be recycled through oral intake before it could be absorbed.

However, menaquinones have been found in the liver of humans and animals. Matschiner (1970) demonstrated that menaquinone isoprenologues (MK-10, MK-11, and MK-12) recovered from bovine liver were also present in their rumen contents. In addition, vitamin K-responsive hypoprothrombinemia and clinical bleeding occurred in patients receiving broad spectrum antibiotics capable of eradicating the intestinal flora during illnesses in which oral intake of vitamin K_1 was severely reduced. Frick et al (1967), found that patients on parenteral nutrition (glucose solution) did not become hypoprothrombinemic unless unspecified antimicrobial agents were concurrently admini-

stered. These findings are consistent with the acknowledged difficulty in producing vitamin K deficiency in most animals by simple dietary restriction.

Hypoprothrombinemia appears to develop most commonly when low dietary vitamin K_1 intake is combined with the use of antibiotics capable of suppressing the menaquinone producing intestinal flora.

Although bioavailability has not been demonstrated, these studies suggested that the intestinal flora may be an important source of vitamin K. In order to determine the role of menaquinone in maintaining normal coagulation homeostasis, this study was undertaken with the following objectives in mind:

(1) Survey of members of the normal intestinal flora for their menaquinone profile to determine which are the major producers. Menaquinones have been shown to be present in a number of different bacteria but most of these studies have been done for biochemical or taxonomic reasons. Only limited information is available on the production of menaquinones by the intestinal flora.

(2) Examine fecal samples from neutropenic patients receiving empiric antimicrobial therapy to determine the effect of these agents on the menaquinone producing flora and to quantitate their menaquinone content. The objective was to correlate changes in the menaquinone producing flora with changes in menaquinone content and occurrence of hypoprothrombinemia.

(3) Induce vitamin K_1 and K_2 deficiency in healthy human volunteers by placing them on a vitamin K_1 deficient diet while on antibiotics capable of suppressing the intestinal flora and correlate

changes in fecal flora and vitamin K content with the incidence of hypoprothrombinemia.

(4) The mechanism of production of hypoprothrombinemia associated with the administration of antimicrobial agents has not been clearly established. There are two possible mechanisms suggested to explain hypoprothrombinemia commonly seen with use of the newer third generation cephalosporins. It is thought that hypoprothrombinemia occurs secondary to removal of menaquinone producing intestinal flora and the endogenous menaquinone pool during periods of low dietary vitamin K_1 intake. An alternative and more widely quoted hypothesis is that the N-methylthiotetrazole (NMTT) side chain of some cephalosporins directly inhibits the microsomal carboxylase and prevents the formation of functional vitamin K-dependent coagulation factors.

To study the contribution of menaquinones to coagulation homeostasis and determine the mechanism of antibiotic associated hypoprothrombinemia an animal model was developed using rats. The animals were placed on vitamin K_1 sufficient and deficient diets alone or in combination with antimicrobial agents containing and lacking the NMTT group. Vitamin K_1 and menaquinone concentrations were measured at several levels in the intestinal tract and in the liver and the findings related to the development of hypoprothrombinemia. In addition, an attempt was made to reverse hypoprothrombinemia in these animals by oral ingestion of killed B. fragilis organisms or allowing spontaneous recolonization of the intestine with menaquinone producing bacteria. Finally, the model was also used to attempt to define the mechanism by which hypoprothrombinemia is produced when animals are placed on a diet similar to that used by Mameesh and Johnson (1960a).

3. LITERATURE REVIEW

A. Historical Perspective

1. Discovery of Vitamin K

The discovery of vitamin K resulted from experiments that were carried out by the Danish researcher, Henrik Dam, working with chickens to determine their cholesterol needs. He noticed that chicks fed an ether extracted diet developed spontaneous bleeding and their blood clotted more slowly. Dam's findings were corroborated independently by McFarlane in 1931 in studies with chickens fed ether extracted fish and meat meal diets to determine their vitamin A and D requirements (Suttie, 1978; Almquist, 1975). Holst and Halbrook (1933), found that chickens bled when placed on a diet devoid of green feed and that the condition could be prevented by using fresh cabbage. However, Cribbett and Correll (1934), failed to confirm these findings. Cook and Scott (1935), found increased clotting times and numerous hemorrhages in chicks placed on a fish meal diet, but not when this was replaced by meat scrap. They ascribed the coagulation abnormality to a toxic factor present in the fish meal (Suttie, 1978; Almquist, 1975; Almquist, 1979).

In 1935, Dam proceeded to repeat Cook & Scott's work and found that bleeding was produced when the ether extracted fish meal or meat scrap diet was used. On adding back the ether extract or small amounts of alfalfa, the coagulation abnormality was reversed. These observations were supported by the contemporaneous work of Almquist & Stokstad (Almquist, 1975). From these findings it was obvious that there was a factor present in the diet that was responsible for normal

coagulation. This factor was always present in the fat soluble extract of diets, and was not identifiable with any of the previously known vitamins. Dam continued to study the lipid soluble component in vegetable and animal sources and in 1935, proposed that the fat soluble factor be called vitamin K. The lack of this factor was responsible for the bleeding tendency. K was chosen because it was the next letter in the alphabet after which a vitamin had not been already named and, also, was the first letter in the German word "Koagulation" (Suttie, 1978).

Schonheyder (1935) discovered that vitamin K controlled the prothrombin (factor II) level in blood. A deficiency of vitamin K was linked to the lowering of prothrombin level in the blood of chicks. Subsequently vitamin K was isolated in two forms - vitamin K_1, and vitamin K_2. Vitamin K_1 was first isolated as a pure compound from alfalfa by Dam's group in 1938 and also by Almquist's group and from putrified fish meal by Doisy et al (Suttie, 1978; Almquist, 1975). Vitamin K_2 was isolated from putrified fish meal in 1939 by McKee et al. The compound isolated from fish was characterised as MK-6 by Binkley et al (1940) but was subsequently shown to be MK-7 (the preparation also contained small amounts of MK-6) by Isler et al (1958). Almquist's group also found that the vitamin was synthesized by bacteria and could be present in chick feces, even when the diet was free of the vitamin (Suttie, 1978; Almquist, 1979).

In order to investigate an alternative source of the vitamin, Almquist and co-workers embarked on testing single cultures of bacteria for antihemorrhagic activity using the chick assay and found that Mycobacterium tuberculosis had considerable activity.

In the meantime, a pigment, phthiocol or 2-methyl-3 hydroxy-1,4-naphthaquinone, was isolated and tested with the chick assay for activity by Almquist and Klose. This compound was able to maintain normal prothrombin levels in the chicks. Phthiocol was reputed to be the first pure form of vitamin K isolated (Almquist, 1975; 1979). The synthesis and characterization of vitamin K_1 or 2-methyl-3-phythyl-1,-4-naphthaquinone was accomplished by three groups working separately (Almquist & Klose 1939, Binkley, et al 1939, and Feiser, 1939).

In 1948, vitamin K_2 was isolated from a pure culture of bacteria, in this case MK-7 from Bacillus cereus, by Tishler & Sampson (1948).

B. The K Vitamins

1. Description of the Molecules

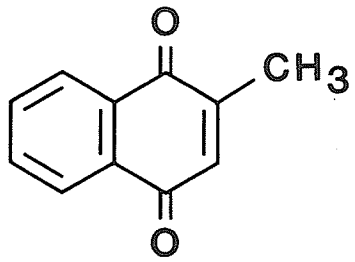
The K vitamins are largely concentrated in the membranes of the cells in which they are found. Vitamin K_1 is found in the green areas of plants, in the lamellae of the chloroplast where it constitutes part of the electron transport apparatus of photosynthesis. Similarly, menaquinones are found in the plasma membrane of bacteria where they function as redox co-factors. In mammalian cells, menaquinones are concentrated in the microsomes or mitochondrial membranes.

Structurally, vitamin K consists of a parent nucleus which is a naphthaquinone ring with a methyl group attached at the carbon-2 position. This compound, 2-methyl-1,4-naphthaquinone is not found naturally but can be chemically synthesized. It is commonly referred to as menadione or vitamin K_3 (Figure 3.1).

Vitamins K_1 and K_2 contain an isoprenoid chain (repeating 5

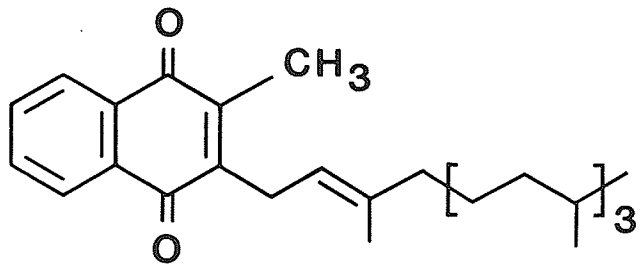
Figure 3.1 Structures of menadione, phylloquinone, menaquinone and dimethylmenaquinone.

1. Menadione, K_3



2-methyl-1, 4-naphthaquinone

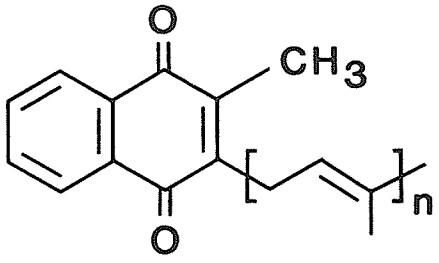
2. Phylloquinone, K_1



2-methyl-3-phythyl-1, 4-naphthaquinone

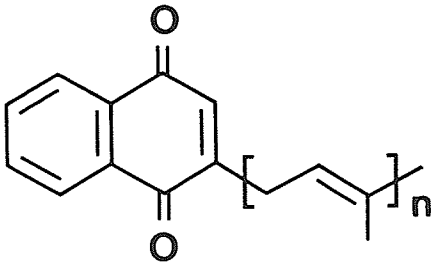
Figure 3.1 (Continued)

3. Menaquinone (MK-n), K_2



2-methyl-3-multiprenyl-1, 4-naphthaquinone

4. Demethylmenaquinone (DMK-n)



2-desmethylmenaquinone

carbon unit) substituted at the carbon-3 position. The isoprenoid chain in phylloquinone contains 4 isoprene units, three of which are fully saturated. The unsaturated unit is the one nearest to the naphthaquinone ring. This chain is called a phythyl group. Vitamin K_1 is referred to as 2-methyl-3 phythyl-1,4-naphthaquinone.

The isoprenoid side chain in menaquinones varies in length giving rise to a series of isoprenologues. As far as has been determined, menaquinones with up to 15 isoprene units have been found in bacteria. The compounds are usually referred to as 2-methyl-3-multiprenyl-1, 4-naphthaquinone and are abbreviated as MK-n, n denoting the number of isoprene units. For example, MK-8 is a naphthaquinone ring having an isoprenoid side chain with eight 5-C units (IUPAC-IUB Commission on Biochemical Nomenclature, 1975).

Menaquinones can also be found unsubstituted at the carbon-2 position giving rise to demethylmenaquinone (DMK), or have a side chain partially saturated in one or more isoprene units. For example, MK-9 (H_2) is a menaquinone with a side chain of 9 isoprene units, one of which is saturated. DMK is apparently an intermediate in the synthesis of menaquinones in bacteria; some organisms may produce DMK alone eg Haemophilus parainfluenzae (DMK-6), or in combination with menaquinones eg. E. coli (DMK-8 and MK-8).

Menaquinones with one or more saturated isoprene units have been found in bacteria. For instance, many of the Mycobacterium and Corynebacterium species contain menaquinones with monosaturated isoprene side chains. On the other hand, Streptomyces sp. produce MK-9 with 3 saturated isoprene units.

2. Physicochemical Properties

Vitamin K is a lipid soluble compound occurring either as cis or trans isomers. The trans form is the naturally occurring isomer as established by nuclear magnetic resonance (NMR) spectroscopy. Vitamin K_1, is a yellow oil at room temperature with a melting point of -20°C. Menaquinones are solids at room temperature, with melting points depending on the length of the side chain (30-60°C). Both forms are light and alkali sensitive but are relatively stable to heat and oxidizing conditions.

Ultraviolet (UV) absorption maxima is a function of the naphthaquinone ring, and is not affected by the length of the isoprenoid side chain. Absorption maxima occur at 242, 248, 260, 269 and 326 nm. The benzenoid moiety is responsible for absorption maxima at 242 and 248 nm and the quinoid moiety at 260 and 269 nm. Variations of the side chain will not affect the UV absorption profile and therefore, fully and partially saturated menaquinones have similar absorption profiles.

Removal of the methyl group from the carbon-2 position, to yield DMK does not affect the benzenoid UV absorption maxima markedly but causes pronounced changes in the quinoid absorption maxima. Thus absorption maxima in menaquinones at 260 and 269 nm are shifted to lower wavelengths at 254 and 263 nm in DMK.

3. Biological Activity

The cis isomer of vitamin K is considered to have low bioactivity; however, as the cis double bond moves further along the polyisoprenoid chain away from the nucleus, its effect on bioactivity is reduced (Parrish, 1980). It also appears that saturation of the side

chain reduces activity. The biological activity of the various forms of vitamin K have been tested primarily with the 18-hour curative chick assay using K-deficient chickens and administering the test solution orally. In this assay 1-2 day old chicks are fed a vitamin K deficient diet usually consisting of an energy source such as glucose and a protein source. After the body stores of vitamin K are depleted in 10-14 days, the chicks are given the test solution at two concentrations. Eighteen to 24 hours after administration, prothrombin (factor II) levels in plasma are determined by a one stage procedure using viper venom as an extrinsic activator (Matschiner and Doisy, 1966). Alternatively, clotting time of whole blood may be used as an indicator of activity of the test sample. The assay is sensitive enough for the measurement of vitamin K levels from plant or bacterial samples but cannot detect the very low levels which are usually found in tissues.

The biological activity of vitamin K_1 and menaquinone isoprenologues are listed in Table 3.1. From the data available, it would appear that the effectiveness of the compounds in normalising coagulation depends on the route of administration. When menaquinones are given orally, those with shorter isoprenoid side chains such as MK-4, MK-5 and MK-6 have the highest activity compared to vitamin K_1. However menaquinones with longer chains, eg. MK-7, MK-9, and MK-10 demonstrate extremely high activity when given by the intracardiac route in rats which are vitamin K deficient (Table 3.1). The low activity of menaquinones with longer chains when given orally, may be due to low absorptivity of these compounds since parenteral administration results in very high activity (Suttie, 1978; Matschiner and Taggart, 1968).

Table 3.1 Bioactivity of Vitamin K Isoprenologues (Matschiner, 1969)

Vitamin K	Relative Activity to Vitamin K_1	
	Oral Administration (Chick)	Intracardiac Administration (Rat)
K_1	100*	100*
MK-2	15	<2
MK-3	40	-
MK-4	100	13
MK-5	>120	15
MK-6	100	170
MK-7	70	1,700
MK-8	68	-
MK-9	60	2,500
MK-10	25	1,700

* The activity of vitamin K_1 was taken as 100%.

C. Occurrence of Vitamin K_1 in the Diet

1. Quantitative and Qualitative Survey of Foods Containing Vitamin K_1

Vitamin K is widely distributed in natural foods, usually at low concentrations. The lack of assay methods sensitive enough to measure low concentrations of the vitamin in food samples has hampered its determination. Until recently, the only methods for determination of vitamin K in foods were bioassays, which were done with only a few types of animals and even though fairly sensitive could be considered unreliable. In addition, bioassays do not distinguish between the different molecular forms of the vitamin (Parrish, 1980).

The vitamin K_1 content of a variety of commercially available infant formula products - both milk based and milk substitute formulas - were determined by Schneider, et al (1974) using TLC and densitometry to quantitate the spots.

Newer chemical methods using multistage HPLC assays of dietary sources have generated more reliable data regarding vitamin levels in the diet. Shearer's group (Shearer, et al 1980; Haroon, et al, 1982) and Barnett, et al (1980) have analysed a variety of vegetables, cow's milk, commercial milk based formulas and milk substitute formulas by this method (Table 3.2).

Plant sources contain vitamin K_1, while animal tissues contain mostly menaquinones. Liver is particularly rich in menaquinones and may contain several types. However, liver also contain measurable levels of vitamin K_1. Other tissues generally contain very low levels of menaquinones.

Table 3.2 Vitamin K content of vegetables by HPLC and chick bioassays*

Vegetable	HPLC ug Vitamin K_1/ 100 g fresh weight	Bioassay ug Vitamin K/ 100 g fresh weight
Potato	<1	4
Parsnip	<1	-
White turnip	<1	-
Mushrooms	<1	7
Red Pepper	2	-
Celery	5	-
Carrot	5	10, 20
Tomato	6	11, 24
Green Pepper	6	-
Leek	10	-
Cucumber	15	<5
Red Cabbage	19	-
Cauliflower	27	<5, 140, 280
Peas	39	7, 19, 36
Dwarf beans	46	-
Cress	88	-
Lettuce	-	200
Round	120	-
Webb's	128	-

Table 3.2 (Continued)

White cabbage	-	37, 46, 95, 250
Outer leaves	137	-
Inner leaves	83	-
Winter cabbage	-	-
Outer leaves	189	-
Inner leaves	52	-
Broccoli	147	65, 200
Brussel sprouts	-	-
Sprouts	177	-
Top leaves	400	-
Spinach	415	130,180,240,330
Spring cabbage	472	-
Rale	724	-
Corn	-	1, 2
Oats	-	10, 75
Wheat	-	4, 36
Soy bean oil	-	450-630

* Taken from Parrish (1980) and Haroon, et al (1982)

It is apparent from table 3.2 that vitamin K levels generated by the bioassay, varies according to the study. There are also discrepancies between levels determined by HPLC compared to bioassays. In general,however, the green vegetables such as spinach, cabbage, kale, broccoli and lettuce contain high concentrations of the vitamin compared to other vegetables regardless of the assay method.

Analysis of infant formula foods, human milk and cow's milk by HPLC has demonstrated a wide variation in vitamin K_1 concentrations. One commercial milk formula product based on cow's milk (Ostermilk) had a vitamin K_1 concentration of 4 ug/L similar to levels found in cow's milk (4.9 ug/L). Another formula product (Goldcap SMA) supplemented with vitamin K_1 contained 35 ug/L, a level much greater than that found in cow's or human milk. Human milk had the lowest concentration of vitamin K_1 at 2.1 ug/L (Shearer, et al, 1980). Vitamin K_1 concentrations generated by the chick bioassay have generally been higher - 15 ug/L and 60 ug/L for human and cow's milk, respectively. It has been pointed out that these concentrations refer to that of menadione and do not take into consideration the different bioactivities of different molecular forms of vitamin K. Using a physicochemical assay, Schneider, et al (1974) determined that vitamin K_1 levels in milk based or milk substitute formula products ranged from 19-118 ug/L. Formulas supplemented with vitamin K_1 had levels ranging from 118-256 ug/L. Also, cow's milk analysed at different times of the year had average vitamin K_1 levels of 10, 47 and 85 ug/L respectively. Haroon, et al (1982), suggested that the discrepancy between these values and HPLC determination may be due to incomplete purification of the vitamin in the physicochemical assay.

D. Menaquinone in Bacteria

1. Discovery of Vitamin K_2 (Menaquinone) in Bacteria

The discovery of vitamin K_2, like vitamin K_1, came about as a result of studies on the nutrition of chickens. Chicks fed ether extracted fish or meat meal suffered from poor growth and prolonged

bleeding. Their symptoms were more severe with fish rather than the meat meal. When the fish meal was moistened with water and fed to chicks, bleeding did not occur. Moistening followed by drying also gave the same results, but alcohol followed by drying again induced bleeding. At this point, Almquist and Stokstad (1935) postulated that the protection afforded by moistening the fish meal was due to bacterial activity in the diet. The mechanism by which this occurred was not explained (Bentley and Meganathan, 1982; Almquist, et al, 1938).

Almquist and Stokstad (1936) subsequently discovered that the droppings of chicks on a vitamin K-free diet yielded an extract which when incorporated in their diet prevented bleeding. This indicated that the antihemorrhagic factor was produced in the intestinal tract of chickens. Almquist et al (1938), then isolated an organism, probably B. cereus, from putrified fish meal and used it to inoculate wet ether extracted fish meal. After incubating 10 days, the meal was once again extracted and the extract incorporated in vitamin K-free chick feed. This diet maintained normal coagulation in the chicks. Other species of bacteria when tested by this model also appeared to contain the antihemorrhagic factor. The factor was finally crystalised as a pure compound from putrified fish meal by McKee, et al (1939) and chemically and spectrally identified as vitamin K. However, since it differed physically from the non-crystalline yellow liquid extractable from alfalfa, it became clear that there were two naturally occurring types of vitamin K--K_1 from plants and K_2 from bacteria.

The material from putrified fish meal was characterized as 2,3 -

disubstituted 1,4-naphthaquinone with the formula of MK-6. Isler, et al (1958) subsequently proved that the compound isolated was really MK-7 with MK-6 being present as a minor component of the mixture. This finding also indicated that there was more than one homologue of K_2 (Bentley and Meganathan, 1982).

Vitamin K_2 was finally isolated from a pure culture of bacteria (Bacillus brevis) by Tishler and Sampson in 1948 and identified based on a comparison of its chemical properties to that of the compound isolated by McKee, et al (1939). In addition, using the chick bio-assay, it was demonstrated that it had antihemorrhagic activity similar to vitamin K_1.

2. Distribution in Bacteria

Bacteria may contain only MK, or DMK, or both. Organisms containing only DMK, are the exception rather than the rule. One of the few that does is H. parainfluenzae, which possesses DMK-6. Menaquinones with side chains containing up to 15 isoprene units and DMK with side chains up to 9 units have been isolated so far. Bacteria may also contain menaquinones with partial saturation of the isoprenoid side chain. Organisms may have a single type of menaquinone with a saturated side chain, more than one with saturated side chains, or a mixture with saturated and unsaturated side chains eg. (Corynebacterium creatinavorans, contains MK-8, MK-9, and MK-10 (H_2). In addition, the unsaturated menaquinone may constitute the major form in a particular organism , with the partially saturated forms being the minor component. Partially saturated menaquinones are widespread in Corynebacterium spp. and Mycobacterium spp.

After the original isolation of vitamin K_2 from a pure bacterial culture by Tishler and Sampson in 1948, the presence of a naphthaquinone in extracts of M. phlei was reported. The newly isolated compound showed UV spectral identity to vitamin K but was different from all the homologues known to that point. This naphthaquinone was subsequently characterised by Gale et al (1963) and shown to contain a side chain with 9 isoprene units. However, on reversed phase paper chromatograms, it behaved more like MK-10. Nuclear magnetic resonance spectroscopy revealed that one of the isoprene units was saturated. Meanwhile Noll in 1958, was successful in isolating a napthaquinone from the fat soluble fraction of M. tuberculosis, which based on infrared spectroscopy and chromatographic analysis was shown to contain a side chain with 7 isoprene units (Mayer and Isler, 1971).

Jacobsen and Dam in 1960 analyzed organisms using paper chromatography. They found MK-7 in B. cereus and B. subtilis and unidentified isoprenologues in Proteus vulgaris, Sarcina flava, Staphylococcus aureus, M. phlei and a Nocardia sp. Bishop, et al, (1962) confirmed the presence of MK-8 in P. vulgaris, and found menaquinones in E. coli (MK-8), C. diphtheriae (MK-9), S. albus (MK-8) and Bacillus spp. (MK-7).

Following this, a series of studies documented the presence of menaquinones in a number of bacterial species. Isolation of the pure quinone was accomplished by a series of chromatographic steps including column and paper chromatography and identification of the pure compound was usually based on the relative mobilities of these compounds to authentic standards on chromatography as well as various spectroscopic analyses such as ultraviolet, infrared and nuclear mag-

netic resonance. As a result of these studies, MK-7 was discovered in B. sphaericus (Gale et al, 1962), DMK-9 in Streptococcus faecalis (Baum and Dolin, 1963, 1965), DMK-6 in H. parainfluenza (Lester et al, 1964), and MK-8 (H_2) in C. diphtheriae (Scholes and King, 1964, 1965).

Using a plate assay with an indicator organism, Bacteroides melaninogenicus which required vitamin K for growth, Gibbons and Engle, (1964) were able to demonstrate that various anaerobic bacteria such as Bacteroides spp., Veillonella spp. and Peptostreptococcus spp. produced substances that satisfied the growth requirement of the indicator strain. This implied that the organisms produced vitamin K, a hypothesis that was tested by examining strains previously identified by chemical means to produce menaquinones. On examination of five organisms previously reported as lacking vitamin K (Bishop et al, 1962), three failed to support growth of the indicator organism. The other two, Serratia marcescens and Aerobacter aerogenes, which supported growth contained MK-8 on examination by chemical methods (Gibbons and Engle, 1965).

Analysis of Micrococcaceae for menaquinones, revealed that organisms in this group produced MK-6, MK-7, MK-8, and MK-9, with menaquinone type varying with the species. S. aureus contained MK-7, MK-8, and MK-9, whereas Micrococcus spp. produced MK-6 and MK-7, only, or in some cases MK-6, MK-7, MK-8, and MK-9 with partially saturated side chains (Jefferies et al, in 1967a, 1967b, and 1969). Menaquinones were further examined in S. aureus by Hammond and White, (1969a) using TLC techniques. They determined that S. aureus synthesized MK-0 through MK-9 with MK-8 being the major isoprenologue (60% of total), MK-7 representing 20% of the total and the others each representing

less than 10% of the total. It was also determined that H. parainfluenzae contained DMK-0 through DMK-9 isoprenologues.

Certain anaerobes have shown the capacity to use electron transport systems for the production of energy. Menaquinones have been found to play a role in electron transport systems in facultative organisms, and because of this a number of anaerobes have been examined for these compounds. Anaerobes shown to produce menaquinones have included Desulfovibrio gigas, D. disulfuricans, and D. vulgaris, all with MK-6 (Maroc et al, 1970; Weber et al, 1970), B. melaninogenicus with MK-6, MK-7, MK-8, and MK-9 (Rizza et al, 1970), Propionibacterium arabinosum with the MK-9 (H_4) (Sone, 1974a), and various Bacteroides spp. with MK-5 through MK-14 depending on species (Shah & Collins 1980). In all cases, the menaquinones were isolated by chemical extraction and identified by chromatographic and spectral methods. Menaquinones have also been reported in two Clostridium species: C. formicoaceticum and C. thermoaceticum. However in these studies, the menaquinones were not isolated; instead crude extracts of organisms were spectrophotometrically assayed for menaquinones (Gottwald et al, 1975).

As a group, the anaerobes that would normally be found in the human intestine have not been extensively surveyed for menaquinones. Collins et al (1980a), and Shah & Collins (1980), demonstrated MK-9, MK-10, and MK-11 in type strains of B. fragilis, B. thetaiotamicron and B. vulgatus. Other anaerobes present in the intestine, such as Lactobacillus mali contained MK-8, MK-9, and MK-10 (Collins et al, 1980a) and L. casei, subsp. rhamnosus, an uncharacterised menaquinone isoprenologue, (Hess et al 1979). In Hess's study various other

anaerobes including a number of Clostridium spp. and a Bifidobacterium sp. were tested and found not to contain menaquinones. Because of the distinct profiles of menaquinones found in certain species of bacteria, Jefferies et al, (1969), suggested that menaquinone profiles may be a useful taxonomic tool to divide organisms into groups.

Two groups have carried out extensive surveys with this in mind. Yamada and Collins and their respective co-workers have attempted to use menaquinone profile of various genera as a tool for the classification of their species (Yamada et al 1976a, 1976b, 1977a, 1977b; Collins and Jones, 1979, 1981; Collins et al, 1977, 1979a, 1979b, 1980a, 1980b, 1982, 1985; Nahaie et al 1984; and Alderson et al 1985).

More recently HPLC methods have been used to analyse bacterial extracts for the presence of menaquinones. Tamaoka et al, (1983) have analysed members of various genera and shown the presence of a variety of saturated and unsaturated forms, as has Collins et al, (1984), Carlone and Anet (1983) (MK-6 in Campylobacter spp.) and Collins and Shah (1984) (MK-6, MK-7 and MK-8 in Rothia dentocariosa).

It is clear that many bacteria contain a mixture of menaquinone isoprenologues usually at different proportions in the cell. Table 3.3 adapted from the extensive review of Collins and Jones (1981) on menaquinone profiles in bacteria, lists the various genera in which these compounds are found. It is apparent that most bacterial species contain a multiplicity of menaquinone isoprenologues. In only a few cases is a single menaquinone found in certain species, eg. MK-7 in B. subtilis.

Several genera of the family Enterobacteriaceae (Escherichia,

Table 3.3 Distribution of Menaquinones in Bacteria

Group	Major Isoprenolgues	Minor Isoprenologues	Representative Strains
1. Archebacteria	MK-8, MK-8 (H_2)	MK-7, MK-7 (H_2) MK-8, MK-8 (H_2)	Halobacterium halobium, MK-8
2. Mycoplasmas	MK-4, MK-7, MK-n	-	Acholeoplasma axanthum, MK-4
3. Gm -ve gliders	MK-6, MK-7, MK-8, MK-n	MK-7, MK-8 MK-9	Myxococcus pulvus MK-8
4. Phototrophs	MK-7	-	Chlorobium thiosulfatilum MK-7
5. G +ve spore forming rods	MK-7	MK-2,3,4,5,6	Bacillus sp., MK-7
6. G +ve non-spore forming rods	MK-6,7,8,9	MK-5,6,7,8,9,10	Listeria monocytogenes, MK-7
7. Coryneform group	MK-7,8,9,10,11,12	MK-6,7,8,9,10,11,12,13,14	
a) without mycolic acids	MK-8 (H_2) MK-9 (H_2) MK-8 (H_4) MK-9 (H_4)		Brevibacterium fermentans MK-9 (H_4) Arthrobacter citreus MK-9 (H_2)
b) with mycolic acids	MK-8 (H_2) MK-9 (H_2)	MK-7,8,9 MK-7 (H_2), 8 (H_4), 9 (H_4)	C. diptheriae, MK-8 (H_2) M. tuberculosis, MK-9 (H_2) N. asteroides, MK-8 (H_2)
8. G +ve cocci	MK-6,7,8,9	MK-5,6,7,8,9	
i) Micrococcus	MK-7 (H_2) MK-8 (H_2)		S.aureus, MK-8 M.lysodiekticus MK-8
ii) Peptococcaceae	MK-8, MK-9, MK-n		Peptococcus magnus, MK-n
iii) Streptococcaceae	MK-8, MK-9, DMK-9	MK-6,7,8, DMK-7, DMK-8	S.fecalis, DMK-9 S. cremoris, MK-9

Table 3.3 (Continued)

9. Gm -ve rods			
a) facultative rods			
i) Enterobacteriaceae	MK-8, DMK-8	MK-6,7,9,9, DMK-n	*E.coli*, MK-8, DMK-8
ii) Hemophilus sp.	DMK-n, DMK-6	-	*H. parainfluenzae*, DMK-6
b) Obligate anaerobes	MK-9,10,11,12,13	MK-7,8,9,10,11,12,13	*B.fragilis*, MK-10, MK-11 *B. assacharolyticus* MK-9 *B. ruminicola* MK-11, MK-12 *B. oralis* MK-12, MK-13 *B. oralis*, ss *mel*. MK-10,MK-11 ss *lev*, MK-9

Klebsiella, Proteus) contain MK-8 but in addition, also, DMK-8. These organisms also produce ubiquinone. The relative quantitites of quinone compounds in the cell may vary with the species and a number of other environmental factors such as degree of aeration. Generally speaking, it appears that cells produce MK, DMK and ubiquinone under aerobic conditions, with ubiquinone being in larger molar amounts than either MK or DMK. Under anaerobic conditions, however, the concentration of MK increases, while that of ubiquinone drops. Also in some cases, the MK/DMK ratio changes.

Bacteroides spp., Desulfovibrio spp. and Capnocytophaga spp. are the genera of gram negative obligate anaerobes that have been analyzed for menaquinones. Bacteroides spp. contain MK-7 through to MK-13. Members of the B. fragilis "group" contain MK-10 or MK-10 and MK-11 as the major menaquinones with MK-7, MK-8, and MK-9 as minor ones. On the other hand, rumen strains of Bacteroides ruminicola sub sp. rumincola contain MK-11 and MK-12 as the major menaquinones with MK-8, MK-9, MK-10, and MK-13 as minor ones.

The family Micrococcaceae produces a range of saturated and unsaturated menaquinones. M. luteus produces MK-8 (H_2) while M. lysodiekticus produces MK-8. The majority of organisms tested in the genus Staphylococcus have MK-7 as the major menaquinone but some species may have MK-6 or MK-8. Streptococcaceae may have MK-8 or MK-9 or DMK-9 depending on species tested, eg. MK-9 in S. lactis and DMK-9 in S. faecalis.

The corynebacteria which include Arthrobacter spp., Brevibacterium spp., Corynebacterium spp., Nocardia spp., and Mycobacterium spp. among others are replete with menaquinones that are partially

saturated. In fact, the major menaquinones in these organisms are almost exclusively the longer chain ones with one or two double bonds in the side chain. The common major menaquinones in this group are MK-8 (H_2) or MK-9 (H_2). The Actinomycetes are similar to the coryneform bacteria in that they also have menaquinones with partially saturated side chains. However in the actinomycetes, the degree of saturation is greater and thus members of this group tend to have menaquinones with tetra-, hexa- and even octa hydrogenated side chains, eg. Actinomyces israelii, MK-10 (H_4), Streptomyces sp., MK-9 (H_6).

3. Concentration or Amount Recovered From Bacteria

The amount of menaquinone recovered from bacteria varies with species and also with environmental conditions under which they are grown. In some cases, the recovered amounts may be suspect since the accuracy of assay methods are questionable. In many studies, menaquinones were measured by UV absorption of a purified fraction of cell extract. However, the purity of these fractions have often not been established beyond doubt and thus there are questions arising as to whether all the UV absorbing material consisted solely of menaquinones. In other cases, menaquinones have been isolated as a pure compound by eluting it from thin layers after separation and then measuring UV absorption of the eluate. However, errors generated by incomplete elution of quinone from thin layers or losses during manipulation were not accounted for. In few instances, concentrations have been determined by using radioactive precursors of menaquinones in cell systems and isolating the pure labelled quinone (Hammond and White, 1969b).

HPLC probably offers the simplest and most accurate way of measuring menaquinones in bacterial cells. However this methodology has not been widely used in measurement of menaquinones, probably due to the restricted area of interest in microbiology.

Jacobsen and Dam (1960) used UV spectrophotometry to estimate the concentration of menaquinone in bacteria. Menaquinones were purified by chromatography, eluted from the paper and assayed at 248 nm. MK-7 was used as the standard and therefore the quantity of menaquinone was expressed, as ng K_2 (MK-7)/g of culture. Menaquinones in bacteria were also assayed using the chick bioassay with vitamin K_3 as the standard. In order to convert the results to activity of K_2 (MK-7), the data was multiplied by the ratio of molecular weights of MK-7/K_3. This, however, does not take into account the difference in bioactivities between different forms of vitamin K_2. B.cereus contained 2.6 ug/g culture by UV absorption, which agreed closely with that obtained by the bioassay. Other microorganisms such as B. subtilis, and P.vulgaris contained much lower quantities of vitamin K_2. The UV test and bioassay results agreed closely with the exception of P. vulgaris where there was a 4-fold higher concentration with the bioassay (17 ug/g culture).

Bishop, et al (1962) found micromolar quantities per g dry weight in Corynebacterium sp. and S. albus. Most of the bacteria examined, however, contained menaquinones in range of 0.3 to 0.7 umole/g dry weight. The estimation was carried out by UV measurement of the fraction eluted by 2% ether in petroleum ether from the column fractionation of crude bacterial extract. It was pointed out that correction was not made for other UV absorbing material that may have been

present. However, UV spectral examination of the material assayed, usually showed four absorbance peaks characteristic of vitamin K. Using the same method to assay menaquinone in C. diphtheriae, Scholes and King (1965) found 10 times as much menaquinone (6.6 umole/g dry weight) in this organism. The differences in values between these two studies were not explained.

Hess et al (1979), quantitatively examined 73 strains of bacteria for the presence of respiratory quinones. Concentrations of menaquinones or DMK's in organisms ranged from 10 to 4000 nmole/g protein. In the majority of cases, the levels were in the order of 1000-1500 nmole/g protein. Based on these measurements it appeared that most bacteria contain submicromolar or micromolar concentrations of menaquinones. These concentrations appeared adequate for the function of the quinone in electron transport processes.

As mentioned above, one of the factors that may influence the concentration of menaquinones in cells is the degree of aeration during the growth cycle. Many bacteria, particularly the facultative gram-negative rods contain both menaquinone and ubiquinone. Those cells which are able to use fumarate as a terminal electron acceptor can have different quinone patterns such as MK alone, DMK alone, MK plus ubiquinone or DMK plus ubiquinone. It is generally recognized that in these bacteria, anaerobiosis increases the amount of menaquinones present in conjunction with a decrease in ubiquinone concentration. This effect, however, varies with other culture conditions and between strains tested.

Lester and Crane (1959), demonstrated that E. coli B_4 when grown aerobically produced both ubiquinone and vitamin K_2 (assayed qualita-

tively by UV absorption spectrophotometry). Under anaerobic conditions, there was no ubiquinone produced. On the other hand, Bishop et al (1962), found similar quantities of menaquinone and ubiquinone regardless of whether the cells were grown aerobically or anaerobically. Polglase et al, (1966) found that vigorous aeration of E. coli B/V caused ubiquinone levels to be 20 times that of menaquinone levels. In the resting phase, ubiquinone concentrations were half that found during log phase growth. However, these lag phase concentrations were still up to ten times greater than menaquinone concentrations. The concentrations of menaquinone in both log phase and lag phase cultures during aeration remained unchanged.

Whistance and Threlfall (1968), pointed out that DMK was not measured as a separate entity and was probably measured as menaquinone in the foregoing E. coli studies. They, therefore examined the menaquinone concentrations in Escherichia freundii, Proteus mirabilis, and Aeromonas punctata during aerobic and anaerobic growth. The quinones were isolated and identified by chromatography and assayed by UV absorption spectrophotometry. Ubiquinone concentrations in the aerobically grown cultures were up to three times greater than in the anaerobic ones for all organisms. Also, ubiquinone concentrations were greater than MK plus DMK during aerobic growth but this was reversed during anaerobic growth except in the case of A. punctata. The MK/DMK ratio also changed with aeration, increasing in E. freundii and P. mirabilis, but decreasing in A. punctata. Similar results were demonstrated with E. coli strain AN98, where MK-8 increased from 104 nmole/g wet weight of cells to 240 nmole/g wet weight of cells when switched from aerobic to anaerobic growth. Additionally, there was a

concomittant drop of ubiquinone from 252 nmole/g wet weight to 104 nmole/g wet weight (Newton et al, 1971). In another strain of E. coli, AN387, the differences between ubiquinone and menaquinone levels under aerobic conditions were more marked: 210 nmole/g wet weight ubiquinone to 5 nmole/g wet weight menaquinone. Under anaerobic conditions, ubiquinone concentrations fell to 43 nmole/g wet weight (about 20% of the aerobic value) while MK-8 increased to 59 nmole/g wet weight. DMK concentrations increased from 38 nmole/g wet weight to 58 nmole/g wet weight. Thus the DMK/MK ratio decreased from 7.6 to 1.0 (Wallace and Young, 1977).

Effects of aeration on menaquinone production in S. aureus was demonstrated by Frerman and White, (1967). Total menaquinone concentration increased by 1.6 times on shifting from anaerobic to aerobic growth. MK-9 increased, MK-7 decreased, and MK-8 levels remained the same during this shift.

Conditions other than degree of aeration also affect the concentration of quinones found in bacterial cells. During exponential growth of S. aureus, the total menaquinone content stayed constant at 2.0 ± 0.1 umole/g dry weight. At the same time MK-8 (the major isoprenologue) increased from 36% to 70% of the total (representing an increase of 0.68 umole/g dry wt) while MK-0, MK-1, MK-5, MK-6, and MK-7 decreased by a total of 0.34 umole/g dry weight (Hammond & White, 1969a).

Effects of temperature on cellular menaquinone concentrations were seen when S. aureus growing exponentially at 37°C was rapidly switched to 25°C. There was a slight increase in total menaquinones (16%) reflected in increases in menaquinone isoprenologues, MK-0

through to MK-6 (increased from 4.3% to 15%) and MK-9 (increased from 7.5% to 10.6%). MK-8 decreased from 69.4% to 55.3% of the total at the new temperature (Joyce et al, 1970).

Diphenylamine suppresses the formation of carotenoids and interferes with ubiquinone biosynthesis. The possible effects of diphenylamine on menaquinone biosynthesis in M. lysodiekticus (MK-9), S. lutea (MK-9), B. licheniformis, B. megaterium and B. subtilis (all containing MK-7) were examined. Diphenylamine concentration of 50 ug/ml decreased membrane concentrations of menaquinone in S. lutea and M. lysodiekticus from 3 to 23%. At 12.5 ug/ml diphenylamine there was actually an increase in menaquinone levels 12-34% higher than control levels. In B. megaterium, 12.5 ug/ml and 25 ug/ml diphenylamine, caused a 10% decrease in MK-7 (Salton & Schmitt, 1967).

Hammond and White (1970) also looked at the effect of diphenylamine on S. aureus grown both aerobically and anaerobically. Under anaerobic conditions, 74 uM diphenylamine reduced menaquinone concentrations by approximately 50% without affecting the growth rate; under aerobic conditions, the effect was the same.

Menaquinone concentrations also changed during exponential growth in certain bacteria. Concentration of MK-7 increased two fold between mid log and stationary phase growth in B. subtilis cultures but fell to a constant level by four hours into the stationary phase (Farrand & Taber, 1974).

4. Function in Bacteria

Menaquinones are found entirely in the plasma membrane of bacteria, acting as redox components between energy coupling compounds

such as dehydrogenases and iron-sulphur proteins, cytochromes or reductases. Ubiquinone may also be found in the plasma membrane of bacteria either with or without menaquinones depending on the species.

Ubiquinone functions at high redox potentials and is therefore the functional quinone in electron transport chains of organisms growing under aerobic conditions. Menaquinones, on the other hand, function under reduced redox conditions because of its low redox potential ($E_{o'}$ = -74 mv) and can therefore be used by bacteria growing in aerobic or anaerobic conditions (Taber, 1979). Thus, E. coli with both ubiquinone and menaquinone can grow under both aerobic or anaerobic conditions whereas, Pseudomonas aeruginosa possessing only ubiquinone is unable to grow anaerobically (unless nitrate is supplied as a terminal electron acceptor). E. coli produces both menaquinone and ubiquinone under aerobic conditions but much higher levels of the latter compared to the former. Under anaerobic conditions, higher concentrations of menaquinone are present (Newton et al, 1971); this increase of menaquinone is related to its function as a redox cofactor linked to fumarate reduction.

Menaquinones function in a number of ways in bacteria as listed below:

1. Electron carrier in electron transport chains giving rise to an electrochemical proton gradient which is utilized for ATP production or active transport of nutrients.
2. Redox cofactor linked to biosynthetic reactions such as:
   a) pyrimidine (uracil) biosynthesis
   b) heme biosynthesis

c) sphingolipid biosynthesis

3. Redox cofactor in transformation of steroids.

(i) Role of Menaquinones in Electron Transfer Systems. The early approaches to the study of the role of menaquinone in cellular metabolism took advantage of its sensitivity to UV light. Because of this sensitivity, naphthaquinone bound to membranes could be destroyed by irradiation with a UV source. Using this technique with M. phlei, Brodie and Ballantine, (1960), irradiated particulate and cell free fractions which on recombination lost the ability to couple oxidation to phosphorylation. Activity could be restored on addition of vitamin K_1 or the menaquinone extracted from M. phlei; but not if the vitamin was irradiated prior to addition. Similar results have also been obtained with M. lysodiekticus using MK-4 and MK-5. These results suggested that a quinone was an essential component of the electron transport chain in the organism.

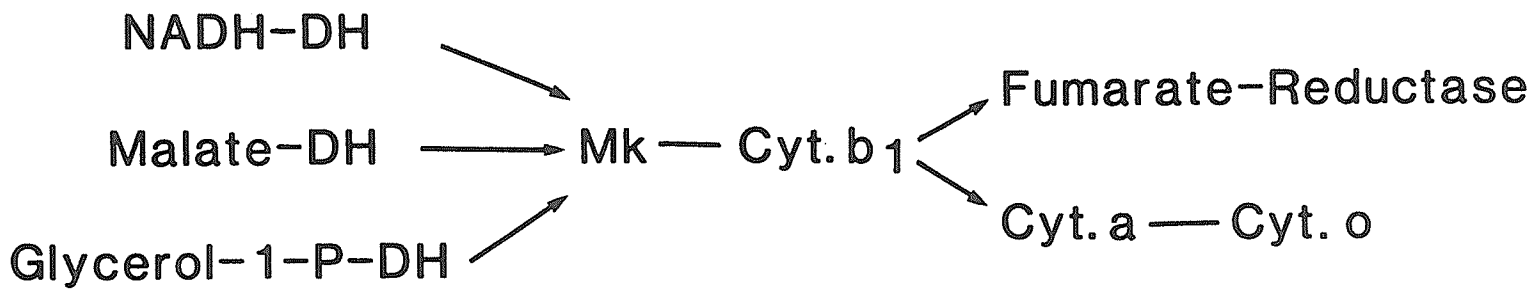
In order to determine the function of menaquinone in B. megaterium, Kroger and Dadak (1969) used membrane systems depleted of its quinone by pentane extraction then added back MK-7 to the extracts. They were able to establish that MK-7 participated in electron transfer from alpha-glycerophosphate, malate and NADH dehydrogenases to oxygen or fumarate. Membrane fractions depleted of menaquinone by pentane extraction caused the loss of electron transport activity for each substance to each acceptor. Addition of MK-7 restored activity to the same extent as the natural pathway. Also, addition of MK-7 led to the reformation of the original pathway as both reconstituted and original membrane preparations showed similar

sensitivity to 2-n-heptyl-4-hydroxyquinolone-N-oxide. MK-7 was established as occurring between the dehydrogenase and cytochrome b_1 to which fumarate reduction or cytochrome oxidase is linked. The scheme of electron transport system of B. megaterium as deduced from the results of this study is presented in Figure 3.2.

Kroger et al (1974) used similar extraction and reincorporation techniques to define the role of quinones (ubiquinone and menaquinone) in electron transport in the facultative gram-negative organism, Proteus rettgeri. Examination of the relative contents of the quinones in the membrane, indicated that they depended largely on the terminal electron acceptor used during growth. For instance the Q/MK ratio was 10 when maximum oxygen was supplied but dropped to 0.27 under anaerobic conditions, and further to 0.12 under anaerobic conditions with fumarate as the electron acceptor. Using membrane preparations from cells grown either aerobically or anaerobically, reincorporation experiments indicated that with O_2 as the terminal electron acceptor and succinate, formate, or NADH as substrates, only ubiquinone and not menaquinone could restore electron transport activity. With fumarate as the electron acceptor and formate or NADH as substrate, menaquinone restored activity and this restoration was more pronounced in membrane preparations from anaerobically grown cells. These results suggested that electron transfer to oxygen was specific for ubiquinone, and to fumarate, specific for menaquinone. The conclusion drawn from these results was that P. rettgeri grown anaerobically with fumarate as the sole carbon and energy source would produce ATP via fumarate reduction rather than substrate level phosporylation.

An alternative approach to study the function of quinones in

Figure 3.2 Schematic diagram showing the role of menaquinone in the respiratory system of B. megaterium.



electron transport was taken by Wallace and Young (1977) who used isogenic E. coli strains with mutations in the ubiquinone and/or menaquinone genes (men+ ubi+ - parent strain; men- ubi+ - MK deficient; men+ ubi- - Q deficient and men- ubi -MK and Q deficient). These strains were used to study the role of O_2 and nitrate as terminal electron acceptors with NADH, D-Lactate, alpha-glycerophosphate and formate as reducing substrates. They found that with O_2 as the electron acceptor, there was normal activity in electron transport with menaquinone using alpha-glycerophosphate as reducing substrate but reduced activity with D-lactate.

With nitrate as the electron acceptor, menaquinone was effective with any of the 4 substrates, formate being the most active. Thus, from the studies of Kroger et al (1974) and Wallace and Young (1977), organisms with ubiquinone will not grow anaerobically with fumarate, but those with menaquinone will; on the other hand, organisms with either menaquinone or ubiquinone will grow in the presence of nitrate with formate as the electron donor.

Sasarman et al (1974) also demonstrated the requirement for menaquinone as the redox cofactor in nitrate respiration in S. aureus using wild type and menaquinone mutants. They were able to demonstrate that mutant strains could not reduce nitrate to nitrite, whereas wild type strains did. Supplementation of media with shikimic acid allowed the aro- (aromatic deficient mutants unable to synthesize menaquinone unless shikimic acid is added) mutants to reduce nitrate at almost the same level as the wild type.

P. arabinosum which possess an electron transfer system linked to fumarate reduction was studied to determine the function of menaquinone in electron transfer (Sone, 1974b). Membrane fragments were prepared and the menaquinone inactivated by irradiation at 360 nm. Menaquinone inactivated membranes lost the ability to oxidise NADH; additionally, glycerol phosphate dehydrogenase, lactate dehydrogenase and NADH-fumarate reducing activity were lost. The latter was fully reversed on addition of MK-4 or MK-9 (H_4) (native to P. arabinosum) to the membrane preparations. Both these menaquinones also partially restored glycerol phosphate-fumarate and lactate-fumarate reducing activities. Thus fumarate reducing activity linked to NADH, glycerol phosphate and lactate depended on the presence of menaquinones.

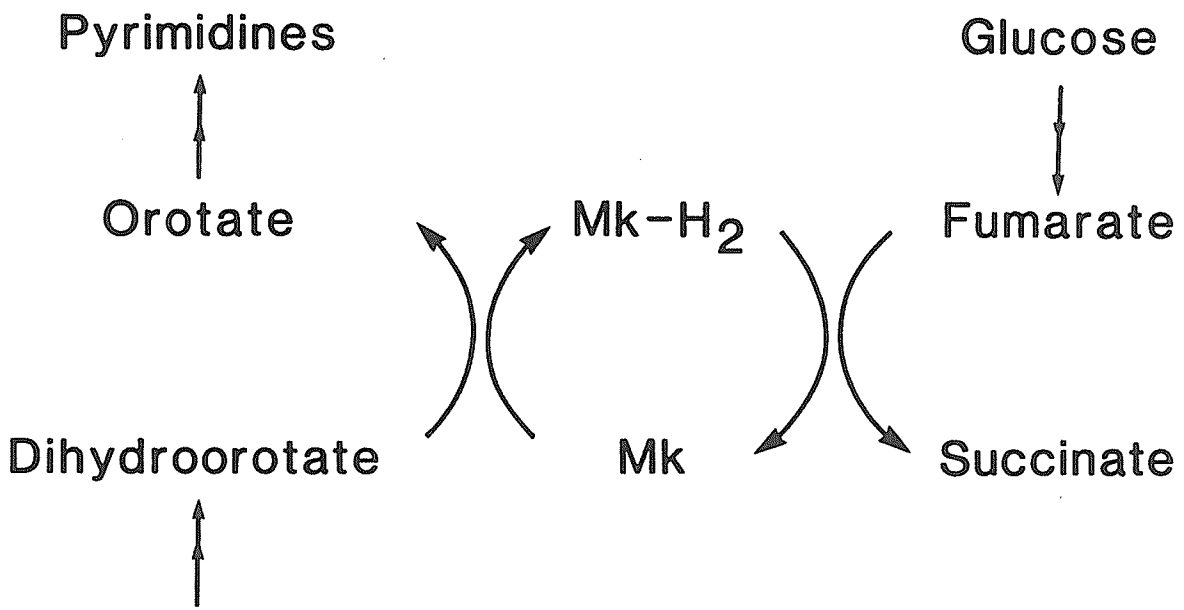
(ii) Menaquinone in Biosynthetic Reactions. The involvement of menaquinone in biosynthetic redox reactions was demonstrated in the work of Newton et al, (1971). Using wild type (men+ ubi+) and mutant strains, it was found that men- ubi- strains would not grow anaerobically unless uracil was supplied. The requirement for uracil was due to the fact that quinones were necessary as redox cofactors for the dehydrogenation of dihydroorotate to orotate during pyrimidine biosynthesis. Using fluorometric methods to detect menaquinone reduction, it was demonstrated that among hydrogen acceptors tested, only fumarate had any effect on menaquinone reduction in membrane preparations of anaerobically grown wild type strains. The link between dihydroorotate oxidation to orotate and reduction of fumarate was demonstrated with wild type (ubi+ men+) and mutant (men- ubi-) strains. Wild type strains grown anaerobically converted

dihydroorotate to orotate until O_2 was exhausted after which the reaction stopped. Further addition of O_2 caused a burst of orotate formation. The same strain grown in the presence of fumarate continued to show production of orotate even after O_2 was exhausted.

Using the double mutant under anaerobic conditions, orotate formation depended on the supply of menaquinone or ubiquinone until the O_2 was exhausted. Thereafter formation was dependent on the presence of both menaquinone and fumarate. These findings demonstrated that the oxidation of dihydroorotate to orotate was linked to the reduction of fumarate via menaquinone where MK-8 in E. coli was the obligatory H+ carrier for the oxidation reaction (Figure 3.3). Under anaerobic conditions, there was an absolute requirement for both menaquione and fumarate; in the presence of O_2 either ubiquinone or menaquinone would suffice as the redox component.

Menaquinones have also been shown to be necessary for the biosynthesis of heme by bacteria growing under anaerobic conditions. The actual step in the pathway of heme synthesis where menaquinone functions as a redox factor is the oxidation of protoporphyrinogen to protoporphyrin. This oxidation is linked to fumarate reduction. Using specially constructed ubi- and men- mutants, Jacobs and Jacobs (1978) demonstrated that ubi- men+ and ubi+ men+ strains produced fumarate dependent oxidation of protoporphyrinogen to protoporphyrin. Contrastingly, ubi+ men- and ubi- men- strains did not. In the latter two strains addition of menadione in the presence of fumarate restored oxidation of protoporphyrinogen; in the absence of fumarate, no activity was seen. In all strains, the absence of fumarate produced minimal activity. They also demonstrated substrate specificity for

Figure 3.3 Schematic diagram showing the role of menaquinone in the anaerobic biosynthesis of pyrimidines in E. coli.



protoporphyrinogen in this reaction by essentially demonstrating lack of oxidation with other substrates such as coproporphyrinogen. Also, the reaction was shown to be non cytochrome dependent as mutants deficient in cytochrome carried out the oxidation with equal facility in the presence of menaquinone and fumarate when compared to cytochrome sufficient strains. It was clear from these findings that the fumarate-linked oxidation of protoporphyrinogen resembled the dihydrootate oxidation linked to fumarate reduction as described previously.

In a series of studies, Lev and his co-workers (Lev and Milford, 1972; 1973; Lev, 1979) demonstrated that the nutritional requirement for vitamin K exhibited by B. melaninogenicus was related to sphingolipid metabolism in these organisms. Cells grown in the absence of vitamin K formed defective cell envelopes and elongated from 0.4 - 0.6 um to as long as 14 um. Since the cells appeared to be unable to divide, the growth rate dropped in the absence of vitamin K -- this rate increased after the addition of vitamin K. Lev and Milford, (1972) first showed that succinate at 2×10^{-2}M concentrations could replace the vitamin K requirement in the growth of B. melaninogenicus but that the organisms grew slowly. These cells had depressed levels of the sphingolipids, ceramide phosphorylethanolamine (CPE) and ceramide phosphorylglycerol (CPG). On supplementation of these cells with vitamin K, phosphosphingolipid biosynthesis was stimulated as shown by increased linear uptake of ^{32}P.

The first step in the sphingolipid biosynthetic pathway is the condensation of an acyl coenzyme A (COA) with serine to form 3-ketodihydrosphingosine. Lev and Milford, (1973) subsequently showed that 3-ketodihydrosphingosine synthetase which catalyses the synthesis of

3-ketodihydrosphingosine from palmitoyl CoA and L-serine, was found in negligible quantitites in membranes from B. melaninogenicus cells grown in vitamin K deficient conditions compared to those grown in vitamin K sufficient culture. Enzyme activity could not be enhanced in cell free systems on addition of vitamin K but increased levels could be induced in whole cells by addition of the vitamin to a vitamin K depleted medium on which the cells were growing. To demonstrate that enzyme activity in the presence of vitamin K_1 was due to denovo synthesis, puromycin or rifampicin and vitamin K were added to culture growing in a vitamin K deficient medium. Compared to controls, there was no production of enzyme in the presence of both inhibitors which indicates that transcription and protein synthesis results from vitamin K dependent induction of the enzyme.

E. Vitamin K in Animal Tissues

1. Distribution in Tissues

The amount of vitamin K in animal tissues is low and as a consequence, the assessment of the quantity and forms becomes quite difficult because of the lack of sensitive assay methods and the large amounts of interfering lipids present.

Information concerning the forms of vitamin K in animal tissue was first obtained through metabolic experiments, carried out by Martius' and co-workers (Martius, 1961). Chickens were placed on a vitamin K_1 free diet, and fed labelled menadione. On extraction of their tissues both the labelled compound and MK-4 was recovered. This was considered evidence that menadione (MK-0) was transformed to MK-4 in the tissues and that the latter was the active form in animals.

When vitamin K_1 labelled with ^3H in the nucleus and ^{14}C in the phythyl chain was fed to chickens and pigeons, MK-4 labelled only at the nucleus was recovered from their tissues. Labelled vitamin K_1 but not vitamin K_2 was found in the liver, whereas the other organs contained only MK-4. These results indicated that the side chain of phylloquinone was being removed in the tissues and the nucleus realkylated with a polyisoprenoid side chain containing 4 isoprene units. Also since only vitamin K_1 was found in the liver this suggested that vitamin K_1 was the functional molecule in the liver and was more important than vitamin K_2 at this site. The removal of the phythyl side chain from vitamin K_1 and addition of a 4 isoprene unit to the naphthaquinone nucleus was confirmed by finding the ^{14}C labelled phythyl chain in extracts of tissues. Also incubation of liver homogenates with labelled methyl naphthaquinone and pyrophosphates of polyisoprenic alcohols led to the formation of menaquinone molecules, in vitro. The enzyme system responsible for the introduction of the 4 isoprenoid unit chain was determined to be localised in the mitochondria or microsomes but was not identified, Dialameh et al, (1970).

The conversion of vitamin K_1 to MK-4 was considered to occur in the liver but this belief was questioned because of data demonstrating that the conversion would only occur if it was administered orally. Such results indicated that bacterial action in the gut may be responsible for the dealkylating and alkylating steps. However, Taggart and Matschiner, (1969) presented data from studies with rats placed on a vitamin K_1 deficient diet and injected intracardially with tritium labelled menadione, to support the contention that conversion to MK-4 occurred in the tissues. After 18 hours, only MK-4 could be isolated

by chromatography from the liver, heart and kidneys. Recently, Lee and Olson (1984) provided evidence that the alkylation of menadione to MK-4 occurs in the microsomal fraction of chicken and rat livers. The alkylation enzyme was located in the smooth endoplasmic reticulum. MK-4 produced from menadione given to vitamin K-deficient chicks was also isolated and its structure confirmed by mass spectrometry (Suttie, 1978).

The discovery that vitamin K_1 can be converted to MK-4 was assumed to indicate that MK-4 is the active form of vitamin K in animal tissues. However this had not been established unequivocally since characterisation of animal tissue for menaquinone content had not been attempted. Examination of livers from different animals for vitamin K_1 and menaquinones have been carried out by Matschiner and his co-workers. By using solvent extraction and a series of chromatographic steps such as silicic acid adsorption column chromatography, reverse phase partition column chromatography and reverse phase and Ag+ TLC, this group was able to isolate MK-10, MK-11 and MK-12 from bovine liver (Matschiner and Amelotti, 1968), vitamin K_1 from horse liver (Duello & Matschiner, 1970), and a series of menaquinones with fully unsaturated and partially saturated side chains (Table 3.4) from pig, dog and human livers (Duello & Matschiner, 1971, 1972). The structures and identities of these compounds as suggested by chromatographic mobility on reverse phase and Ag+ TLC were confirmed by the use of UV absorption spectroscopy and mass spectrometry.

From the available data generated by Matschiner's studies, it appeared that the liver contains those forms of vitamin K which are ingested or present in the intestinal tract. Bovine liver contains

Table 3.4 Summary of K Vitamins Isolated From the Liver (Duello & Matschiner, 1972)

Beef	Rabbit	Chicken	Pig	Dog	Horse	Human
K_1	K_1	K_1	K_1	MK-6	K_1	K_1
MK-10	MK-4 (H_4)	MK-4 (H_4)	MK-4 (H_4)	MK-6 (H_2)		MK-7
MK-11			MK-7	MK-7		MK-8
MK-12			MK-7 (H_2)	MK-7 (H_2)		MK-9
			MK-8	MK-7 (H_4)		MK-9 (H_2)
			MK-8 (H_2)	MK-7 (H_6)		MK-9 (H_4)
			MK-8 (H_4)	MK-8		MK-10
			MK-8 (H_6)	MK-8 (H_2)		MK-10 (H_2)
			MK-9	MK-8 (H_4)		MK-10 (H_4)
			MK-9 (H_2)	MK-8 (H_6)		MK-11
			MK-9 (H_4)	MK-9		
			MK-9 (H_6)	MK-9 (H_2)		
			MK-10	MK-9 (H_4)		
			MK-10 (H_2)	MK-9 (H_6)		
				MK-10 (H_2)		
				MK-11		
				MK-12		
				MK-13		

MK-10, MK-11 and MK-12 which are predominant forms recovered rumen of these animals (Matschiner, 1970). This finding supports the concept that forms of the vitamin absorbed in the intestine make up the composition of menaquinones in the liver and would suggest that in beef liver, the composition is determined by the supply of vitamin K from the microorganisms in the gut (rumen). The recovery of vitamin K_1 as the only form of vitamin K in the liver of the horse, a herbivore also supports the view that the liver contains forms of vitamin K which are absorbed from the gut rather than forms produced by metabolic interconversion in the liver.

Distribution of vitamin K in other parts of the body of animals have been studied by whole body radiography. After the administration of labelled vitamin K_1, menadione, and MK-4, by intravenous injection there was an equal distribution of vitamin K_1 and MK-4, after 24 hours. There were also higher levels of MK-4 than vitamin K_1 in the intestine reflecting higher biliary excretion of MK-4 compared to vitamin K_1. Vitamin K was found to be concentrated in organs such as bone marrow, lungs, kidneys, lymph nodes and adrenal glands.

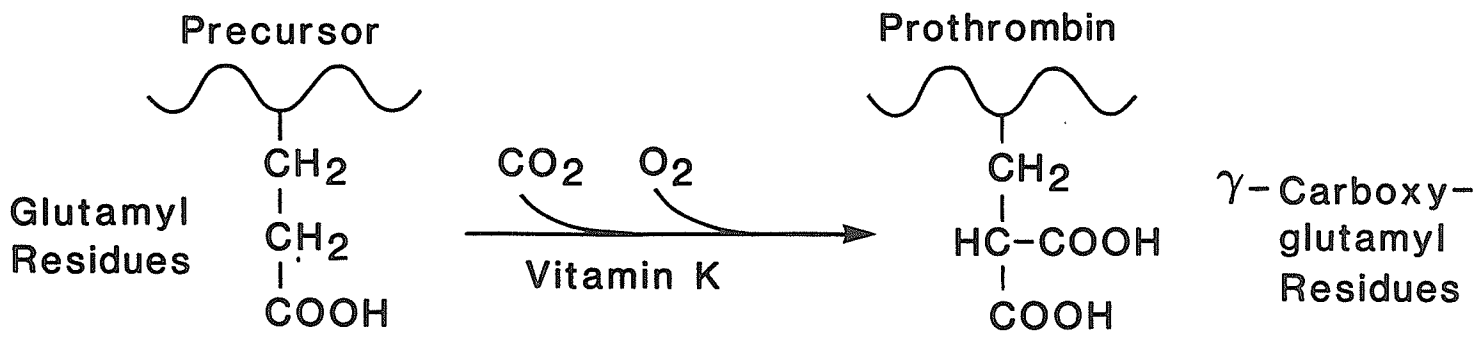
In a similar type of study, Matschiner fed vitamin K depleted rats doubly labelled phylloquinone (^3H label in the naphthaquinone nucleus and ^{14}C in the side chain) for 4 days. On assessing radioactivity in various organs, ^3H activity was highest and similar in the liver and spleen. High concentrations were also present in the lung, kidney and cartilage, and the lowest concentrations were in skeletal muscle. However, since vitamin K was not chemically recovered from the tissues, levels of radioactivity may not be a reflection of structurally intact vitamin molecules.

2. Function of Vitamin K in the Liver

Mammalian coagulation involves a number of protein factors in the form of a cascade system. The production of the active form of four of these proteins--Factor II (prothrombin), VII (proconvertin), IX (Christmas factor), and X (Stuart factor) is dependent upon vitamin K. The protein precursors of these factors are produced in the liver and in order for them to become active, certain glutamic acid (Glu) residues must be post-translationally carboxylated to gamma-carboxy-glutamic acid (Gla). Vitamin K is part of a membrane bound carboxy-lase system that is responsible for the carboxylation reaction. The enzyme system requires vitamin K as the reduced hydroquinone, carbon dioxide, oxygen, NADH and the substrate containing Glu residues (Figure 3.4).

In the initial work to determine the function of vitamin K in mammals (reviewed by Olson and Suttie, 1977), it was first thought that vitamin K acted at the transcriptional level, perhaps as a repressor to regulate the synthesis of the coagulation proteins. However, a series of studies using inhibitors to transcription (actinomycin D) and protein synthesis (puromycin or cyclohexamide) determined that the vitamin acted at a post-transcriptional point in protein synthesis. Administration of vitamin K to severely hypoprothrombinemic rats caused the appearance of prothrombin in plasma after a delay of 30-60 minutes from the time the vitamin was given. In addition, it was demonstrated that the appearance of plasma prothrombin was preceded by transient increase of prothrombin in rat liver microsomal preparations. Microsomal prothrombin peaked after 10 minutes and then decreased as prothrombin appeared in the plasma,

Figure 3.4 Function ofvitamin K in the carboxylation of glutamic acid.



suggesting the prothrombin produced was not due to de novo synthesis of protein. This finding implied the presence of a precursor protein pool in the liver which was being converted to prothrombin, the conversion being vitamin K mediated. Further evidence was supplied by the study of Shah and Suttie, (1971), who demonstrated that hypoprothrombinemic rats given vitamin K and labelled amino acids produced unlabelled prothrombin, suggesting the existence of a precursor pool.

Using immunochemical methods, separate groups were able to show the presence of abnormal prothrombin or the precursor prothrombin molecule in plasma that were antigenically similar but lacking biological activity. This finding led to a series of experiments that culminated in the discovery of gamma-carboxyglutamic acid. The abnormal prothrombin molecule has been also referred to as PIVKA (protein induced by vitamin K absence) or descarboxyprothrombin among other names. Abnormal prothrombin did not adsorb to barium salts and was unable to bind Ca++ required for the phospholipid stimulated activation by factor Xa. The Ca++ binding region of the molecule was determined to be that of fragment I and shown to be vitamin K-dependent region. The nature of this region upon investigation demonstrated a high proportion of acidic-amino acid residues.

Two carboxyglutamic acid (Gla) residues were first discovered by Stenflo et al, (1974) in a peptide digest of prothrombin at positions 7 and 8, followed by two more in a different part of the molecule (Nelsestuen and Zytkovicz, 1974). Magnusson et al, (1974), sequenced the first 42 residues of prothrombin and showed the presence of 10 Gla residues. Gla has also been shown in factors VII, IX and X, the other

vitamin K dependent coagulation proteins.

The in vitro production of prothrombin was initially demonstrated by Shah and Suttie, (1974), using post-mitochondrial supernatants from vitamin K deficient rats. Addition of vitamin K to the system elicited the formation of prothrombin measured by the two stage assay. The vitamin K step was shown to be the formation of gamma-carboxyglutamic acid from glutamic acid. This system was used to demonstrate that the addition of vitamin K and H $^{14}CO_3^-$ promoted the carboxylation of endogenous microsomal proteins. Radioactive prothrombin could be isolated with all the incorporated label being present in the Gla residues (Esmon et al, 1975).

The vitamin K-dependent carboxylation reaction has been studied using 3 systems: (i) post-mitochondrial supernatant suspension containing microsomes, (ii) washed microsomes, and (iii) microsomes solubilized with detergents. These systems differ in a number of properties such as differences in vitamin K requirement, ATP requirement, prothrombin yield and warfarin sensitivity (Suttie, 1980). Using the post-mitochondrial suspension in various studies, it was found that, there was a requirement for both HCO_3^- and O_2 and NADH or NADPH. Furthermore, the requirement for NADH and vitamin K could be replaced by reduced vitamin K (KH_2) or the hydroquinone. In terms of specificity for vitamin K homologues, vitamin K_1, MK-3 and MK-4 demonstrate the best activity, with reduced activity by MK-1 and MK-2 (Uotila and Suttie, 1982). The washed microsome system differs from the post-mitochondrial suspension in that dithiothreitol (DTT) is more effective than NADH or NADPH as a reductant for vitamin K. Also CO_2 was preferentially taken at rates 5-10 times higher than HCO_3^-. The

major difference in the solubilized microsomal system is that it does not require ATP for activity.

In searching for artificial synthetic substrates for carboxylase, a number of low molecular weight peptides have been identified that will serve in this capacity. Suttie, et al (1976), reported the use of Phe-Leu-Glu-Glu-Val (analogous to amino acid residues 5-9 in bovine precursor prothrombin) and Houser, et al (1977) used Phe-Leu-Glu-Glu-Ile or Leu which is similar to the rat prothrombin precursor. With the soluble rat microsomal system, in-vitro carboxylation can be demonstrated at a higher level using solubilized vitamin K-deficient rat liver microsomes compared to normal ones. Also the pentapeptide containing Ile showed higher activity than the one containing Val. The products of carboxylation have not been definitely characterized and there is no data to indicate the extent of carboxylation of the endogenous precursors. The major product of carboxylation of Phe-Leu-Glu-Glu-Leu is Phe-Leu-Gla-Glu-Leu.

In terms of molecular activity the role played by the vitamins is not known. Current theories hold that the vitamin may function to transfer CO_2 for the carboxylation; it may function to stabilize the hydrogen at the gamma carboxyl of the precursor so that it will accept CO_2 or may function as an activator of an enzyme in the reaction. However, there is no evidence to support these hypotheses.

Vitamin K-2,3-epoxide was first discovered by Matschiner, et al (1970), studying the metabolism of vitamin K_1 in rats given warfarin. They demonstrated a build up of this metabolite in rat livers as vitamin K_1 was being metabolized to the K_1-2,3 epoxide. The production of the epoxide was postulated to be an obligatory step in

the action of the vitamin in prothrombin biosynthesis and was based on the observation that epoxidase activity increased in the liver under various conditions paralleling the concentrations of prothrombin precursor. This was further supported by Sadowski, et al (1977) who showed that the requirements for carboxylation and epoxidation were similar, and that the substrate for the epoxide formation is the reduced form of the vitamin. Also, the O_2 incorporated into the epoxide ring arises from molecular oxygen. In general, conditions that favor epoxide formation also favor carboxylation. Normally about 10% of the vitamin in the liver is in the form of the epoxide but this increases and can become the predominant form during warfarin therapy. The reduction of the vitamin K-2,3-epoxide to the quinone is carried out by a vitamin K-epoxide reductase. Like the other enzymes in the vitamin K interconversion cycle, the reductase activity is also membrane bound and stimulated by thiol compounds. Using crude liver homogenates, Matschiner, et al (1970) demonstrated that the reduction of epoxide to the vitamin was stimulated by DTT in vitro and inhibited by warfarin.

The vitamin K related activities in the liver can be generalized as follows: the pool of reduced vitamin K (KH_2) serves as a cofactor for the carboxylation of Glu residues to Gla. The product of vitamin oxidation is uncertain but it is thought to be the epoxide. Epoxide formation from the hydroquinone can also be produced by a microsomal internal monooxygenase. The epoxide is then used as a substrate for the 2,3-epoxide reductase to regenerate the quinone, utilizing an unidentified sulfhydryl compound as reductant. In in-vitro systems, DTT serves as the reductant. Studies have indicated that this

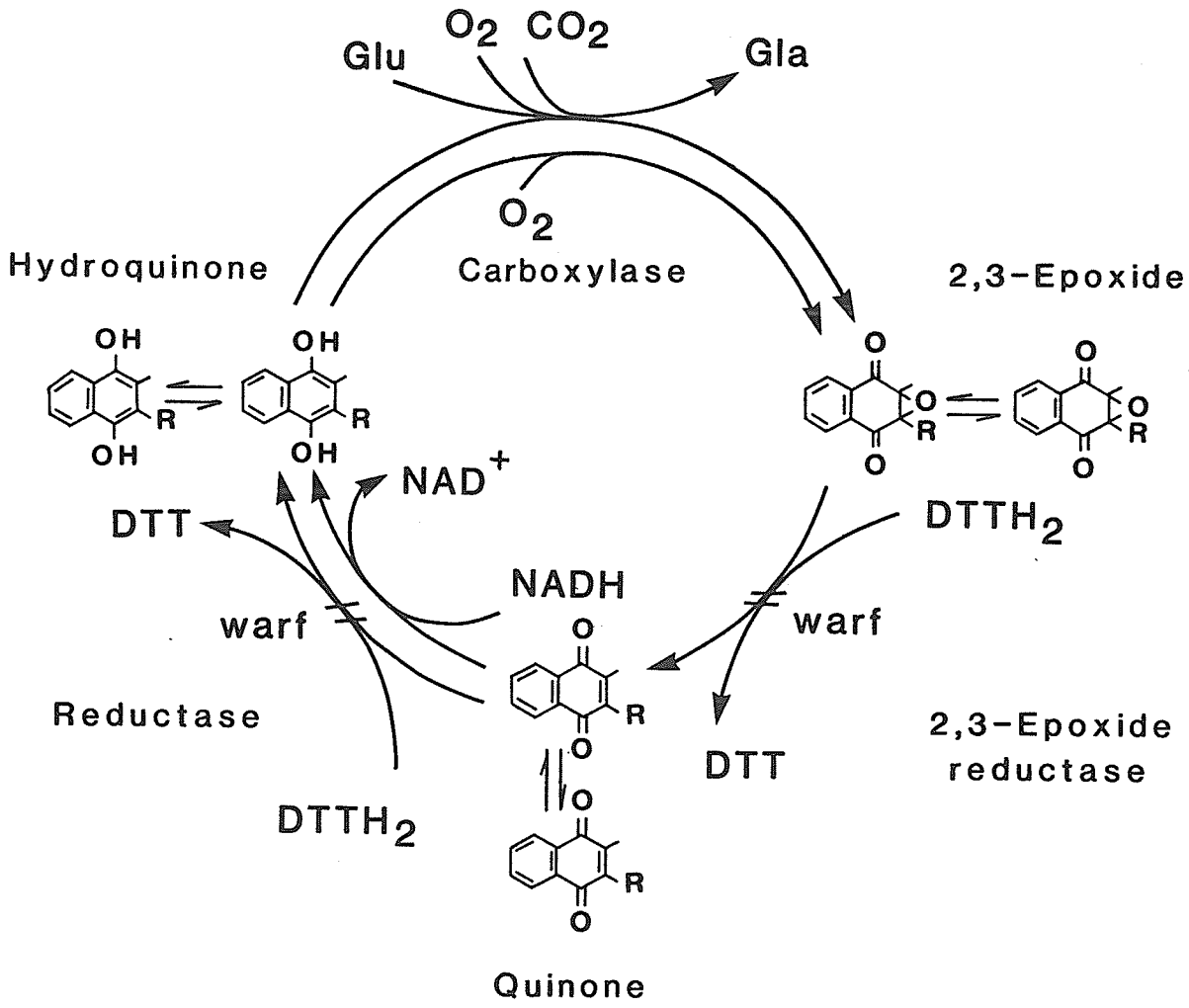
reaction appears to be the site of action of hydroxycoumarin anti-coagulants. The reduction of the quinone to its hydroquinone is carried out by an NAD(P)H linked reductase, but DTT can also be used to drive the reaction (Figure 3.5) (Suttie, 1980; Gallop et al, 1980; Uotila and Suttie, 1982).

The metabolism of vitamin K therefore involves the cyclic reduction of the vitamin K quinone to the hydroquinone, oxidation to the epoxide and reduction back to the quinone. All the enzymes catalysing these reactions, the vitamin K dependent carboxylase, epoxidase, epoxide reductase and vitamin K reductase are in the rough endoplasmic reticulum in rat liver.

Activity of the differing forms of vitamin K have been measured in a number of in-vitro systems. Using microsomes suspended in buffer, Freidman & Shia (1976) found that MK-2 had 10 times and MK-3, 80 times the activity of vitamin K_1 in carboxylating endogenous precursors. Cis vitamin K_1,DMK and MK-0 had little or no activity. In assessing activity, Jones et al, (1976), measured prothrombin production rather than carboxylation activity using post-mitochondrial supernatant from vitamin K-deficient rats. They found that MK-2, MK-3, MK-4, MK-5, MK-6 and MK-7 at a concentration of 4.4×10^{-5}M had roughly the same activity in terms of inducing prothrombin synthesis. MK-0 was inactive. In detergent solubilized microsomal systems vitamin K_1, MK-1, MK-2, MK-3 and MK-4 have similar activity at 10^{-4} M.

In in-vivo systems with intact animals only 2-methyl 3-isoprenyl-1,4-naphthaquinone demostrates activity. Cis vitamin K_1 shows no activity, and demethyl forms also show very low activity. Activity is therefore related to the 2-methyl -1,4-naphthaquinone ring

Figure 3.5 The vitamin K-dependent carboxylation cycle.



warf = warfarin

with substitution at the C-3 site a necessity.

In-in vitro systems, the vitamin K requirement also depends on the presence of cytosolic proteins, the requirement being lower in those systems containing the proteins. In the post-mitochondrial system, when measuring prothrombin formation, half maximal activity was observed at 10^{-7} M as opposed to 10^{-5} M with washed microsomes (measuring incorporation of $^{14}CO_2$) and 5 x 10^{-7} M on addition of the cytosolic fraction back to the system. Similarly, when measuring incorporation of CO_2 with the detergent solubilized system, half maximal activity was achieved at 5 x 10^{-6} M.

F. Vitamin K Deficiency and Hypoprothrombinemia

1. Hypoprothrombinemia in Humans and Other Mammals

In mammals, vitamin K is necessary for the synthesis of four coagulation factors (II, VII, IX and X). Depletion of vitamin K reserves results in coagulation abnormalities which may lead to bleeding. The actual daily requirement in man has not been conclusively determined, but Frick et al (1967) found that doses of vitamin K_1 between 0.03 and 1.5 ug/kg body weight administered to hypoprothrombinemic patients, were sufficient to normalise prothrombin time. They concluded that the minimal daily requirement was probably slightly above 0.03 ug/kg body weight. Barkhan and Shearer (1977), using similar methods, estimated that vitamin K_1 requirement may lie in the range of 0.1 - 0.5 ug/kg body weight. Based on the available data, Olson and Doisy (1980) concluded that 1 ug/kg body weight would be sufficient for healthy persons. The U.S. 1980 Recommended Dietary Allowances has determined that safe and effective daily levels of

vitamin K for healthy adults is in the order of 1-2 ug/kg body weight/day.

At various times it has been stated that "synthesis of vitamin K in the gut is capable of supplying the total needs of animals under ordinary conditions" (Gustaffson, 1959); and "vitamin K in man is ordinarily obtained from the diet, relatively little coming from bacterial action in the gut" (Udall, 1965). It appears that the general belief is that human requirements for vitamin K are fulfilled by dietary sources (green leafy vegetables) only, and that bacterially produced menaquinones are less important in this respect, even though there is evidence to the contrary. The early work delineating the relative roles of vitamin K_1 and K_2 was mostly done with germ free rats. The importance of bacterially produced menaquinone as a possible source of the vitamin stems from the original work of Greaves (1939). He found that only 12 of 77 rats fed a diet of rice and ether extracts of yeast and fish meal developed abnormal prothrombin values (5-50% of normal), which corrected spontaneously, or by feeding vitamin K. Spontaneous correction of hypoprothrombinemia suggested that the animals were acquiring vitamin K in spite of the deficiency in their diet. The fact that rats on the vitamin K deficient diet did not develop severe hypoprothrombinemia indicated that bacterially produced vitamin K was available from the intestine.

Gustaffson (1959), working with germ free and normal rats fed a vitamin K_1 deficient diet (largely starch and casein), found that only the former developed hypoprothrombinemia within 5-9 days. The severity of the deficiency (42 of 44 rats had prothrombin times greater than 200 seconds) was such that rats either had bleeding

symptoms or prothrombin levels less than 10% of normal. Since only the germ free rats became hypoprothrombinemic, it suggested that the absence of bacteria and hence menaquinones in the animals' intestines was the factor that led to the coagulopathy. Further evidence to support this contention was provided in the same study. Hypoprothrombinemia in rats was corrected by transferring them to an infected area (cages that had been occupied by normal animals). As well, in a follow-up study Gustaffson et al (1962), monoinfected animals with lactobacilli, streptococci, micrococci, diphtheroids, Bacteroides, E. coli and Proteus sp. Only E. coli and Micrococcus sp. were shown to reverse hypoprothrombinemia. Why the Bacteroides sp. failed to do so is uncertain. However, colonization of the intestinal tract of the animals by the obligate anaerobe may have been unsuccessful. These findings implied that vitamin K produced by bacteria was sufficient to supply the needs of the animals. However, this evidence, supporting the role of bacterially produced menaquinone in human coagulation, has been regarded as not convincing enough, since Gustaffson did not show whether the animals had become colonized by menaquinone producing bacteria.

This uncertainty was further compounded by the work of Barnes and Fiala (1958) which suggested that menaquinone produced in the intestines is only available to rats through coprophagy and not by absorption in situ. They demonstrated that rats on a vitamin K-deficient diet, and prevented from ingesting their feces, developed severe deficiency in 4 weeks. Rats with normal access to their feces, only had moderate hypoprothrombinemia (prothrombin times of 20 seconds). If the coprophagy prevented rats were allowed to subsist on the diet

for up to 12 weeks, prothrombin times rose steadily, peaking at 6 weeks then falling to normal by 12 weeks. This confusing observation was noted consistently. It was thought that perhaps the prolonged period of preventing coprophagy may have been sufficient time to allow microbial growth in the small intestine--a site where vitamin K could be absorbed. Since the coprophagy prevented rats developed more severe hypoprothrombinemia, vitamin K could not be absorbed to an appreciable extent, but must be obtained by ingestion of feces (Barnes and Fiala, 1959).

Mameesh and Johnson, (1960a,1960b), using a totally synthetic diet consisting largely of sucrose and soy protein, found that rats allowed access to their faeces became hypoprothrombinemic (32 seconds), and when coprophagy was prevented, this condition worsened considerably (92 seconds). These findings were very similar to observations by Barnes and Fiala. Hypoprothrombinemia was reversed by exogenous doses of vitamin K_2 or vitamin K_1. Using serial doses of vitamin K_1 they showed that the dietary vitamin K requirement of a non-coprophagic rat was between 0.1 and 0.15 ug/g of diet. These data, as well as the findings of Barnes and Fiala, suggested that vitamin K synthesized by intestinal bacteria could satisfy the requirements of rats, but to an extent depending on the degree of coprophagy. However, the authors did not explain why coprophagic rats also became hypoprothrombinemic albeit to a lesser extent.

Udall, (1965), studied the role of endogenous vitamin K in humans, and found that vitamin K_1 did not appear to be absorbed from the large intestine (the site where it is expected that the highest quantity of vitamin K would be synthesised by bacteria). He found

that healthy human subjects, on a diet consisting largely of rice, showed a slight but significant increase in prothrombin times from 14.8 to 16.0 seconds after 3 weeks. Subjects placed on a vitamin K_1 sufficient diet or oral vitamin K_1, after becoming hypoprothrombinemic, responded with normal prothrombin times. In order to determine whether vitamin K was absorbable from the human colon, Udall (1965), using a weighted nasointestinal tube, delivererd 400 mg vitamin K_1 directly into the colon of warfarinized hypoprothrombinemic subjects, but was unable to reverse their hypoprothrombinemia. Vitamin K_1 delivered orally to a similar patient caused rapid correction of the defect. Udall concluded from these studies that endogenous vitamin K was not absorbable from the large bowel, though he did concede that the endogenous source may be of some value in fulfilling the human requirement, because of the inability of the vitamin K_1 deficient diet alone to generate a rise in prothrombin times to hemorrhagic levels.

Udall's conclusions were countered by the findings of Frick et al (1967), in a study involving 10 human subjects on glucose intake only. Seven of the subjects on unspecified antibiotics became hypoprothrombinemic with reduced levels of Factors II, VII, IX and X after 21-28 days while the other 3 not on antibiotics showed no defects. These observations implied that in the absence of dietary source of vitamin K_1, hypoprothrombinemia will occur secondary to removal of menaquinone producing intestinal flora. It therefore seemed logical to conclude that endogenous vitamin K (menaquinones) are important in fulfilling human requirements.

There have been numerous reports of hypoprothrombinemia in patients receiving antibiotics. In some cases, hypoprothrombinemia

has been associated with clinical bleeding (Ansell et al, 1977; Hooper et al, 1980; Reddy and Bailey, 1980; Weitekamp and Aber, 1983). The earlier reports of antibiotic associated hypoprothrombinemia involved the use of a variety of agents such as chloramphenicol (Klippel and Pitsinger, 1968), ampicillin alone (Ham,1971) or in combination with cloxacillin and gentamicin (Pineo et al, 1973) or tetracycline (Colvin and Lloyd, 1977, Olson & Doisy 1980), among others. The most recent reports seemed to be confined to the use of the broad-spectrum cephalosporins especially moxalactam (Pakter et al 1982, Weitekamp and Aber 1983, Panwalker and Rosenfeld, 1983, MacLennan et al, 1983; Joehl, et al, 1983; Fainstein et al, 1983; Barza et al, 1986) but also cefamandole (Hooper et al, 1980; Chang, 1983; Clancy & Glew, 1983; Fainstein et al, 1983; Sanburg et al, 1985), cefoperazone (Reddy and Bailey, 1980, Chang 1983, Alitalo et al 1985) or cefoxitin (Reddy and Bailey, 1980; Sattler et al, 1986).

Reports of coagulation defects in patients have usually focused on increased prothrombin times and clinical bleeding episodes in association with the use of antimicrobial agents. The relative importance of reduced dietary intake of vitamin K_1 and/or eradication of the menaquinone producing intestinal flora to the development of the hypoprothrombinemia has not been assessed.

In most cases, patients, in whom bleeding or prolongation of prothrombin times occur, are elderly, debilitated or severely ill and are on reduced or no vitamin K_1 intake even though this has not been quantified. For instance in Hamm's study (1971) all 5 patients showing increased prothrombin times in association with the use of ampicillin were in intensive care on intravenous fluids only. Like-

wise subjects in Pineo's study of 1973, were either post-surgical or medical patients on no vitamin K_1 intake and those of Alitalo et al (1985), and Clancy and Glew, (1983), were elderly malnourished patients on parenteral nutrition with reduced vitamin K_1 intake.

Unfortunately these studies did not assess the effect of the antibiotics or diet on the menaquinone producing organisms of the intestines and therefore no conclusion can be drawn with respect to any correlation between available menaquinone stores, during low dietary intake of vitamin K_1 and the incidence of hypoprothrombinemia.

In spite of this, the predisposing factors to hypoprothrombinemia seem to be that subjects are in a hospital setting, elderly, malnourished and on antimicrobial therapy with reduced vitamin K_1 intake.

Hypoprothrombinemia occurs because of decreased synthesis of "active" vitamin K-dependent factors, II, VII, IX and X. This occurs when there is a deficiency in vitamin K as it is a necessary co-factor in the post-translational modification of precursor factors via carboxylation of glutamic acid residues on the amino terminal end of the polypeptide chains. Vitamin K deficiency results in increased levels of descarboxyprothrombin (abnormal prothrombin) in the circulation. Clinically the defect is reflected in increased prothrombin times with the potential for bleeding episodes to occur when prothrombin levels decreased sufficiently (usually less than 10% of normal).

Antibiotic associated vitamin K deficiency is thought to occur by suppression of the menaquinone producing bacteria in the intestines by these agents during reduced vitamin K_1 intake and consequently the reduction of menaquinone stores. This seems very likely, since

cefamandole, cefoperazone, cefoxitin and moxalactam, are all excreted extensively into the intestines via the bile and have the capacity to profoundly affect the flora (Reddy and Bailey et al 1980, Hooper et al 1980).

Alternatively the possibility exists that reduced synthesis of vitamin-K dependent clotting factors is caused by direct inhibitory effect of the antibiotic on the carboxylation reaction. This is a very attractive hypothesis, especially, since it is felt by some researchers that menaquinone synthesized in the gut is not absorbed and therefore the association of hypoprothrombinemia with antimicrobial agents cannot be due to its removal.

The evidence for drug inhibition of the carboxylation reaction has been somewhat contradictory. Certain cephalosporins such as cefmetazole, cefamandole, cefmenoxime, cefoperazone and moxalactam contain a 1-methyl-1H-tetrazol-5-yl-thiomethyl (commonly referred to as N-methylthiotetrazole or NMTT) group at position 3 of the dihydrothiazine nucleus. It is felt that this group rather than the antibiotic itself is responsible for the inhibition of the carboxylation reaction in the liver that produces active vitamin K-dependent clotting proteins. The reaction is mediated by a membrane bound microsomal carboxylase. The cephalosporins which have been associated with vitamin K responsive hypoprothrombinemia and clinical bleeding have been primarily moxalactam (Pakter et al 1982; Weitekamp and Aber 1983; Panwalker and Rosenfeld 1983), cefamandole (Hooper et al 1980) and cefoperazone (Reddy and Bailey, 1980). These three agents all contain the N-methylthiotetrazole moiety.

Lipsky (1983), using rat liver microsomal preparations, observed

that NMTT inhibited the gamma carboxylation of glutamic acid in-vitro. The 50% inhibitory concentration was determined to be 1.1 mMole/L. Antibiotic preparations themselves did not cause inhibition of the reaction up to concentrations of 2 mMole/L.

In order to validate his results in an in-vivo system, Lipsky et al (1984), examined the effects of moxalactam, cefotaxime (a non-NMTT containing agent) and NMTT on prothrombin times in rats fed a vitamin K_1 deficient diet for 10 days. It was observed that both moxalactam at 3 g/kg and NMTT at 225 mg/kg produced hypoprothrombinemia in rats (3 to 4 fold increase in prothrombin time over controls) within 48 hours. Cefotaxime on the other hand did not cause prolongation of prothrombin times under the same conditions.

The findings from both the in-vitro and in-vivo studies seemed to indicate that NMTT molecule rather than the intact antibiotic, is responsible for inhibition of the carboxylation reaction. This conclusion is supported by the fact that cefoxitin which yields high intestinal levels does not appear to cause hypoprothrombinemia (Neu 1983). Also cefotaxime and ceftizoxime, which contain an acetoxy and hydrogen group, respectively, on the 3 position of the dihydrothiazine nucleus, are not associated with hypoprothrombinemia. In addition to this, there is evidence indicating that with cefmetazole, free NMTT is released in the intestine (Lipsky 1983), and urine (Lipsky et al 1984). Also, NMTT has been found in the plasma of humans on moxalactam administration (Lipsky et al 1984).

NMTT is located in beta-lactam antibiotics at a site known to be a "leaving group" site for such drugs. Lipsky (1983) speculates that the intact antibiotic is secreted via the bile into the lumen of the

intestine where intestinal bacteria may be responsible for its degradation to release the NMTT group. After release, it can then be reabsorbed and reach the liver via the hepatic-portal circulation where it exerts its effect on the carboxylase enzyme.

Lipsky's hypothesis as to the mechanism by which NMTT containing antibiotics caused hypoprothrombinemia has been challenged by a number of studies.

Bang et al (1982), using human volunteers dosed with moxalactam at 3 g, achieved plasma levels of 700 ug/ml. They observed that under these conditions, there was no change in prothrombin times or concentrations of Factors II, VII, IX and X. Using the Echis carinatus assay, the levels of descarboxyprothrombin levels stayed within the normal range.

Wold et al (1983), using doses of 2700 mg/kg moxalactam in dogs, rats and monkeys and NMTT up to 500 mg/kg in rats were unable to demonstrate increased prothrombin times after 31 days. The possibility that NMTT may have a transient effect on the coagulation defect was evaluated by PT measurements at timed intervals after dosing rats with the compound at 410 mg/kg. Once again there was no observed hypoprothrombinemia.

Uotila and Suttie (1983), using an in-vitro rat liver microsomal system, found that cefamandole as well as a structural analogue lacking NMTT inhibited carboxylase activity at 6-10 uM. The significant finding of this study was that both cefamandole and its analogue produced inhibition at similar concentrations, suggesting that NMTT is not necessary for inhibition of the carboxylase system. In addition, NMTT itself was found to produce 50% inhibition of carboxylase acti-

vity at 36.5 mM. Smith and Sundboom (1984) using a similar microsomal system from both vitamin K_1 deficient and sufficient rats found essentially no inhibition of carboxylase at 1 mM NMTT and only about 25% at 10 mM. These data indicated that it was probably unlikely that concentrations required for significant in-vivo inhibition of the carboxylase system could be achieved especially since it has been demonstrated that peak serum levels of NMTT achieved on administraton of a 1 g bolus of cefamandole or 3 g moxalactam every 8 hours for 4 doses (Uotila & Suttie 1983, Smith and Sundboom 1984) was 0.3 mM. Wold et al (1983), calculated that the maximum potential exposure to NMTT on administration of a 12 g dose of moxalactam could be 7.6 mg/kg and Uchida et al, (1984b), found that the maximal concentration achieved on an injection of 300 mg/kg was 23 uM. These data indicate that in as much as NMTT can cause inhibition of the carboxylase system, the concentrations achievable in-vivo are much lower than what would be considered pharmacologically possible.

The situation is further complicated by the findings of Shimada et al (1984), that prolongation of prothrombin times and clinical bleeding in patients were associated with both NMTT (moxalactam, cefoperazone) and non-NMTT containing (cefazolin, ceftazidime) antibiotics. Shimada observed that all the agents associated with prolongation of prothrombin times in this study were ones that are released in high concentrations in the bile and therefore the intestinal lumen and are therefore capable of suppressing the normal flora of the intestine.

Further work by Lipsky et al (1986), seemed to indicate that prolongation of prothrombin time associated with cefazolin may be due to

the presence of its leaving group - 2-methyl-1,3,4-thiadiazole-5-thiol (MTD)-which is structually similar to NMTT. Using rats on an unspecified vitamin K_1 deficient diet, it was observed that hypoprothrombinemia only occurred with cefazolin (IV or orally) and MTD but not with cefotaxime, a drug that does not contain the MTD group. Using in vitro microsomal system, it was also shown that MTD was able to yield 50% inhibition of the enzyme activity at 0.1 mM. However intact cefazolin was fairly ineffective producing 20% inhibition at 10 mM. Lipsky concluded that for hypoprothrombinemia to occur in association with the use of cephalosporins, it was sufficient that the antibiotic contain a thiol group.

Lipsky et al (1984), speculated that the lack of vitamin K intake may be a necessary contributing factor for hypoprothrombinemic episodes to occur while on NMTT containing antibiotics. This would explain why they were able to demonstrate elevated prothromin times in rats fed oral NMTT and vitamin K_1 deficient diet whereas Wold et al (1983) were unable to accomplish the same in rats given 2700 mg/kg NMTT with a vitamin K_1 sufficient diet. Vitamin K_1 deficiency as a factor for the predisposition to hypoprothrombinemia during antibiotic treatment would also resemble the clinical situation, since the antibiotic associated defect usually occurs in subjects on reduced dietary vitamin K_1 intake.

The hypothesis that vitamin K_1 deficiency predisposes inhibition of carboxylation reaction is further strengthened by the findings of Uchida et al (1984a), who demonstrated that only rats on a vitamin K_1 deficient diet (similar to Mameesh and Johnson) and not those on a vitamin K_1 sufficient diet became hypoprothrombinemic during treatment

with NMTT containing antibiotics. However, they were unable to show inhibition of carboxylase activity. In a follow-up study, Uchida et al (1985), observed that both conventional and germ free rats on a vitamin K_1 deficient diet demonstrated increased prothrombin times and decreased plasma concentrations of Factors II and VII in response to NMTT. The experimental factors leading to these conflicting results are as yet unexplained.

There has also been some suggestion that enzymes in the liver cycle other than carboxylase may be affected by the NMTT group. Uotila and Suttie (1983) found inhibition of vitamin K-2,3-epoxide reductase by cefamandole at the same concentration for carboxylase inhibition. However no inhibition was found when reduced vitamin K was used in the assay. Smith & Sundboom (1984) found that 10 mM NMTT in liver microsomal preparations caused the destruction of NADH. Therefore, inhibition of carboxylase may not be due to direct inhibition of the enzyme but perhaps due to decrease of the NADH mediated reduction of vitamin K_1 to the hydroquinone. Smith & Sundboom hypothesized that the weak inhibition with NMTT and the much larger inhibition reported by Lipsky probably reflects interference of processes in the carboxylase assay other than a direct effect on the enzyme. This could be (1) interference with NADH mediated reduction of vitamin K_1 by affecting NADH concentration or (2) direct inhibition of reductase enzymes.

Bechtold et al (1984) provided evidence that indicated it is the regeneration of vitamin K_1 from the epoxide that is affected by NMTT. Eight patients on no oral intake of vitamin K and on cefamandole and moxalactam showed increased prothrombin times and serum descarboxyprothrombin levels between 3-5 days after administration. The defect was

treated with 10 mg vitamin K_1 which induced a transient appearance of vitamin K_1-2,3- epoxide in plasma. This metabolite is not normally detected in the plasma of normal individuals; its appearance in this case suggested that regeneration of vitamin K_1 from its metabolite was the step affected by NMTT. However, this conclusion has been countered by subsequent findings of Suttie et al (1986), indicating that the effect of NMTT is on the carboxylase rather than the quinone reductase. They concluded that significant NMTT inhibition is probably an NADH-dependent slow inactivation of the enzyme. The data taken together points to an NMTT directed inhibition of the carboxylase under specific conditions in-vitro but it is still questionable whether this is significant under physiological conditions in-vivo.

2. Vitamin K Deficiency in Newborns and Infants

The pattern of vitamin K deficiency in the newborn leading to hemolytic disease of the newborn (HDN) also seems to suggest that endogenous menaquinone pools play an important role in maintaining coagulation homeostasis. At birth, human neonatal sera usually contain low concentrations of vitamin K_1 and in some cases may be deficient. This is primarily due to the fact that the placenta is relatively resistant to transfer of lipids such as vitamin K. Shearer et al (1982), using an HPLC assay to analyse cord blood of babies and their healthy mothers, found that the latter had a mean plasma vitamin K_1 concentration of 0.20 ug/ml, but undetectable concentrations in the cord plasma. The situation is further complicated by the fact that human milk contains low concentrations of vitamin K_1 (1-2 ug/L)

whereas, formula feeds and cow's milk (5-17 ug/L) contain higher levels which are more adequate to fulfill the needs of the neonate (Lane and Hathaway, 1985; Haroon et al, 1982). Thus, babies that are predominantly breast fed are at a higher risk of developing vitamin K deficiency than formula fed babies, because of the low vitamin K_1 intake and the delay in colonization of the gut (or substrate mediated proliferation of intestinal bacteria) with bacteria that produce menaquinones.

The problem in newborns was demonstrated in the study of Sutherland et al (1967), who reported that bleeding occurred with twice the frequency in breast fed babies, 1-5 days old, compared to those fed cow's milk. Chaou, et al (1984), also showed that bleeding occurred in older infants 10-40 days of age, totally or predominantly breast fed. In this study, 18 infants were tested for prothrombin times, and they all showed prolonged times ranging from 22->120 seconds.

In older infants having their full complement of intestinal flora, bleeding and hypoprothrombinemia seem to occur secondary to diarrhea or use of antibiotics in conjunction with low vitamin K_1 intake. For instance, Rapaport and Dodd (1946), studied 7 infants with diarrhea, (all on milk or intravenous fluids), and found that they all had prolonged prothrombin times (33-77 seconds). They speculated that the diarrhea probably caused malabsorption of vitamin K thus limiting supply to the subjects. In addition, acute diarrhea could perhaps cause changes in the normal flora, selecting for members which produce none or less vitamin K.

Matoth, (1950), on examination of 21 infants with diarrhea, while on oral streptomycin found they all had decreased prothrombin levels. In a study of 5 infants, 2-4 months of age, Goldman and Desposito (1966) reported hypoprothrombinemia and bleeding. All the infants were on a low vitamin K_1 intake; three had diarrhea and four were on unspecified antibiotic therapy. In a subsequent study of infants 1-18 months of age, a review of 15 cases that showed bleeding after the first 10 days of life, demonstrated that 12 were on antibiotics such as neomycin, tetracycline, penicillin, streptomycin and sulfa and that 9 had diarrhoea (Goldman and Amadio, 1969). They concluded that, vitamin K deficiency does not develop with low intake of vitamin K_1 alone since menaquinones produced in the intestine can supply infant needs. However, hypoprothrombinemia can occur when low dietary intake of vitamin K_1 and low intestinal vitamin K_1 and K_2 supplies co-exist.

In summary, hypoprothrombinemia in the human newborn and older infants seems to depend on the following predisposing conditions which may occur simultaneously: (a) secondary to acute diarrheal episodes, which probably interferes with absorption of vitamin K from the intestine, (b) sterile intestine in neonates or use of antimicrobial agents that perhaps have the capacity to sterilise it and therefore interfere with menaquinone production, (c) low oral vitamin K_1 intake as seen in infants that are breast fed or on formulas containing low vitamin K_1 levels.

G. Absorption of Vitamin K in Humans and Mammals

Vitamin K is a fat-soluble vitamin and like the other fat-soluble vitamins, A, D and E is insoluble in aqueous solutions but soluble in

lipid solvents. In this way they are similar to other biological molecules such as cholesterol and fatty acids. These compounds exist as emulsions in an aqueous environment like the intraluminal contents of the gut. The inability of fat soluble vitamins to dissolve in the aqueous environment presents a problem in terms of absorption from the intestine. Their dissolution is facilitated by the formation of mixed micelles between the vitamin and amphoteric molecules such as the bile acids. Bile acids contain both hydrophobic and hydrophilic ends and at the critical micellar concentration aggregate to form micelles. Those micellar particles are formed by the orientation of the hydrophilic ends of the molecules outwards into the aqueous environment with the nonpolar portion oriented inwards. The non-polar aspects of the micellar particle provide the ideal environment for fat soluble molecules such as vitamin K to be dissolved.

It is thought that transfer of fat soluble vitamin across the microvillus cell membrane is facilitated by these micellar particles which convey the solute towards the cell membrane. Collision of the particles with the membrane enables the partitioning of vitamin into the phospholipid membrane.

The controversy regarding whether bacterially synthesized menaquinone in the gut is available to mammals is partly due to the inability to demonstrate absorption of vitamin K_2 from the cecum or large bowel, areas where bacteria that synthesize vitamin K_2 are in the largest numbers but where bile salts or bile acids are in lower concentrations than found in the lower small bowel.

Dam's early experiments using rats, dogs and guinea pigs showed that the animals were resistant to vitamin K deficiency while on a

vitamin K_1 deficient diet (Dam et al, 1937). Greaves (1939), further showed that rats continued to excrete vitamin K while on such a diet. These data, coupled with the knowledge that some gut bacteria produced vitamin K_2, led to the conclusion that vitamin K in mammals was obtained from bacterial sources in the gut.

Shearer et al (1970) using ^3H labelled vitamin K_1 fed to normal human subjects was able to recover 80% of the label in the serum, 2-4 hours after ingestion. Using TLC, 98% of the labelled compound was shown to co-chromatograph with pure vitamin K_1 standard indicating that it was absorbed in the intestine.

Transport of vitamin K after absorption was investigated by Mezick et al (1968), using labelled vitamin K_3, 2-(methyl-^{14}C)-1,4-naphthaquinone. The vitamin was administered to rats or dogs by direct injection into closed duodenal (rats) or jejunal (dogs) loops. The recovery of label in the portal vein indicated absorption of the vitamin into blood; direct evidence for lymphatic transport was obtained from experiments with the dogs by recovery of label in the thoracic duct lymph of these animals.

In order to determine the exact location and possible mechanisms whereby vitamin K is absorbed, Hollander's group conducted a series of studies to show absorption of vitamin K_3 (Hollander & Truscott, 1974a, 1974b), vitamin K_1, (Hollander, 1973; Hollander et al, 1977a) and vitamin K_2 (Hollander et al 1976, Hollander & Rim 1976, and Hollander et al, 1977) in the rat. There were two approaches used in these experiments: (1) the in-vitro everted gut sac model and (2) the in-vivo intestinal perfusion model. The everted gut sac model involved dissecting out a section of the gut, everting and preparing

sacs which are then incubated in a mixture containing the solute (labelled) under study. After incubation, the sac is assayed for uptake of label from the incubation mixture. This method suffers from an obvious limitation since material taken up is not being removed and therefore it becomes difficult to maintain a constant rate of uptake for a prolonged period of time. The in-vivo perfusion method involves the insertion of inflow and outflow polyethylene catheters in a section of the gut, which is then perfused with an incubation mixture containing the labelled solute (tritiated vitamin K_2) at a constant rate. The mixture at the outflow catheter is collected and measured for label, uptake being the difference between the perfusate and the effluent (Hollander, 1981).

Hollander (1973), demonstrated absorption of ^{3}H-vitamin K_1 in-vitro by both the proximal and distal sections of the small intestine, with the rate of uptake being three-fold higher at the proximal end. Using 2,4-dinitrophenol (energy decoupler) resulted in a marked decrease in uptake in the proximal small intestine suggesting that uptake of vitamin K_1 at the proximal end is energy mediated. Using the in-vivo perfusion method, Hollander et al, (1977a), confirmed the findings of the previous study by showing absorption of labelled vitamin K_1 from similar segments of the small intestine. At nanomolar concentrations in the perfusate the rate of uptake of the vitamin was linear. However, at micromolar concentrations, uptake demonstrated saturation kinetics, implying that absorption was carrier mediated. The rate of uptake in either sections of the intestine was not different and was unaffected by changes in bile salt (sodium tauro cholate) concentration but decreased with an increase in H^+ concen-

tration. Hollander and his group speculated that decrease in absorption at high pH may be due to the high negative charge on the micelles which would inhibit diffusion of the particles towards the negatively charged luminal wall. Another significant finding of this study was that the rate of uptake was dependent on the rate of perfusion of the segments -- as the rate of perfusion increased, so did vitamin uptake, indicating that the unstirred water layer provides an effective barrier against diffusion of the vitamin.

Using the same approach, and a purified preparation of bacterially produced MK-9 labelled at the 2-methyl position with ^{3}H, Hollander's group was able to show absorption of vitamin K_2 both in-vitro and in-vivo (Hollander et al 1976, Hollander and Rim 1976, Hollander et al 1977b). MK-9 was shown to be absorbed in both small and large intestinal everted sacs. In the small intestine, absorption occurred both in the proximal and distal segments of the bowel (Hollander and Rim, 1976) with the rate of uptake greater in the former segment. Increasing concentration of the vitamin up to 900 nM yielded an increase in rate of uptake that was linear. This, together with the fact that rate of uptake was unaffected by 2,4-dinitrophenol, sodium azide and potassium cyanide, indicated that absorption of menaquinones from the small bowel was by passive diffusion and not carrier mediated. Absorption of MK-9 was enhanced by decreasing the concentration of sodium taurocholate, and increasing the concentration of medium chain (octanoic) but not long chain (linoleic) fatty acids.

Absorption of MK-9 by the colonic everted sac (Hollander et al 1976) like that of the small intestine was also by passive diffusion and increased linearily with respect to concentration of the vitamin.

Absorption of MK-9 in-vivo was demonstrated both in the small and large intestine (Hollander et al, 1977b) occurring by passive diffusion, with no difference in rates between the two.

As with vitamin K_1 absorption, factors such as pH, bile salt concentration and fatty acids affected rates of uptake of menaquinone from both areas of the intestine. Increasing bile salt concentration caused increases in the rate of MK-9 uptake in both the ileum and colon. Saturated medium (butyric) and long (stearic) chain fatty acids decreased uptake of MK-9 in the ileum but did not affect it in the colon. Unsaturated long chain (linoleic) fatty acids caused uptake rates to decrease in both intestinal segments. Increase H+ concentration also caused increased uptake of MK-9 in both segments. Increased perfusion rate positively affected uptake in the ileum but had no effect on colonic rates.

In summary, vitamin K_1 can be absorbed both in the proximal and distal small intestine with the proximal area probably the major site. Absorption at this site is energy mediated. Menaquinones can be absorbed both in the small and large intestines by passive diffusion.

H. Assays for K Vitamins in Bacterial and Biological Samples

1. Extraction of Lipid

(i) Extraction From Tissue. The isolation and purification of fat soluble vitamins, especially vitamin K from tissues, presents a problem because of large quantities of contaminating lipids present, and because of the small amount in the samples. There is also the additional problem of having several molecular forms with biological

activity in the same sample. Tissues containing large quantities of triglyceride can usually be extracted by alkaline saponification followed by organic solvent partitioning. The saponification step removes some of the bulk lipid contaminants such as glycerides and phosphatides and has been used quite successfully for isolation of other fat soluble vitamins (Shearer, 1986).

Alkaline saponification is not useful for the extraction of vitamin K because of the latter's lability to alkali. Extraction from tissue has therefore been accomplished by using dehydrating solvent systems such as that of the Folch, chloroform-methanol procedure or the Bligh & Dyer modification of this method (Folch et al, 1957; Bligh and Dyer, 1959). Alternatively tissues could also be extracted by grinding with anhydrous Na_2SO_4 and then extracting with ether, hexane or acetone (Suttie, 1978; Shearer, 1986). Extensive work on the extraction of vitamin K from liver was done by Matschiner's group in the late 1960's. In extracting vitamin K from cow's liver, Matschiner et al, (1967), evaluated several solvent systems as follows: 1) dehydration of the tissue with 95% ethanol followed by acetone extraction, 2) extraction with alcohol:ether (3:1 v/v) and, 3) tissue dehydrated with Na_2SO_4 followed by ether extraction. In all three cases, yields of vitamin were low and similar. Livers that had been sonicated or cooked before extraction did not influence yield of vitamin on ethanol:acetone extraction. However cooking of the tissue before extraction reduced the amount of extractable vitamin K present, suggesting destruction of the compound by heat. In subsequent studies by Matschiner's group to isolate vitamin K from pig, dog and horse livers, the tissue was first ground, then dehydrated with absolute

ethanol. After removal of the ethanol, the residue was exhaustively extracted with acetone. The acetone extracts were then partitioned between hexane and water and the hexane layer fractionated. For extraction of vitamin K from human liver, the procedure was similar with the exception that the alcohol dehydration step was deleted.

Other extraction methods for liver that have been employed include hexane:isopropranol (3:2 v/v) extraction of rat liver, (Elliot et al, 1976), for the isolation of vitamin K_1 and vitamin K_1-epoxide and hexane extraction of rat livers macerated with anhydrous Na_2SO_4 for the isolation of vitamin K_1 (Haroon and Hauschka, 1983).

Recently, Hara and Radin, (1978) have described a new procedure for the extraction of lipids from tissues using a binary solvent mixture consisting of hexane:isopropanol (3:2 v/v). Briefly, one g of tissue is homogenised with 18 ml of solvent, the suspension filtered and the extract partitioned into 2 phases by the addition of Na_2SO_4 solution. The resulting hexane phase contains all the neutral lipids extractable by other solvents such as chloroform:methanol but is superior because of the reduced amounts of contaminating proteolipids, proteins, pigments and other non-lipid tissue materials. This extract, can be chromatographed through a column with less danger of clogging due to settling of non lipids at the top (Radin, 1981).

In their study to determine absorption and excretion of labelled vitamin K_1, levels were determined in feces by extraction of the sample using the Bligh and Dyer method (Shearer et al, 1970).

(ii) Extraction From Serum. Extraction of serum or plasma usually entails the initial deproteinating of the sample with an

alcohol followed by extraction with a highly non-polar solvent such as hexane (Shearer, 1986). In some cases, however, the deproteination step is ignored and the samples simply extracted with a non-polar solvent, eg Bjornsson et al (1978) and Akman et al (1984) triple extracted plasma with ether, whereas Ikenoya et al (1979) and Kusube et al (1984), used hexane. In another study, Pietersma-de Bruyn and van Haard (1985) adapted the Bligh and Dyer method for serum extraction. One ml of serum was deproteinated with 3 ml of methanol followed by extraction with 3 ml dichloromethane and 3 ml water to yield a biphasic system. The dichloromethane layer containing the vitamin was then subjected to HPLC. The most common solvent systems for serum or plasma extraction, however, have involved the use of either ethanol or methanol followed by hexane extraction. Ueno & Suttie (1983) extracted human serum by deproteinating with ethanol followed by hexane. The ratio of serum to ethanol and hexane was 1:2:6 (v/v). In preliminary experiments they found that keeping the serum:hexane ratio constant and varying ethanol from 0.5 to 2.0 caused an increase in recovery of vitamin K_1 from 23-95%. Further increase in ethanol did not improve recovery. There are numerous other studies where serum or plasma vitamin K_1 measured by HPLC methods was extracted using an ethanol:hexane extraction system with the ratio of solvents to serum being similar to that used by Ueno and Suttie. Others have substituted methanol or isopropanol in place of ethanol. Wilson and Park (1983) used 1 ml of methanol to deproteinate 1 ml of serum followed by extraction with 5 ml of hexane. Langenburg and Tjaden (1984a) on the other hand deproteinated 2 ml of serum with 3 ml isopropanol followed by extraction with 10 ml of hexane.

(iii) Extraction From Dietary Sources. Extraction of dietary sources such as milk for vitamin K has been done largely by the method of Folch (Folch et al, 1957) using chloroform:methanol (2:1 v/v) primarily in studies done by Shearer's group. However, any of the other organic solvents such as petroleum ether, ethyl ether, acetone, or hexane, traditionally associated with lipid extraction from tissue will suffice. Shearer et al (1980) in the assay of dietary sources for vitamin K levels extracted vegetables, milk and milk formulas using the Folch chloroform:methanol procedure.

Thompson et al (1978) in their assay of dietary sources for fat soluble vitamins including vitamin K, extracted samples by homogenizing with isopropanol followed by acetone and then extracting the homogenates with hexane. The organic phase contained the vitamin K fraction.

Barnett et al, (1980) in their determination of fat soluble vitamins including vitamin K in milk, milk products and infant formula foods, extracted the samples with ethanol followed by pentane after they had been treated with lipase in an alkaline environment. All these extraction methods yield a complex mixture of lipids which must be subjected to multiple chromatographic steps in order to isolate pure vitamin K compounds.

(iv) Extraction From Bacteria. Alkaline saponification as well as acid hydrolysis have been used for the extraction of vitamin from bacteria in some of the older work. Briefly, alkaline saponification involves refluxing the cells with an aqueous alcohol solution such as 50% ethanol and a base such as KOH or NaOH. Pyrogallol is also added

to reduce oxidation of labile lipids. This is then extracted with an organic solvent such as hexane, the extract washed repeatedly with water to remove the base and then dried with Na_2SO_4. Alkaline saponification was used successfully to extract MK-9 (H_2) from M.phlei (Gale et al, 1963), MK-7 from B. sphaericus (Gale et al, 1962), and menaquinones from various bacterial species (Bishop et al, 1962).

Acid hydrolysis involves heating the cells with 6N HCl for about 2 hours. Water is then added and this mixture extracted with ether. After washing the ether extract with water to remove the acid, it is dried with Na_2SO_4 and evaporated (Bishop et al, 1962).

Both of these methods have been superceded by solvent extraction which is less harsh and more efficient in terms of vitamin K recovery. In addition, alkaline saponification is unsuitable because vitamin K is labile under such conditions. In comparing five methods of extracting lipids from bacterial cells, Bishop et al (1962) reported the lowest yield of menaquinone results from alkaline saponification. Extraction of menaquinone from bacteria is best accomplished by taking advantage of the solubility of vitamin K in organic solvents such as hexane, chloroform, ether, acetone and alcohols. Solvent systems that have been used to extract bacterial cells have included the use of ethanol or methanol:ether, iso-octane:isopropanol, acetone:methanol, or acetone:light petroleum.

All these methods are based on the same principle, that is, extraction of lipids from the samples with a single phase mixture of the organic solvents followed by partitioning of the extract into aqueous and organic phases by either water or a salt solution such as Na_2SO_4 solution. The aqueous phase contains the more polar lipids

while the organic phase contains the neutral fraction including the quinones.

Procedures that have been used extensively for vitamin K extraction have been the method developed by Folch et al (1957) and later modified by Bligh and Dyer (1959). This method uses a mixture of chloroform and methanol for extraction.

Chloroform:methanol extraction by Folch et al (1957), entails homogenizing a 1 g sample for 3 minutes with 19 volumes of a single phase mixture of chloroform:methanol, (2:1 v/v). The fine suspension obtained is filtered through a sintered glass funnel and the filtrate partitioned into aqueous and organic phases with 0.2 volumes of water or salt solution such as 0.04% $CaCl_2$ or 0.03% $MgCl_2$. Partitioning can be aided where necessary by centrifugation--this also reduces contamination of the phases with microdroplets. The lower chloroform phase may be washed with the Folch upper phase solvent mixture consisting of chloroform:methanol:water (3:48:47 v/v). The aqueous phase contains much of the non-lipid contaminants as well as the polar lipids such as membrane phospholipids, while the non-polar and neutral lipids settle into the organic phase.

The Folch method has also been used extensively by Collins' group in a number of studies to determine the menaquinone profiles of a variety of bacteria. Dried cells (100 mg) were mixed with 20 ml chloroform:methanol (2:1 v/v) and the suspension stirred overnight. After filtering to remove cell debris, the extract was evaporated to dryness. Unlike the original Folch method, however, the extract was not partitioned by the addition of water before being evaporated (Collins, et al, 1977).

The Folch method was subsequently modified by Bligh and Dyer, (1959) for the extraction of lipids from cod fish. The advantage of the latter lies in that much smaller volumes of extracting solvents are used while still retaining complete lipid extraction and removal of lipid impurities afforded by the Folch extraction.

In Bligh and Dyer's modified procedure of the Folch extraction, 100 g of tissue is blended with 100 ml of chloroform and 200 ml of methanol. It is essential that the tissue contains 80% water (water is added to make up 80% if this is not so) as the ratio of water:chloroform:methanol in this single phase extract must be (0.8:1:2 v/v) for maximum retrieval of lipids. The homogenate is then partitioned into a biphasic mixture by the further addition of 100 ml chloroform and 100 ml water to bring the final concentrations of these fluids to (1.8:2:2 v/v), water:chloroform:methanol.

The organic phase contains neutral lipids including the quinones--this phase can be dried by the use of anhydrous Na_2SO_4 or $MgSO_4$. The Bligh and Dyer method as applied to extraction of bacterial cells was described by Hammond & White (1969b) in the extraction of menaquinones from S. aureus. Bacterial cells were suspended in 30 ml of phosphate buffer and 75 ml methanol and 37.5 ml chloroform were added. The single phase mixture was shaken, allowed to stand for complete lipid extraction to take place, and then 37.5 ml of chloroform and 37.5 ml of water were added to partition the mixture into two phases. The lower organic phase was then collected and dried with anhydrous Na_2SO_4 before the solvent was vacuum evaporated.

2. Chromatography of Vitamin K

Historically, methods for separating and purifying vitamin K have made use of two principal systems: (1) adsorption or solid-liquid and, (2) partition (reversed phase) chromatography. The basic principles underlying these modes of chromatography have been applied in traditional methods such as column, thin layer and paper chromatography and more recently in the newer chromatograhic techniques exemplified by high pressure liquid chromatography (HPLC).

(i) Adsorption Chromatography (solid-liquid). This type of separation involves the use of a solid matrix or adsorbent, eg silica gel, or alumina, which is very polar in nature, and a mobile eluting solvent, usually a non-polar compound such as hexane.

In principle, the solute molecules compete with the solvent molecules for sites on the adsorptive surface. Because of its polar nature, molecules of a similar type have an extremely strong affinity for sites in the matrix and are strongly retained. At the same time non-polar groups have little affinity for the surface and will be eluted ahead of their polar counterparts.

Adsorption systems do not usually resolve lipids having a similar polarity to vitamin K, and because of this, groups of lipids such as waxes, unsaturated hydrocarbons and esters of long chain fatty acids will co-elute with it. In addition, generally, adsorption chromatography does not separate the members of a homologous series such as the K vitamins. This mode is chiefly used as a means of providing a preliminary clean up of the sample by removing some of the major contaminating lipids.

Adsorption chromatography has been applied either as column chromatography or thin layer. Columns with adsorbents such as deactivated alumina, silicic acid, Florisil (precipitate of magnesium and silica) or Decalso have been widely used as an initial step in the separation of lipid extracts into the major classes. This way, adsorption columns are used to remove contaminating materials. Because of large sample loading capacity, this type of separation is used as a preparative step from which the vitamin K containing fraction can be eluted and subsequently analyzed. Adsorption TLC has also been used as a preparative step, instead of columns. In this case the adsorbent is layered on a solid support. The thickness of the layer of adsorbent will determine the sample load. Usually, preparative thin layers have a thickness of at least 0.5 mm and can be as thick as 2-3 mm. Further and more complete purification can then be carried out by chromatography on columns, paper or thin layer plates.

(ii) Partition Chromatography (liquid-liquid). Separation of the individual members of the vitamin K homologous series was achieved by use of partition chromatography where separation is achieved by partitioning of the solute between two solvents. One solvent called the stationary solvent is usually bonded to a solid matrix, usually silica. The mobile solvent constitutes the second solvent, with the solute partitioning between these based on its partition coefficient.

Partition chromatography can be carried out in two modes--normal phase or reverse phase. In the former, the solvent coated into the solid matrix is polar, eg water, and the eluting solvent, non-polar,

eg hexane. This mode is usually applied to the separation of polar lipids such as phospholipids. With reverse phase, the stationary phase is non-polar, eg $C_{18}H_{37}$ (octadecane) alkane chemically bonded to silanol groups on silica particles. The mobile solvent is usually polar. This mode is applied to the separation of non-polar groups of dafaxh tejarv h"edd xassnonrbnh ar "cdnbpdeo znavtt, eg members of a tc"cdcvcph hnoanhW In this case non-polar compounds are strongly retained and eluted last.

Reverse phase chromatography is the ideal method for separation of the homologous series of vitamin K as separation is based on length of the polyisoprenoid side chain.

Vitamin K separation was initially achieved using reversed phase chromatography on silicone impregnated paper, silicone being the stationary phase. This was subsequently modified to column chromatography and eventually TLC. Other stationary phases that have been used include paraffin oil and petroleum jelly. The mobile, eluting solvent has usually been an alcohol:water or acetone:water mixture. Since reversed phase TLC is usually an analytical step with very small sample loads, these plates unlike adsorption TLC plates are in the order of 0.25 mm thickness. This allows for lower sample loads but increased detection of small concentrations.

Another mode of chromatography which has been used on a limited basis for the separation of vitamin K homologues, is silver-ion chromatography. The underlying principle governing separation is based on the number, type, and position of unsaturated bonds present in the molecule. The unsaturated double bonds form a complex with the silver ion which is usually bonded to a solid matrix such as silica

gel. In addition to separation of menaquinones based on the polyisoprenoid chain length, this method has the added dimension of being able to resolve partially saturated forms of homologues containing the same chain length but differing in the number of unsaturated double bonds. So powerful is silver ion chromatography, a pair of menaquinones differing by a single double bond can be separated. Silver ion chromatography can be carried out both in columns or on TLC plates, with the latter being the more common mode.

The adsorption procedure generally resolves a mixture of complex lipids into the various classes with minor separation of the classes in some cases. Partition and silver-ion chromatography brings about separation of the members of a class of lipid and is therefore ideally suited to the resolution of vitamin K isoprenologues.

3. Column Chromatography

(i) Adsorption Columns. The most commonly used adsorbents for gravity column chromatography of vitamin K have been aluminum oxide (deactivated), silicic acid, Florisil, Decalso, permutit and silica gel. Elution has usually been accomplished with diethyl ether in petroleum ether at about 2-4% v/v, with variation of this concentration depending upon conditions and adsorbent. Fractions are collected and examined for vitamin K by colour or flourescence tests. The adsorption mode separates the naphthaquinones from the majority of the lipid classes with the exception of esters of long chain fatty acids, hydrocarbons and other hydrophobic quinones (Dunphy and Brodie, 1971).

As an adsorbent, alkali treated alumina, presents a serious dis-

advantage as the compounds are alkali sensitive. It has been shown with ubiquinones, that alkali causes the cyclization of the molecule to its chromenol form.

Florisil, a coprecipitate of magnesium and silica, separates neutral lipids into classes with high recovery rates.

Decalso, or magnesium aluminosilicate, is another adsorbent used to separate napthaquinones by adsorption, column chromatography. This particular adsorbent was used to isolate DMK-9 from S. fecalis (Baum and Dolin, 1965). They found that a major problem with using Decalso lay in the instability of the naphthaquinone molecule in the presence of the adsorbent. They estimated that at least 50-60% of the menaquinone extracted from the organism decomposed within 2-3 hours of loading onto the column.

Silicic acid is by far the most popular adsorbent that has found use in chromatography of vitamin K. Resolution of vitamin K_1 and menaquinones are also possible with this adsorbent as is separation of K_1 from MK-6 and MK-7. Diethyl ether:hexane (0.5:99.5 v/v) elutes vitamin K_1 whereas (10:90 v/v) yields MK-6 and MK-7 from columns. Silicic acid column chromatography has been used as an initial step in the fraction of menaquinone from bacterial lipids.

Matschiner has also used it to fractionate crude lipid extracts of animal livers and the contents of cow's rumen. Silicic acid used in these separations contained about 8% water. The initial fractionation of lipid extracts on silicic acid is designed to remove the major classes of contaminating lipids after which the vitamin K containing fraction can be resolved into its components by other chromatographic systems.

(ii) Partition Columns. Partition column chromatography in the reverse phase mode has been used in a semi-preparative manner for the separation of vitamin K from associated lipids in extracts of liver and tissues from a variety of animals.

Matschiner and Taggart (1967), initially developed the chromatographic system to separate pure vitamin K standards from other fat soluble vitamins and other neutral lipids that would normally be found associated with the vitamin in various tissues. Using a column support consisting of equal parts of celite and polyethylene powder and stationary phases of ether in hexane or isooctane, fat soluble vitamins and other lipids could be separated and eluted from such a column with a mobile solvent mixture consisting of isopropanol, acetic acid and water. In this study, column size was varied to provide flexible loading capacity and elution was started with the mobile solvent at a concentration of isopropanol:acetic acid:water (62.5:5:32.5 v/v). The concentration of isopropanol was increased, and water concentration decreased, leaving acetic acid unchanged, for the stepwise elution of different lipids. This system successfully separated vitamin K_1 and menaquinone from other fat soluble vitamins and other neutral lipids such as retinoic acid, alpha-tocopherolquinone and cholesterol palmitate. When menaquinone isoprenologues (MK-4 to MK-9) were tested alone separation was also achieved.

It was necessary to replace hexane with isooctane as the stationary phase for the separation of compounds more lipophillic than MK-9, since with the former, these compounds did not resolve but co-eluted. For the isolation of vitamin K from tissues in

Matschiner's studies, reverse phase gravity columns were used to resolve the vitamin K containing fraction obtained from silicic acid adsorption chromatography. For beef liver, hexane was used as the stationary phase with most of the vitamin K being eluted with isopropanol at a concentration of 72.5%, keeping acetic acid at 5% and water at 22.5%. Similar partition columns were used in the purification of vitamin K_1 from horse liver and a variety of fully unsaturated and partially saturated menaquinones from pig, dog, and human liver.

4. Thin Layer Chromatography (TLC)

(i) Adsorption TLC. Adsorption thin layer chromatography is an alternative to columns for prepurification of lipid extracts. The obvious advantage lies on the fact that unlike column methods, TLC plates do not demand the kind of monitoring needed with column methods to determine when elution of the fraction of interest occurs. Even though the results are similar, TLC systems are more rapid, and frequently may produce better separations. The principle underlying separation in adsorption columns are the same for adsorption TLC. Besides generally separating naphthaquinones from other classes of lipids, there are cases where using TLC, separation of cis and trans isomers of vitamin K_1 have been achieved. Adsorption TLC systems used in chromatography of vitamin K have included silica gel G with mobile solvents such as petroleum ether:diethylether (1:1 v/v), or (4:1 v/v), and methylene chloride or hexane:di-n-butyl ether (92:8 v/v) (Mayer and Isler, 1971). Adsorption TLC has been employed in some studies as

the first step in the fractionation of lipids for the isolation of menaquinones. In other cases, it has been used as a second prepurification step following gravity column adsorption chromatography (Dunphy et al, 1968; Phillips et al, 1969).

In the fractionation of bacterial lipids for the isolation of menaquinones a number of adsorption TLC systems have been used as follows: 0.5 mm silica gel G eluted with chloroform:methanol (2:1 v/v) for MK-8 from S. aureus; silica gel G eluted with chloroform:-isooctane (2:1 v/v);0.25 mm silica gel G eluted with benzene or iso-octane:ether (100:30 v/v); 0.5 mm silica gel G eluted with disopropyl ether:petroleum ether (1:9 v/v); hexane:benzene (1:1 v/v); or n-butyl ether:hexane (1:9 v/v); and 1 mm silica gel G eluted with hexane:ben-zene (1:1 v/v) (Dunphy and Brodie, 1971).

In Hammond and White's 1969b study, after developing the thin layer chromatogram with chloroform:isooctane (2:1 v/v), the menaqui-none fraction (Rf=0.6) was well separated from the phospholipids, polar carotenoids (solvent front) and non-polar carotenoids (origin). Collins et al, (1977) analysed extracts of a number of bacterial species for their menaquinone profiles using 0.5 mm, Kieselgel H thin layers developed with petroleum ether:diethyl ether (85:15 v/v). In this system the menaquinone fraction migrated with an Rf of approximately 0.7.

In comparing a number of adsorption TLC systems for the fractionation of vitamin K_1, Dunphy et al, (1971), used either Kieselgel G or Kieselguhr G, and mobile phases consisting of n-butyl ether:hexane (10:90 v/v); diethyl ether:pentane (10:90 v/v) or hexane:benzene (25:75 v/v), and obtained resolution between cis and

trans forms of vitamin K_1 or a particular menaquinone isoprenologue. The desmethyl form of that isoprenologue could also be resolved from the cis and trans forms. There was some separation between menaquinone isoprenologues if they differed by at least 3 or more isoprene units in their side chain. The degree of separation also depended on the solvent systems used.

(ii) Silver Ion TLC. Silver is able to form co-ordination complexes with the pi bonds of unsaturated materials. This property enabled silver ion chromatography to separate vitamin K isoprenologues depending on the length of the chain and degree of saturation. Ag+ impregnated silicic acid in a column has been used to separate MK-3 (H_2) from vitamin K_1, and DMK-3 (H_2) from DMK-1. However, most of the silver ion chromatography applied to vitamin K purification has been carried out with thin layers. Silver ion TLC, like reversed phase TLC, is used after the preliminary preparative stage for fractionation of vitamin K containing lipid mixtures.

Using methyl ethyl ketone:hexane (3:97, 4:96, 10:90, or 15:85 v/v) (Mayer and Isler, 1971), or methanol:benzene (5:95 v/v), mobile eluting phases, separation of menaquinone isoprenologues, as well as partly saturated members and vitamin K_1 have been achieved. Silver ion TLC does not separate cis and trans isomers.

As can be seen from Table 3.5, there is a clear separation between the fully unsaturated menaquinone isoprenologues as well as the partially saturated forms. In addition, there is separation between a fully saturated form and its corresponding partially saturated equivalent, eg. MK-9 is well separated from MK-9 (H_2).

Table 3.5 Rf values (x 100) for a number of naphthaquinones on silica gel G impregnated with $AgNO_3$ (Dunphy and Brodie, 1971).

Compound	Methyl Ethyl Ketone:Hexane (15:95 v/v)	Methanol:benzene (5:95 v/v)
Cis/trans K_1	63	80
MK-2	54	75
MK-3	48	70
MK-4	43	65
MK-5	36	59
MK-6	30	53
MK-7	25	46
MK-8	21	38
MK-9	15	33
MK-10	11	27
MK-9 (2-H)	26	45
MK-9 (4-H)	37	55
MK-9 (6-H)	54	69
MK-9 (8-H)	62	75

Dunphy et al, (1971) compared three solvent systems, (methanol:benzene (5:95 v/v); 2-butanone:hexane and acetone:pentane with the ketone varied between 10-20% v/v) for the elution of 5% Ag+ impregnated TLC plates. Menaquinone isoprenologues were separated based on the number of double bonds present in the isoprene side chain as well as length of the isoprene side chain. Saturation of a double bond resulted in a positive shift in Rf value as did loss of an isoprene unit in the side chain. Since it is possible to distinguish between a menaquinone isoprenologue having one saturated double bond and its fully unsaturated counterpart, eg. MK-9 and MK-9 (H_2), it would appear that the number of double bonds in the isoprene side chain is a determining factor for resolution.

Silver ion TLC has generally been used in conjunction with reversed phase TLC for the separation and identification of natural mixtures of menaquinone isoprenologues. Since menaquinones vary both in length and saturation of the isoprene side chain, a single chromatographic system such as reversed phase TLC cannot unambiguously define the composition of a mixture of these compounds. Together, however, reverse phase and silver ion TLC provide a simple procedure for characterization of menaquinone isoprenologues.

Sone, (1974), used reversed phase TLC in conjunction with 25% $AgNO_3$ impregnated silica gel H plates to determine that the menaquinone isoprenologue of P. arabinosum was one containing a partially saturated side chain. Mass spectrometric data confirmed that the compound was MK-9 (H_4).

Thin layer chromatography in both the reverse phase and silver ion modes have been used largely in identification of vitamin K

components isolated from animal livers. TLC was usually preceeded by silicic acid and reverse phase column chromatography for the removal of bulk lipid contaminants and resolution of the different forms of vitamin K_1.

Matschiner and Amelotti, (1968), used 5% paraffin impregnated silica gel G (eluted with acetone:water 96:4 v/v) and 0.5% $AgNO_3$ impregnated silica gel (eluted with benzene:heptane 4:1 v/v, benzene: heptane 1:1 v/v, or benzene) to characterise the menaquinones of bovine liver as MK-10, MK-11 and MK-12. These conclusions were confirmed with ultraviolet (UV) and mass spectral analysis.

(iii) Reverse Phase TLC. This method separates components of a mixture based on the relative solubilities of these compounds in different solvents. Partition chromatography for the analyses of menaquinone mixtures has been used primarily in the TLC mode. The principal adsorbent has been silica gel, though Kieselgel or or cellulose has been used. The most popular non-polar stationary phases have been paraffin oil, silicone oil or petroleum jelly. More recently, an 18-carbon alkane has been used with good results for separation of menaquinones (Collins et al, 1977).

The mobile solvent is usually a polar solvent mixture of alcohol:water. Since reverse phase TLC is used to analyze the homologous menaquinone profile of a sample, the plates have been analytical in nature, which means that the thickness of the layers are in the order of 0.20-0.25 mm.

Separation in reverse phase chromatography depends on the length of the side chain and the extent of its saturation--thus molecular

size and polarity are the primary determining factors underlying separation. Reverse phase TLC systems utilised for vitamin K purification have included: 1) silica gel G impregnated with paraffin oil and eluted with acetone:water (95:5 v/v), 2) silica gel G with petroleum jelly and acetone:water, (95:5 v/v) saturated with petroleum jelly or propanol:acetic acid:water (98:2:1 v/v) (Mayer & Isler, 1971).

As with silver ion TLC, reverse phase TLC achieves good resolution of menaquinone isoprenologues (Table 3.6). MK-9 (H2) from M. phlei and MK-8 (H2) from C. diphtheriae have been chromatographed on petroleum jelly impregnated silica gel plates and eluted with acetone:water (95:5 v/v) saturated with petroleum jelly. Other systems used have included hexadecane impregnated Kieselguhr developed in acetone:water (95:5 v/v) for determination of menaquinone isoprenologues in B. melaninogenicus; 5% paraffin impregnated silica gel G plates developed with dimethylformamide:water (98:2 v/v) or acetone:water (95:5 v/v) both saturated with paraffin; 0.25 mm Kieselguhr plates impregnated with 5% liquid paraffin and developed with acetone:water (97:3 v/v) saturated with liquid paraffin for the separation of MK-9, MK-9 (H_2), MK-9 (H_4), MK-9 (H_6) and MK-9 (H_8) in an unspecified Streptomyces sp. (Phillips et al, 1969).

Hammond and White (1969b) separated the menaquinone isoprenologues of S. aureus with Kieselguhr G adsorbent impregnated with hexadecane rather than complex hydrocarbons or silicone oils. Hexadecane provides an advantage as a stationary phase for reversed phase TLC since it gives better separation than previously used stationary phases. More importantly, in cases where it is necessary to elute the

Table 3.6 Rf values (x100) of naphthaquinones on reversed phase TLC: (Dunphy and Brodie, 1971).

Compound	Acetone:water (93:7 v/v)	Dimethylformamide:water (97:3 v/v)
As-trans K_1	61	56
MK-2	91	87
MK-3	85	83
MK-4	78	77
MK-5	69	66
MK-6	58	54
MK-7	44	42
MK-8	33	28
MK-9	21	16
MK-10	16	10
MK-9 (2H)	17	11
MK-9 (4H)	14	7
MK-9 (6H)	8	3
MK-10 (8H)	6	2

menaquinone isoprenologues from reverse phase TLC plates for spectral analyses, the hexadecane which co-elutes from the adsorbent on treatment with organic solvents does not contain contaminants which would interfere with these analyses. Thus, menaquinone isoprenologues recovered from hexadecane impregnated TLC plates can be assayed by ultraviolet spectroscopy without further purification. However should it be necessary to acquire a pure preparation of a menaquinone isoprenologue, the hexadecane can easily be separated from the menaquinone isoprenologues by further adsorption TLC. Development of the hexadecane impregnated TLC plates was carried out using a solvent system consisting of acetone:water (95:5 v/v) saturated with hexadecane.

Collins et al (1980a), have used a high performance reverse phase plate (commercially available) in which the stationary phase, rather than being a hydrocarbon such as liquid paraffin or hexadecane, is an 18-carbon non-polar compound chemically bonded to silica gel. Development of the plate is carried out with a mobile solvent of acetone:water (99:1 v/v). Separation of menaquinone isoprenologues was excellent and the system facilitated the resolution of menaquinones with the same number of isoprene units but differing degrees of saturation, eg. MK-9, MK-9 (H_2), MK-9 (H_4), MK-9 (H_6). Saturation of one double bond caused a negative shift of Rf value approximately 0.7 times the effect of adding one isoprenic unit, eg. MK-9 (H_2) migrated to a position between MK-9 and MK-10.

(iv) Elution of Lipid Fraction From TLC Plates. Unlike column chromatography where particular fractions are eluted by the flow of

solvent under gravity and collected, recovery of such fracations from adsorption TLC plates is a more cumbersome process. The fraction (band) of interest is usually located with reference to pure standards and the adsorbent constituting this band scraped from the plate. The lipid components are eluted from the adsorbent using an organic solvent in which the lipids are soluble. After separating the solvent from the adsorbent particles by centrifugation or filtration, the solvent can be evaporated to leave the lipid fraction of interest.

A number of solvent systems have been used successfully for the elution of lipids from adsorbents. These have either been a single solvent or a mixture of 2 or more solvents. White and Frerman, (1967), and Hammond and White, (1969b) eluted the menaquinone fraction from silica gel G by consecutively using 5 ml chloroform:methanol (1:1 v/v) containing 4% water followed by chloroform:methanol (2:1 v/v) containing 2% water and chloroform:methanol (1:1 v/v) containing 4% water. Because these are binary systems, the menaquinones would remain in the non-polar organic phase upon partitioning.

Salton and Schmitt (1967), Dunphy et al (1968) and Phillips et al (1969), on the other hand used diethyl ether for the elution of menaquinone from silica gel G after adsorption TLC and Collins et al (1971), have used chloroform, acetone or diethyl ether.

Hammond and White (1969b) eluted menaquinone isoprenologues from hexadecane impregnated Kieselguhr G reversed phase plates using 3 ml chloroform followed by 3 ml methanol and then 3 ml chloroform. Since the hexadecane impregnated in the adsorbent was also eluted by the solvent treatment, in order to separate the hexadecane from the menaquinones, further adsorption TLC needed to be performed. This was

done using silica gel G with a mobile solvent system consisting of 5% chloroform in hexane (v/v) or 10% chloroform in methanol (v/v).

Quantitative studies of recovery of vitamin K from TLC plates have been carried out by Dunphy et al, (1971). Using vitamin K_1, MK-4 and MK-9, the elution efficiency from adsorption silver ion and reversed phase plates was determined. Diethyl ether gave the best recovery (90-96%) for all the TLC systems with the exception of MK-9 from Ag+ plates, where recovery was 27%. This was probably because of the strong complexes formed between MK-9 and the Ag+ on the TLC plate. Ethanol and acetone also resulted in 80-90% elution efficiency of the vitamin from the different TLC systems. However with the latter solvents, dyes placed in the adsorbent for visualization of components, eg. rhodamine co-eluted with the menaquinone isoprenologue.

5. Paper Chromatography

Reverse phase paper chromatography has been used extensively in early studies to purify vitamin K. The various stationary and mobile solvents used have included: 1) silicone oils with methanol:water (95:5 v/v), propranol:water (4:1 v/v), isopropanol:acetic acid:water (60:2.5:37.5 v/v), 2) paraffin oil with dimethylformamide:water or 3) petroleum jelly with dimethylformamide:water (98:2 v/v) (Mayer and Isler, 1971). This technique has been generally superseded by TLC or HPLC methods.

6. High Performance Liquid Chromatography (HPLC)

Because of increased resolution and sensitivity, HPLC presents

the ideal technology for measuring small quantities of compounds in samples. When compared to TLC or paper chromatography, HPLC provides much greater sensitivity, and improved precision and accuracy for measuring the low concentrations of vitamin found in biological samples.

The first reported separation of vitamin K by HPLC was in 1972 by Williams et al who used adsorption HPLC to resolve vitamin K from a mixture of other fat soluble vitamins. Detection limit was less than 10 ng. Donnahey, et al (1979), used HPLC to detect 5-10 ng compared to detection of 250-500 ng by TLC. Haroon et al, (1980), improved upon this by being able to detect 500 pg of vitamin K_1.

Donnahey concluded that achieving the same level of separation and detection with TLC as that of HPLC would entail using at least two successive chromatographic systems.

The use of HPLC in the separation and purification of vitamin K has centered around two modes - adsorption HPLC as a preparative stage followed by reverse phase HPLC for analysis of the partially purified fraction containing vitamin K.

In some cases, eg. assay of liver samples, a preliminary purification step prior to adsorption HPLC may be needed to remove excessive amounts of lipids present in the sample extract. This is usually a gravity column adsorption chromatography using silica gel as the adsorbent. The adsorption column retains the bulk of fat, eg. triglycerides, sterols, and phospholipids. The eluted vitamin K fraction can then be subjected to preparative adsorption HPLC followed by reverse phase HPLC. This assay design was used successfully by Shearer et al, (1982), and Thompson et al, (1978), to measure vitamin

K_1 content in foods. In Thompson's study, the preliminary gravity column step was carried out on hydroxyalkoxypropyl Sephadex (HAPS) rather than silica gel. The use of the traditional gravity column has now been superceded by the quicker and more efficient Sep-Pak® silica cartridge (Waters) designed for rapid clean up of samples.

(i) Adsorption HPLC. Adsorption HPLC, like TLC, has been used primarily in the prepurification stage in the analysis of vitamin K. Elution of the vitamin K containing fraction is usually accomplished with a hydrocarbon such as hexane, moderated by a more polar solvent. The moderator is usually of a high eluotropic strength, and with the exception of methylene chloride, is needed in very small concentrations. The commonly used moderators have included alcohols, acetonitrile and methylene chloride. Alcohol concentrations have been in the order of 0.1%, and acetonitrile at concentrations of 0.05 - 0.5%. Methylene chloride on the other hand is needed at 5-30% concentrations, and therefore methylene chloride in hexane should be considered more as a solvent mixture rather than a moderated system (Engelhardt, 1977).

Moderated systems are necessary to achieve deactivation of the adsorption column in order to have uniform elution characteristics. Adsorption sites on a silica gel column are represented by silanol groups which are of different strengths because of their different spatial arrangement on the surface of silica particles. These sites can be classified as strong or weak sites. Deactivation of the strong sites leaving only weak sites for separation is necessary for reproducibility of separation and constant retention times of the

components being separated.

Water, because it can act as a strong moderator, influences elution characteristics of adsorption columns especially when non-aqueous elution systems are being used. With non-aqueous solvents, small amounts of water in the solvent causes large changes in the retention behaviour of the sample component. In order to minimize the effect of water in the solvents, one of the solvents in such a solvent mixture is water saturated. Even though it is difficult to saturate organic solvents, this is easier than the alternative of trying to remove all water (Saunders, 1976). Haroon et al, (1981), used a 50% water saturated methylene chloride:hexane mixture (25:75 v/v) for the separation of cis and trans vitamin K_1 and cis and trans MK-4 and MK-10.

Acetonitrile is superior to water as a moderator in solvent systems since it allows faster equilibration and allows better reproducibility of retention times. Alcohols have been used as moderators but to a lesser extent than methylene chloride or acetonitrile. Alcohols suffer from the disadvantage of low column efficiencies and slow column equilibration.

In adsorption HPLC, the longer chain menaquinones which are more lipophilic elute first followed by shorter chains. However, vitamin K_1 which has 3 saturated isoprene units, elutes ahead of the most lipophillic menaquinones. Also, menaquinones having the same chain length but different degrees of saturation can be separated by this method.

Cyanosilica columns have also been used in the HPLC separation of vitamin K. The principle underlying separation is also adsorption

chromatography and the column gives the same order of elution as silica columns.

Even though adsorption HPLC columns are usually used as a pre-purification step in the separation and analysis of the various forms of vitamin K, Lichtenthaler and Prenzel, (1977) achieved separation of vitamin K_1 from the other neutral lipids of plant extract by using two adsorption columns in series eluted with hexane:dioxane (99.7:0.3 v/v). In the elution scheme, the early peaks from column #1 consisting of carotenoids, vitamin k_1 and plastoquinone are fed into column #2. Using a switching valve the late peaks of column #1 are then fed into the detector - after their elution, the early peaks are eluted from column #2.

Haroon et al, (1980) used 5 um silica particles of either an irregular or spherical shape to resolve cis, trans and chloro-K_1 using 50% water saturated dichloromethane:hexane (25:75 v/v). Also dichloromethane (dry):hexane (25:75 v/v) resolved cis and trans vitamin K_1 and cis and trans vitamin K_1-epoxide.

Using the same systems of columns and mobile solvents, MK-4 is resolved from MK-10 but the isoprenologues with intermediate side chain lengths are only partially resolved. The menaquinones were however resolved from cis and trans vitamin K_1. When cyano bonded columns containing the same particle types as before were used with water saturated dichloromethane:hexane mobile solvents, similar results were obtained. A silica column eluted with dichloromethane:-hexane provided, separation of menaquinones with unsaturated and partially saturated isoprenoid side chains, eg. MK-8, MK-8 (H_4).

The major problem with using adsorption HPLC as a preparative stage stems from the difficulties in collection of the vitamin K containing fraction. Adsorption HPLC of samples are monitored by ultraviolet absorption and often the vitamin K containing peak is masked by other ultraviolet absorbing material. In order to solve this problem, the vitamin K containing fraction can either be: 1) collected via reference to the retention time of the vitamin K standard or, 2) collected in reference to the ultraviolet absorbing pattern given by contaminants in the sample. The former method is inconsistent since it is always difficult to retain a constant retention time for particular components during adsorption HPLC because of continual deactivation and equilibration of the column with time. The latter presents a better alternative for collection of vitamin K as its retention time remains the same relative to the ultraviolet contaminating material. This method can be further enhanced by using an external marker added to the sample and having the same or similar elution characteristics of vitamin K during chromatography. The marker ideally, should elute, just before the vitamin K peak serving as a guide for starting of fraction collection. Shearer (1983), has successfully used chloro-K, a vitamin K_1 analogue, as a marker.

Having collected the partially purified vitamin K containing fraction, resolution into its components and internal standard, whenever used, is accomplished by reversed phase HPLC. Thompson et al (1978), used a linear gradient, aqueous methanol, to methanol to resolve vitamin K_1 while Shearer (1983) and Lefevere (1979) have used isocratic systems with the same results.

(ii) Reverse Phase HPLC. Reverse phase HPLC presents the best method for separating and analysing menaquinone isoprenologues and vitamin K_1. The columns used consist of a silica support, which in the earlier columns was pellicular in nature; the later columns have contained 5-10 um spherical porous silica particles. In any event, the stationary mobile phase has been exclusively octadecylsilane (ODS), an 18-carbon alkane chemically bonded on to the silica. The mobile phase like that of reverse phase TLC is a binary system consisting of a mixture of polar and less polar solvents, eg. water:alcohol mixture. The nature and type of the mobile solvent pair varies with the type of column used and are governed by the following characteristics of the column.

a) particle type of the support to which the stationary phase is bonded, ie. pellicular vs porous, shape (spherical), porosity.

b) carbon load, ie. percentage of carbon load bonded to silica support.

c) type of stationary phase, ie. C_8 or C_{18} hydrocarbon.

d) capped or uncapped columns.

These factors also govern the retention characteristics of the column.

In addition to water:alcohol mobile phases, purely non-aqueous eluants such as an acetonitrile:alcohol have also been used. This is seen especially with the commercially available Zorbax ODS (DuPont) column which contains ODS bonded onto 6 um particles. This column retains some non-polar compounds strongly and therefore needs a non-aqueous solvent system, unlike other microparticulate and pellicular packing for elution of these components. Haroon et al, (1980)

utilised three different C 18 columns eluted with totally organic mobile phase, and obtained separation of vitamin K_1, K_1- epoxide and chloro-K_1. Retention of these components varied with column type, probably due to different carbon loads on the columns.

Haroon et al (1981) examined the behaviour of menaquinones on C 18 columns under isocratic and gradient elution. Separation of the menaquinone isoprenologues was achieved with 30% dichloromethane in acetonitrile. MK-6 was not separated from vitamin K_1, and only partially so, when 30% dichloromethane in methanol was used. A gradient of 20-50% dichloromethane in methanol resolved all the menaquinones and vitamin K_1 and in a shorter time compared to isocratic elution. Similarly, Haroon et al (1981), were able to obtain separation of vitamin K_1 and MK-4-10 standards on a Zorbax ODS column using a methylene chloride:methanol linear gradient. Lefevere, et al (1979), obtained the best resolution of vitamin K_1, MK-2, MK-4, and MK-9 using a C18 column with high carbon load and 5 um particulate support.

(iii) Detection Systems in HPLC. Detection systems for vitamin K in HPLC assays are of 3 different types--UV absorption, electrochemical or fluorometric detection. UV detection represents the least sensitive or selective of the three, because of the large variety of UV absorbing material that is present in samples. These often mask peaks of interest and also reduce the loading capacity of the columns.

Fluorometry is more sensitive and certainly more selective than UV absorption because of lesser amounts of fluorescing material present. However, since vitamin K is not a naturally fluorescing com-

pound, post column derivatization of the separated quinones is necessary. This has been achieved by electrochemical, photochemical, or wet chemical reduction with $NaBH_4$.

Electrochemical detection has also been used for detection of separated vitamin K components. The problem with this mode usually lies with detector drift and high background noise.

(iv) Quantitation of Vitamin K. A valid quantitative assay for vitamin K must have high precision, accuracy and recovery. Being able to correct for incomplete recovery after extraction or losses during chromatographic steps can be achieved with an internal standard. The internal standard is usually a compound structurally related to vitamin K, or in some cases tritiated vitamin K. Lefevre (1979) in measuring vitamin K_1 in blood, used tritium labelled vitamin K_1 as an internal standard (Shearer, 1983). Other alternatives have been vitamin K_1 epoxide and chloro-K_1. If detection of the internal standard is by the same means as the vitamin , eg. photometric detection, then it is essential that it has the same retention volume as vitamin K during adsorption HPLC, but is resolved during reverse phase HPLC. In addition, the internal standard must be a compound that is not found endogenously in the sample being assayed or if it is, at low enough levels that it would not be normally detected.

Vitamin K_1 epoxide is a useful internal standard for vitamin K assays as it co-elutes with vitamin K_1 during adsorption HPLC. However, it is a metabolite of vitamin K_1 in animals, usually in low concentration except in cases where coumarin anticoagulants are administered causing a build up.

Chloro-K does not occur endogenously, but elutes slightly ahead

of vitamin K_1 in adsorption HPLC. For this reason it is an excellent choice as an external marker to aid in fraction collection during preparative absorption HPLC, but could present problems as an internal standard (Shearer, 1983).

7. Design of Assay Systems for Vitamin K

(i) Liver. Purification of vitamin K from liver is probably the most difficult of all the vitamin K separations to achieve.

To assay vitamin K in tissues such as the liver, multistage chromatographic procedures are necessary to achieve spectrally pure isolates of the various molecular forms. For instance, an assay of liver for vitamin K_1 would entail preliminary purification of the extract by either gravity adsorption columns or using silica Sep-Pak® cartridges followed by adsorption and reverse phase HPLC.

The assay of vitamin K in whole livers, in this case the rat, was first achieved by Haroon and Hauschka, (1983). After hexane extraction of tissue under dehydrating conditions, the extract was first subjected to conventional adsorption chromatography on silica Sep-Pak® columns. This step served to remove most of the contaminating lipid material, yielding a vitamin K containing fraction which was then further purified by adsorption HPLC on a Partisil 5 um column (Whatman). A vitamin K containing fraction was collected on elution of the column with 50% saturated dichloromethane:hexane (2:8 v/v). Resolution of vitamin K_1 was achieved by a C18 column eluted with methanol:dichloromethane (3:1 v/v), with UV detection. Livers of male rats fed a diet with no alfalfa contained 8.0 ng/g wet weight of vitamin K_1 while female rat livers contained 7.3 ng/g wet weight.

Livers of chow fed rats contained 44 ng/g wet weight of vitamin K_1. The values for vitamin K_1 obtained by the HPLC assay were lower than what had been previously reported by Matschiner & Doisy, (1966). This group reported vitamin K_1 levels in female rat liver at 100 ng/g (phylloquinone equivalent). However these data were generated from the chick bioassay and higher values may have reflected the presence of menaquinones which were not accounted for, but which are active in the bioassay.

Measurement of liver vitamin K_1 concentrations was further improved in terms of sensitivity and selectivity by using an electrochemical detection system rather than UV absorption. The minimum level of detection for vitamin K_1 was 100 pg compared to 500 pg with UV detection (Haroon et al, 1984).

(ii) Dietary Samples. Analyses of dietary sources also presents problems because of the quantity of contaminating lipids present. The design of assays for vitamin K in foods follows the same procedure as that for liver--a preliminary gravity adsorption column clean up, followed by adsorption then reverse phase HPLC. In most cases the initial gravity column is a silica gel column. However Thompson et al, (1978), chromatographed their extract on a hydroxyalkoxypropyl Sephadex (HAPS) column followed by the HPLC steps. The reverse phase column was eluted using a gradient of methanol:water with methanol varied from 85-100%, while the adsorption column was eluted with 0.03% isopropanol in hexane.

Shearer et al, (1980), developed an assay along these lines for the determination of vitamin K_1 and menaquinone in vegetables. The

silica gel column was eluted with diethyl ether:hexane (3:97 v/v) to yield a vitamin K containing fraction which was then subjected to adsorption HPLC and eluted with hexane:50% water saturated dichloromethane (8:2 v/v). In a few instances a cyano bonded column was employed in this stage instead of the silica adsorption column. Analytical reverse phase HPLC was carried out on a C18 column eluted with methanol:dichloromethane (8:2 v/v) or (9:1 v/v)or acetonitrile dichloromethane (7:3 v/v), or (17:3 v/v) for the separation of vitamin K_1. Menaquinones were resolved using a gradient elution system of 20-50% dichloromethane in methanol.

(iii) HPLC of Menaquinone in Bacteria. Reverse phase partition TLC as described by Collins et al (1977) adequately facilitates the separation of menaquinone isoprenologues in bacterial extract. However, HPLC has the advantage over TLC, of speed, sensitivity, resolving power and most importantly the ability to provide quantitative analysis.

HPLC has only recently, been applied to the analysis of bacterial extracts for menaquinones. Tamaoka et al (1983) first chromatographed bacterial extracts on adsorption thin layers to remove the bulk of contaminating lipids, then resolved the menaquinones on a Zorbax ODS column eluted with methanol:isopropyl ether. Detection was by UV absorption. Identification of menaquinones was based on mass spectral analysis.

The pattern of menaquinone elution on HPLC was quite similar to that of TLC, ie. retention time of menaquinones increased with chain length. Separation of menaquinones with unsaturated side chains (MK

6-12) as well as partially saturated side chains (up to 4 double bonds saturated) was easily accomplished. Determinations of the menaquinone profiles of a number of strains was carried out.

Collins et al, (1984) and Collins and Shah (1984) used a similar assay system with an ODS column eluted with methanol to determine the menaquinone profiles of a number of Campylobacter species as well as Rothia dentocariosa (formerly of the genus Nocardia). In combination with mass spectral analyses, it was determined that the Campylobacter sp. contained MK-6 with a partially saturated side chain; while the Rothia sp. contained MK-7 as the major menaquinone with minor amounts of MK-6 and MK-8.

(iv) Vitamin K_1 in Serum. Most of the developmental work for the HPLC assay of vitamin K has been done with serum. Purification of vitamin K_1 in serum or plasma can usually be achieved by use of successive chromatographic steps with adsorption and reverse phase HPLC columns.

Bjornsson et al (1978) and Ikenoya et al (1979) were able to resolve vitamin K_1 from the lipids of plasma using a single reverse phase step. Using vitamin K_1 epoxide as the internal standard, Bjornsson was able to recover 100.7% of vitamin K_1 by this method from plasma. Wilson and Park, (1983) were also able to achieve resolution of vitamin K_1 in rabbit plasma by use of a single column HPLC assay. In this case, separation of vitamin K_1 from the lipid extract could be achieved either with an adsorption or reverse phase column. The Partisil-10 silica column eluted with 0.2% acetonitrile in hexane allowed complete separation of vitamin K_1 from other peaks such as the

MK-4 internal standard without interference of any background UV absorbing material co-extracted from rabbit plasma. The adsorption column also resolved cis and trans forms of vitamin K_1, but the limit of sensitivity was only 3 ng.

The reverse phase C18 column eluted with 30% dichloromethane in acetonitrile also allowed separation of vitamin K_1 without interference of background UV absorbing material.

Lefevere et al, (1979) detected 500 pg/ml vitamin K_1 in serum using adsorption and reverse phase HPLC separations with UV detection. Using 0.2% acetonitrile in hexane to elute the adsorption column allowed separation of cis and trans isomers of vitamin K_1 as well as MK-2 and MK-4 from MK-9. Alternatively, methylene chloride did not allow separation of cis and trans forms of vitamin K_1.

To determine the best column parameters for separation of the various forms of vitamin K, MK-2, MK-4, MK-9, vitamin K_1 and vitamin K_1 epoxide were chromatographed on C8 or C18 columns with high and low carbon loads. High carbon load C18 columns gave the best results. Lefevere et al, (1982) subsequently used a similar system, consisting of adsorption chromatography on a silica column eluted with 3% diisopropyl ether in hexane followed by reverse phase chromatography to determine vitamin K_1 levels in fasting human volunteers. Mean serum concentration in this group was 2.6 ng/ml.

Shearer's group adapted an HPLC assay system developed for the determination of vitamin K_1 in dietary samples to measure levels in healthy fasting adults, healthy mothers and newborns at term. The mean concentration of vitamin K_1 in plasma of fasting adults was 0.26 ng/ml which represents a ten fold lower level than that reported by

Lefevere's group. Mothers had a mean concentration of 0.20 ng/ml, with undetectable levels in the babies' cord blood. The sensitivity of this assay was of the same order (0.5 - 1 ng) as Lefevere's assay (Lefevere, 1979).

Sann et al, (1985) studied the plasma vitamin K_1 status of newborns supplemented or unsupplemented with vitamin K at birth. Analysis was carried out by multistage adsorption and reverse phase HPLC with UV detection. Supplemented babies had a median value of 55 ng/ml vitamin K_1 while unsupplemented ones contained a median value of 9 ng/ml. It was suggested that the differences between these findings and those of Shearer et al (1982), may be due to technical problems in the assay systems used. Shearer's study entailed use of an additional preliminary purification step and the sample was heated during extraction.

Pietersma-de Bruyn and van Haard, (1985) used C18 Sep-Pak® as a preliminary step in purification of plasma extracts before subjecting the vitamin K containing fraction to adsorption HPLC with UV detection. Elution of the adsorption column was carried out with dioxane:hexane (2.5:97.5 v/v). The level of detection was 0.45 nmole/L. Assay of serum for vitamin K_1 in 103 neonates revealed an average value of 2.0 ± 0.7 nmole/L (mean ± S.D.). Vitamin K_1 levels of 22 mothers were 4.3 ± 2.0 nmole/1 (mean ± S.D.).

Ueno & Suttie, (1983) suggested that the limiting factor in the measurement of vitamin K_1 in serum depended on the detection system. Applying similar techniques as Lefevere, ie. separation by adsorption HPLC followed by reverse phase HPLC but with electrochemical detection, this group was able to achieve a lower limit of detection

estimated at 300 pg/ml. Fasting vitamin K_1 levels in 26 individuals were found to give a mean value ± S.D. of 1.11 ± 0.42 ng/ml. Mummah-Schendel and Suttie, (1986) subsequently adapted this assay system to assess the vitamin K_1 levels of normal healthy blood donors. After reverse phase HPLC, the column effluent was electrochemically reduced and the reduced vitamin determined by fluorescence spectrophotometry. The mean serum vitamin K_1 levels was 1.3 to 64 ng/ml agreeing with the results of a previous study of human volunteers (Ueno & Suttie, 1983).

In order to simplify the assay system for vitamin K_1 in plasma to the use of a single column, Langenberg and Tjaden (1984a) postulated that selectivity in the detection system (which would reduce interference by other materials present and therefore reduce chromatographic manipulation to a single step) was essential. Using a reverse phase Hypersil-MOS 5 um column eluted with 92.5-95% methanol solution, and post-column electrochemical reduction followed by detection of the reduced quinone, they were able to detect 25 pg of vitamin K_1 in plasma. There was complete resolution of the vitamin K_1 peak. Langenberg and Tjaden, (1984b),subsequently adapted this method to the determination of vitamin K_1 epoxide in human plasma.

Haroon et al, (1986) criticised Langenberg and Tjaden's (1984a) method of electrochemical reduction because of contamination in the electrochemical reactor cell, low efficiency (60%) for the reduction of the vitamin, and quenching of the fluorescence response due to oxygen in the system. To determine vitamin K_1 in serum using a single chromatographic step, they extracted plasma samples by standard methods and then subjected the extract to silica Sep-Pak® chromato-

graphy. The vitamin K containing fraction in hexane was then reduced to the hydroquinone using a reductant consisting of $ZnCl_2$ in acetic acid:acetonitrile (3:97 v/v) and metallic Zn. The upper hexane layer contained all the lipid contaminants while the lower acetonitrile layer had the reduced vitamin K. The reduced vitamin K was reoxidized, chromatographed on a C18 column, and eluted with dichloromethane:methanol (2:8 v/v). The effluent was then subjected to post-column on line reduction to the hydroquinone with metallic Zn and detected by fluorescence spectroscopy. Using this method the limit of detection was 50 pg/ml. A survey of vitamin K_1 levels in 22 healthy fasting adults revealed a mean level of 550 (range 90-2120) pg/ml, (Haroon et al, 1986).

Kusube et al, (1984) used a similar detection system in their study to determine vitamin K_1 and MK-4 levels in rat plasma. After chromatography of extracts on a C18 column eluted with acetonitrile:isopropyl alcohol (9:1 v/v), the quinones were electrochemically reduced to the hydroquinones and measured by fluorometry. The method was applied to determination of the vitamin K forms after oral or intravenous administration. Separation of the peaks were complete with no interfering material detected.

HPLC fractionation of blood samples with fluorometric detection after post column electrochemical reduction has been used to determine the vitamin K_1 status of newborns and from human and animal plasma. In the study of van Haard et al, (1986), plasma of adults and newborns was cleaned up with Sep-Pak® and then subjected to reverse phase HPLC. The detection system was as described by Langenberg and Tjaden (1984a). Hirauchi et al, (1986) applied the same method to measure

vitamin K_1 and MK-4, MK-5, MK-6, MK-7 and MK-8 in plasma. Extracts were subject to Sep-Pak® clean up and then reverse phase HPLC. Concentrations of vitamin K_1 in human plasma was 1.16 ng/ml; MK-4, 0.30 ng/ml; MK-5, 0.08 ng/ml; MK-6, 0.21 ng/ml; MK-7 0.37 ng/ml and MK-9, 0.20 ng/ml. This method was described as an improvement on Langenberg and Tjaden's procedure and estimated to be 5 times more sensitive. The results also indicated that the assay system was suitable for the determination of endogenous K vitamins from plasma of various sources, as it was the first successful determination of menaquinone from plasma.

Wet-chemical post column reduction with fluorescence detection has been documented in the study of Lambert et al, (1986). After serum extracts had been subject to semipreparative adsorption HPLC with UV detection, the K fraction was chromatographed on a C18 column, elution being carried out with methanol:ethyl acetate (96:4 v/v). The column effluent was then reacted with tetramethylammonium octahydridotriborate to yield the flourescing hydroquinone. Another chemical reducing agent for quinones, $NaBH_4$, was tried without success due to incompatibility in refractive index characteristics between the mobile solvent (methanol) and the ethanol used to dissolve $NaBH_4$. The limit of detection for the assay was 150 pg. In a survey of 10 human sera, the vitamin K_1 level was determined to be of the order of 30 pg/ml.

Wet chemical post column reduction with fluorometric detection has also been used for the determination of menadione (K_3) in animal feed and premixes (Speek et al, 1984).

4. MATERIALS AND METHODS

A. Strains Used for Determination of Menaquinone Profiles

The ninety-two organisms that were tested for menaquinone profiles, included eighteen strains obtained from the American type culture collection (Rockville, MD) and one strain, Peptostreptococcus magnus WAL 2508, from the Wadsworth Anaerobic Laboratory (Los Angeles, CA). The remaining organisms were obtained from stool cultures of neutropenic patients with cancer at the time of their enrollment in a study of empiric antimicrobial therapy for febrile episodes or from cultures of clinical specimens from patients hospitalized at the Health Sciences Centre in Winnipeg. All organisms were maintained on 5% sheep's blood agar supplemented with 1 ug/ml vitamin K_1 (BAK) for anaerobes. Cultures were incubated at 37°C under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2) in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI).

B. Identification of Bacterial Strains

1. Anaerobic Gram Negative Rods. The B. fragilis group organisms, consisting of the six species, B. fragilis, B. distasonis, B. vulgatus, B. ovatus, B. thetaiotaomicron and B. uniformis were presumptively identified by their ability to grow in the presence of 20% bile. The members of the group were speciated by using the Minitek Anaerobe II system (BBL, Cockeysville, MD). B. bivius was identified by inhibition of growth in 20% bile and by the Minitek system. Fusobacterium spp. were differentiated from Bacteroides spp. by gas liquid chromatography analysis (GLC) of the end products of

glucose fermentation. Fusobacterium spp. produces butyric acid whereas Bacteroides spp. does not. Speciation was achieved by using the Minitek identification system and by previously established criteria (Lenette et al, 1980; Holdeman et al, 1977).

2. Anaerobic Gram-Positive Rods. The members of the genus Clostridium are gram positive, spore-forming bacilli. With the exception of C. perfringens, this genus was speciated both by GLC analysis and the Minitek system. C. perfringens was identified by the combined egg yolk/Nagler's test which demonstrates the ability of this organism to produce lecithinase. The gram-positive non spore-forming bacilli comprises of five major genera: Proprionibacterium, Lactobacillus, Actinomyces, Eubacterium and Bifidobacterium. These genera were differentiated, based on GLC analysis of the end products of glucose fermentation and the species identified by the Minitek system.

Anaerobic gram positive cocci were presumptively identified by GLC analysis of the products of glucose fermentation and by the Minitek system. The gram-negative facultative anaerobes, Escherichia coli, Citrobacter spp. and Klebsiella spp. were plated on McConkey agar and speciated by using the API 20E system (API Analytab Products, St. Laurent, PQ) (Holdeman, et al., 1977).

C. Growth and Harvesting of Bacteria for Menaquinone Analysis

Strains stocked in 10% Bacto Skim milk (Difco, Detroit, MI) supplemented with 10% glycerol were subcultured to brain heart infusion broth (BHI) (Gibco Diagnostics, Madison, WI) supplemented with 5 g/L yeast extract (Difco, Detroit, MI), 5 ug/ml hemin (Eastman

Organic Chemicals, Rochester, NY) and 0.5 g/L cysteine hydrochloride (Fisher Scientific, Fairlawn, NJ) and maintained on BAK plates. Strains obtained fresh from the clinical laboratories of the Health Sciences Centre were maintained on BAK plates. All organisms examined, except Lactobacillus spp., were grown in BHI broth; Lactobacillus spp. were grown in broth listed in Appendix 1. Pure cultures from BAK plates were used to inoculate 2 litres of broth and this was incubated at 37°C in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI) under an atmosphere of 10% H_2, 10% CO_2 and 80% N_2) for two to seven days with the interval depending on growth rates.

Cells in the stationary growth phase were harvested and washed twice with 0.05 M Phosphate buffer, pH 7.0 by centrifugation at 6,000 x g for 10 minutes at 4°C.

The pellet of approximately 5-10 g packed wet cells was resuspended in phosphate buffer, pH 7.0 at approximately 5 ml/g wet weight of cells and extracted for lipids as described.

Purity of batch cultures was established by Gram stain and subculture of washed cells to BAK.

D. Extraction of Samples for Lipids

1. Bacteria.

Extraction of cells for menaquinones was carried out by a modified Bligh & Dyer method (Hammond and White, 1969b). Thirty ml of the cell suspension were mixed with 75 ml methanol and 37.5 ml chloroform in a 250 ml separatory funnel (0.8:2:1 v/v) and after vigorous shaking for 5 minutes, allowed to stand for 15 minutes. 37.5 ml, each, of

chloroform and distilled water were then added to partition the mixture (1.8:2:2 v/v) into an upper aqueous phase and a lower organic phase. Separation of the phases was aided by centrifugation at 100 x g for 10 minutes. The lower phase was collected, dried over Na_2SO_4 crystals and concentrated under vacuum and with a stream of N_2 gas at 35°C.

This extract was used for adsorption chromatography on thin layers or with silica Sep-Pak® cartridges.

2. Human feces.

Two methods were used throughout these studies for the extraction of lipids from fecal material. Initially, extraction was accomplished with a modified Bligh and Dyer method using chloroform:methanol. Even though this method is very effective for lipid extraction from biological samples, it suffers from the disadvantage of dissolving appreciable amounts of non-lipid material, resulting in an extract with a high content of contaminants. As a result, extensive prepurification must be carried out before the sample can be analysed. The second method used, utilises a mixture of hexane:isopropanol (Hara and Radin, 1978) which is of less polarity than chloroform:methanol and is therefore superior to the latter as the extract contains less contaminants. In addition, hexane extracts almost no protein material at the same time yielding the full complement of neutral lipids.

Chloroform/Methanol Method. 0.5 - 1.5 g of fecal material was suspended in 10.0 ml distilled water and to this was added 25.0 ml methanol and 12.5 ml chloroform. After homogenizing for 1 minute

(Sorvall Omni-Mixer, Sorvall Inc., Newton, CT), the mixture was allowed to stand at room temperature for approximately 3 hours, with intermittent shaking. It was then centrifuged at 150 x g for 5 minutes and the supernatant collected. The residue was resuspended in 10.0 ml distilled water and re-extracted for 15 minutes with chloroform and methanol as before. The supernatant was again collected after centrifugation, pooled in a 250 ml separatory funnel and partitioned into two phases by the addition of 25.0 ml chloroform and 25.0 ml of 5% Na_2SO_4 solution (w/v). Separation into upper aqueous and lower organic phases was aided whenever necessary by centrifugation at 150 x g for 5 minutes and the lower organic phase collected and concentrated under vacuum in a rotary evaporator or with a stream of N_2 gas at 35°C.

Hexane:Isopropanol (HIP) Method. 0.5 - 1.5 g of fecal material was suspended in 30.0 ml hexane:isopropanol (3:2 v/v) solution and homogenized for 1 minute (Sorvall Omni-Mixer, Sorvall Inc., Newton, CT). After standing for approximately three hours with intermittent shaking, the mixture was centrifuged at 150 x g for 5 minutes and the supernatant collected. The residue was re-extracted with 30.0 ml HIP for 15 minutes, centrifuged and the supernatant collected and pooled. This liquid was transferred to a 250 ml separatory funnel and partitioned into two phases by the addition of 0.5 volumes of 5% Na_2SO_4 solution (w/v). The upper hexane layer was collected and concentrated under vacuum in a rotary evaporator and by a stream of N_2 gas at 35°C.

3. Serum.

Extraction of serum for vitamin K was achieved by first deproteinating the sample with an alcohol followed by extraction with a non-polar organic solvent. 1.0 - 3.0 ml rat serum was mixed with MK-4, used as the internal standard, and allowed to stand for 15 minutes to allow the vitamin to enter the matrix. Four volumes of absolute ethanol were then added and the mixture vortexed. This was followed by 6 volumes of hexane, and after vortexing, allowed to stand until two phases separated. The upper hexane layer was collected and concentrated under vacuum in a rotary evaporator or with a stream of N_2 gas at 35°C.

4. Extraction of Rat Intestinal Contents.

The contents of rat intestines were removed by extrusion and 0.2-0.5 g wet weight was extracted using HIP as previously described, with the exception that samples were only extracted once, using 25.0 ml of solvent mixture.

5. Rat Livers.

Whole rat livers of approximately 5-10 g wet weight were suspended in 25.0 ml hexane and homogenized for 1 minute (Sorvall Omni-Mixer, Sorvall Inc., Newton, CT). The mixture was centrifuged at 150 x g for 5 minutes and the supernatant collected. The residue was re-extracted as before with 25.0 ml hexane, centrifuged and the supernatant collected and pooled. This was dried by passing over Na_2SO_4 crystals and then concentrated under vacuum in a rotary evaporator and with a stream of N_2 gas at 35°C.

E. Chromatography

A number of chromatographic methods have been used to separate menaquinones from other lipids in bacterial extracts.

Adsorption chromatography is a good preliminary stage for preparative purification of vitamin K from other contaminating lipids. Since adsorption chromatography does not resolve menaquinones into the different isoprenologues, this was achieved by subsequent analysis by reverse phase chromatography.

1. Adsorption TLC.

Preparative adsorption TLC was carried out on 20 cm x 20 cm, 2 mm thick Silica gel 60, F_{254} plates (Merck, Darmstadt, Federal Republic of Germany).

Lipid extracts of bacterial cells were resuspended in approximately 0.1-0.3 ml of the eluting solvent and applied as a line to the origin of the plates (approximately 2 cm from the edge). The plates were developed in chloroform:isoctane, (2:1 v/v) in the ascending mode, with the solvent being allowed to travel approximately 10 cm up the plate. Vitamin K_1 (Sigma Chemical Co., St. Louis, MO) was used as a standard for the identification of the menaquinone band. In this system the two forms of vitamin K have similar Rf values (0.6).

The menaquinone band was visualized by irradiation of the thin layers with a UVG-11 mineralight lamp (Ultra Violet Products Inc., San Gabriel, CA) at 254 nm.

Adsorption systems do not allow for the separation of menaquinone isoprenologues, which elute as a single band. In order for the lipid extract to be analysed for its menaquinone profile, the sample must be

first chromatographed on adsorption thin layers and the menaquinone band recovered for reverse phase chromatographic analysis.

Menaquinones were recovered from the adsorption thin layers by scraping the band corresponding to the standard and eluting the compound from the adsorbent with chloroform. After scraping and collecting the silica gel, 10.0 ml chloroform was added, mixed and the suspension filtered. The residue on the filter paper was washed with another 10.0 ml of chloroform and the filtrates pooled and concentrated under vacuum in a rotary evaporator and with a stream of N_2 gas at 35°C.

2. Reverse Phase TLC.

The band recovered from the adsorption thin layer plates was analysed for its menaquinone profile by reversed phase TLC. The samples were dissolved in 5 ul of acetone and spotted at the origin of either 20 cm x 20 cm, 0.25 mm thick reverse phase silica gel, F_{254} plates (Supelco, Bellefonte, PA) or 10 cm x 10 cm, 0.20 mm thick, HPTLC RP-18 F_{254} plates (Merck, Darmstadt, FRG).

Plates were developed in acetone:water, (99:1 v/v) in the ascending mode and menaquinones visualized by irradiating the plates with the UV lamp at 254 nm.

Menaquinones were identified based on a comparison of the Rf values of the unknown spots to those of pure standards MK 4-10 (kindly supplied by Hoffman La Roche, Basle, Switzerland) run simultaneously on the plates.

3. Silica Sep-Pak® Column Chromatography.

Silica Sep-Pak® cartridges (Waters Associates, Milford, MA) are single use minicolumns packed with silica gel and used in preparative adsorption chromatography, instead of the more traditional gravity columns or thin layers. The sample is loaded onto the cartridges and elution carried out by negative or positive pressure.

Lipid extracts were dissolved in 1.0 ml of hexane and loaded on the cartridge using a syringe fitted with a luer-lok tip. After washing the columns with 8.0 ml hexane, a vitamin K fraction was eluted using 8.0 ml hexane:diethyl ether (97:3 v/v). The eluate was collected and concentrated with a stream of N_2 gas at 35°C.

4. High Performance Liquid Chromatography (HPLC)

HPLC was carried out in both the adsorption (semi-preparative) and reverse phase (analytical) modes.

Apparatus

A Waters M-45 solvent delivery system was used to deliver the mobile solvent, and a U6K universal injector used to load the samples (Waters Associates, Milford, MA).

For adsorption HPLC of liver extracts, two types of columns were used. These were (a) Spheri-5, silica cartridge, 5 um particle size, 10 cm x 4.6 mm i.d. (Brownlee Labs, Santa Clara, CA) protected by a 3 cm x 4.6 mm i.d. guard cartridge of the same type (Brownlee Labs, Santa Clara, CA). (b) a Partisil 10, 10 um particle size, 25 cm x 4.6 mm i.d. silica column (Whatman Chemical Separation Inc., Clifton, NJ), protected with a Spheri-5, 3 cm x 4.6 mm i.d. guard cartridge (Brownlee Labs, Santa Clara, CA).

Initially the 10 cm, Spheri-5 column was used for semi-preparative analysis of liver extracts. However, this was subsequently superseded by the use of the longer Partsil-10 column to improve sample load. For analytical HPLC, two types of reverse phase columns were used: (a) a C18 Microbondapak, 30 cm x 3.9 mm i.d., 10 um particle size, (Waters Associates, Milford, MA) protected by a 3 cm x 3.9 mm i.d. guard column dry packed with Corasil C18 (Waters Associates, Milford, MA). The guard column was repacked periodically as necessary. (b) a Spheri-5 ODS, RP-18 cartridge, 5 um particle size, 10 cm x 4.6 mm i.d. protected with a 3 cm x 4.6 mm i.d. guard cartridge of the same type (Brownlee Labs, Santa Clara, CA). For adsorption chromatography, the samples were monitored with an SPD-2A UV spectrophotometric detector (Shimadzu Corporation, Kyoto, Japan), equipped with a 12 ul flow cell at 254 nm. For reverse phase chromatography, quinones were detected using an M-420 fluorescence monitor, equipped with an 8 ul flow cell and fitted with 338 nm band-pass excitation filter and a 425 nm long pass emission filter (Waters Associates, Milford, MA).

Since the quinones are in the oxidized state during separation and because they do not naturally fluoresce, in order for fluorescence detection to be carried out, it was necessary to reduce the vitamin K compounds post column, before they entered the detector. Reduction of quinone to hydroquinone can be accomplished by reacting it with an ethanolic solution of $NaBH_4$. On line, post column reduction of vitamin K was achieved by delivering a 0.1% ethanolic $NaBH_4$ solution (w/v) to a 200 cm x 0.5 mm i.d. reaction coil connected between the column and detector. An Eldex A-30-S minipump (Eldex Labs. Inc.,

Menlo Park, CA) was used to deliver the $NaBH_4$ solution at 0.30 ml/min. Length of reaction coil and flow rate and concentration of the $NaBH_4$ solution was optimised prior to sample analysis.

Elution profiles were displayed and analysed using an HP3392A integrator (Hewlett Packard, Avondale, PA).

Adsorption HPLC. Bacterial, fecal, serum and intestinal extracts could all be successfully analysed for menaquinone profile after a single prepurification step by either silica TLC or silica Sep-Pak cartridges. However with liver extracts it was found that after the initial silica Sep-Pak prepurification, the sample still retained enough contaminating lipids that interfered with reverse phase HPLC analysis by either co-eluting with menaquinone isoprenologues or reducing loading capacity of the column thus decreasing the sensitivity of the method. In order to achieve a preparation that could be analysed with high sensitivity and specificity, it was necessary to further clean up the silica Sep-Pak eluates of liver extract by subjecting it to adsorption HPLC. Adsorption HPLC using silica columns seemed to remove most of the contaminating lipids allowing for analysis by reverse phase HPLC.

The menaquinone fraction collected from Sep-Pak® purification was redissolved in 100-500 ul of hexane and injections of 100 ul made. Elution was accomplished by a binary solvent mixture of hexane:acetonitrile (99.8:0.2 v/v) delivered at 1.0 ml/min.

Vitamin K_1 and menaquinone fractions were identified based on a comparison of retention times (Rt) of peaks in the sample to those of pure standards. Fractions corresponding to these peaks were collected

and concentrated by a stream of N_2 gas at 35°C. They were then analysed for menaquinone profile by reverse phase HPLC.

Reverse Phase HPLC. The fractions obtained from preparative TLC, Sep-Pak® or adsorption HPLC (in the case of liver extracts) were dissolved in 100 ul of HPLC grade ethanol and 25-50 ul injected onto the reverse phase column. Menaquinones and vitamin K_1 were eluted using a mobile solvent consisting of ethanol:water (95:5 v/v) at a flow rate of 0.7 ml/min. Post column derivatization of quinones was carried out with ethanolic $NaBH_4$ solution (0.1% w/v), pumped into the mixing coil at 0.3 ml/min. To determine optimal conditions (flow rate and concentration) for quinone reduction, assays were done using a mixture of MK 4-10 pure standards. With the length of the reaction coil fixed at 200 cm, the flow rate of $NaBH_4$ solution was varied between 0.1 ml/min and 0.8 ml/min. Similarly, the concentration of $NaBH_4$ solutions was varied between 0.015% and 0.2%. The best response was obtained with the concentration of the reductant set at 0.1% and at a flow rate of 0.3 ml/min. Identity of menaquinone peaks in samples were based on comparison of their Rt to those of pure standards.

To determine linearity of detector response for the fluorescence monitor, a standard mixture of vitamin K_1 and MK 4-10 was chromatographed on the C18 column over a range of concentrations and linear regression curves determined.

To determine concentrations of menaquinones in samples of bacterial extracts, feces, intestinal contents and liver, peak area comparisons were made to those of known concentrations of standard

mixtures of vitamin K_1 and MK 4-10. Concentrations of vitamin K in serum were determined in the same way, with correction for recovery based on recovery of the internal standard.

Duplicate runs of standards were carried out before and after each batch of samples done on the same day.

F. Assays for Vitamin K_1 and Menaquinone

Complete separation of lipid extracts of the various samples assayed could not be achieved by a single chromatographic step. All samples had to be subjected to at least two and sometimes three chromatographic separations - one or more preparative adsorption step to remove as much contaminating lipids as possible followed by analysis with reversed phase HPLC.

To achieve separation and purification to the degree necessary for accurate quantitative analysis, adsorption column or thin layer chromatography in combination with reversed phase TLC or HPLC was employed.

1. Analysis of Menaquinone in Bacteria.

Initially, extracts of bacterial cells were chromatographed on adsorption thin layers and the vitamin K containing band corresponding to the vitamin K_1 standard, scraped from the plate. The vitamin K fraction was then eluted from the silica gel with chloroform, and analysed on reversed phase thin layers. Species were only analysed for their qualitative menaquinone profile. Identification of the major menaquinone isoprenologue was based on the relative intensity of spots on the TLC plates. Subsequently, a number of bacterial speci-

mens were reanalysed by using silica Sep-Pak® for preparative adsorption chromatography followed by reversed phase HPLC. In this case, quantitative determination of the menaquinone content of these species were made.

2. Analysis of Vitamin K_1 in Human Feces and Rat Intestinal Contents.

Human feces were initially extracted using a modified chloroform:methanol method of Bligh and Dyer (1959). However becasue of the high quantity of lipid material being extracted with this solvent mixture, subsequent samples of fecal matter and all the rat intestinal contents were extracted using the hexane:isopropanol method of Hara & Radin (1978).

The lipid extracts were then chromatographed on silica Sep-Pak® using hexane:diethyl ether, (97:3 v/v) to elute the vitamin K containing fraction. The eluate was then analysed by reversed phase HPLC for its vitamin K_1 and menaquinone profile. Since the assay did not have an internal standard, no correcton was made for extraction efficiency. However, from determination of spiked stool samples, it was apparent that the efficiency of the assay was in the order of 70-80% depending on the particular menaquinone isoprenologue.

3. Analysis of Vitamin K_1 in Rat Livers.

Livers were extracted with hexane and the extract chromatographed with silica Sep-Pak® as described earlier. Because the Sep-Pak® eluate still contained a high quantity of lipid material that interfered with resolution of menaquinone peaks on reverse phase HPLC

analysis, it was next subjected to adsorption HPLC and the vitamin K containing fractions collected. Collection of vitamin K containing eluates from the HPLC column was based on the retention time of pure standards run at regular intervals during the assay of a batch of livers. The eluates were pooled and analysed by reversed phase HPLC for vitamin K_1 and menaquinone.

4. Analysis of Vitamin K_1 in Human and Rat Serum

Determination of vitamin K_1 in serum was carried out using an internal standard. The internal standard was MK-4, which eluted before vitamin K_1 on reverse phase HPLC. MK-4 was considererd an appropriate choice as it was completely separated from vitamin K_1 on reverse phase analysis, but co-eluted with it from Sep-Pak®. Also at the sensitivity of the assay, endogenous MK-4 could not be detected in serum.

Serum samples were mixed with the internal standard at an appropriate level detectable by HPLC and after extraction with ethanol:-hexane, the extract was applied to silica Sep-Pak® cartridges. Elution of vitamin K from the cartridges was accomplished with hexane:diethyl ether, (97:3 v/v), and the eluate analysed by reverse phase HPLC. Vitamin K_1 levels were determined after corrections were made for recovery of MK-4.

5. Accuracy and Precision of Vitamin K Assays

To determine the quantity of vitamin K (K_1 and MK) recoverable by the assay method ie. after extraction and chromatography, samples of feces, cecal contents and liver, were divided into two portions,

vitamin K_1 and MK-4 to MK-10 added at known concentrations to one portion, and both assayed as described. The quantity of menaquinone recovered was expressed as a percentage of that added after correcting for endogenous vitamin K. The efficiency of recovery was determined at 3 separate concentrations of each form of the vitamin, with assays being done in duplicate.

$$\% \text{ Recovery} = \frac{\text{Vitamin K recovered (nmole)} - \text{Vitamin K in sample (nmole)}}{\text{Vitamin K added (nmole)}} \times 100$$

To determine precision of the assay for the same sample measured on the same day and on different days, human stool or rat intestinal contents were extracted and assayed by HPLC as described. Five determinations were done on the same day and five on consecutive days. For serum, 2.0 ml of pooled serum was spiked with 108.67 pmol of vitamin K_1. This high concentration was used because it was uncertain whether pooled sera contained measurable quantities of vitamin K_1. Sera were assayed as described for stool and rat intestinal contents.

G. Bioassay for Antimicrobial Levels

Levels of antibiotics in the cecal contents, and in some cases, serum of rats, were determined by a microbiological assay (with the exception of gentamicin) as described by Louie et al., (1976). The assay is an agar diffusion method which utilises the inhibition of hemolysis of blood by Clostridium perfringens to determine the level of antimicrobial activity. In the presence of an antibiotic with activity against C. perfringens, the organism fails to grow and hence

a zone without hemolysis (appears opaque) is observed.

C. perfringens SAL #249 (Sepulveda Anaerobic Lab, 1975), was maintained on BAK plates in an anaerobic chamber (Coy Manufacturing, Ann Arbor, MI) under an atmosphere of 10% CO_2, 10% H_2 and 80% N_2 at 37°C. To prepare a seed culture in BHI broth, a single colony from BAK plates was innoculated into broth which was incubated at 37°C for 6 hours in the anaerobic chamber.

Trypticase soy broth (Gibco Diagnostics, Madison, WI) was prepared by dissolving the powder in distilled water and then agar added to yield a final concentration of 1.1% (w/v). This solution was solubilised by autoclaving at 121°C for 15 minutes and dispensed into 23.0 ml lots. After cooling to exactly 44°C, 1.25 ml defibrinated sheep's blood and 0.75 ml of C. perfringens SAL #249 seed culture were added, mixed and poured into 150 x 15 mm plates. After the agar had solidified at room temperature 4 mm diameter wells were cut and the agar aspirated. These wells were used to load standards and samples.

Cefoxitin, moxalactam and clindamycin were determined by this method. Cefoxitin and clindamycin standards were made at concentrations that varied from 2-64 ug/ml, whereas moxalactam standards were at concentrations ranging from 4-64 ug/ml. Cecal samples were prepared by adding an equal volume of distilled water to a known weight of sample, mixed by vortexing and then centrifuged at 1500 x g for 10 minutes. The supernatant was collected and tested as is, or diluted further to 1:10 for samples containing moxalactam or clindamycin. Samples containing cefoxitin were diluted 1:10 or 1:100 and tested. Serum samples were tested undiluted or at 1:10. The diluent used in standards and samples was VPI salts solution.

Wells were filled by capillary action with a micropipette. For each plate, standards were run in duplicate, samples in triplicate at each dilution, and also VPI salts control in triplicate. Plates were incubated for 4-6 hours at 37°C in an anaerobic chamber (10% CO_2, 10% H_2 and 80% N_2) (Coy Manufacturing, Ann Arbor, MI). Zone sizes were read with vernier calipers, averaged and plotted vs concentration in a semi-logarithmic fashion. Using the standard curve generated, drug levels were calculated after correction for dilution factors.

Gentamicin levels in serum or cecal samples were done using the TDX Automated Fluorescence Polarization Analyser (Abbott Labs., Irving, TX). Standards were run at 1, 4 and 8 ug/ml for each batch of assays to yield a standard curve. Samples were assayed in duplicate, as is, or diluted, and levels determined using the standard curve.

H. Quantitative Cultures of Human Feces and Rat Intestinal Contents

0.5 to 1.0 g wet weight of fecal material or rat intestinal contents were diluted ten fold in VPI salts solution and this serially diluted to 10^{-2}, 10^{-4}, 10^{-6} g/ml. 0.1 ml of the dilutions were plated on selective and non-selective media to yield final concentrations of 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} g. Media included 5% sheep's blood agar (BA), McConkey agar, inhibitory mold agar (IMA), 5% sheep's blood agar supplemented with 1 ug/ml vitamin K_1 (BAK) (Sigma Chemical Co., St. Louis, MO), phenylethyl agar (PEA), kanamycin-vancomycin laked sheep's blood agar (LKV) and Bacteroides bile esculin agar (BBE).

Plates were incubated under aerobic conditions at 37°C and under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2) at 37°C in a Coy anaerobic chamber (Coy Manufacturing, Ann Arbor, MI).

Plates (BA, McConkey, IMA) incubated aerobically were examined after 18-24 hours. Total aerobic counts were determined from BA plates. Staphylococcus spp. were presumptively identified by colonial morphology on BA plates. The coagulase test was used to differentiate S. aureus from other Staphylococcus spp. Streptococcus fecalis and other group D streptococci were presumptively identified by colonial morphology on BA plates. Growth in 6.5% NaCl solution (w/v) and bile with the hydrolysis of esculin were used as confirmatory tests. Gram negative facultative aerobes were tested by the API 20E system (API Analytab Products, St. Laurent, PQ) and whenever necessary, this was supplemented by additional tests such as motility or the O/F test.

Bacillus spp. were identified based on aerobic growth patterns and microscopic (gram positive rods with spores) and colonial morphology.

Anaerobically incubated media (BAK, PEA, LKV, and BBE) were initially examined after 48 hours. Total count of anaerobes and facultative anaerobes were determined from BAK plates. Clostridium perfringens was presumptively identified from BAK plates by the characteristic double zone of hemolysis, and confirmed by a positive Nagler test. Other Clostridium spp. were identified based on colonial morphology, gram stain, and by GLC profiles.

Bacteroides spp. were identified as B. fragilis group organisms or Bacteroides spp. Organisms from BAK plates were subcultured to BBE plates. Growth with blackening on this medium was taken as positive result for B. fragilis group. Identity to species level was determined by the Minitek system.

Plates were again examined after 5 days, by which time gram

positive non-spore forming bacilli would have grown. This group of organisms (Propionibacterium spp., Lactobacillus spp., Actinomyces spp., Bifidobacterium spp., and Eubacterium spp.), were identified usually to the genus level primarily by GLC analysis of fermentation products from PYG broth. Peptococcus spp., Peptostreptococcus spp., and Fusobacterium spp., were identified to genus level using criteria as outlined by Sutter et al, (1980) in the Wadsworth Anaerobic Bacteriology manual.

I. Gas Liquid Chromatography (GLC) Identification of Anaerobes

GLC analysis of the fermentation products of anaerobic cultures was used for the identification of these organisms to the genus level.

Pure cultures of bacteria from BAK plates were used to inoculate PYG broth (Appendix 1) and this incubated in an anaerobic chamber in an atmosphere 10% CO_2, 10% H_2O, 80% N_2 (Coy Manufacturing, Ann Arbor, MI) at 37°C for 48 hours.

Volatile fatty acid (VFA) (acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic) analysis was carried out on these broth cultures using a Pye Unicam 104 gas liquid chromatograph (PYE Unicam Ltd., Cambridge, England) equipped with dual hydrogen flame ionisation detectors (FID). The conditions for analysis were as follows: oven temperature set at 180°C with injector and detector temperatures at 220°C; carrier gas (N_2) flow rate at 40.0 ml/min, H_2 at 40.0 ml/min and air at 600 ml/min. Separation was achieved on glass columns, 1.83 m x 4 mm i.d. packed with Chromosorb

101, 80/100 mesh (Supelco Inc., Bellefonte, PA). Sample preparation was according to method of Carlsson (1973).

To 1.0 ml of bacterial culture, 0.4 ml of cationic exchange resin AG 40W-X4, 200-400 mesh (Biorad Laboratories, Richmond, CA) and 0.1 ml isopropranol was added and mixed by vortexing. The mixture was allowed to stand and after the resin had settled, 2 ul of the supernatant injected onto the column. Peaks were identified by comparison of Rt with those of pure standards.

Non volatile fatty acids (NVFA) (succinic and lactic) analysis was carried out using a Capco 700 dual column chromatograph fitted with a thermal conducting detector (TCD) (Capco Clinical Analysis Products Co., Sunnyvale, CA). Stainless steel columns, 1.83 m x 6.35 mm i.d., were packed with Supelco-1000 (SP1000, 1% H_3PO_4, 100-200 mesh, Chromosorb, WAW) packing material (Supelco, Inc., Bellefonte, PA). Conditions for analysis were as follows: oven temperature and injector temperatures were set at 137°C with the carrier gas (He) flow rate set at 120 ml/min.

Samples were prepared by the method described by Holdeman et al, (1977). Methyl ester derivatives of the NVFA were made by reacting 1.0 ml sample with 2.0 ml of methanol and 0.4 ml of 50% H_2SO_4 at 60°C for 30 min. One ml water and 0.5 ml chloroform were then added, and the mixture allowed to partition into two phases. The lower chloroform layer, was injected on to the column, and peaks identified by comparison of Rt with those of pure standards.

J. Study Design and Approach

1. Analysis of Intestinal Microflora for Menaquinone Profiles.

Pure cultures of representative members of the intestinal flora were grown in-vitro and the cells extracted as described above. The extracts were then analysed using TLC techniques.

2. Hypoprothrombinemia in Neutropenic Patients.

Febrile neutropenic patients receiving several empiric antimicrobial regimens had their feces examined for menaquinone producing flora and menaquinone concentrations. These findings were correlated with hypoprothrombinemia. Antimicrobial regimens and dosages used are described in the Results section.

3. Hypoprothrombinemia in Normal Human Volunteers.

The objectives of this study were similar to that described above. Demographic data on the volunteers, antimicrobials used, diet and experimental details are presented with the results for purposes of clarity.

4. Animal Studies.

These experiments tested the following: (i) effect of diets on the intestinal menaquinone producing flora, the intestinal and hepatic menaquinone concentrations and prothrombin times, (ii) effects of antibiotics in combination with the diets on the above mentioned parameters, and (iii) restoration of normal prothrombin times in hypoprothrombinemic rats with menaquinone producing bacteria. The diets used, antimicrobials and dosages and experimental design are all described in the Results section for improved clarity of presentation.

K. Statistical Analysis

Alterations in $\log_{10}$ CFU/g dry weight of flora and quantitative differences in menaquinones were statistically analysed by Tukey's studentized analysis of variance procedure using an SAS statistical package for biological sciences (SAS Institute Inc., Cary, N.C.). Whenever bacterial counts or menaquinone levels were below the limits of detection for their respective assays, the SAS statistical program was used to randomly generate values between 0 and the detection threshold. For example, since the limit of detection for the quantitative culture determinations was $\leq 10^3$ CFU/g weight, values between 0 and 2.99 were randomly generated for statistical purposes.

5. RESULTS

A. Analysis of the Intestinal Microflora for Menaquinone Profiles

Menaquinones have been reported in a wide variety of bacteria. The majority of those tested, however, have been for taxonomic reasons and are not members of the human intestinal flora. Also, most are of little clinical importance (Collins and Jones, 1981). Organisms belonging to the Bacteroides fragilis "group" as well as certain genera of gram-positive non-spore forming rods such as Eubacterium spp. and Bifidobacterium spp. make up a major part of the intestinal flora (Finegold, 1977). With respect to analysis of these types of bacteria for menaquinones, only a few Bacteroides spp. have been tested (Collins and Jones, 1981) and information on menaquinone profiles of the other gram-positive non-spore forming rods have been sparse. It is therefore unclear which members of the intestinal flora are major producers of menaquinones. The objective of this study was to examine representatives of the human intestinal flora, especially the anaerobes, to determine their menaquinone profiles. As well, the isolates to be analysed would be those obtained from clinical specimens or from human stool.

1. Analysis of Bacteria for Menaquinone by TLC.

Bacteria were grown in pure culture, extracted and chromatographed for menaquinone profile by TLC as described in materials and methods. Subsequently, extracts of a number of these organisms were reanalysed using silica Sep-Pak® cartridges for preparative chromato-

graphy followed by analysis with HPLC. HPLC analysis was carried out on selected strains to determine the accuracy of the TLC findings, since the former affords greater sensitivity for the detection of menaquinones. Also, a few additional organisms were analysed for menaquinones by HPLC only.

Menaquinone isoprenologues on TLC were identified whenever possible by comparison of Rf values of the samples to those of known standards. Since only fully unsaturated menaquinone standards were available, those with saturated side chains or demethylated at the carbon-2 (DMK) position could not be identified. In addition, the standard for MK-11 was unavailable and identification of this isoprenologue was based on the correlation between change of Rf value and increase in isoprenoid chain length (Figure 5.1).

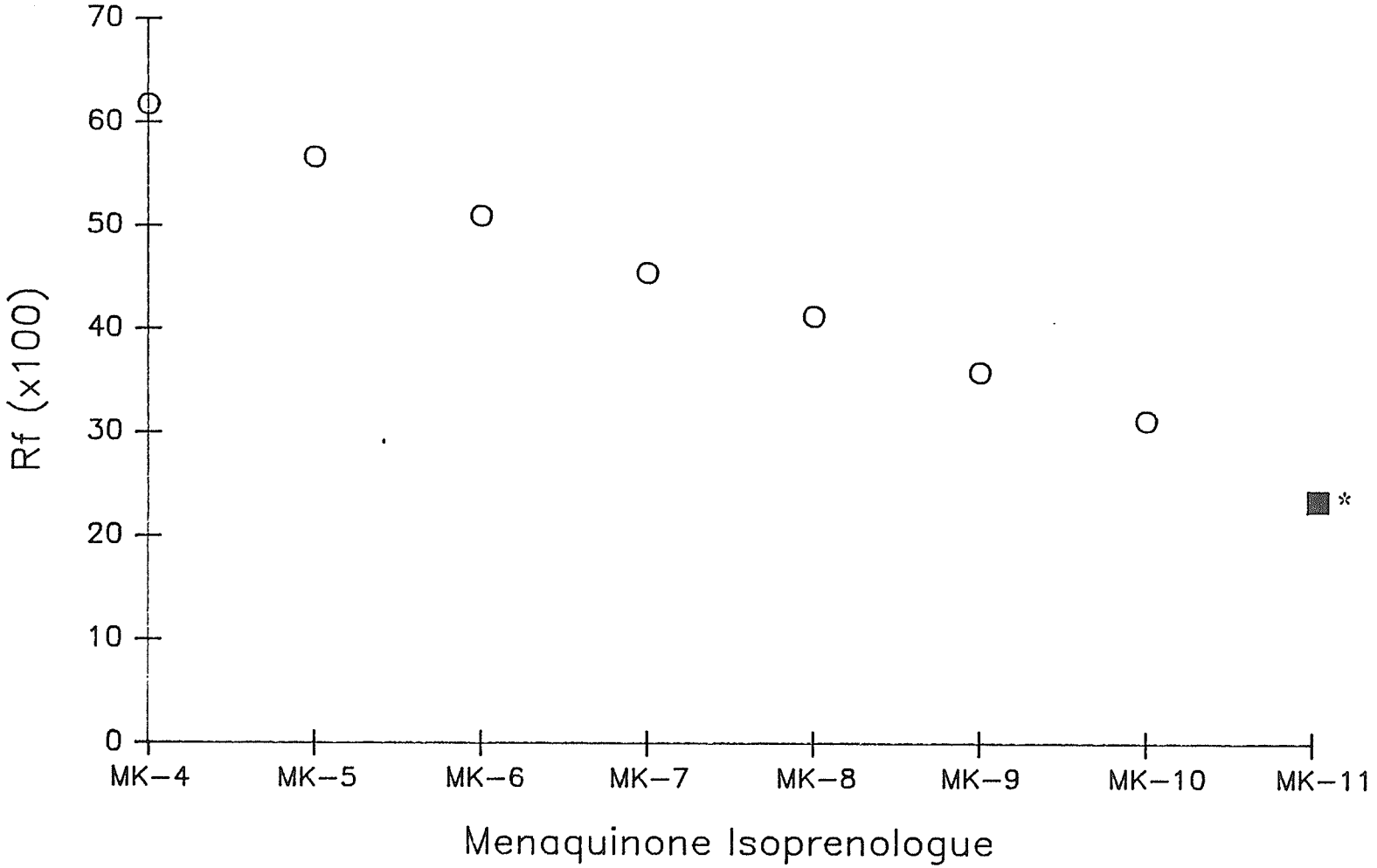
As can be seen from Table 5.1, the Rf values obtained for standards agreed fairly well with published values (Collins et al, 1980a). When the Rf values of menaquinone isoprenologues were plotted against length of isoprenoid chain, the relationship was "linear" in an inverse fashion, that is, as the length of the menaquinone side chain increased, the Rf value decreased by a constant value (Figure 5.1). The coefficient of correlation for the plot of Rf value vs length of side chain was -0.9994. Using a linear regression curve generated by this plot, the predictive Rf value for MK-11 would be 25.7. When organisms from the genus Bacteroides was analysed for menaquinones, the spot identified as MK-11 had an Rf value of 26.1 agreeing closely with the predicted value.

In reviewing the organisms tested for menaquinone profiles it is evident that only a few anaerobic genera of the normal intestinal

Table 5.1 Rf value (x 100) of menaquinones on reverse phase TLC withacetone:water (99:1 v/v) as mobile phase.

Menaquinone Isoprenologue	Rf values (x 100)	
	Standards	Samples
MK-4	61.7 ± 0.25	-
MK-5	56.6 ± 0.28	-
MK-6	50.9 ± 0.35	50.6
MK-7	45.4 ± 0.35	45.7
MK-8	41.2 ± 0.29	40.1
MK-9	35.8 ± 0.25	35.3
MK-10	31.1 ± 0.36	30.7
MK-11	-	26.1

Figure 5.1 Reverse phase TLC of menaquinone standards. Solvent system; acetone:water, 99:1 (v/v).



*Predicted Rf for MK-11 based on linear regression analysis.

flora produce menaquinones. These, essentially, include Bacteroides spp., Veillonella spp., Propionibacterium spp., Eubacterium spp., and Arachnia spp. (Tables 5.2-5.5).

With the exception of the single B. uniformis strain tested, which contained no menaquinone, all the other members that constitute the B. fragilis group contained MK-10 and MK-11 as the major isoprenologue with some strains having MK-9 in minor quantities (Tables 5.2, 5.6). The estimation of major and minor isoprenologue in these organisms was arbitrarily based on MK-9 appearing qualitatively less intensely than MK-10 and MK-11 on thin layer plates when irradiated by a short wave ultraviolet source. The single isolate of B. disiens and both isolates of B. bivius contained menaquinones. The latter, like those numbers of the B. fragilis group contained MK-9, MK-10 and MK-11 with the last two being the major isoprenologues. B. disiens, however unlike all the other Bacteroides sp. contained MK-11 and MK-12 as major isoprenologues (Figure 5.2). None of the four strains of Fusobacterium sp. examined contained menaquinones (Table 5.6).

Of the gram-positive anaerobes, none of the spore formers (all Clostridium spp. contained menaquinones (Table 5.4, 5.7). Only three genera of the non-spore formers contained menaquinones: Propionibacterium spp., Eubacterium spp., and Arachnia propionica. Within the Proprionibacterium and Eubacterium species, only certain members contained menaquinones (Table 5.4, 5.8). The isoprenologue predominating in these genera could not be identified based on Rf values (TLC) as it did not co-elute with any of the pure standards available. However, on TLC plates, this isoprenologue tended to run

Table 5.2 Menaquinone profiles of obligate anaerobic gram negative bacterial species.

The major MK isoprenologue for each organism is denoted by +++. Minor isoprenologues are denoted by ++ or +. Relative quantities were qualitatively determined based on the intensities of spots on the plates after irradiation with a short wave (254 nm) ultraviolet source.

Organism	MK-9	MK-10	MK-11	MK-12
Bacteroides fragilis "group"				
B. fragilis ATCC23745	+	+++	+	-
B. fragilis 3606	+	+++	++	-
B. fragilis 79	±	+++	++	-
B. fragilis 3602	-	+++	++	-
B. fragilis 527	-	+++	+++	-
B. distasonis 11	-	+++	++	-
B. distasonis 234	-	+++	++	-
B. distasonis 3	-	+++	++	-
B. distasonis 47299	+	+++	++	-
B. distasonis ATCC 8503	+	+++	++	-
B. distasonis 24	-	+++	+++	-
B. vulgatus 9	-	+++	++	-
B. vulgatus 17	-	+++	++	-
B. vulgatus 649	+	+++	++	-
B. vulgatus 551	+	+++	++	-
B. vulgatus 554	-	+++	+++	-

Table 5.2 (Continued)

Organism	MK-9	MK-10	MK-11	MK-12
B. ovatus 19	-	+++	++	-
B. ovatus 235	-	+++	++	-
B. ovatus 236	-	+++	++	-
B. ovatus 2852	-	+++	+++	-
B. ovatus 3133	-	+++	+++	-
B. ovatus ATCC 8483	-	+++	+++	-
B. thetaiotaomicron ATCC 29741	-	+++	+++	-
B. thetaiotaomicron ATCC 29148	-	+++	+++	-
B. uniformis 25	-	-	-	-
B. disiens ATCC29425		+	+++	++
B. bivius ATCC 29303	+	+++	++	-
B. bivius 75	+	+++	++	-
Fusobacterium spp.				
F. nucleatum 9685	-	-	-	-
F. nucleatum 8959	-	-	-	-
F. nucleatum ATCC 25586	-	-	-	-
F. necrophorum ATCC 25286	-	-	-	-

Table 5.3. Menaquinone profile of facultative gram negative bacterial species

The major MK isoprenologue for each organism is denoted by +++. Minor isoprenologues are denoted by ++ or +. Relative quantities were qualitatively determined based on the intensities of spots on the plates after irradiation with a short wave (254 nm) ultraviolet source.

Organism	MK-8
Escherichia spp.	
E. coli R 4-6	+++
E. coli 0111	+++
E. coli ATCC 23744	+++
E. coli 5	+++
E. coli 90	+++
Citrobacter spp.	
C. freundii	-
Klebsiella spp.	
K. pneumoniae 1	+++
K. pneumoniae 2	+++
K. pneumoniae 8170	+++
K. pneumoniae 7351	+++

Table 5.4 Menaquinone profile of obligate anaerobic gram-positive bacterial species

The major MK isoprenologue for each organism is denoted by +++. Minor isoprenologues are denoted by ++ or +. Relative quantities were qualitatively determined based on the intensities of spots on the plates after irradiation with a short wave (254 nm) ultraviolet source.

Organism	MK-7	MK-n
Clostridium spp.		
C. perfringens 14209	-	-
C. perfringens 37	-	-
C. perfringens 1	-	-
C. perfringens 67	-	-
C. perfringens 2210	-	-
C. butyricum 9151	-	-
C. bifermentans 438	-	-
C. sordellii 1125	-	-
C. innocuum 522	-	-
C. ramosum 23	-	-
C. ramosum 5	-	-
C. sporogenes 1522	-	-
C. paraputrificum 5577	-	-
C. tertium	-	-
C. difficile	-	-
Bifidobacterium spp.		
B. longum A02	-	-
B. adolocentis 267	-	-
B. longum A650	-	-
B. breve A291	-	-
Propionibacterium spp.		
P. acnes 7808	-	+++
P. acnes 16406	-	+++

Table 5.4 (Continued)

Organism	MK-7	MK-n
P. acnes A634	-	+++
P. acnes A679	-	+++
P. jensenii 9990	-	+++
P. acidi-propionici 265	-	-
P. acidi-propionici 266	-	-
P. acidi-propionici 2411	-	-
Arachnia spp.		
A. propionica	-	+++
Lactobacillus spp.		
L. acidophilus ATCC 4356	-	-
L. acidophilus 268	-	-
L. casei ATCC 393	-	-
L. casei ATCC 4646	-	-
L. casei ATCC 15008	-	-
Eubacterium spp.		
E. lentum 615	-	+
E. lentum 517	-	+
E. alactolyticum ATCC 17927	-	-
E. limosum ATCC 8486	-	-
E. lentum ATCC 25559	+++	-
Actinomyces spp.		
A. naeslundii A653	-	-

Table 5.5 Menaquinone profiles of gram-positive and gram-negative cocci

The major MK isoprenologue for each organism is denoted by +++. Minor isoprenologues are denoted by ++ or +. Relative quantities were qualitatively determined based on the intensities of spots on the plates after irradiation with a short wave (254 nm) ultraviolet source.

Organism	MK-6	MK-7	MK-8
Peptostreptococcus spp.			
P. anaerobius A648	-	-	-
Peptostreptococcus sp. 45	-	-	-
Peptostreptococcus sp. 46	-	-	-
Peptostreptococcus sp. 6574	-	-	-
Peptococcus spp.			
P. magnus WAL 2508	-	-	-
Peptococcus sp. 6782	-	-	-
Peptococcus sp. 9739	-	-	-
Peptococcus sp. 46294	-	-	-
Veillonella spp.			
V. parvula ATCC 10790	+	+++	+
Staphylococcus spp.			
S. aureus 45845	-	+++	-

Table 5.6 Menaquinone in anaerobic gram-negative bacilli

Organism	No. tested	No. with MK	MK isoprenologues	
			Major	Minor
B. fragilis "group"				
B. fragilis	5	5	MK-10,11	MK-9(3)[a]
B. ovatus	6	6	MK-10,11	-
B. distasonis	6	6	MK-10,11	MK-9(2)
B. vulgatus	5	5	MK-10,11	MK-9(2)
B. thetaiotaomicron	2	2	MK-10,11	-
B. uniformis	1	0	-	-
Bacteroides spp.				
B. disiens	1	1	MK-11,12	MK-10
B. bivius	2	2	MK-10,11	MK-9 (2)
Fusobacterium spp.				
F. nucleatum	3	0	-	-
F. necrophorum	1	0	-	-

[a] = Number in brackets denote number of strains with MK-9.

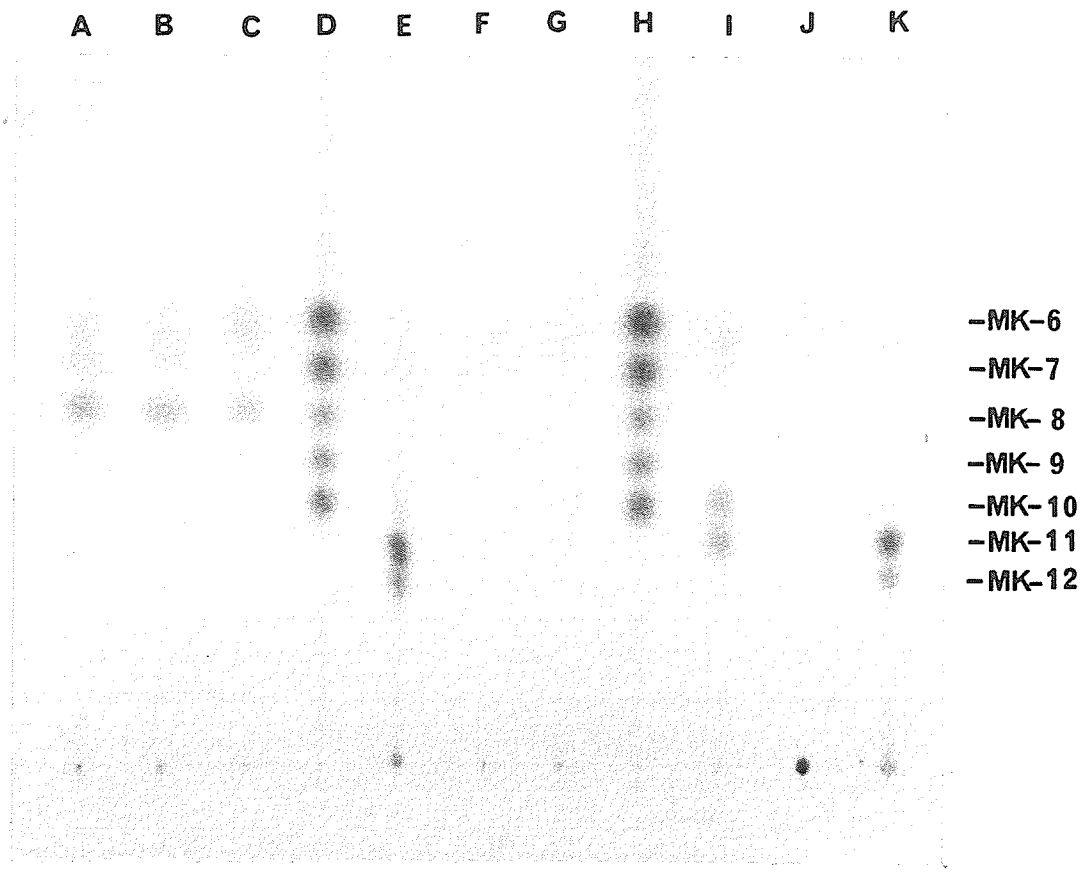


Figure 5.2 Reverse phase TLC of bacterial menaquinones. Solvent system; Acetone:water, 99:1 (v/v). (A) Klebsiella pneumoniae, (B) Escherichia coli, (C) Staphylococcus aureus, (D) MK-6 - MK-10 standards, (E) Bacteroides disiens, (F) Bifidobacterium longum, (G), Lactobacillus casei, (H) MK-6 - MK-10 standards, (I) Bacteroides theatiotaomicron, (J) Clostridium sordelli, (K) B. disiens.

Table 5.7 Menaquinones in anaerobic gram-positive spore forming bacilli

Organism	No. tested	No. with MK	MK isoprenologues	
			Major	Minor
C. perfringens	5	0	-	-
C. butyricum	1	0	-	-
C. bifermentans	1	0	-	-
C. sordellii	1	0	-	-
C. innocuum	1	0	-	-
C. ramosum	2	0	-	-
C. sporogenes	1	0	-	-
C. paraputrificum	1	0	-	-
C. tertium	1	0	-	-
C. difficle	1	0	-	-

Table 5.8 Menaquinones in anaerobic gram-positive non-spore forming bacilli

Organism	No. tested	No. with MK	MK isoprenologues	
			Major	Minor
Bifidobacterium spp.				
B. longum	2	0	-	-
B. adolocentis	1	0	-	-
B. breve	1	0	-	-
Propionibacterium spp.				
P. acnes	4	4	MK-n	-
P. jensenii	1	1	MK-n	-
P. acidi-propionici	3	0	-	-
Arachnia spp.				
A. propionica	1	1	MK-n	-
Lactobacillus spp.				
L. acidophilus	2	0	-	-
L. casei	3	0	-	-
Eubacterium spp.				
E. lentum	2	2	MK-n	-
E. lentum	1	1	MK-7	-
E. alactolyticum	1	0	-	-
E. limosum	1	0	-	-
Actinomyces spp.				
A. naeslundii	1	0	-	-

between MK-10 and MK-11 suggesting that it could probably be partially saturated menaquinone (Figure 5.3). Five of the eight Propionibacterium spp. contained MK-n; the three that did not were all P. acidipropionici species. The only Arachnia sp. tested also contained a menaquinone isoprenologue similar to that seen in the Proprionibacterium spp. Of the Eubacterium spp. containing menaquinones, two contained MK-n similar to that found in Proprionibacterium spp. based on similar Rf values on TLC and a third contained MK-7. One other strain of E. limosum tested had no menaquinone.

The other gram-positive anaerobic genera, Bifidobacterium spp., Lactobacillus spp., Actinomyces spp., Peptococcus spp., and Peptostreptococcus spp., were all devoid of menaquinones (Table 5.4, 5.9). Actinomyces species have all been reported to contain menaquinones (Hess et al, 1979; Collins et al, 1977), but the particular strain tested here had not been previously examined. The facultative gram-negative organisms E. coli and Klebsiella spp., all contained MK-8 (Table 5.3, 5.10).

2. Analysis of Bacteria for Menaquinone by HPLC

When the menaquinone profiles of organisms obtained by TLC analysis is compared to that obtained by HPLC analysis, it is evident that the profiles obtained by both methods are essentially the same, at least in terms of the major isoprenologue(s) found. When organisms were analysed by TLC, the major menaquinone isoprenologues were subjectively determined based on the relative intensities of the spots. Subsequent analysis of a number of strains by HPLC confirms

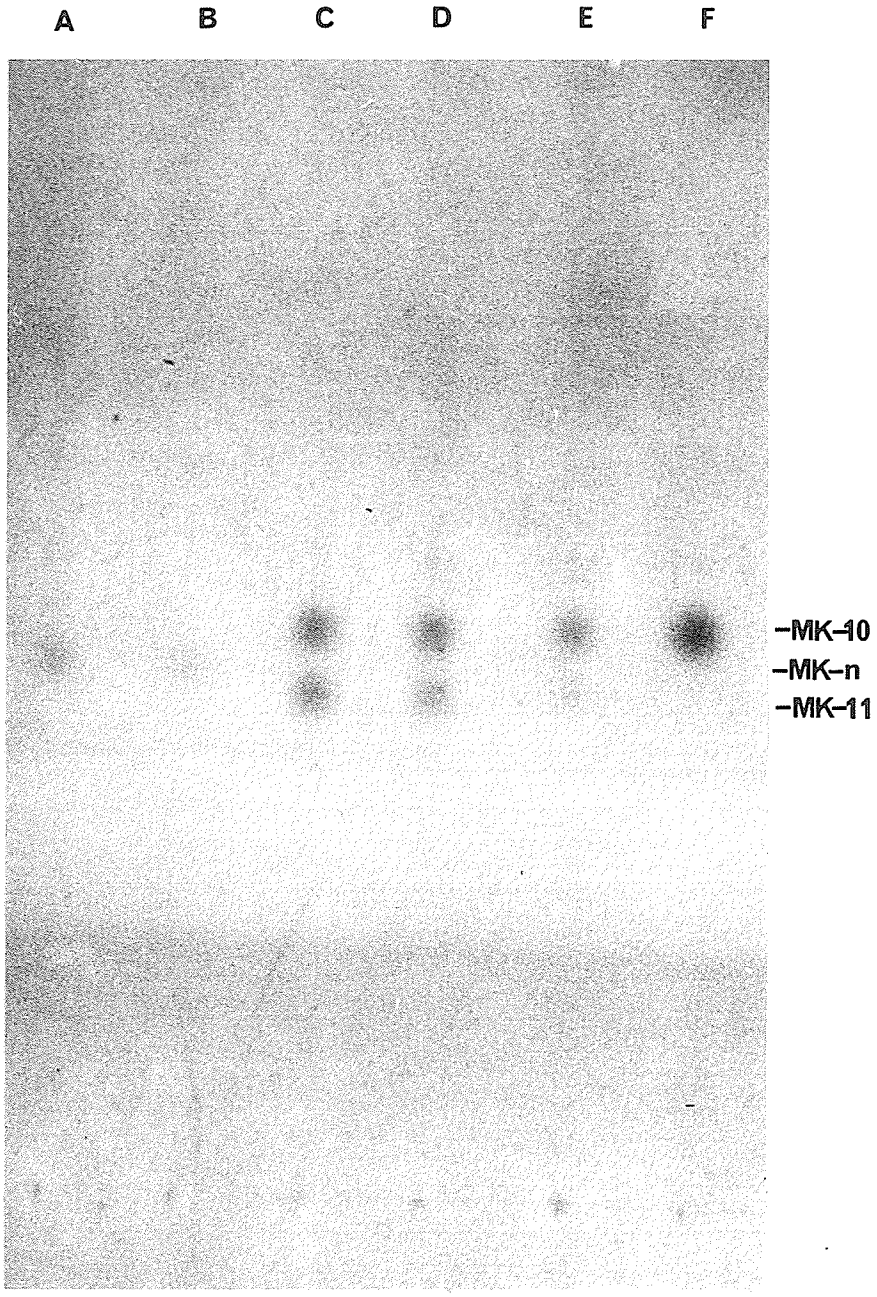


Figure 5.3 Reverse phase TLC of bacterial menaquinones. Solvent system; Acetone:water, 99:1 (v/v). (A) and (B) Propionibacterium acnes, (C) Bacteroides fragilis, (D) B. vulgatus, (E) B. distasonis, (F) MK-10 standard.

Table 5.9 Menaquinones in anaerobic cocci

Organism	No. tested	No. with MK	MK isoprenologues	
			Major	Minor
Peptostreptococcus spp.				
P. anaerobius	2	0	-	-
Peptostreptococcus spp.	3	0	-	-
Peptococcus spp.				
P. magnus	1	0	-	-
peptococcus spp.	3	0	-	-
Veilonella spp.				
V. parvula	1	1	MK-7	
			MK-6,8	

Table 5.10 Menaquinones in facultative gram-negative bacilli

Organism	No. tested	No. with MK	MK isoprenologues	
			Major	Minor
Escherichia spp.				
E. coli	5	5	MK-8	-
Citrobacter spp.				
C. freundii	1	0	-	-
Klebsiella spp.				
K. pneumoniae	4	4	MK-8	-

that these isoprenologues were in fact the major ones since the relative quantities of each isoprenologue as a percentage of the total for a particular bacterium could be determined based on peak area calculations (Table 5.11). The only advantage HPLC had over TLC is that with the former method, very minor isoprenologues could be detected whereas they were not by TLC. However, these minor isoprenologues were in such low quantities as to be almost negligible compared to the major ones, eg. MK-8 is the major isoprenologue in E. coli and makes up 85% of the total, whereas the minor menaquinones, MK-6, MK-7, and MK-9 contribute only 15%. Only MK-8 was detected in E. coli using TLC, whereas with HPLC, MK-6, MK-7 and MK-9 were also detected. Similarly with B. fragilis 3606, using TLC, MK-9, MK-10, and MK-11 were detected, whereas with HPLC, MK 6-11 were seen. However, MK-9, MK-10 and MK-11 constituted 88% of the total in this organism.

Quantitative analysis of certain bacteria for menaquinone content indicates that bacteria contains varying amounts of menaquinones (Table 5.12). This ranged from as low as 1 nmole/g dry weight to as much as 200 nmole/g dry weight under the growth conditions described. Most of the organisms examined appeared to contain between 50-100 nmole/g dry weight. B. ovatus and K. pneumoniae 2 had less than 1 nmole/g dry weight of menaquiones. On the other hand, a second strain of K. pneumoniae 26 contained 30 nmole/g dry weight. E. coli 90 had the highest level of menaquinone at 178 nmole/g dry weight.

Table 5.11 Comparison of menaquinone profiles in organisms analysed by both TLC and HPLC

Bacteria	MK Profile													
	TLC[a]							HPLC[b]						
	MK-6	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12	MK-6	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12
B. fragilis 3606	-	-	-	+	+++	++	-	2.8	3.2	6.3	11.7	58.1	17.9	-
B. ovatus 8483	-	-	-	-	+++	+++	-	-	-	-	5.9	49.4	44.9	-
B. vulgatus 649	-	-	-	+	+++	++	-	-	4.1	10.6	9.0	46.8	29.5	-
B. bivius 29303	-	-	-	+	+++	++	-	6.01	4.5	6.4	15.4	60.2	7.5	-
B. disiens 29426	-	-	-	-	+	+++	++	4.5	3.1	5.2	5.0	11.3	51.4	19.5
B. uniformis	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. coli 90	-	-	+++	-	-	-	-	0.8	12.0	84.5	2.7	-	-	-
K. pneumoniae 2	-	-	+++	-	-	-	-	-	3.9	95.5	0.6	-	-	-
C. tertium	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. sordelli	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P.acidipropionici	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. aureus 49548	-	+++	-	-	-	-	-	11.7	88.3	-	-	-	-	-
V. parvula	+	+++	+	-	-	-	-	10.5	82.3	7.2	-	-	-	-

a: plus sign denotes relative quantities of menaquinone isoprenologues
b: figure denotes % of total menaquinones

Table 5.12 Menaquinone content (nmole/g dry wt) in bacteria, measured by HPLC

Bacteria	MK Profile (nmole/g dry wt)					
	MK-6	MK-7	MK-8	MK-9	MK-10	Total
B. fragilis	-	4.63	3.16	24.85	132.36	165.00
B. ovatus ATCC 8483	-	-	-	0.18	1.11	1.29
B. vulgatus 649	-	2.27	7.0	7.36	29.56	46.19
E. coli 90	0.63	18.35	153.26	6.17	-	178.41
K. pneumoniae 2	-	1.00	28.76	0.21	-	29.97
K. pneumoniae 26	-	-	0.24	-	-	0.24
A. hydrophila	-	1.41	70.30	0.58	-	72.29
Morganella morganii	1.43	3.71	76.20	0.84	-	82.18
C. sordellii	-	-	-	-	-	-
C. tertium	-	-	-	-	-	-
C. paraputrificum	-	-	-	-	-	-
C. sporogenes	-	-	-	-	-	-
E. alactolyticum	-	-	-	-	-	-
S. aureus ATCC 29242		12.92	49.96	5.65	0.45	68.98

B. Hypoprothrombinemia in Febrile Neutropenic Cancer Patients on Broad Spectrum Antimicrobial Agents.

Vitamin K_1 acquired from the diet has been generally presumed to be the sole source of vitamin K in humans for the carboxylation of descarboxyprothrombin to active prothrombin; menaquinones synthesized by bacteria were considered unavailable to the host and therefore unimportant in maintaining normal coagulation (Barkhan and Shearer, 1977).

However, Matschiner and Amelotti (1968) were able to correlate the menaquinone profile of bovine liver (MK-10, MK-11 and MK-12) with that of the rumen contents (MK-10, MK-11, MK-12 and MK-13), suggesting that the origin of these menaquinones was from the rumen. In addition, Duello and Matschiner (1972) isolated a series of fully unsaturated and partially saturated menaquinones from human liver.

Another factor that argues in favor of menaquinones being clinically important is that it is difficult to induce vitamin K deficiency and clinical hypoprothrombinemia in humans and animals on a vitamin K_1 deficient diet alone unless antimicrobial agents capable of suppressing the intestinal flora are used concurrently. This suggests that only in the presence of agents that can remove the menaquinone producing flora of the intestine and therefore the endogenous source of the vitamin, is K deficiency likely to occur. Gustaffson (1959) and Gustaffson, et al (1962) were able to demonstrate hypoprothrombinemia in germ free rats on a vitamin K_1 deficient diet but not in those contaminated with various bacteria. Also Frick et al (1967) using human subjects, found hypoprothrombinemia only in those receiving dextrose

solution for up to 5 weeks ("vitamin K_1 deficient diet") and on unspecified antibiotics. Finally, clinical reports have demonstrated that vitamin K_1 responsive hypoprothrombinemia tends to occur more often in association with the use of broad spectrum antimicrobial agents during low vitamin K_1 intake.

Having determined the members of the intestinal flora that constitute the major producers of menaquinones (B. fragilis group, E. coli), feces of febrile neutropenic patients on empiric antimicrobial therapy were examined to determine: (1) the effect of antimicrobial regimens on the intestinal menaquinone-producing microflora, and (2) their menaquinone content. The objective was to correlate any changes in menaquinone producing flora due to antimicrobial pressure with changes in menaquinone content and the occurrence of hypoprothrombinemia (HPT). Since these patients are on cancer chemotherapy, they are generally undernourished and are on a reduced caloric and dietary vitamin K_1 intake. The patients from whom samples were obtained were hospitalized at the Health Sciences Centre and St. Boniface General Hospital, Winnipeg, Manitoba, and were part of several multicentre* trials to test the efficacy of empiric antimicrobial therapy during febrile neutropenic episodes.

Patients were randomly allocated to receive the following antimicrobial regimens: aztreonam plus cloxacillin (AC), aztreonam plus tobramycin (AT), moxalactam plus tobramycin (TM), moxalactam plus ticarcillin (MT), and ticarcillin plus tobramycin (TT). Aztreonam, moxalactam, and cloxacillin were administered 2 g IV every 6 hours

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*infections in cancer, trial #4

(q6h); tobramycin, 1.25 mg/kg q6h and ticarcillin 300 mg/kg/day. The first 3 regimens were the arms of the IC-4 trial evaluating the efficacy of a control regimen which destroys the endogenous microflora (TM) and two sparing regimens (AT and AC) (Louie et al, 1985). On the basis of selective activity of aztreonam against coliforms and Pseudomonas organisms only, it was expected that the anaerobes would be spared in the AT and AC regimens. The TT and MT regimens were the arms of the IC-2 trial (Feld et al, 1985) evaluating a double beta-lactam regimen (MT and comparing it to the standard beta-lactam/aminoglycoside regimen (TT). Aztreonam was obtained from E.R. Squibb and Sons, Princeton, NJ; moxalactam and tobramycin, Eli Lilly Inc., Scarborough, Ont.; cloxacillin, Ayerst Laboratories, Montreal, PQ; and ticarcillin from Beecham Labs., Pointe Claire, Quebec.

Fecal samples were obtained at or within 48 hours of study entry and at day 7 or thereafter. Quantitative cultures were carried out immediately or within 24 hours of collection of feces, and an aliquot frozen at -70°C for subsequent analysis for menaquinones and phylloquinone.

Plasma was also collected at the same time for determination of prothrombin times (PT).

1. HPLC Assay of Vitamin K_1 and Menaquinones

HPLC has been used successfully to measure vitamin K_1 and menaquinones in a number of different types of biological material. Vitamin K_1 has been quantitated in liver, serum, green vegetables, natural and artificial milk and milk products, and menaquinones in bacteria. The measurement of menaquinone in feces, has been a diffi-

cult problem primarily because of lack of selectivity and sensitivity in assay methodologies.

Fecal samples contain low levels of menaquinone and in addition, a large variety of contaminating lipid material that must be removed so as to detect the compound. In order to successfully measure menaquinones and vitamin K_1 in feces, successive chromatographic steps were necessary. The lipid extract of the feces was first chromatographed on an adsorption silica column (Silica Sep-Pak® cartridges) and a vitamin K containing fraction collected. This was then analysed by reverse phase HPLC and menaquinone isoprenologues identified and quantitated by comparison with known standards.

(i) Separation

In developing the HPLC assay, separation of a standard mixture of menaquinone isoprenologues (MK-4 to MK-10) and vitamin K_1 was attempted on a 30 cm, C18 Microbondapak column with absolute ethanol as the mobile solvent. Elution was in the isocratic mode. Use of this solvent system gave incomplete resolution; addition of water and increasing the proportion up to 5% allowed resolution of all the components of the mixture with the exception of vitamin K_1 and MK-5 (Figure 5.4). In order to improve separation of these two compounds, the water concentration of the mobile phase would have to be increased further but total elution time of the chromatographic run would increase to impractical lengths. With a mobile phase of ethanol:water (95:5 v/v) under isocratic conditions, total run time was approximately 30 minutes (Table 5.13). Subsequent use of a 10 cm C18 column with 5 um silica particles gave similar results as the 30 cm column under the same conditions.

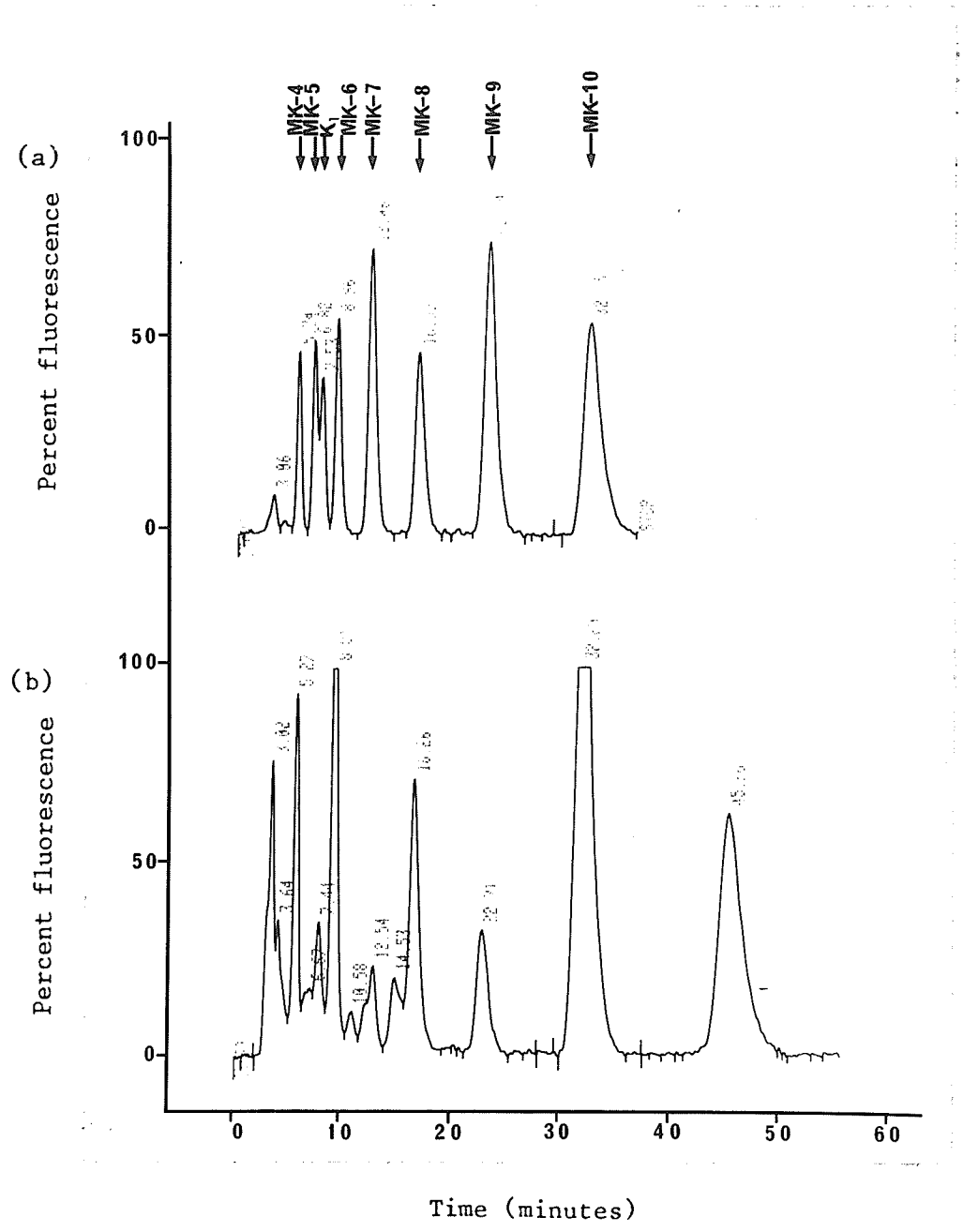


Figure 5.4 Chromatogram of (a) menaquinone standards and (b) cecal contents of a chow fed rat after HPLC separation on a C18 column with ethanol:water (95:5 v/v) as mobile phase.

Table 5.13 Retention time for menaquinone standards on reverse phase HPLC

a) C18 Microbondapak Column (30 cm x 3.9 mm i.d.)

Menaquinone	Rt (min)*
MK-4	8.0 ± 0.14
MK-5	9.5 ± 0.36
MK-6	11.3 ± 0.46
MK-7	13.6 ± 0.64
MK-8	16.8 ± 0.79
MK-9	21.3 ± 1.17
MK-10	27.9 ± 1.80

b) C18 Spheri-5 Column (10 cm x 4.6 mm i.d.)

Menaquinone	Rt (min)
MK-4	5.32 ± 0.03
MK-5	6.60 ± 0.02
K_1	7.23 ± 0.02
MK-6	8.36 ± 0.02
MK-7	10.87 ± 0.03
MK-8	14.39 ± 0.00
MK-9	19.44 ± 0.02
MK-10	26.59 ± 0.02

*Mean + SD of 4 determinations
Conditions for run are as described in Materials and Methods

(ii) Detection

Fluorescence detection after post column reduction was used in the assay because fluorometric detection is more selective and sensitive than UV methods. Enhanced selectivity is achieved because many contaminating components of the sample that absorb in the UV range do not fluoresce. Since the K vitamins do not possess native fluorescence, a reduction to the highly fluorescent hydroquinone is necessary. In this assay reduction was achieved by use of an ethanolic solution of $NaBH_4$.

The effect of reagent concentration and flow rate on fluorescence response was investigated. While keeping the flow rate of the reagent fixed at 1.0 ml/min, $NaBH_4$ concentration was varied between 0.01% and 0.15%. As can be seen in Figure 5.5 fluoresence response of a mixture of menaquinone standards increased steadily with concentration of $NaBH_4$. At concentrations beyond 0.1%, bubbles were produced in the reagent and this tended to produce wide variations in background response. A $NaBH_4$ concentration of 0.1% was therefore chosen for assay conditions.

To determine optimal flow rate of $NaBH_4$ reagent, while keeping concentration at 0.1%, flow rate was varied between 0.08 ml/min to 0.9 ml/min. As can be seen in Figure 5.6, the best response was achieved at approximately 0.15 ml/min. At reagent delivery rates higher than the optimum, response tended to fall off in a manner that was linear with increase in flow rate. Under assay conditions, reagent delivery rate was set at 0.3 ml/min. This is because the Eldex pump tended to produce wide variations in flow at rates lower than 0.3 ml/min.

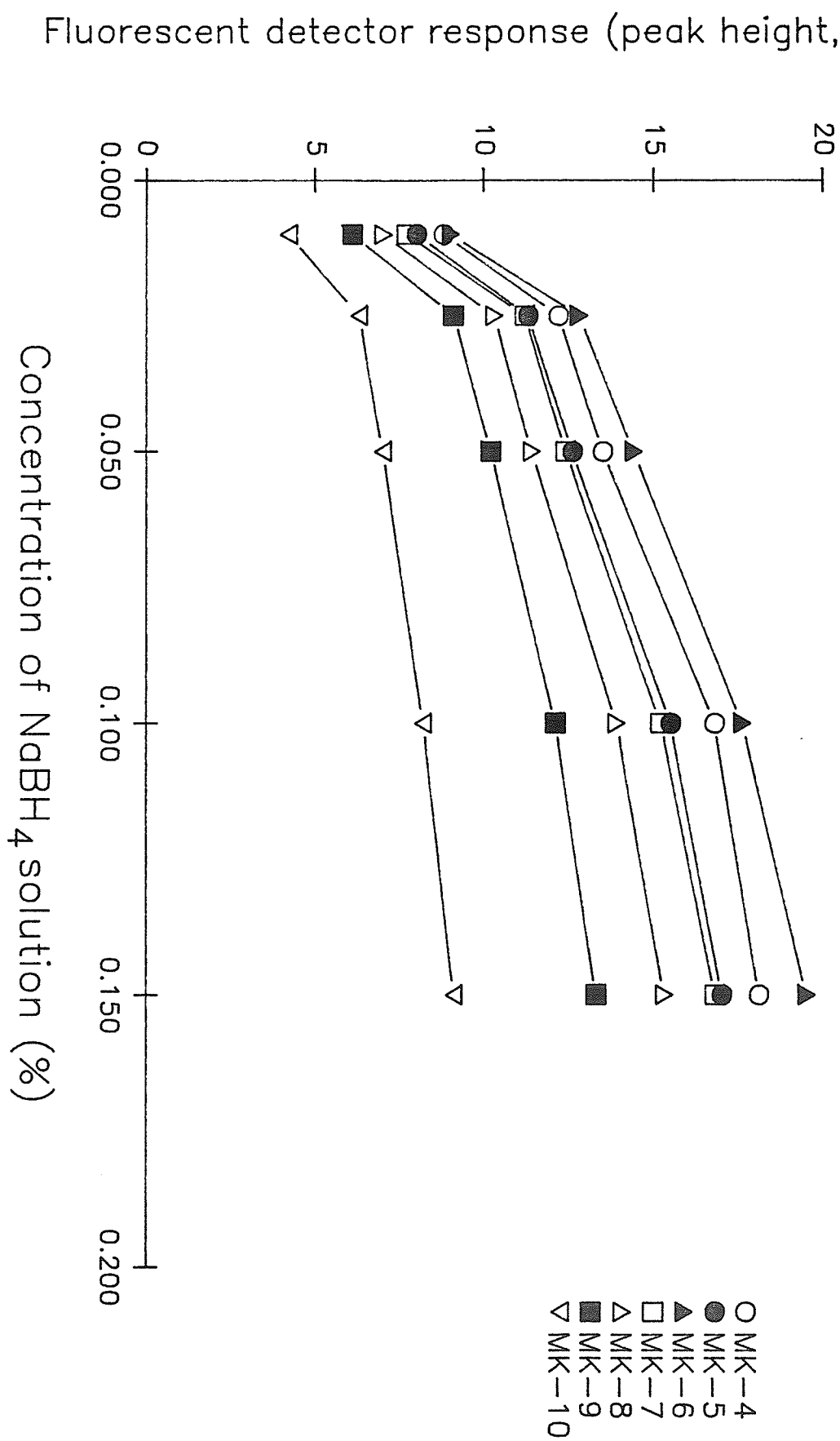


Figure 5.5 Fluorescent detector response (peak height) for MK 4-10 as a function of $NaBH_4$ concentration for MK 4-10.

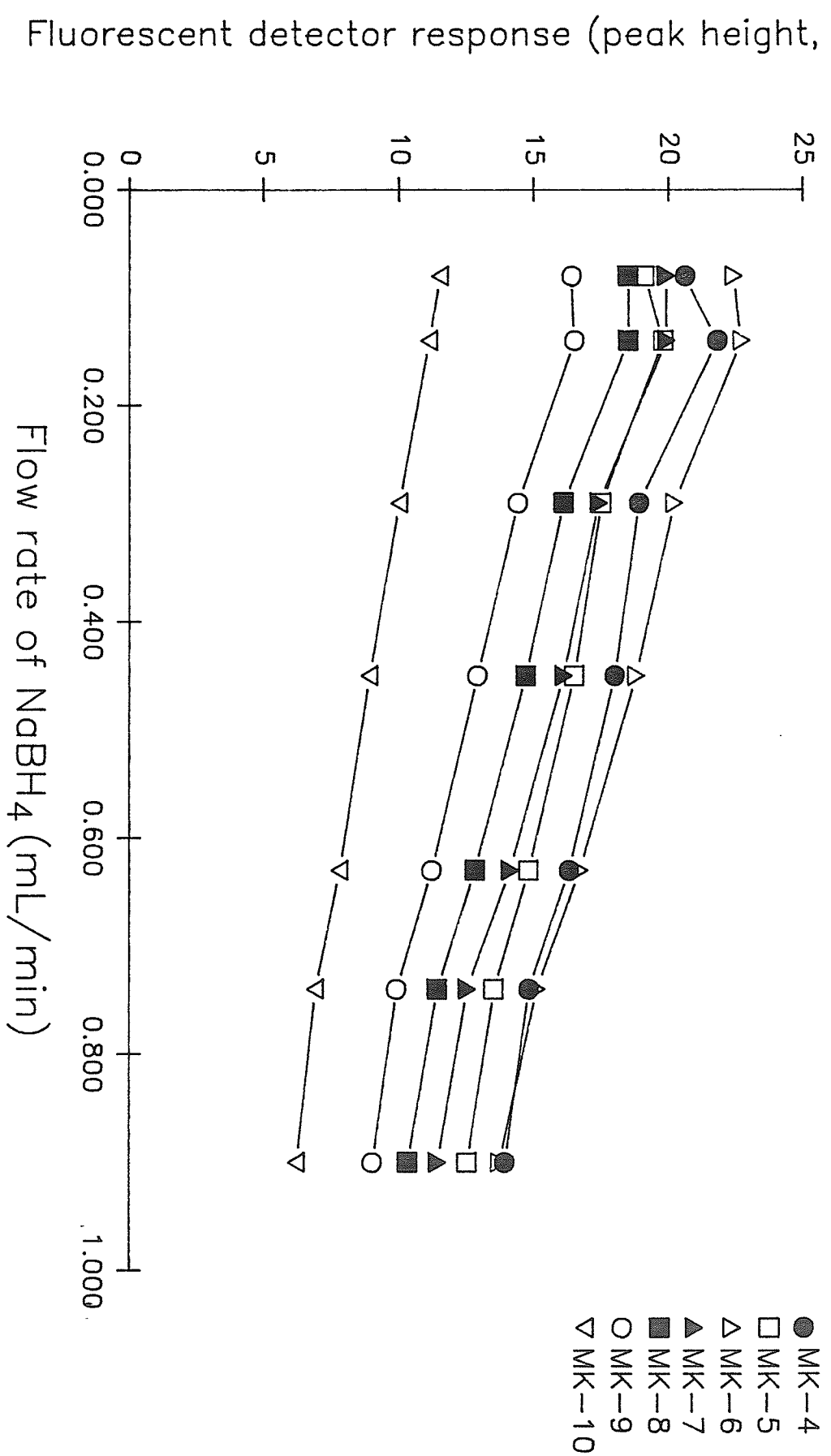


Figure 5.6 Fluorescent detector response for MK 4-10 as a function of flow rate of $NaBH_4$.

Having optimised the conditions for separation and detection response, the linearity of detector response was next determined. This was accomplished by injecting dilutions of a mixture of standard menaquinone preparation over the range of detector sensitivities and generating an on-scale response.

A plot of detector response against concentration for each component yielded a linear relationship (Figure 5.7-5.10) with coefficients of correlation of 0.99 in all cases (Table 5.14). Thus the detector response was essentially linear at all measurable concentrations.

The lowest concentration at which a clearly identifiable peak could be detected was 0.05 nmole for MK-4, MK-5 and MK-6, while it was approximately 0.1 nmole for MK-7, MK-8, MK-9 and MK-10.

When an extract of feces was chromatographed on reverse phase HPLC columns after Sep-Pak® clean up, all the menaquinone isoprenologues were well separated from each other and, with the exception of MK-8, from other contaminating material (Figure 5.4). Detection of MK-8 tended to be inconsistent in human feces (but not rat intestinal contents) due to the presence of fluorescing material eluting quite near it on the chromatogram, and in most cases completely masking or being incompletely resolved from it. It appeared that the contaminating compound were of similar polarity to the quinones, so manipulating elution conditions of either adsorption (Sep-Pak®) or reverse phase chromatography did not improve separation to any great extent.

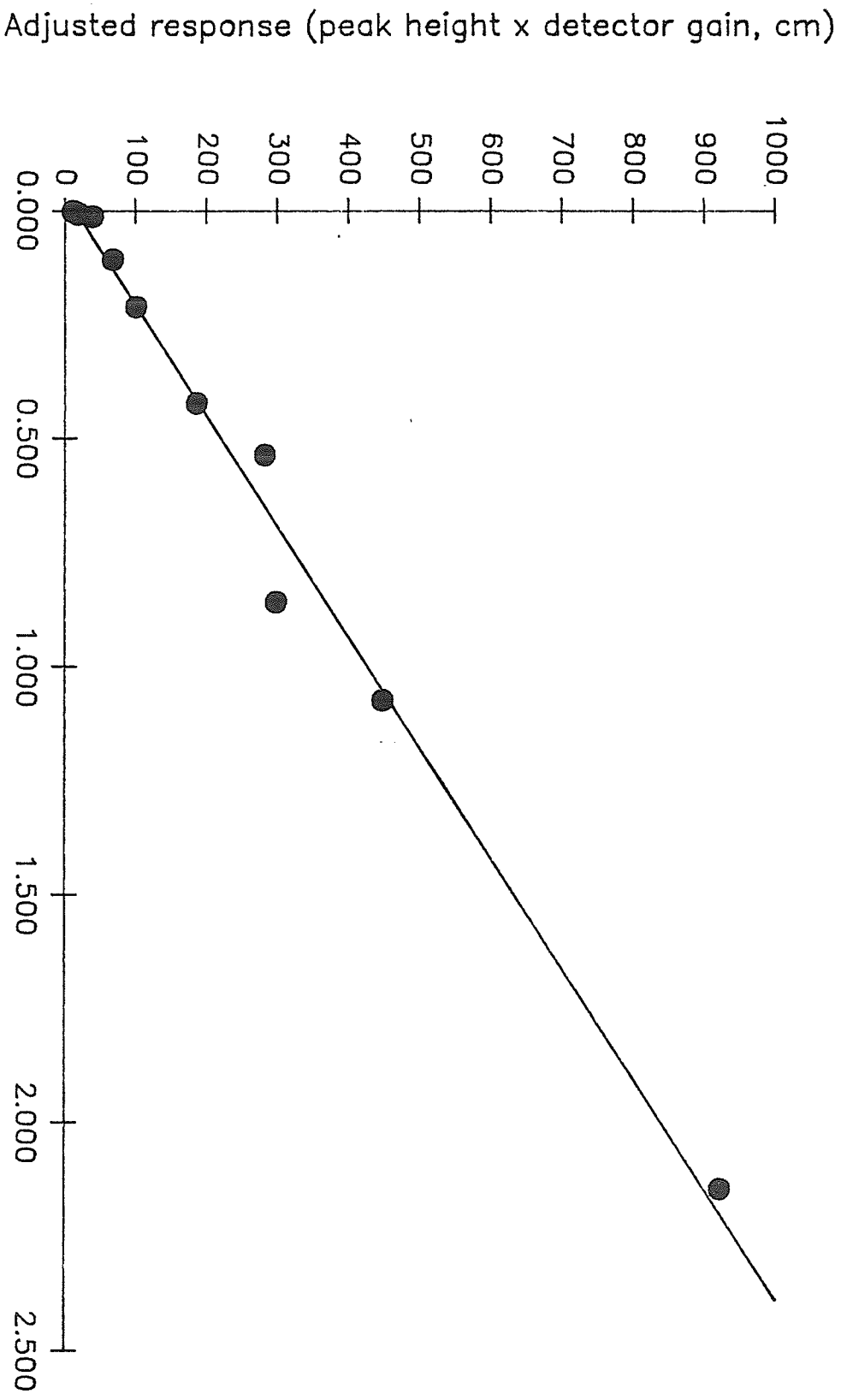


Figure 5.7 Fluorescent detector response as a function of vitamin K_1 concentration.

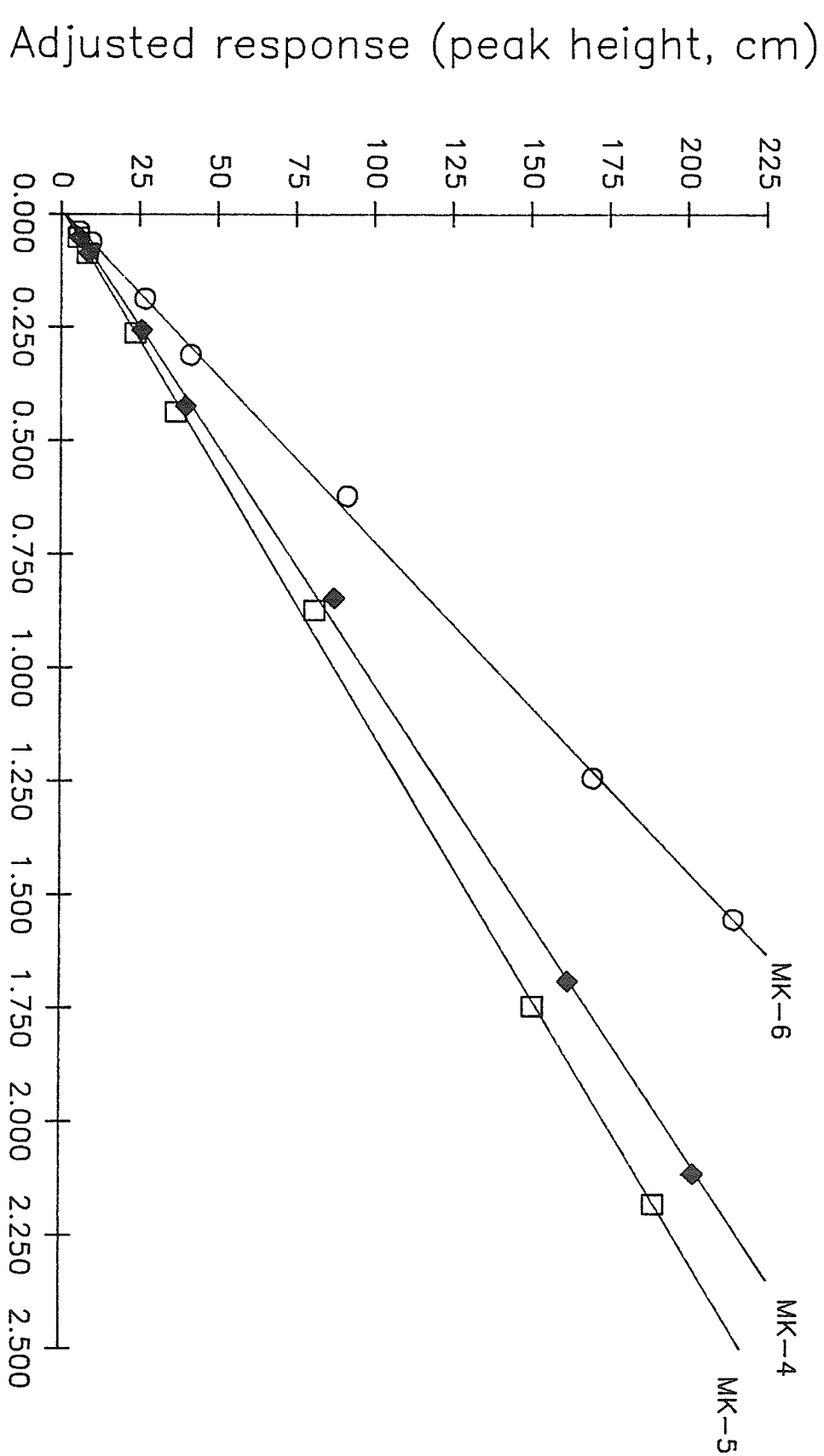


Figure 5.8 Fluorescent detector response as a function of MK-4, MK-5 and MK-6 concentrations.

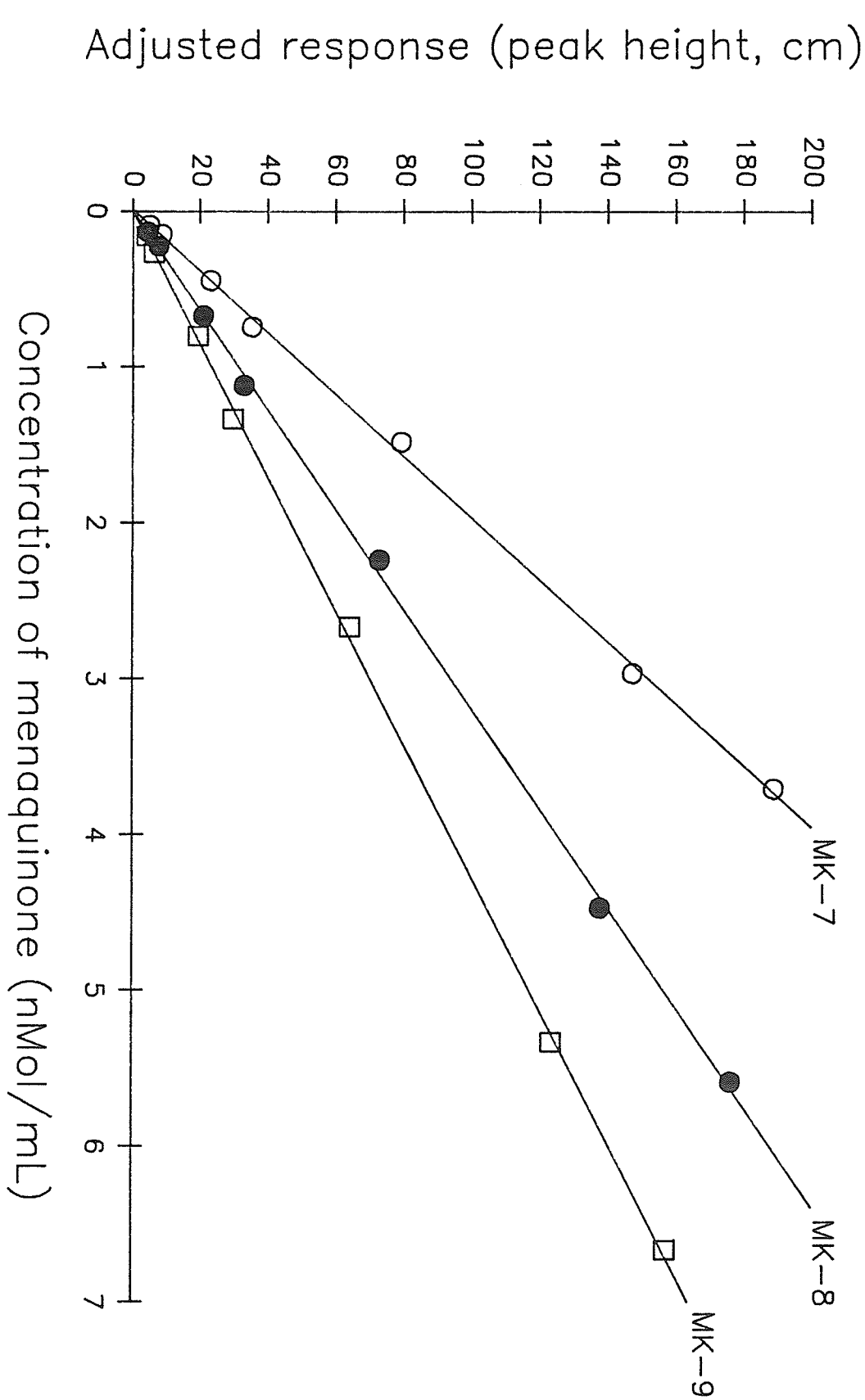


Figure 5.9 Fluorescent detector response as function of MK-7, MK-8 and MK-9 concentrations.

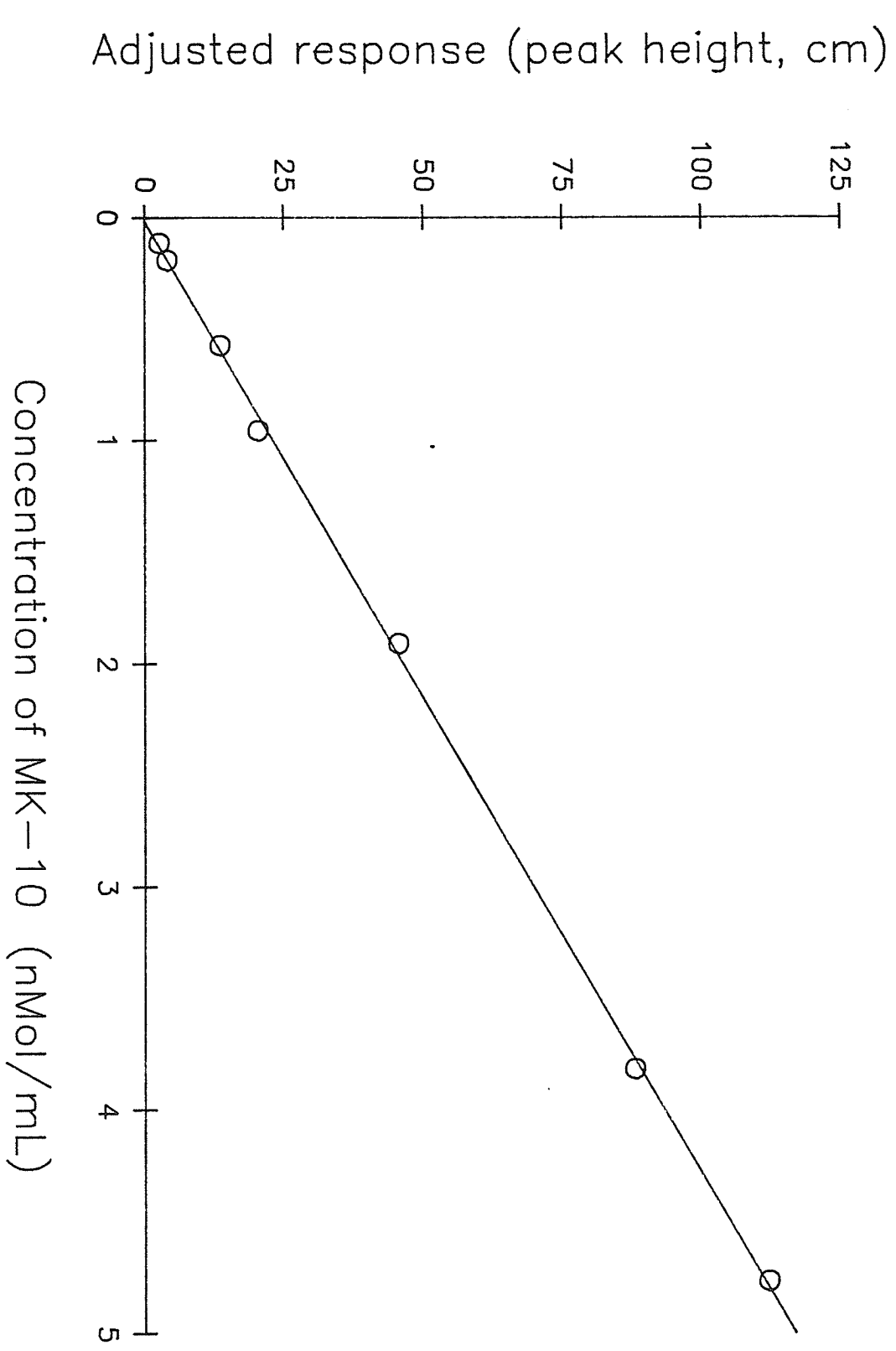


Figure 5.10 Fluorescent detector response as a function of MK-10 concentration.

Table 5.14 Ranges and Correlation Coefficients (r value) for Detector Response Curves of Vitamin K_1 and Menaquinones.

MK/K_1	Range (nmole)	r Value
K_1	5.362-0.002	0.9950
MK-4	2.113-0.052	0.9995
MK-5	2.180-0.052	0.9996
MK-6	1.552-0.038	0.9996
MK-7	3.704-0.090	0.9995
MK-8	5.587-0.134	0.9996
MK-9	6.665-0.159	0.9998
MK-10	4.765-0.115	0.9997

(iii) Recovery of Menaquinones and Vitamin K_1 From Feces

Since the assay of menaquinones and vitamin K_1 in feces did not involve the use of an internal standard, the efficiency of extraction or accuracy of the assay, and precision, were determined prior to analysis of samples.

The quantity of vitamin K_1 recoverable from feces by the HPLC assay was estimated as described previously.

As can be seen from Table 5.15, the recovery of menaquinones and vitamin K_1 added to feces, ranged from 70-95% for the different compounds. Recovery of each compound at different concentrations was similar in most cases, eg. at 3 different concentrations MK-9 was recovered at 78.7 ± 6.22%, 87.2 ± 3.6% and 81.8 ± 1.13% respectively. Recovery was generally on the order of 80% for all the menaquinones and vitamin K_1.

(iv) Precision of HPLC Assay of Feces for Menaquinones and Vitamin K_1

An estimate of the precision of the HPLC assay for menaquinones in feces was determined for a sample on the same day (n=4) and for the same sample on different days (n=5). This enabled the within run and between run precision for the assay to be calculated.

Table 5.16 demonstrates that the co-efficient of variation of each component measured in assay was similar for runs done on the same day and on different days. This was usually in the order of 10% with the exception of MK-4 which was much higher.

Table 5.15 Efficiency of extraction: Recovery of menaquinones added to normal feces

MK-n	nmole Added	nmole Recovered		% Recovery	
		Exp 1	Exp 2	Exp 1	Exp 2
K_1	0.80	0.72	0.70	89.7	87.0
	1.29	1.32	1.34	102.5	103.7
MK-4	0.43	0.37	0.37	85.9	85.4
	0.86	0.68	0.72	79.1	84.2
	1.69	1.52	1.55	90.1	92.0
MK-5	0.45	0.38	0.37	85.1	81.6
	0.88	0.73	0.75	83.0	85.2
	1.76	1.62	1.58	92.2	90.0
MK-6	0.31	0.26	0.25	82.9	81.2
	0.62	0.56	0.53	90.8	85.1
	1.24	1.10	1.12	88.6	90.5
MK-7	0.74	0.60	0.60	81.3	80.7
	1.85	1.74	1.57	93.8	85.1
	2.96	2.61	2.55	88.2	86.0
MK-8	1.12	0.87	0.88	78.1	78.6
	2.24	2.19	1.95	97.9	87.1
	4.47	3.92	3.80	87.8	85.0
MK-9	1.34	0.99	1.11	74.2	83.1
	3.04	2.76	2.60	90.8	85.4
	5.33	4.40	4.32	82.6	81.1
MK-10	0.95	0.67	0.63	70.0	66.3
	3.05	2.87	2.78	94.1	91.0
	3.81	3.19	2.92	83.8	76.7

Table 5.16 Menaquinone content of feces analysed on the same and different days

	Same Day		Different Days	
	nmole/g dry wt (n=4)		nmole/g dry wt (n=5)	
MK-n	mean ± S.D.	CV%	mean ± S.D.	CV%
K_1	6.200 ± 0.500	8.1	6.462 ± 0.316	4.9
MK-4	0.896 ± 0.266	29.7	1.552 ± 0.351	22.6
MK-6	1.836 ± 0.184	10.0	1.876 ± 0.201	11.9
MK-7	1.627 ± 0.008	5.2	1.818 ± 0.205	11.3
MK-9	1.404 ± 0.189	13.4	1.788 ± 0.195	10.9
MK-10	5.313 ± 0.288	5.4	5.714 ± 0.168	8.5

(v) Precision of the HPLC Assays for Serum

To determine within run and between run precision of the serum vitamin K_1 assay, 2.0 ml of pooled serum were spiked with 108.67 pmole of vitamin K_1 and, after adding the internal standard (MK-4), assayed by HPLC as described.

Given in Table 5.17 are mean and standard deviations for 5 measurements done on the same and different days for the same pool of serum. The coefficient of variation was about 10%.

Feces from human volunteers or neutropenic patients assayed for menaquinones by HPLC were usually determined in a single run with a few exceptions where duplicate runs were carried out. Duplicate runs were not usually possible because of the limited quantity of sample available.

Shown in Table 5.18 is the result of the analysis of a stool sample that was determined in duplicate. It is evident that the variation between runs for the assay was quite small.

2. Vitamin K Content of Human Feces

An examination of the vitamin K_1 content of human feces shows that there was no difference in the levels of normal subjects compared to those at study entry (Table 5.19). In this study, no attempt was made to monitor the vitamin K_1 intake of the subjects on antimicrobial therapy, hence their dietary vitamin K_1 status was uncertain. However, at day 7 all the groups with the exception of AT had lower levels of vitamin K_1 than at study entry. These ranged from 1.04 ± 0.04 nmole/g dry weight in the AC group to 2.07 ± 1.00 nmole/g dry weight in the MT group (Table 5.19). In a previous study in our

Table 5.17 Precision of the serum vitamin K_1 assay

	Mean ± S.D. (pmole/2 ml)	CV%
Same day (n=5)	107.26 ± 10.66	9.94%
Different days (n=5)	111.32 ± 12.34	11.08%

Table 5.18 Vitamin K content of a normal human feces assayed in duplicate by HPLC method.

MK-n/K_1	nmole/g dry wt	
	Exp 1	Exp 2
K_1	1.66	1.66
MK-4	1.00	0.85
MK-5	1.65	1.48
MK-6	3.59	3.62
MK-7	1.38	1.41
MK-9	6.08	5.97
MK-10	7.41	8.53

Table 5.19 Menaquinone content and B. fragilis group counts in stools of neutropenic patients on antibiotics (mean ± S.D.)

		nmole/g dry weight				
Group	n	K_1	MK 4-7	MK 9+10	MK TOTAL	B.fragilis $\log_{10}$ CFU/g dry wt
Normal Volunteers	29	3.62 ± 2.77	8.29 ± 5.63	9.07 ± 5.98	17.36 ± 8.99	9.4 ± 1.2
Study entry (day 0-1)	10	3.35 ± 2.29	6.68 ± 3.02	8.19 ± 3.68	14.86 ± 5.68	8.38 ± 1.25
AC (day 7-10)	5	1.04 ± 0.04	3.24 ± 2.26	8.66 ± 6.66	11.90 ± 7.72	4.18 ± 2.18
AT (day 7-10)	5	3.52 ± 1.28	2.35 ± 1.84	5.51 ± 3.22	7.86 ± 4.57	6.57 ± 2.26
TT (day 7-10)	5	1.78 ± 0.92	2.62 ± 1.65	4.64 ± 5.65	8.40 ± 6.29	6.82 ± 2.74
MT (day 7-10)	5	2.07 ± 1.00	1.21† ± 1.62	0.41* ± 0.75	1.61† ± 1.67	2.14† ± 3.13
TM (day 7-10)	5	1.51 ± 0.92	0.72† ± 1.11	0.06* ± 0.04	0.78† ± 1.10	0.68† ± 0.53

† Denotes significant difference from study entry values at $p \leq 0.0001$.
* Denotes significant difference from study entry values at $p \leq 0.001$.

institution (unpublished) it was observed that most patients had a reduced dietary intake of vitamin K_1, ranging from 10-30% of recommended daily intake (70-140 ug/day).

Analysis of feces from subjects on a regular diet demonstrated a total menaquinone concentration of 17.36 nmole/g dry wt (Table 5.19). These stools contained isoprenologues ranging from MK-4 to MK-12. However MK-11 and MK-12 were not quantitated because of the unavailability of pure standards. In these subjects, MK 9 + 10 made up 50% of the total menaquinones.

Neutropenic patients, at the time they were started on empiric parenteral antimicrobial therapy (day 0), had a slightly reduced total level of menaquinones (14.86 ± 5.68 nmole/g dry weight) compared to normal subjects. The reduction was not significant and was reflected both in reduced quantities of MK-9 and MK-10 as well as MK 4-7. The patients comprising the group at study entry were subsequently administered one of the different antimicrobial regimens. The study entry values from different groups were collapsed together since no differences were observed between groups at the onset of intravenous antibiotic treatment.

After seven to ten days on antimicrobial agents, there was a reduction in the menaquinone content of stools of patients on all the regimens. Only a slight reduction occurred in patients on AC. These patients had a total menaquinone concentration of 11.90 nmole/g dry weight, representing a reduction of 20% over study entry totals. This reduction was reflected in the decreases of MK-4, MK-5, MK-6 and MK-7. MK-9 and MK-10 remained the same. Patients on AT and TT had total menaquinone concentrations about half that of study entry

totals. This decrease was reflected in decreases in the complete spectrum of menaquinone isoprenologues. However the reduced totals found in these patients were not significantly different from study entry.

Patients on MT and TM showed marked reductions in total menaquinones. In both cases these reductions were statistically significant. Total menaquinones were in the order of 1 nmole/g dry weight which represented a 90-95% decrease. MK 9 + 10 decreased by 95% in the MT group and by 99% in the TM group. The decreases in MK 9 + 10 were also significant compared to study entry (day 0) values (Table 5.19, Figure 5.11).

The change in menaquinone content paralleled the change in B. fragilis group levels (Table 5.19). Subjects on a normal diet contained 9.4 ± 1.2 $\log_{10}$ CFU/g dry weight and this was slightly reduced to 8.4 ± 1.3 $\log_{10}$ CFU in study entry specimens. Stools of patients on MT and TM contained 2.1 ± 3.1 and 0.7 ± 0.5 $\log_{10}$ CFU/g dry weight B. fragilis group organisms, respectively. These levels were significantly lower from study entry levels. Patients in AC, AT and TT had 4.2 ± 2.2, 6.6 ± 2.3 and 6.8 ± 2.7 $\log_{10}$ CFU/g dry weight B. fragilis group organisms,respectively. It should be pointed out that even though stools of the AC group had a higher MK-9 + 10 level (8.7 ± 6.7 nmole/g dry weight) compared to AT and TT, the B. fragilis group counts were actually lower. This discrepancy may have been due to differences in metabolic activity between organisms in different groups or more dead organisms with extracted menaquinone in the AC group compared to the other two. In-vitro synergy between aztreonam and cloxacillin was observed for B. fragilis organisms possessing the beta-lactamase enzyme.

Figure 5.11 Menaquinone content in stools of neutropenic patients on antimicrobials (mean ± S.D.)
Data from table 5.19 reproduced as graph. See Table 5.19 for statistical comparison.

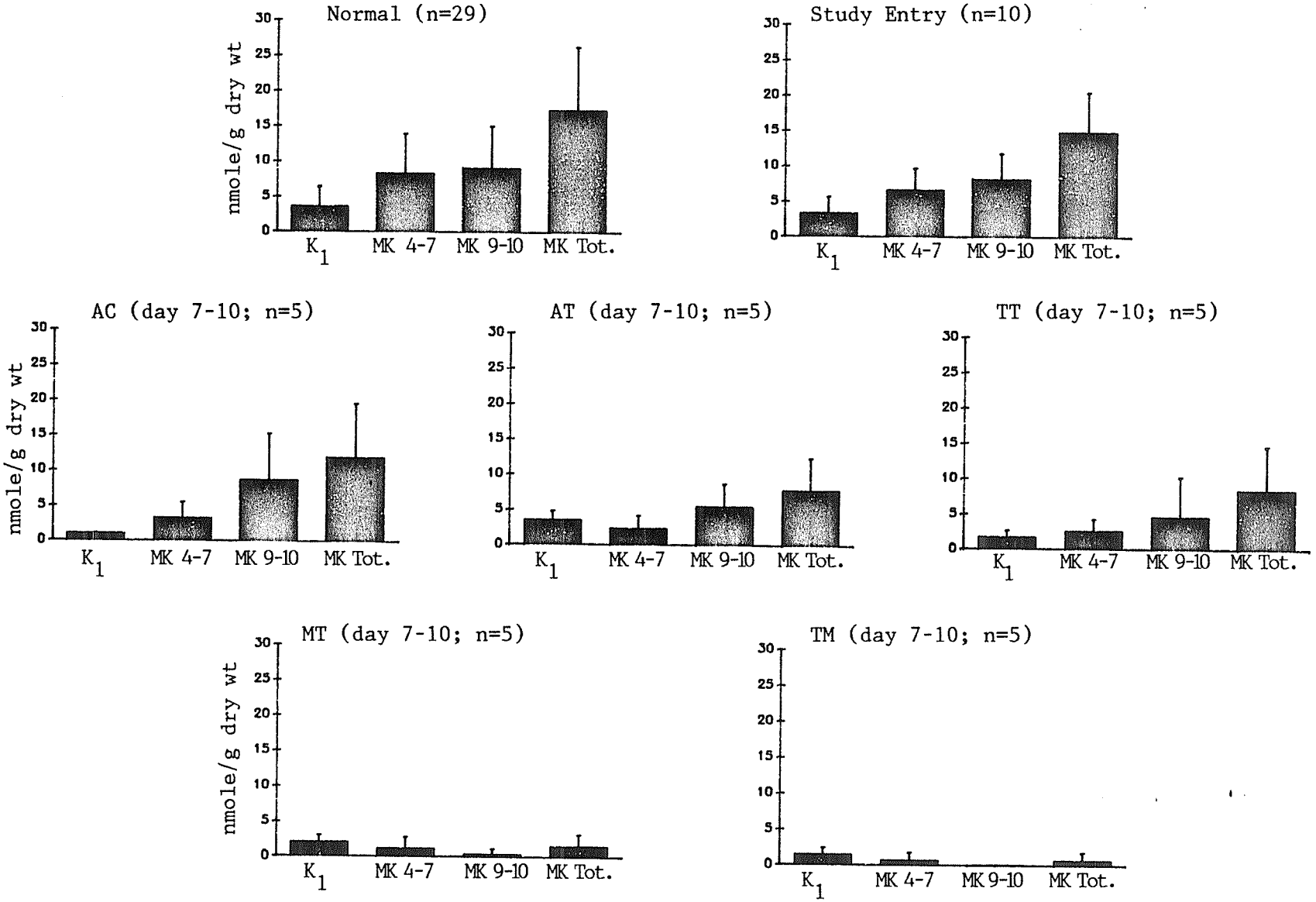


Table 5.20 demonstrates that there is a corresponding decrease of menaquinone content in conjunction with decreases in B. fragilis group counts. This implies that the MK 9 + 10 content of human feces is contributed solely by these organisms.

The incidence of hypoprothrombinemia (defined as an increase in prothrombin time 3 seconds beyond control) was highest in patients in MT (30%) and TM (19%) (Table 5.21). These groups were the ones that had the lowest level of menaquinones and B. fragilis group organisms in the stool. Thus it appears, the suppression of B. fragilis group organisms which are producers of MK-9 and MK-10 is correlated with the reduction of these menaquinone levels and the occurrence of hypoprothrombinemia in the clinical setting.

C. Hypoprothrombinemia in Healthy Human Volunteers on Ciprofloxacin, Co-trimoxazole or Placebo

Eradication of the menaquinone producing microflora and reduction of fecal menaquinone levels in neutropenic patients on broad spectrum antimicrobials was shown to be correlated with hypoprothrombinemia. However, there is some controversy as to the mechanism by which antimicrobial agents produce hypoprothrombinemia. There are thought to be two possible mechanisms: (1) eradication of the menaquinone producing organisms, removes the endogenous source of vitamin K_2 in the face of low dietary vitamin K_1 intake, thus making the animal both vitamin K and menaquinone deficient. This mechanism presupposes that menaquinones produced by bacteria in the gut are available for absorption, (2) N-methylthiotetrazole (NMTT) leaving group of some cephalosporin antibiotics inhibits the carboxylation reaction during

Table 5.20 Reduction of MK-9 + 10 concentrations with B.fragilis group counts

Group	B. fragilis group Mean Log_{10} CFU/g dry weight	% MK 9 + 10
Normal volunteers (n=29)	9.4	100*
Study entry (n=10)	8.4	90.3
AC (n=5)	4.2	95.5
AT (n=5)	6.6	60.7
TT (n=5)	6.8	51.2
MT (n=5)	2.1	4.5
TM (n=5)	0.7	0.7

* Concentration of MK 9 + 10 in the stool of normal volunteers was taken as 100%

Table 5.21 Incidence of hypoprothrombinemia (HPT) in neutropenic patients on antibiotics

Regimen	No of Pts. with elevated PT/Total No. of Pts.	%
Tobramycin/Ticarcillin	5/54	9
Moxalactam/Ticarcillin	16/54	30
Aztreonam/Tobramycin	2/24	8
Aztreonam/Cloxacillin	2/25	8
Tobramycin/Moxalactam	5/26	19

Hypoprothrombinemia is defined as:

Prothrombin time $\geq$ 3 seconds beyond control.

the final stages in the production of active vitamin K dependent coagulation factors.

To test the importance of the menaquinone produced by intestinal microflora in maintaining normal coagulation and attempt to determine the mechanism whereby antibiotic associated hypoprothrombinemia is produced, healthy human volunteers were administered an antimicrobial agent lacking the NMTT leaving group and which had some in-vitro activity against B. fragilis group organisms. These volunteers were simultaneously placed on a vitamin K_1 deficient diet.

The objective was to (1) examine the effect of the drug on the major menaquinone producing fecal flora and (2) to examine the relationship between the change in flora, menaquinone levels and hypoprothrombinemia in volunteers being deprived of dietary vitamin K_1.

(i) Subjects

The study group consisted of thirty male volunteers between the ages of 18-40 years, in good health by physical examination, history and lab testing (Table 5.22), who had taken no antibiotics or regular medication 28 days previously. The laboratory tests to confirm health were as follows:

(a) hemogram including hemoglobin, red cell morphology and indices, (b) white cell differential and platelet counts, (c) serum creatinine, (d) 24 hour creatinine clearance and protein excretion, (e) serum bilirubin, lactic acid dehydrogenase, alkaline phosphatase and glutamic/oxalic acid transferase concentration, and (f) urine for glucose, protein and sedimentation abnormalities.

Table 5.22 Demographic features of volunteers on ciprofloxacin, co-trimoxazole or placebo

Placebo Data, Mean ± SD	Group (n=5 per group)					
	Vitamin K_1 deficient diet					Normal diet
	Cipro	Cipro 100 mg	Cipro 250 mg	Cipro 500 mg	TMP/SMX	500 mg
age (yr)	24 ± 5	31 ± 6	30 ± 4	29 ± 8	33 ± 7	33 ± 7
weight (kg)	72.6 ± 9.6	75.6 ± 6.1	67.9 ± 5.4	72.1 ± 8.5	75.8 ± 10.1	76.6 + 8.7
creatinine clearance ($ml.min^{-1}$/ 1.73 m^2)	107 ± 6	133 ± 36	112 ± 11	128 ± 40	113 ± 36	127 ± 23

(ii) Diet:

Twenty five individuals were placed on vitamin K_1 deficient diet which included cooked white rice, egg albumin, jello, black coffee and Coca Cola® (O'Reilly, 1971) for 7 days. Five individuals were on their regular diet.

(iii) Antibiotic Regimens:

(1) ciprofloxacin (Miles Laboratories, Rexdale, Ontario) at doses of 100 mg, 250 mg and 500 mg respectively.

(2) trimethoprim/sulfamethoxazole (TMP/SMX), 160/800 mg (Bactrim®) (Hoffman La Roche, Toronto, Ontario).

(3) placebo tablet indistinguishable from the 100 mg dose of ciprofloxacin tablet.

(iv) Experimental Design:

Twenty five subjects on the vitamin K_1 deficient diet were randomly allocated into groups of 5 by a computer generated list of random numbers.

They received medication at 12 hour intervals for 13 doses as follows:

(a) placebo

(b) ciprofloxacin at 100 mg (C-100)

(c) ciprofloxacin at 250 mg (C-250)

(d) ciprofloxacin at 500 mg (C-500[a])

(e) TMP/SMX

Another five subjects were on their regular diet and received ciprofloxacin at 500 mg (C-500b) as described above.

(v) Sample collection

Individuals fasted 8 hours before (12 midnight to 8 am of day 0) study entry. At study entry, before the first dose of drug was administered, the following samples were collected and frozen at -70°C until analysed: (a) feces for quantitative culture and menaquinone analysis, (b) blood for serum vitamin K_1 levels. (c) blood for PT determinations which were done immediately. (d) pharmacokinetic profiles for C-100, C-250, C-500 were determined after the 1st and 13th doses.

Prior to ingestion of the final (13th) dose on day 7, similar samples were again collected.

From an examination of the demographic features of the volunteers (Table 5.22.), it can be seen that there were no differences in groups receiving ciprofloxacin, placebo or TMP/SMX.

Serum vitamin K_1 concentrations were determined on 90 serum samples from volunteers enrolled in the study. It was felt that by monitoring the serum vitamin K_1 levels at intervals during the study, inferences could be made regarding the vitamin K_1 status of the subjects. Of the 90 samples examined, 73 were obtained after an overnight fast at day 0, 4 and 7, and 17 randomly selected mid-day (1000-1600 h) from volunteers ingesting both the vitamin K_1 deficient or regular diet. The mid-day samples were analysed and compared to fasting values for those subjects to determine the effect of the diets on serum vitamin K_1 concentration. As well, another 14 samples obtained from healthy individuals not enrolled in the study and who had ingested their regular meal were analysed to determine non-fasting levels. The results of serum vitamin K_1 analysis are presented in

Figure 5.12 Serum vitamin K_1 concentrations in non-fasting individuals on a normal diet ( ) and fasting subjects on a vitamin K_1 deficient diet ( ).

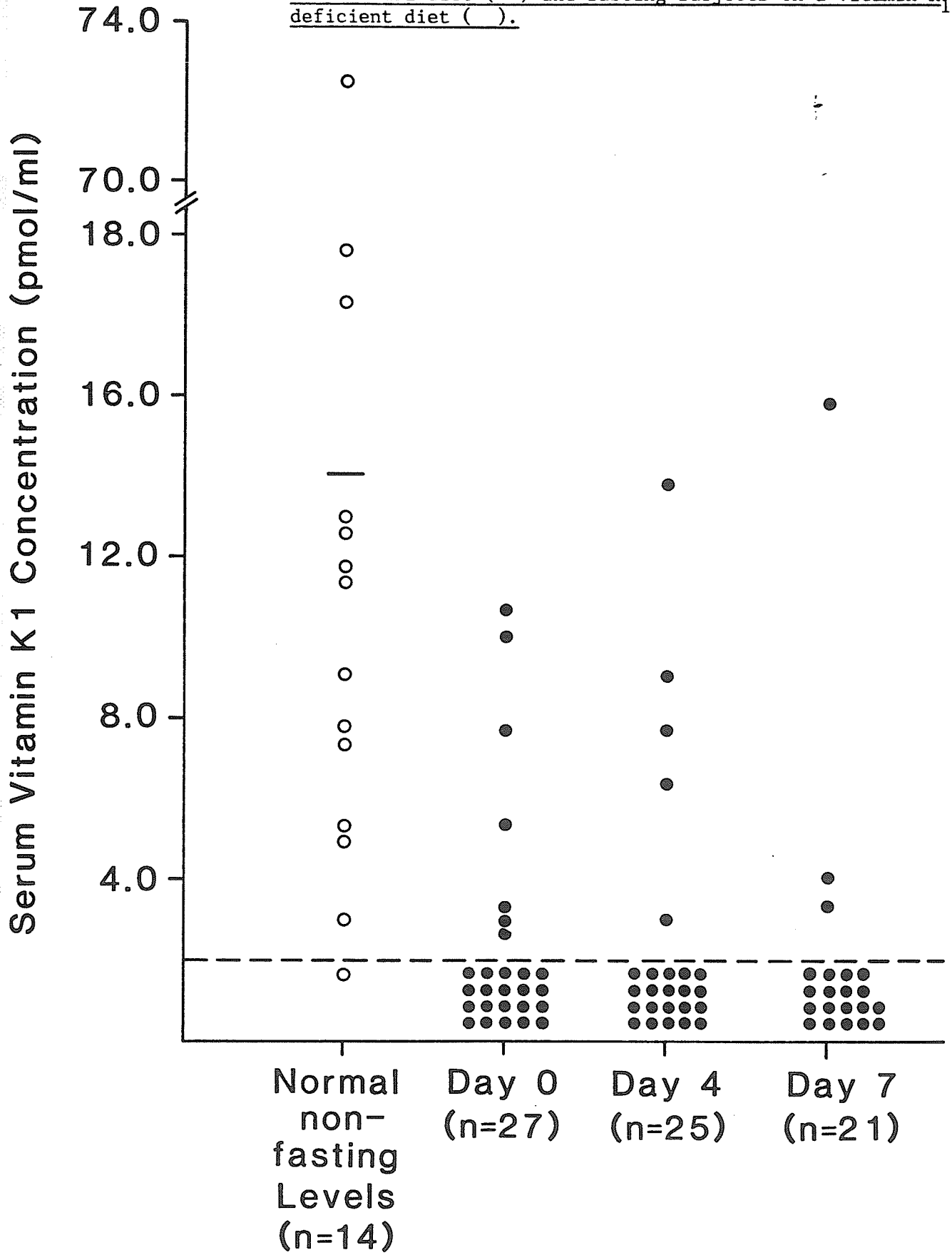


Figure 5.12. In these subjects, the serum vitamin K_1 level was 14.07 ± 17.67 pmole/ml with a range of <1-72.48 pmole/ml. The level of detection for phylloquinone in serum was 2 pmole/ml.

An assessment of vitamin K_1 in serum obtained from volunteers at day 0, 4 and 7 after an overnight fast showed that 80-85% of samples contained levels below the level of detection (Figure 5.12). In samples where vitamin K_1 was detected, the value ranged from 1-16 pmole/ml. There also appeared to be no differences between fasting levels of serum vitamin K_1 between groups on the vitamin K_1 deficient diet and the group on their regular diet. There were 17 serum samples obtained at mid-day from volunteers in the study. Eight of these were from subjects on the vitamin K_1 deficient diet, and of these, 6 had undetectable vitamin K_1 levels. The remaining 9 samples were from subjects on their regular diet. Of these, one subject showed a rise in serum vitamin K_1 level over fasting levels - from undetectable to 6.73 pmole/ml.

Quantitative cultures for fecal levels of B. fragilis group organisms at day 0 demonstrated that all groups contained similar counts in the order of 9 $\log_{10}$ CFU/g dry weight (Table 5.23). After 7 days of ciprofloxacin treatment, the only regimens that produced significant decreases in counts were ciprofloxacin at the 250 mg and 500 mg doses in subjects on the vitamin K_1 deficient diet. The reductions in counts were by means of 3.2 and 2.7 $\log_{10}$ CFU/g dry weight, respectively. In all the other groups, B. fragilis organisms were preserved at the same levels as study entry. It should be noted that whereas, 500 mg ciprofloxacin reduced B. fragilis counts by approximately 3 $\log_{10}$ CFU in subjects on a vitamin K_1 deficient diet, the

Table 5.23 Concentrations of B. fragilis group organisms in feces of normal volunteers before and after antimicrobial treatment

Group (n=5/group)	Log_{10} CFU/gram dry weight feces ± S.D. Study entry	Day 7	Change in mean Log_{10} CFU
Placebo & diet	9.3 ± 1.5	8.4 ± 1.5	-0.9
C-100 & diet	9.4 ± 1.3	8.5 ± 0.8	-0.8
C-250 & diet	9.8 ± 0.8*	6.5 ± 2.4*	-3.2
C-500 & diet	9.7 ± 1.6†	6.9 ± 2.8†	-2.7
TMP/SMX & diet	9.2 ± 1.8	9.1 ± 0.5	-0.1
C-500 & normal diet	8.9 ± 0.6	8.0 ± 1.1	-0.9

* $P < .001$ compared to study entry.

† $P \leq 0.05$ compared to study entry.

same dosage had no effect on subjects on their regular diet (Table 5.23).

Vitamin K_1 and menaquinone levels of fecal samples were measured to determine the effect of diet and antimicrobial administration on the vitamin K reservoir in the intestine. Vitamin K_1 levels were determined for 29 samples at day 0 and 7 (Table 5.24). Taken as a group at day 0, the 29 samples had a mean vitamin K_1 level of 3.62 ± 2.77 nmole/g dry weight (Table 5.25). By group, vitamin K_1 levels ranged from 1.44 ± 0.53 nmole/g dry wt (C-500 with regular diet) to 6.46 ± 3.32 nmole/g dry weight (C-100) (Table 5.24).

For all groups at day 7, fecal vitamin K_1 levels were reduced, when compared to day 0 levels in each group and reduction ranged from slight in TMP/SMX, C-500 and C-500 with regular diet groups (6-17%), to nearly 70-85% in the other three groups. Thus, the vitamin K_1 deficient diet appeared to reduce fecal concentration of the vitamin but reduction was also observed in volunteers on a normal diet (Table 5.24, Figure 5.13).

Menaquinone analysis was also performed on 29 paired (day 0 and 7) specimens. MK 4-7 and MK 9 + 10 were quantitated and grouped, the latter representing the isoprenologues produced primarily by Bacteroides fragilis group organisms. MK-8, produced by gram-negative facultative organisms such as E. coli, could not be consistently detected because of the presence of an interfering peak co-chromatographing with it. As a result MK-8 was not quantitated. MK-11 (27), MK-12 (10) and MK-13 (7) were also detected in a number of fecal samples (indicated by the number in parenthesis) but not quantitated because of unavailability of standards.

Table 5.24 Fecal vitamin K content at day 0 and day 7 in human volunteers on placebo, TMP/SMX or ciprofloxacin at different doses

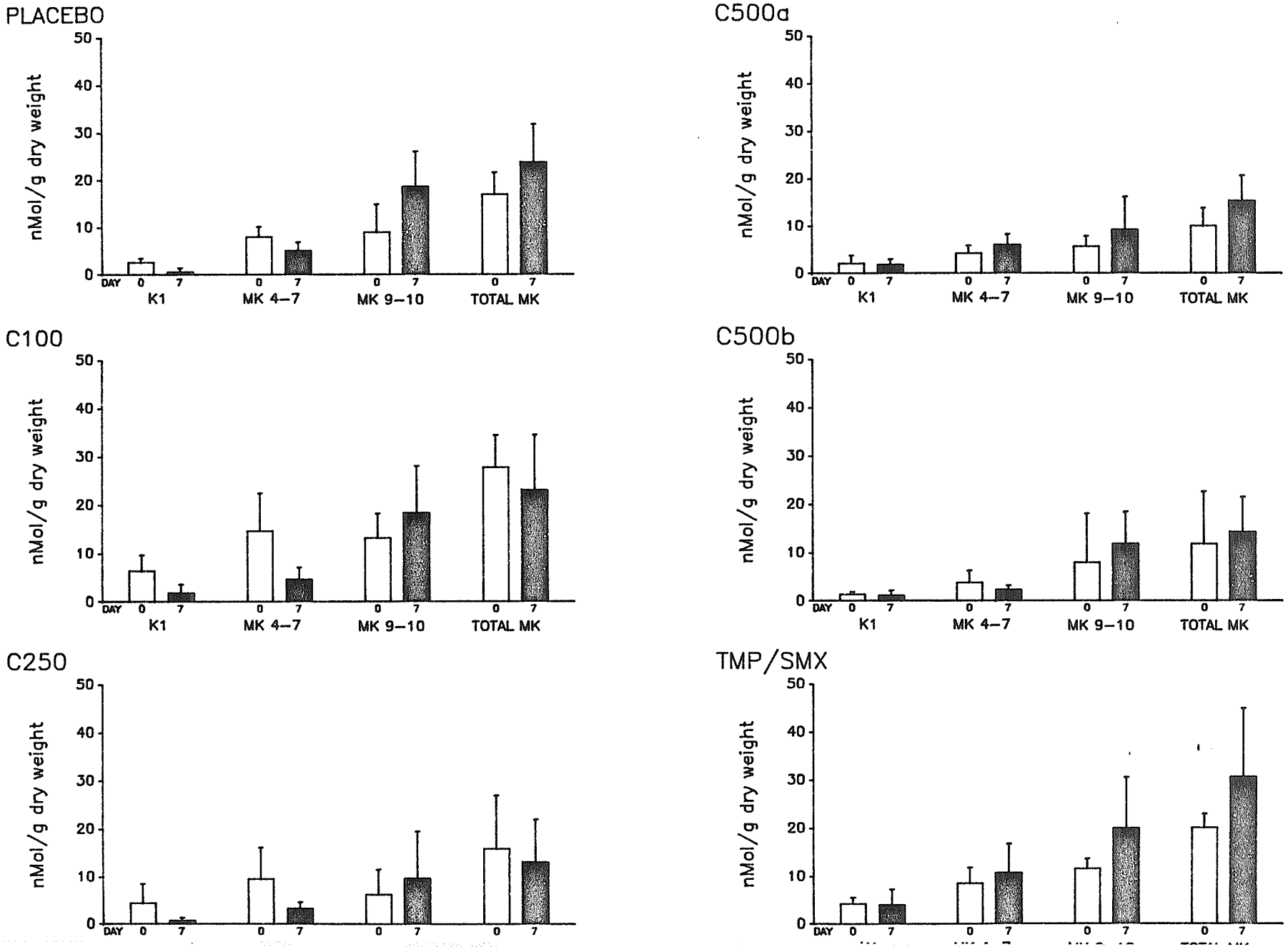
Group	nmole/g dry weight (mean ± S.D.)							
	K_1		MK 4-7		MK 9-10		Total MK	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
Placebo (n=5)	2.63 ± 0.90	0.64 ± 0.85	8.03 ± 2.11	5.14 ± 1.83	8.98 ± 5.98	18.62 ± 7.40	17.01 ± 4.61	23.75 ± 8.05
C_{100} (n=5)	6.46 ± 3.32	1.93 ± 1.79	14.64 ± 7.85	4.71 ± 2.50	13.18 ± 5.09	18.40 ± 9.72	27.84 ± 6.67	23.12 ± 11.46
C_{250} (n=5)	4.44 ± 4.10	0.75 ± 0.68	9.54 ± 6.58	3.30 ± 1.38	6.24 ± 5.28	9.66 ± 9.80	15.77 ± 11.13	12.96 ± 8.94
C_{500}(a) (n=4)	2.16 ± 1.71	1.91 ± 1.17	4.30 ± 1.64	6.09 ± 2.23	5.75 ± 2.19	9.25 ± 6.97	10.04 ± 3.79	15.34 ± 5.30
C_{500}(b) (n=5)	1.44 ± 0.53	1.19 ± 1.05	3.85 ± 2.50	2.39 ± 0.92	8.01 ± 10.08	11.86 ± 6.63	11.86 ± 10.75	14.26 ± 7.25
TMP/SMX (n=5)	4.28 ± 1.33	4.05 ± 3.25	8.57 ± 3.27	10.72 ± 6.10	11.60 ± 2.08	20.04 ± 10.69	20.18 ± 2.86	30.76 ± 14.24

Table 5.25 Fecal vitamin K_1 and menaquinone content for subjects on placebo, TMP/SMX and ciprofloxacin at day 0 and day 7

nmole/g dry weight (mean ± S.D.)

	Day 0	Day 7					
	(n=29)	n=5	n=5	n=5	n=4	n=5	n=5
		Placebo	C_{100}	C_{250}	C_{500a}	C_{500b}	TMP/SMX
K_1	3.62 ± 2.77	0.64 ± 0.85	1.93 ± 1.79	0.75 ± 0.68	1.91 ± 1.17	1.19 ± 1.05	4.05 ± 3.25
MK 4-7	8.29 ± 5.63	5.14 ± 1.83	4.71 ± 2.50	3.30 ± 1.38	6.09 ± 2.23	2.39 ± 0.92	10.72 ± 6.10
MK-9	2.94 ± 1.92	3.95 ± 1.56	3.76 ± 2.21	1.28 ± 0.71	1.72 ± 1.05	1.67 ± 0.78	6.84 ± 3.06
MK-10	6.13 ± 4.29	14.66 ± 6.25	14.65 ± 7.51	8.39 ± 9.19	7.53 ± 6.28	10.19 ± 5.94	13.20 ± 7.78
MK 9 + 10	9.07 ± 5.98	18.62 ± 7.40	18.40 ± 9.72	9.66 ± 9.80	9.25 ± 6.97	11.86 ± 6.63	20.04 ± 10.69
Total MK	17.36 ± 8.99	23.75 ± 8.05	23.12 ± 11.46	12.96 ± 8.94	15.34 ± 5.30	14.26 ± 7.25	30.76 + 14.24

Figure 5.13 Vitamin K content of stools (mean ± S.D.) at day 0 and day 7 from human volunteers on placebo, TMP/SMX and ciprofloxacin. These data are presented in numerical fashion on table 5.24. See text for discussion of data.



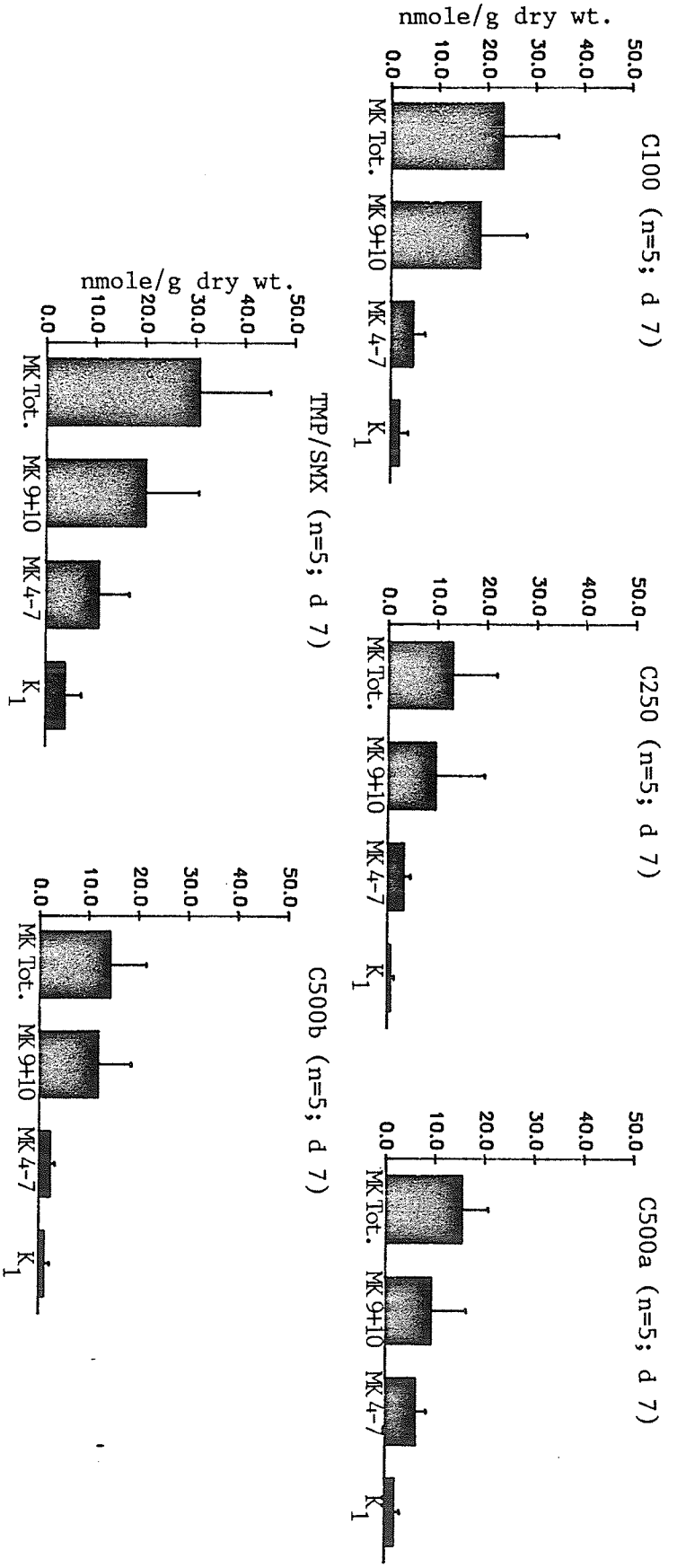
When the paired samples were examined by group, changes in menaquinone levels that were statistically significant could not be discerned. This was due to small sample numbers and large variances in the data (Table 5.24, Figure 5.13).

At study entry (day 0) combining the data from all 29 individuals into a single group revealed that MK 4-7 constitutes 50% with MK-9 and 10 the other 50% of total menaquinone. At day 7, because of the increase in total menaquinone in all groups and the decrease in MK 4-7, the latter then constitutes only 16% (C-500 with regular diet) to 40% (C-500) of total menaquinone. Thus there appears to be a shift towards production of longer chain menaquinones in the gut by bacteria after diet and drug treatment (Table 5.25, Figure 5.14). Comparison of total menaquinone levels at day 7 to day 0 showed slight reduction in the C-250, C-500[a], and C-500[b] groups and increases in the others.

When day 7 values of MK 9 + 10 for each group, are compared to the mean for all 29 subjects at day 0, taken together (Table 5.25, Figure 5.14), it is readily apparent that MK 9 + 10 has increased by approximately 50% in placebo, TMP/SMX and low dose ciprofloxacin (C-100). These were the groups where there was no reduction in B. fragilis group organisms (Table 5.23). In the groups when there were a 3 $\log_{10}$ CFU reduction (C-250, C-500), MK 9 + 10 concentrations virtually remained the same at 9.66 ± 9.80 and 9.25 ± 6.97 nmole/g dry weight respectively, compared to day 0 at 9.07 ± 5.98 nmole/g dry weight. The lack of increase of menaquinone in the C-250 and C-500 groups likely reflects drug suppression.

A correlation between counts of B. fragilis group and MK 9 + 10 levels in stool at day 7 in subjects on the vitamin K_1 deficient diet

Figure 5.14 Vitamin K content of stools (mean ± S.D.) of volunteers on placebo, TMP/SMX and ciprofloxacin at day 7.



was observed ($r=0.47$, $p< 0.025$) (Figure 5.15).

Of the 30 subjects in the study, abnormal prothrombin times were observed in 4, all of whom were ingesting the vitamin K-deficient diet. One subject each was observed in the C-500 and TMP/SMX groups, and two in the C-250 group (Table 5.26). Prothrombin time was considered abnormal when it was >2.0 seconds beyond control values (a value considered to be >95% outside of normal limits). In all the other volunteers prothrombin times remained within normal limits.

Table 5.26 summarises data on prothrombin times, quantitative culture, serum vitamin K_1 levels and intestinal vitamin K levels.

Increase in PT ranged from 2.0 to 3.5 seconds after 7 days. E. coli was suppressed, as were Bacteroides spp., but to variable extents. Serum vitamin K_1 levels at day 7 were undetectable in all cases. However, changes in fecal menaquinone levels were variable. In 3 subjects there was actually an increase in MK 9 and 10 at day 7 over day 0, reflecting the effect of the rice diet. Only in one subject (#21) in the C-500 group was there an apparently clear correlation between increase in PT, reduction of menaquinone-producing flora, and reduction in fecal menaquinones. Thus there was no clear cut correlation between suppression of menaquinone-producing flora, reduction in menaquinone content and hypoprothrombinemia in this study, since the unforseen effect of diet on menaquinone concentrations confounded results. Furthermore, ciprofloxacin did not have sufficient suppressive activity against gut anaerobes, especially menaquinone producing ones to produce the desired effect of reducing the endogenous menaquinone pool to low enough levels.

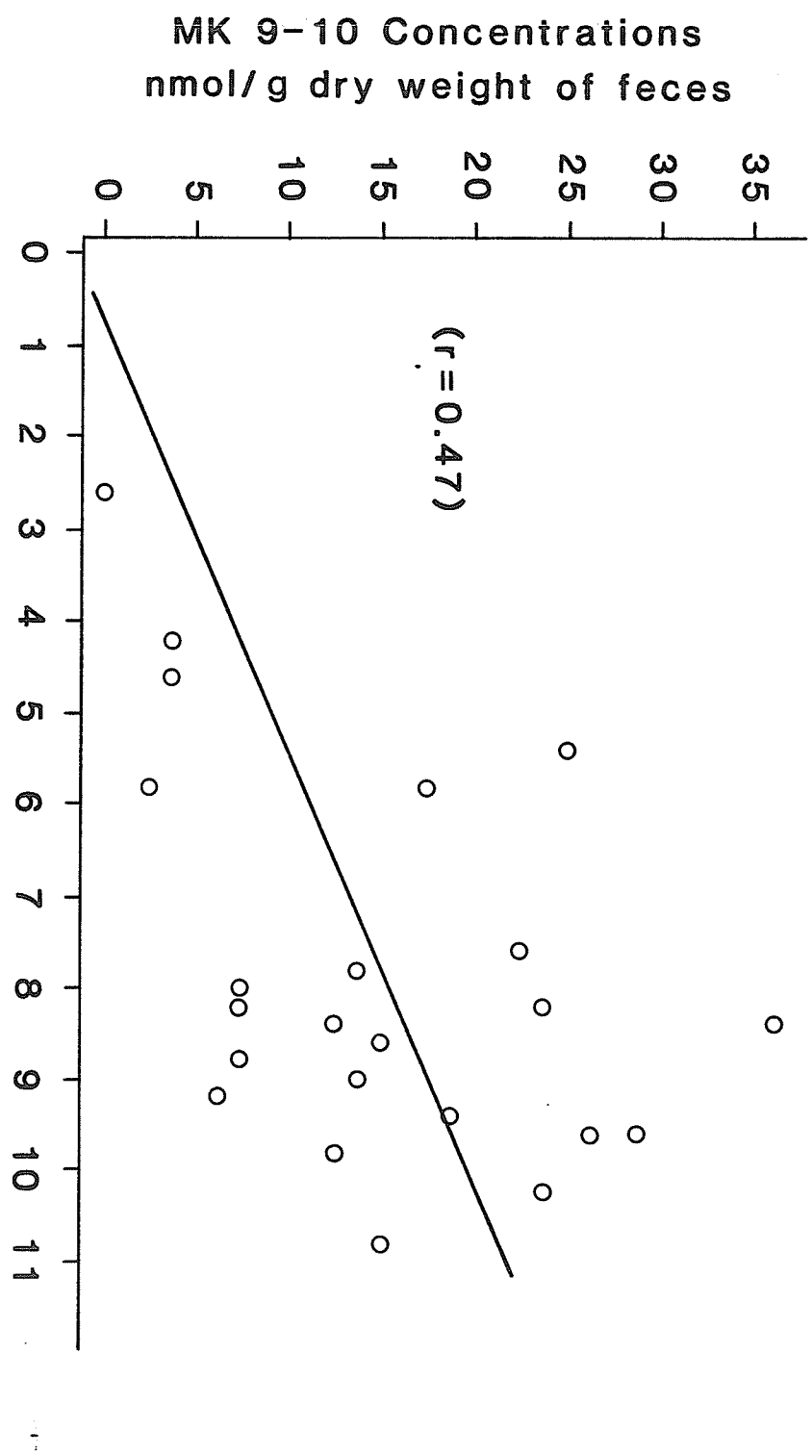


Figure 5.15 Correlation of MK 9+10 concentrations with B. fragilis group counts in feces at day 7 from subjects on a vitamin K_1 deficient diet.

Table 5.26 Correlation of prothrombin abnormalities, changes in bacterial counts, and menaquinone profile in volunteers ingesting a vitamin K_1 deficient diet. Data are expressed as day 0/day 7.

	Group (Volunteer #)			
	C-250 (#5)	C-250 (#18)	C-500 (#21)	TMP/SMX (#9)
Increase in PT(s) over control @ day 7	2.5	2.0	2.5	3.5
Log_{10} CFU/g dry weight feces				
E. coli	7.8/0	8.5/0	4.5/0	4.1/0
B. fragilis group	10.4/9.6	9.7/4.5	7.5/0	10.7/8.4
Bacteroides spp	10.3/7.5	4.7/0	4.4/0	9.8/4.3
Fasting serum vitamin K_1 concentrations (pmole/ml)				
Day 0	<1.00	10.62	5.33	<1.00
Day 4	<1.00	NT*	6.31	<1.00
Day 7	<1.00	<1	<1.00	<1.00
Vitamin K_1 & menaquinone content (nmole/g dry weight feces)				
K_1	0.69/ND†	2.75/0.27	4.71/3.33	4.36/8.56
MK 4-7	3.65/1.84	10.07/3.16	5.32/9.19	3.95/12.36
MK 9-10	2.04/25.79	2.53/3.88	6.85/ND	14.87/35.66
Total MK	5.69/27.63	12.60/7.05	12.17/9.19	18.82/48.02

† ND = not detected, $\leq$0.05 nmole/g

* NT = not tested

D. Use of the Rat Model to Study Hypoprothrombinemia

The failure to show a clear association between hypoprothrombinemia and decreased menaquinone levels in human volunteers led to the development of an animal model using rats. The model was used (1) to determine the effect of vitamin K_1 deficient and sufficient diets on menaquinone producing flora in the intestines, menaquinone and K_1 stores in the intestines and liver, and on prothrombin times, (2) to determine the effect of a vitamin K_1 deficient diet and antibiotics capable of reducing the menaquinone producing flora in the intestines on the same parameters above, and (3) to produce hypoprothrombinemia in rats using a vitamin K_1 deficient diet and an antimicrobial agent capable of removing the intestinal menaquinone producing flora, then reversing the coagulation abnormality by recolonization or feeding the animals killed menaquinone producing bacteria. It was expected that these studies would explain the mechanism whereby antibiotic associated hypoprothrombinemia occurs in mammals on a low dietary vitamin K_1 intake, ie, was hypoprothrombinemia occurring secondary to removal of the menaquinone producing flora producing K deficiency, or was it due to the drug or a part of it inhibiting the post-translational carboxylation of vitamin K_1 dependent proteins in the liver?

1. Experimental Design

Animals: Rats used in all studies were Sprague-Dawley males housed singly in coarse wire meshed cages. The mesh size of the cage was such that it would allow fecal material to fall through, making it inaccessible to the animals. Feed and liquids were

provided ad libitum. Ingestion of liquids, diet, stool output and weight were monitored daily.

Rats were killed by exsanguination after collecting blood for prothrombin time (PT) determinations and serum for vitamin K_1 levels. PT determinations were carried out immediately; serum for vitamin K_1 determination was frozen at -70°C for later analysis. Animals were immediately dissected and the liver and intestines removed. The intestines were usually divided into 4 sections: upper small intestine, lower small intestine (comprising of the last 20 cm of the small bowel), cecum and colon. These samples were stored in vials and usually frozen at -70°C for subsequent analysis, or were assayed immediately. The liver was analysed for menaquinone and vitamin K_1; the contents of the intestinal segments were quantitatively cultured for flora as well as analysed for menaquinone and vitamin K_1 as described. To obtain the contents of intestinal segments for analysis, they were thawed and then the contents aseptically extruded. Quantitative culture and menaquinone analysis were then carried out simultaneously.

(ii) Effect of Diet. The effect of three separate diets were examined. Rats were randomized into groups of 5 and were fed either rat chow (Ralston Purina, St. Louis, MO), lean ground beef (Canada Safeway Ltd.) or a rice diet comprised of cooked white rice and egg albumin (O'Reilly, 1971) accompanied by a 4% glucose solution (w/v).

After seven days rats were killed and samples were collected as described above.

(iii) Effects of Diets and Various Antibiotics. Rats were randomized into groups of 5 and were given the following combinations

of diets and antibiotics for seven days: (a) rat chow or rice plus moxalactam, (b) rat chow or rice plus clindamycin, in combination with gentamicin, (c) rat chow or rice plus cefoxitin in combination with gentamicin. One group of rats was also fed a meat diet. Moxalactam was administered daily at 25 mg, 3 times per day (q8h), by subcutaneous injection; clindamycin-gentamicin mixture at 30 mg and 2 mg per day, respectively, by oral intake with drinking water or glucose solution and the cefoxitin-gentamicin mixture at 40 mg and 4 mg per day, respectively, by oral intake with water or glucose solution. In all cases, where antibiotics were administered orally, total daily liquid and therefore drug intake was monitored.

(iv) Effect of Synthetic Diet. Rats were randomized into two groups of 5 and fed the rice diet as previously described or the diet of Mameesh and Johnson (1960a,b), slightly modified. The diet is listed below:

Ingredients	% (w/v)
Glucose	71.4
Soya Protein	20.0
Methionme	0.5
Vitamin Mix*	0.1
Salts	4.0
Wheat germ	0.5
Cod-liver oil	1.5
Glycerol	2.0

* The vitamin mix comprised of the following:

	mg/kg diet
Thiamine HCl	10.0
Riboflavin	10.0
Calcium panthotenate	50.0
Pyridoxine HCl	5.0
Nicotinic acid	20.0
Folic acid	1.0
Vitamin B_{12}	0.10
Biotin	0.10
Choline chloride	1000.0

After 14 days, rats were killed and blood, intestines and liver samples recovered.

(v) Restoration of Normal PT by Oral Feeding of B. fragilis or Recolonization of the Intestine. Rats were placed on the rice diet and cefoxitin-gentamicin at 40 and 4 mg respectively as previously decribed. After seven days they were randomized into groups of five and treated as follows: One group (control #1) was killed immediately, and blood for PT as well as the small intestine, cecum and liver removed for menaquinone and vitamin K_1 analysis. This group was being used to demonstrate that the rats were in fact hypoprothrombinemic at this time as well as containing low liver and intestinal stores of vitamin K_1 and menaquinones.

A second group was kept on the rice diet in combination with the antibiotic regimen (control #2) and killed at day 12. This was to demonstrate the continuing effect of the diet and drugs at sacrifice of the other groups.

Two other groups were kept on the rice diet with the antibiotic regimen and, in addition, fed a pellet of B. fragilis ATCC 23745 cells as part of their diet. The pellet (containing 10^{11} CFU representing the bacterial load in the colon) was mixed with the cooked rice and then with egg white, which was then coagulated by cooking so that cakes were produced. The cakes were fed to the rats.

The B. fragilis ATCC 23745 was grown in BHI broth and harvested as described in the methods section.

The bacterial pellet was added to the rice at 10.4 g packed wet cells per 1 kg. This was equivalent to total MK-8, MK-9 and MK-10 of

718.3 nmole. At an average intake of approximately 50 g of rice per day, the total menaquinone intake was approximately 35.92 nmole/day. These two groups were kept on the diet containing the B. fragilis ATCC 23745 pellet for another 3 days (Test #1) and 5 days (Test #2) respectively, before being killed. These test groups would determine whether menaquinones contained in bacteria would be available and absorbed in the gut to restore normal coagulation in rats that were on the vitamin K_1 deficient diet.

A fifth group was kept on the rice diet but taken off the antibiotic regimen (Test #3). This group would determine whether recolonization with menaquinone producing bacteria occurred and whether normalization of coagulation times resulted. These rats were killed after 5 days and because of the short period, no recolonization had occurred nor had coagulation times normalised. A sixth group was, therefore, allowed to go for 20 days on rice without the drug, and then killed. During the 20 days the animals were without antimicrobial suppression, their fecal material was monitored every other day by swabbing a fecal sample to BBE plates and observing it for growth of any B.fragilis group organisms. In all cases, after the rats were sacrificed, blood was taken for PT and serum vitamin K_1 determination. The animals were dissected, the liver, small intestine and cecum removed and analysed for menaquinones and vitamin K_1. In addition, the groups that were being allowed to recolonize for 5 and 20 days also had their intestinal contents quantitatively cultured as described previously.

2. Accuracy and Precision of HPLC Assay for Vitamin K

Before serum and intestinal samples were analysed for vitamin K_1 and menaquinones, the precision of the assay was determined. Cecal contents of a normal rat was assayed on the same day (n=4) and on different days (n=5). The coefficient of variation for the same day determinations ranged between 6-17% for the different vitamin K components and 7-10% on different days (Table 5.27).

The recovery of vitamin K_1 and menaquinones from cecal contents was also determined, since the assay does not use an internal standard. The determination was carried out in a manner similar to that for human feces as described previously. Analyses were done at three separate concentrations for each vitamin K component. Recovery was in the order of 90% with a range of 80-105% (Table 5.28).

3. Effect of Diet and Antibiotics on Menaquionone Levels and Prothrombin Times

The intestines of the animals were analysed as four separate sections for menaquinones so as to determine the site of the highest concentration and perhaps infer their relative importance as endogenous pools. From Table 5.29 and Figure 5.16, it can be seen that the concentration of total menaquinone was much lower in both the upper and lower small intestine, compared to the cecum and colon. The same is true for the individual menaquinone isoprenologues. Thus, rats on rice had a total menaquinone concentration of 22.89 ± 11.87 nmole/g dry weight in the cecum compared to 0.41 ± 0.20 nmole/g dry weight or 1.25 ± 1.66 nmole/g dry weight in the upper and lower small intestines respectively. A comparison of the cecum (22.89 ± 11.87

Table 5.27 Precision of HPLC assay for rat intestinal contents

	Same Day		Different Days	
	nmole/g dry wt (n=4)		nmole/g dry wt (n=5)	
MK-n	mean ± S.D.	CV%	mean ± S.D.	CV%
K_1	1.99 ± 0.13	6.5	4.05 ± 0.41	10.1
MK-4	0.61 ± 0.04	5.9	0.86 ± 0.08	8.8
MK-6	0.78 ± 0.06	7.1	1.63 ± 0.12	7.1
MK-7	0.33 ± 0.06	17.2	0.54 ± 0.05	9.4
MK-9	0.56 ± 0.07	13.2	8.95 ± 0.09	10.3
MK-10	4.09 ± 0.31	7.7	4.12 ± 0.33	8.0

Table 5.28 Efficiency of extraction: Recovery of menaquinones added to rat cecal contents

The quantity of menaquinone uncoverable from samples of rat intestinal contents was determined using cecal matter. Determinations were done as described for feces.

MK-n	nmole Added	nmole Recovered		% Recovery	
		Exp 1	Exp 2	Exp 1	Exp 2
K_1	0.17	0.15	0.16	91.5	94.1
	0.34	0.30	0.26	88.2	77.8
	0.68	0.73	0.69	106.9	100.9
MK-4	0.21	0.19	0.21	88.5	101.4
	0.42	0.43	0.39	102.0	93.8
	0.85	0.92	0.87	108.4	102.2
MK-5	0.23	0.21	0.22	92.6	95.1
	0.46	0.41	0.36	89.6	79.0
	0.92	0.91	0.88	98.9	95.5
MK-6	0.16	0.14	0.16	89.0	102.4
	0.32	0.32	0.26	99.1	80.2
	0.65	0.66	0.63	101.6	97.3
MK-7	0.46	0.42	0.44	90.0	89.1
	0.92	0.85	0.77	92.6	83.7
	1.85	1.72	1.92	92.8	104.2
MK-8	0.84	0.70	0.79	82.9	93.9
	1.68	1.65	1.49	97.9	88.5
	3.35	2.96	3.08	88.3	91.9
MK-9	1.06	0.90	0.81	84.5	76.4
	2.12	1.88	1.67	88.9	78.9
	4.24	3.80	3.56	89.5	83.9
MK-10	0.86	0.73	0.74	85.3	85.7
	1.72	1.55	1.41	90.1	82.0
	3.43	2.74	2.79	80.0	81.2

Table 5.29 Vitamin K and bacterial concentrations of different segments of the rat intestine (mean ± S.D.)

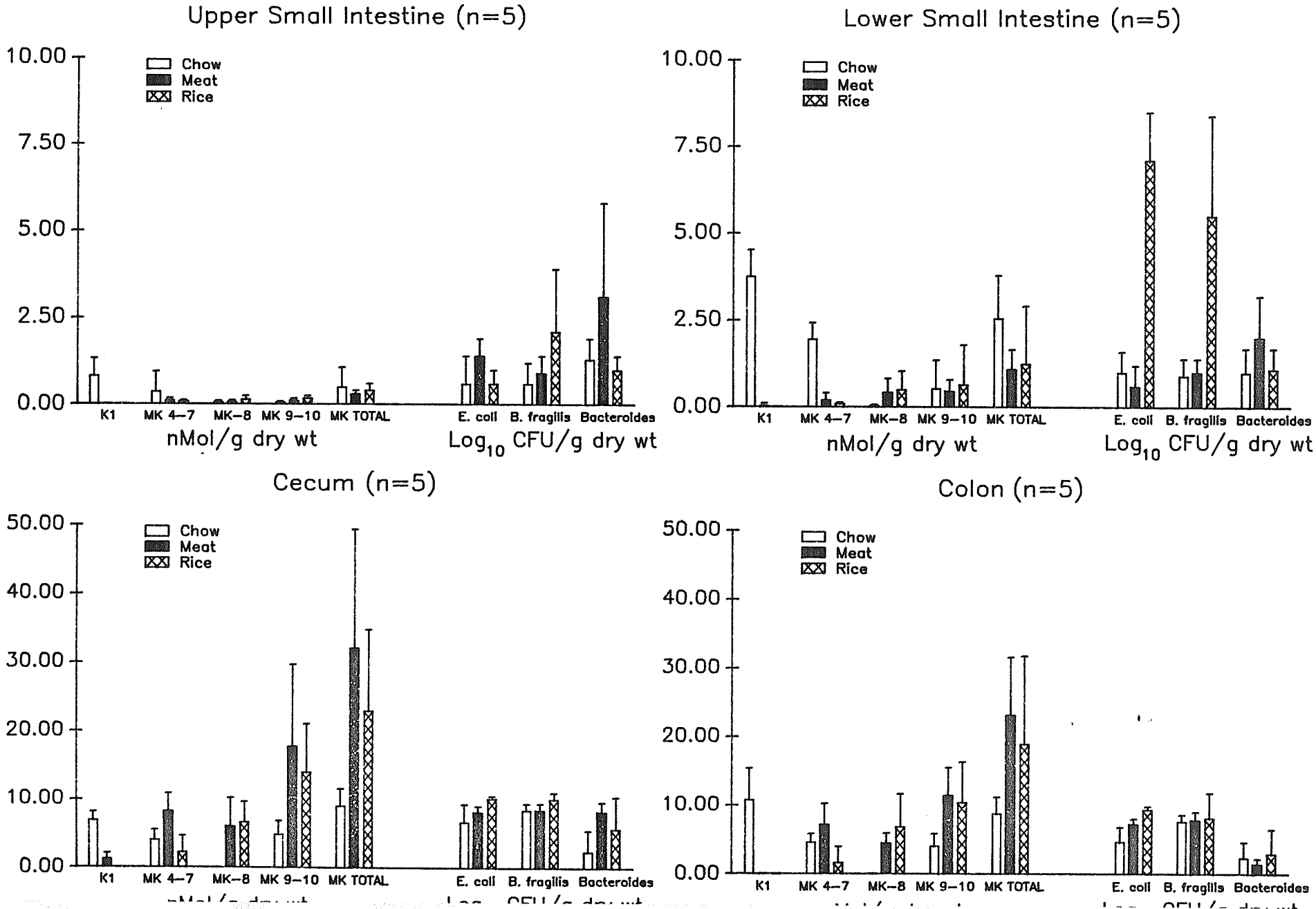
	nmole/g dry wt					Log_{10} CFU/g dry wt		
	K_1	MK 4-7	MK-8	MK 9+10	MK Total	E. coli	B. fragilis	Bacteroides spp.
Upper Small Intestine								
Chow (n=5)	0.82 ± 0.51	0.37 ± 0.59	0.06 ± 0.05	0.06 ± 0.03	0.50 ± 0.58	0.6 ± 0.8	0.6 ± 0.6	1.3 ± 0.6
Meat (n=5)	0.01 ± 0.01	0.12 ± 0.07	0.07 ± 0.05	0.12 ± 0.06	0.31 ± 0.11	1.4 ± 0.5	0.9 ± 0.5	3.1 ± 2.7
Rice (n=5)	0.01 ± 0.01	0.09 ± 0.04	0.14 ± 0.12	0.18 ± 0.08	0.41 ± 0.20	0.6 ± 0.4	2.1d ± 1.8	1.0 ± 0.4
Lower Small Intestine								
Chow (n=5)	3.75 ± 0.77	1.95 ± 0.48	0.05 ± 0.03	0.54 ± 0.82	2.55 ± 1.24	1.0 ± 0.6	0.9 ± 0.5	1.0 ± 0.7
Meat (n=5)	0.04a ± 0.08	0.21b ± 0.21	0.43 ± 0.40	0.46 ± 0.34	1.10 ± 0.57	0.6 ± 0.6	1.0 ± 0.4	2.0 ± 1.2
Rice (n=5)	0.01a ± 0.01	0.10b ± 0.04	0.51 ± 0.54	0.65 ± 1.15	1.25 ± 1.66	7.1 ± 1.4	5.5c ± 2.9	1.1 ± 0.6

Table 5.29 (Continued)

	nmole/g dry wt					Log_{10} CFU/g dry wt		
	K_1	MK 4-7	MK-8	MK 9+10	MK Total	E. coli	B. fragilis	Bacteroides spp.
Cecum								
Chow (n=5)	6.89 ± 1.27	4.06 ± 1.53	0.03 ± 0.01	4.91 ± 2.02	9.01 ± 2.56	6.7 ± 2.6	8.4 ± 1.0	2.4 ± 3.1
Meat (n=5)	1.27a ± 0.86	8.26 ± 2.69	6.08a ± 4.20	17.72a ± 11.97	32.06a ± 17.32	8.1 ± 0.9	8.4 ± 1.0	8.2 ± 1.4
Rice (n=5)	0.01a ± 0.002	2.26c ± 2.46	6.68a ± 3.02	13.95a ± 7.07	22.89a ± 11.87	10.1a,c ± 0.4	10.0d ± 1.0	5.7a ± 4.7
Colon								
Chow (n=5)	10.74 ± 4.64	4.63 ± 1.27	0.08 ± 0.04	4.08 ± 1.83	8.80 ± 2.47	4.7 ± 2.2	7.7 ± 1.0	2.4 ± 2.3
Meat (n=5)	0.01a ± 0.01	7.22a ± 3.09	4.50a ± 1.50	11.50a ± 4.06	23.22a ± 8.48	7.3a ± 0.8	7.9 ± 1.2	1.5 ± 0.8
Rice (n=5)	0.01a ± 0.01	1.69a,c ± 2.32	6.89a ± 4.84	10.41a ± 5.99	18.99a ± 12.88	9.4a ± 0.5	8.2 ± 3.7	3.0 ± 3.6

a $p \leq 0.0001$ compared to chow group
b $p \leq 0.05$ compared to chow group
c $p \leq 0.0001$ compared to meat group
d $p \leq 0.05$ compared to meat group

Figure 5.16 Vitamin K and bacterial concentrations (mean ± S.D.) of intestinal segments of rats fed chow, meat or rice.



nmole/g dry weight) and colon (18.99 ± 12.88 nmole/g dry weight) indicates that the former has slightly higher but not significantly different levels. It therefore appears that the cecum may represent the major endogenous pool of menaquinone in the intestine. Also, coprophagy is unlikely to be significant since the upper small bowel concentrations of menaquinones were very low.

In terms of the major menaquinone producing microflora (B.fragilis and E.coli), their concentration profiles parallel that of the menaquinone concentration, that is low counts in the small intestine compared to 7-10 $\log_{10}$ CFU/g dry weight in the other two sections.

In animals fed rice and meat, vitamin K_1 concentrations were negligible in all sections of the intestines reflecting the fact that these diets were deficient in this form of the vitamin. Chow fed rats contained higher vitamin K_1 levels than the other diet fed rats, with concentrations being lowest in the upper small intestine, becoming progressively higher in the lower small intestine, cecum and colon respectively. The rice diet was more effective than meat in reducing cecal vitamin K_1 concentrations (Table 5.30, Figure 5.16).

Menaquinone concentrations and menaquinone producing bacterial counts (E.coli, B.fragilis group) of the upper and lower small intestine were similar regardless of type of diet fed to the animals. The largest differences in either menaquinones or flora induced by diet were discernible in the cecum of the animals. Total menaquinone concentration in chow fed rats was 9.01 ± 2.56 nmole/g dry weight -- in rice and meat fed rats the concentration had increased 2 and 3 fold respectively. The increase in total menaquinone concentration was

Table 5.30 Effect of diets on PT, bacteria and vitamin K stores in rats (mean ± S.D.)

	Chow (n=5)	Meat (n=5)	Rice (n-5)
Prothrombin time(s)	13.5 ± 1.2	14.2 ± 0.6	15.0 ± 0.5
Serum K_1 (pmole/ml)	4.22 ± 3.41	2.65 ± 3.79	1.24 ± 0.72
Cecum (nmole/g dry wt)			
K_1	6.89 ± 2.26	1.27 ± 0.86*	0.01 ± 0.002*
MK 4-7	4.06 ± 1.53	8.26 ± 2.69*	2.26 ± 2.46††
MK-8	0.03 ± 0.01	6.08 ± 4.20*	6.68 ± 3.02*
MK 9-10	4.91 ± 2.02	17.72 ± 11.97*	13.95 ± 7.07*
MK Total	9.01 ± 2.56	32.06 ± 7.32*	22.89 ± 11.87*
Cecum (Log_{10} CFU/g dry wt)			
E. coli	6.7 ± 2.6	8.1 ± 0.9	10.1 ± 0.4*
B. fragilis grp	8.4 ± 1.0	8.4 ± 1.0	10.0 ± 1.0
Bacteroides spp.	2.4 ± 3.1	8.2 ± 1.4*	5.7 ± 4.7†
Liver (pmole/g wet wt)			
K_1	20.33 ± 13.90	37.66 ± 46.01	13.21 ± 22.87
MK 4-7	52.16 ± 69.55	166.04 ± 14.77	148.79 ± 72.01
MK-8	36.53 ± 16.00	87.22 ± 79.65	111.33 ± 59.01
MK 9-10	13.74 ± 16.00	75.00 ± 40.85§	43.76 ± 37.01
MK Total	102.43 ± 124.77	328.27 ± 255.26†	303.88 ± 109.93

* $p \leq 0.0001$ compared to chow group

† $p \leq 0.05$ compared to chow group

†† $p \leq 0.0001$ compared to meat group

§ $p \leq 0.005$ compared to chow group

reflected in increases in all the isoprenologues, especially MK-8 which was negligible (0.03 ± 0.01 nmole/g dry weight) in the cecum of chow fed rats, increasing 200 fold in both meat and rice fed rats (Table 5.30). At the same time, however, the concentration of E.coli only increased by 1.4 (meat) and 3.4 (rice) $\log_{10}$ CFU/g dry weight, respectively, and B.fragilis group by 1.6 $\log_{10}$ CFU/g dry weight over the chow group. This implied that in the presence of the meat and rice diet, the menaquinone producing organisms were probably metabolically more active than with the chow diet. Differences in cecal concentrations of vitamin K between dietary groups were also reflected in differences in the liver. Vitamin K_1 levels appeared to be similar but spread over a wide range. Menaquinone concentrations on the other hand were 3 fold higher in both meat and rice fed rats. These increases were reflected in increases of all the menaquinone isoprenologues (Table 5.30).

Serum vitamin K_1 levels like cecal levels reflected the vitamin K_1 status of the diet being consumed, that is concentrations were highest in chow fed rats, compared to the other two groups.

None of the groups had elevated prothrombin times (Table 5.30). Since hypoprothrombinemia was not seen in the rice fed group which contained low vitamin K_1 stores, it is apparent that endogenous menaquinone is bioavailable and contributes to maintaining normal coagulation homeostasis.

Concentrations of moxalactam in the cecum of animals given this drug ranged from 146 ± 68 ug/g weight (mean ± S.D.) in chow fed rats to 696 ± 360 ug/g (mean ± S.D.) in rice fed rats. Clindamycin concentrations were much higher in the rice group at 6792 ± 4356 ug/g com-

pared to chow at 2061 ± 1078 ug/g. However gentamicin concentrations were low, measuring 22 ± 20 ug/g weight and 4.3 ± 0.6 ug/g weight (mean ± S.D.) respectively. In contrast, the concentration of cefoxitin and gentamicin in the group of rats ingesting the rice diet with this regimen was 3217 ± 1388 and 206 ± 138 ug/g weight respectively. The higher level of aminoglycoside in this group probably reflects the higher dose of drug given to this group compared to clindamycin/gentamicin treated rats which ingested half the dose of gentamicin. Part of the increase in concentration of antibiotic in rice fed rats compared to chow fed rats is the decrease of fecal biomass in rice fed (low residue) animals. It should be noted that when serum levels of cefoxitin and gentamicin were examined, the mean concentrations were 4.3 and <0.5 ug/ml respectively, indicating the poor absorption of these antimicrobials from the intestines of the animals.

When animals were placed on subcutaneous moxalactan and fed chow, there was a complete eradication of E.coli and B.fragilis group organisms in the cecum and colon (Table 5.31). Cecal and colonic concentrations of menaquinones were reduced to about 20% of that in chow fed group. Cefoxitin/gentamicin combination induced the same changes in the microflora and menaquinone concentration as moxalactam. In fact the cecal menaquinone concentration in the former group was reduced to 5% of normal. The reduction in total menaquinone concentrations was reflected in reductions of all the isoprenologues. Clindamycin/gentamicin combination also eradicated the intestinal flora to a large extent. However, total menaquinone concentrations in the cecum were not as significantly reduced as with the other two

Table 5.31 Vitamin K and bacterial concentrations in the intestines of rats on chow combined with antibiotics (Mean ± SD)

	nmole/g dry wt					Log_{10} CFU/g dry wt		
	K_1	MK 4-7	MK-8	MK 9+10	MK Total	E. coli	B. fragilis	Bacteroides spp.
Upper Small Intestine								
Chow (n=5)	0.82 ± 0.51	0.37 ± 0.59	0.06 ± 0.05	0.06 ± 0.03	0.50 ± 0.58	0.6 ± 0.8	0.6 ± 0.6	1.3 ± 0.6
Chow & Moxalactam (n=5)	2.39c ± 1.15	1.99b ± 0.39	0.08 ± 0.03	0.11 ± 0.07	2.17b ± 0.45	0.6 ± 0.5	0.6 ± 0.5	0.7 ± 0.4
Chow & Clindamycin/ Gentamicin (n=5)	0.81d ± 0.53	0.80f ± 0.24	0.09 ± 0.04	0.11 ± 0.03	0.99 ± 0.24	0.8 ± 0.6	1.1 ± 0.8	1.0 ± 0.8
Chow & Cefoxitin/ Gentamicin (n=5)	0.52 ± 0.36	0.09 ± 0.03	0.11 ± 0.02	0.13 ± 0.02	0.32 ± 0.06	3.2a ± 1.2	1.5 ± 0.4	1.1 ± 0.3
Lower Small Intestine								
Chow (n=5)	3.75 ± 0.77	1.95 ± 0.48	0.05 ± 0.03	0.54 ± 0.82	2.55 ± 1.24	1.0 ± 0.6	0.9 ± 0.5	1.0 ± 0.7
Chow & Moxalactam (n=5)	4.53 ± 0.68	3.02b ± 0.27	0.03 ± 0.03	0.13 ± 0.05	3.18 ± 0.32	0.9 ± 0.5	0.8 ± 0.7	0.9 ± 0.8
Chow & Clindamycin/ Gentamicin (n=5)	2.73a,e ± 0.67	1.27 ± 0.29	0.06 ± 0.03	0.12 ± 0.04	1.46f ± 0.31	1.4 ± 0.5	1.0 ± 0.6	0.7 ± 0.7
Chow & Cefoxitin/ Gentamicin (n=5)	0.24b,f ± 0.22	0.14 ± 0.05	0.08 ± 0.04	0.10 ± 0.05	0.32b,f ± 0.08	3.2a ± 1.6	1.7 ± 0.2	1.5 ± 0.3

Table 5.31 (Continued)

	nmole/g dry wt					Log_{10} CFU/g dry wt		
	K_1	MK 4-7	MK-8	MK 9+10	MK Total	E. coli	B. fragilis	Bacteroides spp.
Cecum								
Chow (n=5)	6.89 ± 2.26	4.06 ± 1.53	0.03 ± 0.01	4.91a ± 2.02	9.01 ± 2.56	6.7 ± 2.6	8.4 ± 1.0	2.4 ± 3.1
Chow & Moxalactam (n=5)	3.76a ± 0.23	1.50c ± 9.28	0.07 ± 0.05	0.12c ± 0.07	1.68c ± 0.34	1.7a ± 0.7	2.1a ± 1.1	1.1 ± 0.9
Chow & Clindamycin/ Gentamicin (n=5)	3.79a ± 1.63	2.58 ± 1.23	3.79a,h ± 2.27	0.26c ± 0.28	6.63 ± 1.98	3.5a ± 1.2	1.4a ± 1.1	0.8 ± 0.8
Chow & Cefoxitin/ Gentamicin (n=5)	1.62afg ± 0.32	0.39 ± 0.25	0.11 ± 0.02	0.17c ± 0.05	0.67c ± 0.28	2.3a ± 0.5	2.9a ± 0.1	2.5 ± 0.4
Colon								
Chow (n=5)	10.74 ± 4.64	4.63 ± 1.27	0.08 ± 0.04	4.08 ± 1.83	8.80 ± 2.47	4.7 ± 2.2	7.7 ± 1.0	2.4 ± 2.3
Chow & Moxalactam (n=5)	4.33a ± 2.27	2.45 ± 1.17	0.06 ± 0.05	0.18c ± 0.14	2.68 ± 1.10	1.6a ± 0.7	0.9a ± 0.8	1.3 ± 1.1
Chow & Clindamycin/ Gentamicin (n=5)	3.19a ± 1.17	1.91c ± 1.40	3.59a,h ± 2.02	0.08c ± 0.03	5.58 ± 3.11	3.8 ± 2.1	2.1a ± 0.9	1.2 ± 0.8
Chow & Cefoxitin/ Gentamicin (n=5)	1.35a ± 0.86	0.11b ± 0.05	0.08 ± 0.02	0.12c ± 0.04	0.31 ± 0.07	2.7 ± 0.4	2.8a ± 0.3	2.5 ± 0.3

a $p \leq 0.0001$ compared to chow group
b $p \leq 0.05$ compared to chow group
c $p \leq 0.01$ compared to chow group
d $p < 0.01$ compared to chow & moxalactam group
e $p \leq 0.005$ compared to chow & moxalactam group
f $p \leq 0.05$ compared to chow & moxalactam group
g $p \leq 0.05$ compared to chow & clindamycin/gentamicin group
h $p \leq 0.0001$ compared to moxalactam group

regimens. The clindamycin/gentamicin group had a total menaquinone concentration of 6.63 nmole/g dry weight -- a reduction to only 75% of normal values. The preservation of such high levels in these animals was in a large part due to the increase in MK-8 concentrations (3.79 ± 2.27 nmole/g dry weight) when compared to the chow group (0.03 ± 0.01 nmole/g dry weight) (Table 5.32, Figure 5.17). It appears that this antimicrobial regimen selected for and preserved certain MK-8 producing flora such as S.aureus (4.6 ± 2.4 $\log_{10}$ CFU/g dry weight) and Bacillus sp. (4.3 ± 0.4 $\log_{10}$ CFU/g dry weight), respectively. Also suppression of coliforms but not clearance of MK-8 could account for the persisting MK-8 concentration in clindamycin/gentamicin treated rats.

Total menaquinone concentrations in the liver were reduced approximately 10 fold in animals fed chow in combination with moxalactam or cefoxitin/gentamicin. Animals on clindamycin/gentamicin contained levels similar to those of chow fed rats (Table 5.32). Detectable serum vitamin K_1 levels were similar in all groups (Table 5.32). Since hypoprothrombinemia was not seen in the groups given moxalactam and cefoxitin/gentamicin even though there were significant reductions of cecal and liver menaquinone stores, this indicated that there were adequate levels of vitamin K_1 available for normal coagulation homeostasis. More importantly, the fact that hypoprothrombinemia did not occur in the rats administered moxalactam indicates that the NMTT chain did not inhibit the microsomal carboxylase in these animals.

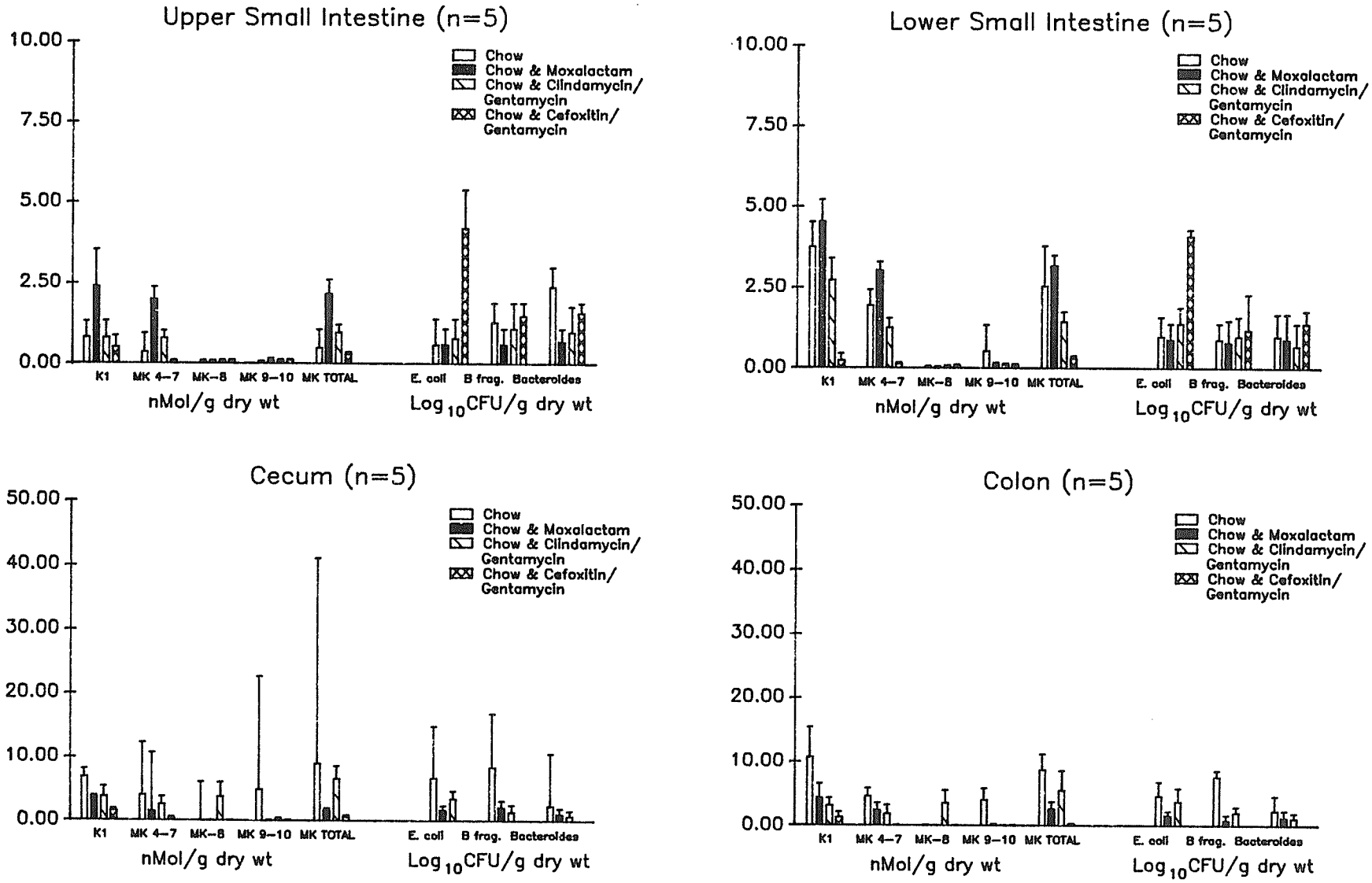
An examination of the intestinal segments of rats placed on rice

Table 5.32 PT, vitamin K and bacterial concentrations in rats on chow alone or in combination with antibiotics (mean ± SD)

	Chow (n=5)	Chow & Moxalactam (n=5)	Chow & Clindamycin/ Gentamicin (n=5)	Chow & Cefoxitin Gentamicin (n=5)
Prothrombin time(s)	13.5 ± 1.2	13.8 ± 1.6	13.4 ± 1.6	12.5 ± 0.5
Serum K_1 (pmole/ml)	4.22 ± 3.41	12.92 ± 7.72	5.88 ± 6.53	6.87 ± 3.64
Cecum (nmole/g dry wt)				
K_1	6.89 ± 2.26	3.76 ± 0.23a	3.79 ± 1.63a	1.62 ± 0.32acd
MK 4-7	4.06 ± 1.53	1.50 ± 0.28e	2.58 ± 1.23	0.39 ± 0.25
MK-8	0.03 ± 0.01	0.07 ± 0.05	3.79 ± 2.27a,f	0.11 ± 0.02
MK 9-10	4.91 ± 2.02	0.12 ± 0.07e	0.26 ± 0.28e	0.17 ± 0.05e
MK Total	9.01 ± 2.56	1.68 ± 0.34e	6.63 ± 1.98	0.67 ± 0.28e
Cecum (Log_{10} CFU/g dry wt)				
E. coli	6.7 ± 2.6	1.7 ± 0.7a	3.5 ± 1.2a	2.3 ± 0.5a
B. fragilis grp	8.4 ± 1.0	2.1 ± 1.1a	1.4 ± 1.1a	2.9 ± 0.1a
Bacteroides spp.	2.4 ± 3.1	1.1 ± 0.9	0.8 ± 0.8	2.5 ± 0.4
Liver (pmole/g wet wt)				
K_1	20.33 ± 13.90	15.70 ± 19.30	33.56 ± 15.02	0.84 ± 1.84
MK 4-7	52.16 ± 69.55	7.14 ± 11.05	68.35 ± 44.65	0.12 ± 0.03
MK-8	36.53 ± 16.00	2.72 ± 5.98	26.10 ± 16.37	1.35 ± 2.80
MK 9-10	13.74 ± 16.00	2.52 ± 5.42	14.19 ± 15.03	10.68 ± 13.43
MK Total	102.43 ± 124.77	12.38 ± 22.31	108.65 ± 66.33	12.15 ± 15.41

a $p \leq 0.0001$ compared to chow group
b $p \leq 0.05$ compared to chow group
c $p \leq 0.05$ compared to chow & moxalactam group
d $p \leq 0.05$ compared to chow & clinda/genta group
e $p \leq 0.01$ compared to chow group
f $p \leq 0.0001$ compared to chow & moxalactam group

Figure 5.17 Vitamin K and bacterial concentrations (mean ± S.D.) of intestinal segments of rats fed chow alone or in combination with antibiotics.



in combination with moxalactam, clindamycin/gentamicin or cefoxitin/gentamicin shows that in all the groups there was complete eradication of E.coli and B.fragilis group organisms. Vitamin K_1 was at negligible levels in all the groups throughout the intestine as were menaquinone levels in the upper and lower small intestine (Table 5.33, Figure 5.18).

Cecal levels of menaquinone were reduced to 2% and 7% of normal values in groups on moxalactam and cefoxitin/gentamycin, respectively. At the same time, liver concentrations were reduced to 5% of normal in both groups. Significantly, in both of these groups, there was elevation of prothrombin times - 28.9 ± 13.1 seconds and 22.9 ± 2.64 seconds in the moxalactam and cefoxitin/gentamycin groups, respectively (Table 5.34).

Even though E.coli and B.fragilis group organisms were eradicated in the clindamycin/gentamicin group, cecal menaquinone levels were preserved to some extent--concentration of total menaquinone was 6.17 ± 7.4 nmole/g dry weight. Once again as with chow plus clindamycin/gentamicin, the shorter chain menaquinones (MK 4-8) persisted. Persistence of menaquinone in the cecum of the clindamycin/gentamicin group was reflected in high levels in the liver (77.83 ± 62.38 pmole/g wet weight) (Table 5.34). MK 4-8 constituted 80% of this total. It therefore appears that, the inability to produce hypoprothrombinemia in this group is due to persistence of liver stores of menaquinone in the absence of vitamin K_1. On the other hand, hypoprothrombinemia is seen in animals on moxalactam and cefoxitin/gentamicin due to reduction of cecal and liver stores of menaquinones with concommitant low vitamin K_1 intake. Since hypoprothrombinemia was seen both with

Table 5.33 Vitamin K and bacterial concentrations in various segments of the intestines of rats placed on rice diet alone in combination with antibiotics (mean ± SD)

	nmole/g dry wt					Log_{10} CFU/g dry wt		
	K_1	MK 4-7	MK-8	MK 9+10	MK Total	E. coli	B. fragilis	Bacteroides spp.
Upper Small Intestine								
Rice (n=5)	0.01 ± 0.01	0.09 ± 0.04	0.14 ± 0.12	0.18 ± 0.08	0.41 ± 0.20	0.6 ± 0.4	2.1 ± 1.8	1.0 ± 0.4
Rice & Moxalactam (n=5)	0.01 ± 0.01	0.33 ± 0.48	0.06 ± 0.02	0.12 ± 0.05	0.51 ± 0.48	0.8 ± 0.6	0.9 ± 0.5	0.6 ± 0.5
Rice & Clindamycin/ Gentamicin (n=5)	0.01 ± 0.01	0.16 ± 0.12	0.07 ± 0.02	0.09 ± 0.05	0.32 ± 0.14	0.8 ± 0.6	1.0 ± 0.4	0.7 ± 0.5
Rice & Cefoxitin/ Gentamicin (n=5)	0.01 ± 0.01	0.12 ± 0.04	0.07 ± 0.05	0.10 ± 0.06	0.29 ± 0.06	0.7 ± 0.3	0.3 ± 0.1	1.0 ± 0.5
Lower Small Intestine								
Rice (n=5)	0.01 ± 0.01	0.10 ± 0.04	0.51 ± 0.54	0.65 ± 1.15	1.25 ± 1.66	7.1 ± 1.4	5.5 ± 2.9	1.1 ± 0.6
Rice & Moxalactam (n=5)	0.11 ± 0.13	0.54 ± 0.82	0.04 ± 0.04	0.23 ± 0.24	0.81 ± 0.81	0.7a ± 0.8	0.8a ± 0.1	1.0 ± 0.8
Rice & Clindamycin/ Gentamicin (n=5)	0.01 ± 0.01	0.08 ± 0.03	0.32 ± 0.51	0.12 ± 0.05	0.53 ± 0.49	0.8a ± 0.4	1.1a ± 0.2	1.2 ± 0.5
Rice & Cefoxitin/ Gentamicin (n=5)	0.01 ± 0.01	0.10 ± 0.05	0.05 ± 0.03	0.08 ± 0.02	0.23 ± 0.67	0.1a ± 0.4	0.7a ± 0.3	0.9 ± 0.7

Table 5.33 (Continued)

	nmole/g dry wt					Log_{10} CFU/g dry wt		
	K_1	MK 4-7	MK-8	MK 9+10	MK Total	E. coli	B. fragilis	Bacteroides spp.
Cecum								
Rice (n=5)	0.01 ± 0.002	2.26 ± 2.46	6.68 ± 3.02	13.95 ± 7.07	22.89 ± 11.87	10.1 ± 0.4	10.0 ± 1.0	5.7 ± 4.7
Rice & Moxalactam (n=5)	0.01 ± 0.01	1.23 ± 1.81	0.19a ± 0.27	0.24a ± 0.07	1.66a ± 2.11	1.5a ± 1.0	1.3a ± 1.1	2.5a ± 0.5
Rice & Clindamycin/ Gentamicin (n=5)	0.01 ± 0.01	3.97b ± 7.65	1.81a ± 1.38	0.38a ± 0.26	6.17a ± 7.40	1.9a ± 0.8	1.3a ± 0.7	0.8a ± 0.7
Rice & Cefoxitin/ Gentamicin (n=5)	0.07 ± 0.05	0.22c ± 0.07	0.03a ± 0.03	0.28a ± 0.36	0.53a ± 0.31	0.8a ± 0.2	1.2a ± 1.0	1.4a ± 1.6
Colon								
Rice (n=5)	0.01 ± 0.01	1.69 ± 2.32	6.89 ± 4.84	10.41 ± 5.99	18.99 ± 12.88	9.4 ± 0.5	8.2 ± 3.7	3.0 ± 3.6
Rice & Moxalactam (n=5)	0.02 ± 0.01	0.90 ± 1.68	0.05a ± 0.04	0.17a ± 0.19	1.12a ± 1.86	1.7a ± 1.1	1.9a ± 0.9	1.3 ± 0.8
Rice & Clindamycin/ Gentamicin (n=5)	0.01 ± 0.005	0.21 ± 0.09	0.78a ± 0.41	0.26a ± 0.15	1.24a ± 0.43	1.5a ± 1.2	1.7a ± 0.9	0.9 ± 0.6
Rice & Cefoxitin/ Gentamicin (n=5)	0.01 ± 0.01	0.10 ± 0.05	0.06a ± 0.04	0.08a ± 0.06	0.24a ± 0.09	1.6a ± 1.0	1.9a ± 1.0	1.7 ± 0.8

a $p \leq 0.0001$ as compared to rice group
b $p \leq 0.01$ as compared to rice & moxalactam group
c $p \leq 0.005$ as compared to rice & clindamycin/gentamicin group

Figure 5.18 Vitamin K and bacterial concentrations (mean ± S.D.) of intestinal segments of rats fed rice alone or in combination with antibiotics.

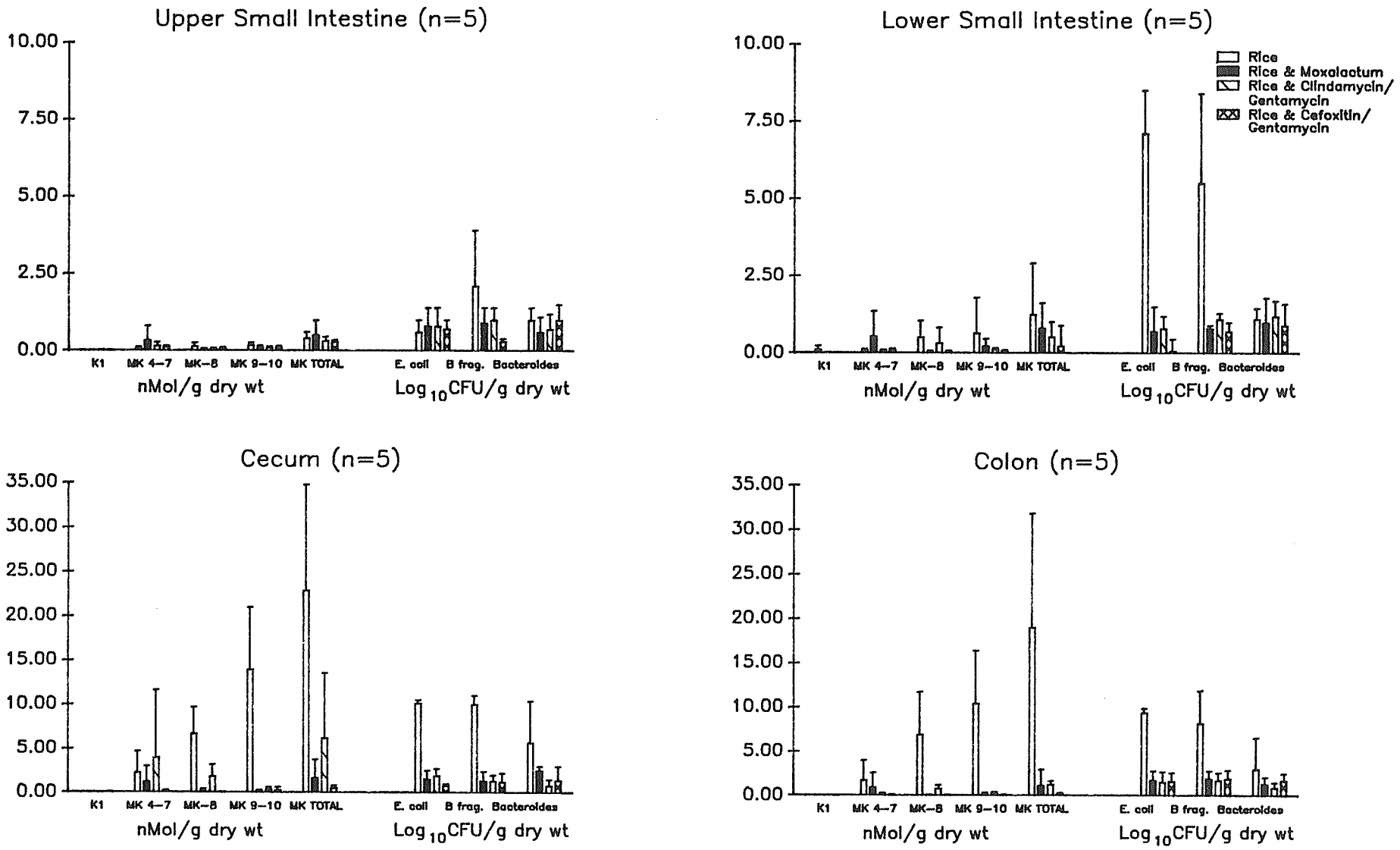


Table 5.34 Pt, vitamin K and bacterial concentration in rats on rice in combination with antibiotics (mean ± S.D.)

	Rice (n=5)	Rice & Moxalactam (n=5)	Rice & Clindamycin/ Gentamicin (n=5)	Rice & Cefoxitin/ Gentamicin (n=5)
Prothrombin Time(s)	15.0 ± 0.5	28.9 ± 13.1	13.5 ± 2.2	22.9 ± 2.6[b]
Serum (pmole/ml)	1.24 ± 0.72	1.22 ± 0.72	1.21 ± 1.00	0.72 ± 0.53
Cecum (nmole/g dry wt)				
K_1	0.01 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.07 ± 0.05
MK 4-7	2.26 ± 2.46	1.23 ± 1.81	3.97 ± 7.65c	0.22 ± 0.07d
MK-8	6.68 ± 3.02	0.19 ± 0.27a	1.81 ± 1.38a	0.03 ± 0.03a
MK 9-10	13.95 ± 7.07	0.24 ± 0.07a	0.38 ± 0.26a	0.28 ± 0.36a
MK Total	22.89 ± 11.87	1.66 ± 2.11a	6.17 ± 7.4a	0.53 ± 0.31a
Cecum (Log_{10} CFU/g dry wt)				
E. coli	10.1 ± 0.4	1.5 ± 1.0a	1.9 ± 0.8a	0.8 ± 0.2a
B. fragilis grp	10.0 ± 1.0	1.3 ± 1.1a	1.3 ± 0.7a	1.2 ± 1.0a
Bacteroides spp.	5.7 ± 4.7	2.5 ± 0.5a	0.8 ± 0.7a	1.4 ± 0.6a
Liver (pmole/g wet wt)				
K_1	13.21 ± 22.87	6.85 ± 15.29	18.03 ± 16.98	0.01 ± 0.01
MK 4-7	148.79 ± 72.01	12.29 ± 13.92	39.65 ± 29.81	6.53 ± 4.69b
MK-8	111.33 ± 59.01	3.62 ± 4.88a	23.15 ± 17.13a	5.51 ± 1.99a
MK 9-10	43.76 ± 37.01	2.87 ± 3.80	15.03 ± 16.06	5.91 ± 5.10
MK Total	303.88 ± 109.93	18.78 ± 17.06e	77.83 ± 62.38	17.95 ± 11.46e

a $p \leq 0.0001$ as compared to rice group
b $p \leq 0.05$ as compared to rice group
c $p \leq 0.01$ as compared to rice & moxalactam group
d $p \leq 0.005$ as compared to rice & clindamycin/gentamicin group
e $p \leq 0.001$ as compared to rice group

an antimicrobial agent containing the NMTT group (moxalactam) and lacking it (cefoxitin), it appears that the coagulopathy is occurring secondary to the removal of the menaquinone producing bacteria and therefore the endogenous pool rather than NMTT inhibition of the microsomal carboxylase.

4. Effect of Synthetic Diet on Menaquinone Levels and Prothrombin Times

In order to determine whether prolonged subsistence on the vitamin K_1 deficient rice diet may induce hypoprothrombinemia, animals were placed on rice for 14 days. In addition, since previous studies had indicated that rats on a synthetic diet of glucose and protein became hypoprothrombinemic, in the absence of antimicrobial administration, the rat model was used to attempt to examine possible mechanism(s) by which coagulopathy occurs while on such a diet. To this end, rats were placed on a glucose-protein diet, as previously described, for 14 days.

Rats on rice for 7 or 14 days or the synthetic diet for 14 days contained concentrations of vitamin K_1 that were uniformly low throughout the intestine--in the order of 0.01 nmole/g dry weight--indicating that the animals were not receiving any vitamin K_1 through their diets. Small intestinal levels of menaquinones were low and similar in all three groups, as were counts of E.coli and B.fragilis group (Table 5.35, Figure 5.19). When cecal levels of total menaquinones and the microflora are compared in rice groups at day 7 and day 14, no major differences are seen in menaquinone concentrations and only slight differences in B.fragilis group

Table 5.35 Vitamin K and bacterial concentrations in the intestinal segments of rats fed rice or a synthetic diet (Mean ± SD)

	nmole/g dry wt					Log_{10} CFU/g dry wt		
	K_1	MK 4-7	MK-8	MK 9+10	MK Total	E. coli	B. fragilis	Bacteroides spp.
Upper Small Intestine								
Rice, d 7 (n=5)	0.01 ± 0.01	0.09 ± 0.04	0.14 ± 0.12	0.15 ± 0.08	0.41 ± 0.20	0.6 ± 0.4	2.1 ± 1.8	1.0 ± 0.4
Rice, d 14 (n=5)	0.01 ± 0.01	0.25 ± 0.26	0.21 ± 0.30	0.39 ± 0.30	0.85 ± 0.83	ND*	ND	ND
Syn Diet, d 14 (n=5)	0.02 ± 0.002	0.35 ± 0.10	0.07 ± 0.06	0.14 ± 0.04	0.56 ± 0.08	ND	ND	ND
Lower Small Intestine								
Rice, d 7 (n=5)	0.01 ± 0.01	0.10 ± 0.04	0.51 ± 0.54	0.65 ± 1.15	1.25 ± 1.66	7.1 ± 1.4	5.5 ± 2.9	1.1 ± 0.6
Rice, d 14 (n=5)	0.01 ± 0.01	1.03[b] ± 0.64	1.06 ± 1.02	2.06 ± 1.77	4.15 ± 2.93	1.8 ± 0.2	3.9 ± 1.6	3.2 ± 2.3
Syn Diet, d 14 (n=5)	0.01 ± 0.004	0.28 ± 0.09	0.30 ± 0.43	0.08a ± 0.05	0.66a ± 0.34	4.8a ± 2.6	1.6a ± 0.2	1.6 ± 0.5

Table 5.35 (Continued)

	nmole/g dry wt					Log_{10} CFU/g dry wt		
	K_1	MK 4-7	MK-8	MK 9+10	MK Total	E. coli	B. fragilis	Bacteroides spp.
Cecum								
Rice, d 7 (n=5)	0.01 ± 0.002	2.26 ± 2.46	6.67 ± 3.03	13.95 ± 7.07	22.89 ± 11.87	10.1 ± 0.4	10.0 ± 1.0	5.7 ± 4.7
Rice, d 14 (n=5)	0.01 ± 0.01	3.26 ± 2.01	6.13 ± 2.88	12.08 ± 4.09	21.46 ± 7.56	9.5 ± 0.8	6.9c ± 0.2	3.4 ± 1.3
Syn Diet, d 14 (n=5)	0.01 ± 0.01	1.68 ± 0.69	3.07a ± 1.11	3.95a ± 1.65	8.69a ± 2.96	6.6a ± 1.1	6.9c ± 0.6	2.5 ± 0.5
Colon								
Rice, d 7 (n=5)	0.01 ± 0.01	1.69 ± 2.32	6.89 ± 4.84	10.40 ± 5.99	18.99 ± 12.88	9.4 ± 0.5	8.2 ± 3.7	3.0 ± 3.6
Rice, d 14 (n=5)	0.01 ± 0.01	1.94 ± 0.74	7.34 ± 3.04	14.93 ± 4.34	24.21 ± 7.34	ND	ND	ND
Syn Diet, d 14 (n=5)	0.01 ± 0.01	1.39 ± 0.16	3.45a ± 1.18	4.72a ± 1.48	9.56a ± 2.41	ND	ND	ND

a $p \leq 0.05$ as compared to rice, d 14
b $p \leq 0.03$ as compared to rice, d 7 group
c $p \leq 0.002$ as compared to rice, d 7
* ND - Not done

Figure 5.19 Vitamin K and bacterial concentrations (mean ± S.D.) of intestinal segments of rats fed rice for 7 or 14 days and a synthetic diet for 14 days.

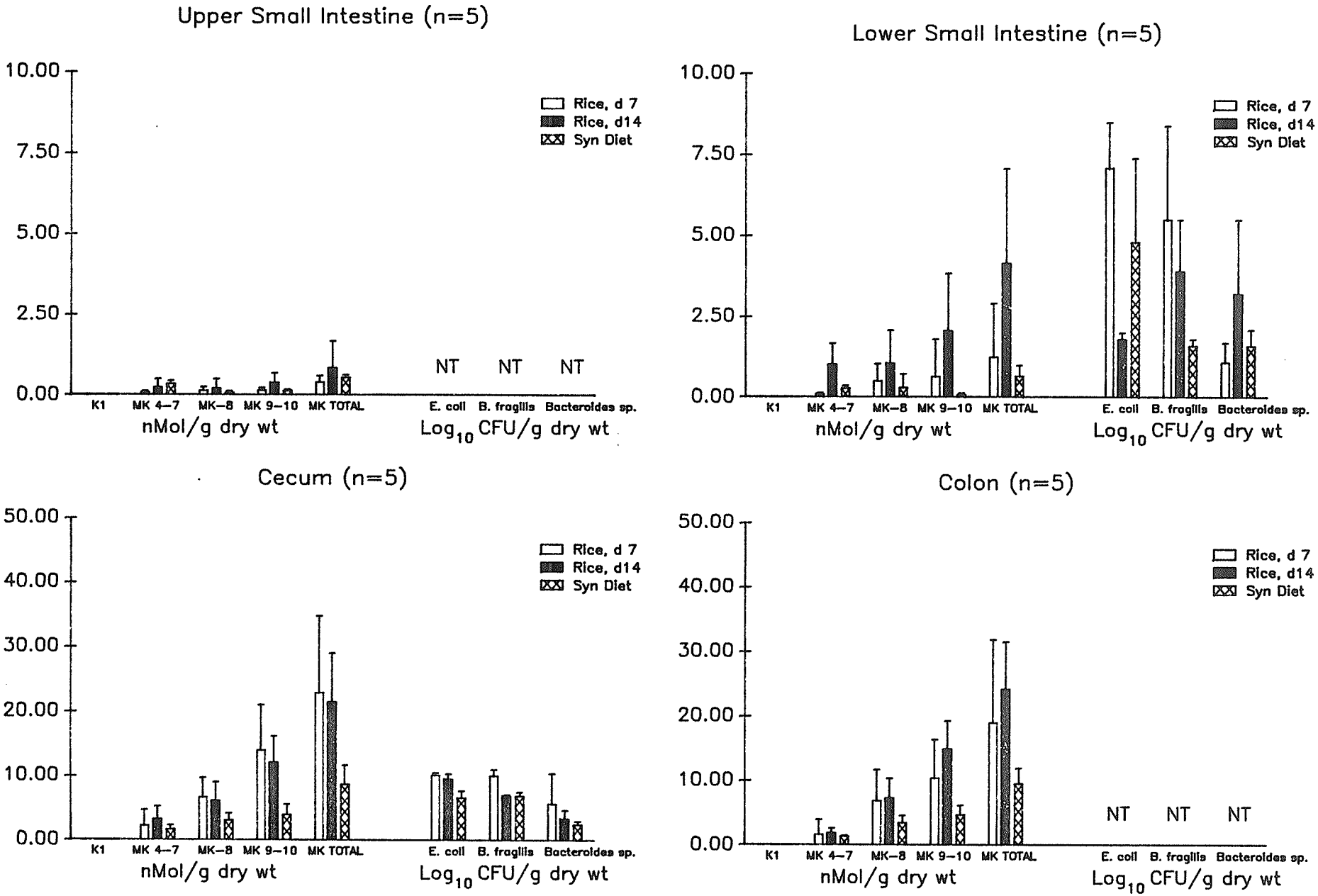


Table 5.36 PT, vitamin K and bacterial concentration in rats fed rice or a synthetic diet (mean ± SD)

	Rice, d 7 (n=5)	Rice, d 14 (n=5)	Synthetic Diet, d 14 (n=5)
Prothrombin Time(s)	15.0 ± 0.5	13.2 ± 1.0	36.6 ± 11.4a
Serum (pmole/ml)	1.24 ± 0.72	1.32 ± 0.72	1.64 ± 1.88
Cecum (nmole/g dry wt)			
K_1	0.01 ± 0.002	0.01 ± 0.01	0.01 ± 0.01
MK 4-7	2.26 ± 2.46	3.26 ± 0.01	1.68 ± 0.69
MK-8	6.67 ± 3.03	6.13 ± 2.88	3.07 ± 1.11b
MK 9-10	13.95 ± 7.07	12.08 ± 4.09	3.95 ± 1.65b
MK Total	22.89 ± 11.87	21.46 ± 7.56	8.69 ± 2.96b
Cecum (Log_{10} CFU/g dry wt)			
E. coli	10.1 ± 0.4	9.5 ± 0.08	6.6 ± 1.1c
B. fragilis grp	10.0 ± 1.0	6.9 ± 0.2d	6.9 ± 0.6d
Bacteroides spp.	5.7 ± 4.7	3.4 ± 1.3	2.5 ± 0.5
Liver (pmole/g wet wt)			
K_1	13.22 ± 22.87	7.48 ± 8.09	6.99 ± 10.07
MK 4-7	148.79 ± 72.01	4.43 ± 8.07	1.69 ± 2.69
MK-8	111.33 ± 59.01	1.63 ± 1.73	1.57 ± 2.08
MK 9-10	43.76 ± 37.01	4.73 ± 4.78	1.33 ± 2.59
MK Total	303.88 ± 109.93	10.79 ± 12.89d	4.59 ± 5.02d

a $p \leq 0.002$ as compared to rice, d 14
b $p \leq 0.05$ as compared to rice, d 14
c $p \leq 0.001$ as compared to rice, d 14
d $p \leq 0.05$ as compared to rice, d 7

counts. Total menaquinone concentration in these two were 22.89 ± 11.87 nmole/g dry weight and 21.46 ± 7.56 nmole g/dry weight, respectively, with MK 9 and 10 constituting 60% of this and MK-8 another 30%. E.coli counts were similar in both groups but rice, day 14 had 3 Log_{10} CFU/g dry weight less organisms (Table 5.36).

Rats on the synthetic diet of Mameesh and Johnson developed prolonged prothrombin times. Total cecal menaquinone concentration was 8.69 ± 2.96 nmole/g dry weight representing almost 3 fold lower levels observed in rats on rice but similar to those on chow (Table 5.30 and 5.36). MK-8, MK-9 and 10 concentrations were 2 and 3 fold lower than oberved in rats on rice. MK 9 + 10 concentrations were similar to those seen in the chow group. Both E.coli and B.fragilis group organisms were reduced approximately 3 log_{10} CFU/g dry weight compared to rice groups. Compared to chow fed rats, E.coli counts were similar but there was a slight decrease in B.fragilis group counts. At 8.69 ± 2.96 nmole/g dry weight total menaquinone concentration, there appeared to be a sufficiently high endogenous pool to maintain normal coagulation. However, these animals contained significantly low menaquinone stores in their livers. It appears that even though there was an adequate pool of menaquinone in the cecum, the extremely low levels in the liver (4.59 ± 5.02 pmole/g dry weight) (Table 5.36) indicates that unknown factors may be interfering with absorption and could explain the effect of this diet.

5. Reversal of Hypoprothrombinemia by Feeding B. fragilis and Recolonization

Using the rice plus cefoxitin/gentamicin model,

hypoprothrombinemia was induced in rats after 7 days and then an attempt made to reverse the coagulopathy by feeding the animals killed B.fragilis or allowing recolonization of the intestine with menaquinone producing bacteria.

After 7 days on the antimicrobial regimen, prothrombin times in a group of 5 animals were elevated to a mean value of 25.6 ± 3.95 (control $\leq$ 15.0s). Since this group was chosen randomly from rats under the same treatment conditions, it was likely that all rats were hypoprothrombinemic at day 7.

Cecal concentrations of MK 4-10 were reduced to 1% of values found in rats ingesting rice alone. Hepatic concentrations of vitamin K_1 and menaquinone were reduced as well (Table 5.37, Figure 5.20).

Continuation of the drug regimen for another 5 days further elevated prothrombin times to levels almost double that at day 7 (46.8 ± 5.6s)(Table 5.37). Cecal menaquinone concentrations remained low and at the same level as rats on antibiotic for 7 days. However, there was a further 5 fold decrease in liver concentrations indicating that the hepatic pools were progressively declining in face of cecal menaquinone deficiency.

Animals that were fed 1 x 10^{11} CFU/day of killed B.fragilis ATCC 23745 for 3 and 5 days, respectively, while being kept on cefoxitin/gentamycin showed normalization of prothrombin times (Table 5.37). Both groups had elevated MK 9 + 10, and total menaquinone concentrations in the small intestine. These concentrations were actually higher than usually seen in rats on rice alone. Rats on rice alone contained a total menaquinone concentration of 1.25 ± 1.66 nmole/g dry weight, about half that found in the groups fed B.fragilis

for 3 and 5 days which had 2.38 ± 0.12 and 2.29 ± 0.58 nmole/g dry weight respectively (Table 5.37).

Cecal menaquinone concentrations also showed similar increases in animals fed B.fragilis. In the case of the cecum, the group fed organisms for 3 days contained 4.94 ± 1.79 nmole/g dry weight while the other fed for 5 days contained 2.49 ± 1.08 nmole/g dry weight. These increases were reflected exclusively in increases of MK 9 + 10 in the cecum and represented approximately 10 fold higher levels in control animals on antibiotics for 7 or 12 days and not fed B.fragilis. However these concentrations were only about 20% of values seen in rats fed rice for 7 days. The ceca of these animals were not cultured as periodic swabbing of their feces during the period they were being fed killed bacteria indicated that there were no live organisms present.

Hepatic levels of MK 9 and 10 and total menaquinones had also increased in a manner paralleling that of the cecum. At 20.32 ± 13.24 pmole/g dry weight and 23.08 ± 19.73 nmole/g wet weight, these levels represented 96% and 84% of total menaquinones in the liver of rats fed B.fragilis for 3 and 5 days respectively. Again, like the cecum, however, the total menaquinone content of the liver in rats fed B.fragilis was only about 6% of the values seen in rats fed rice alone.

Rats taken off the antibiotic suppression for 5 days had prothrombin times that were still elevated and to a degree higher than those on cefoxitin/gentamicin for 7 days, but less so than those on antibiotic for 12 days. Despite no ingestion of the antimicrobial regimen for 5 days, the number of B.fragilis and E.coli remained at

undetectable levels. Hepatic concentrations of MK 9 and 10 and total menaquinone remained low and were similar to concentrations seen in livers of rats receiving 12 days of antibiotic suppression. It appears therefore that 5 days was insufficient time to allow recolonization of the cecum or small intestine and enable the intestinal menaquinone source to restore hepatic menaquinone concentration.

When rats fed cefoxitin/gentamicin for seven days were allowed to receive the rice diet without antibiotic suppression for 20 days, prothrombin times returned to normal values (12.4 ± 0.65 s). The menaquinone producing flora--E.coli and B.fragilis group recovered to concentrations of 5.6 ± 1.8 and 8.7 ± 1.4 $\log_{10}$ CFU/g dry weight, respectively, somewhat lower than seen in rats on rice only. At the same time, the menaquinone concentration in the small intestine increased by 5 times over that which is normally seen in rats fed rice alone. MK 9 and 10 constituted 50% of the total concentration which was 6.42 ± 4.55 nmole/g dry weight. Cecal menaquinone totals also increased to 9.36 ± 3.75 nmole/g dry weight, of which MK 9 + 10 represented 90%. Hepatic menaquinone increased to levels similar to those seen in rats that had been fed B.fragilis but 10 fold lower than that normally seen in rice fed rats (Table 5.37, Figure 5.20).

Since it is probable that oral ingestion of bacterial products in such large numbers is not likely to occur in man, additional reconstitution experiments of a preliminary nature were performed by surgically implanting the same number of live or dead organisms into tied (obstructed) cecal loops. None of the 5 rats corrected their prothrombin times after 24 hours, but one of three did by 72 hours. Thrice daily enemas containing the same number of liver or dead

organisms, suspended in a volume (7-8 ml) sufficient to perfuse the cecum but not the small bowel, corrected prothrombin times in 4/4 rats while they were on cefoxitin/gentamicin treatment with the rice diet.

Table 5.37 Restoration of normal PT in rats by feeding killed B. fragilis or by recolonization with menaquinone-producing flora (mean ± S.D.)

	(n=5) Rice d 7	(n=5) Rice+CFX/G d 7	(n=5) Rice+CFX/G d 12	(n=5) Rice+CFX/G d 10 & Bf d 8-10	(n=5) Rice+CFX/G d 12, & Bf d 8-12	(n=5) Rice+CFX/G d 7, No drug d 8-12	(n=5) Rice+CFX/G d 7, No drug d 8-28
Prothrombin Time(s)	15.0 ± 0.5	25.6a ± 3.9	46.8a ± 5.6	12.0 ± 0.5	12.1b ± 0.7	38.1 ± 15.9	12.4 ± 0.6
*Small Intestine (nmole/g dry wt)							
K_1	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.005	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
MK 4-7	0.10 ± 0.04	0.14 ± 0.03	0.09 ± 0.05	0.12 ± 0.03	0.12 ± 0.06	0.13 ± 0.05	1.08 ± 1.88
MK-8	0.51 ± 0.54	0.05 ± 0.05	0.08 ± 0.05	0.05 ± 0.03	0.08 ± 0.03	0.10 ± 0.08	1.89 ± 2.31
MK 9 + 10	0.65 ± 1.15	0.10 ± 0.07	0.13 ± 0.05	2.22c ± 0.69	2.07b ± 0.61	0.19 ± 0.16	3.45b ± 0.82
MK Total	1.25 ± 1.66	0.29 ± 0.29	0.30 ± 0.07	2.38c ± 0.12	2.29b ± 0.58	0.43 ± 0.13	6.42b ± 4.55

Table 5.37 (Continued)

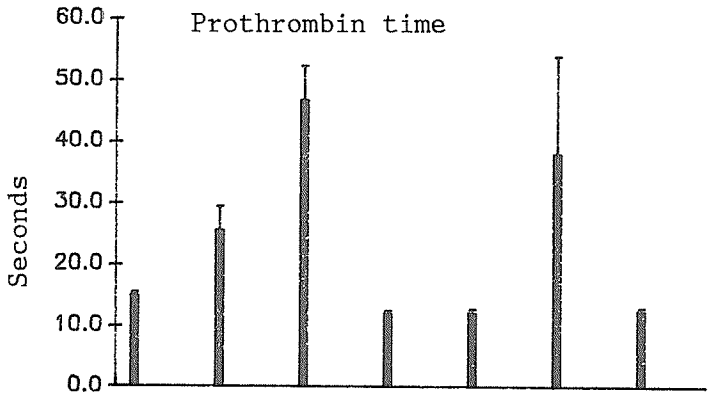
Cecum (nmole/g dry wt)							
K_1	0.01 ± 0.002	0.01 ± 0.01	0.01 ± 0.003	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.003	0.01 ± 0.01
MK 4-7	2.26 ± 2.46	0.11 ± 0.07	0.11 ± 0.06	0.19 ± 0.16	0.15 ± 0.07	0.10 ± 0.06	0.14 ± 0.11
MK-8	6.67 ± 3.03	0.08 ± 0.02	0.08 ± 0.05	0.19 ± 0.16	0.15 ± 0.09	0.35 ± 0.61	0.92 ± 1.14
MK 9 + 10	13.95 ± 7.07	0.13 ± 0.07	0.14 ± 0.06	4.57 ± 1.65	2.19 ± 0.95	1.95 ± 3.99	8.30b ± 3.62
MK Total	22.89 ± 11.87	0.32 ± 0.12	0.33 ± 0.09	4.94 ± 1.79	2.49 ± 1.08	2.40 ± 4.59	9.36b ± 3.75
Cecum (Log_{10} CFU/g dry wt)							
E.coli	10.1 ± 0.4	ND*	2.8 ± 0.3	ND	ND	2.7 ± 0.4	5.6b ± 1.8
B.fragilis	10.0 ± 1.0	ND	2.4 ± 0.4	ND	ND	2.7 ± 0.3	8.7b ± 1.4
Bacteroides spp.	5.7 ± 4.7	ND	2.4 ± 0.5	ND	ND	2.8 ± 0.4	3.2 ± 2.5

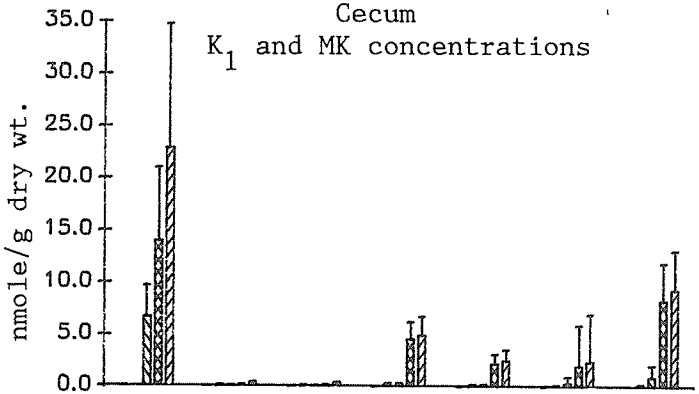
Table 5.37 (Continued)

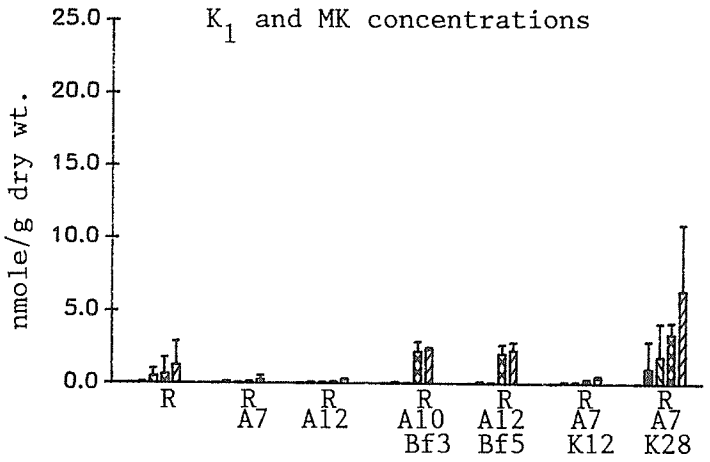
Liver (pmole/g wet wt)							
K_1	13.22 ± 22.87	8.49 ± 14.97	1.39 ± 1.49	0.01 ± 0.01	0.01 ± 0.01	1.20 ± 1.68	0.01 ± 0.01
MK 4-7	148.79 ± 72.01	12.90 ± 15.01	2.14 ± 4.57	0.07 ± 0.04	2.22 ± 3.65	11.07 ± 22.27	15.58 ± 17.74
MK-8	111.93 ± 59.01	0.07 ± 0.05	0.11 ± 0.02	5.20 ± 3.93	2.32 ± 3.86	1.04 ± 2.16	11.25 ± 25.00
MK 9 + 10	43.76 ± 37.01	1.63 ± 3.04	0.14 ± 0.05	20.32 ± 13.24	23.08 ± 19.73	0.14 ± 0.04	2.86 ± 3.91
MK Total	303.88 ± 109.43	14.60 ± 13.55	2.38 ± 4.55	21.17 ± 13.60	27.61b ± 25.86	12.25 ± 22.01	29.69 ± 37.97

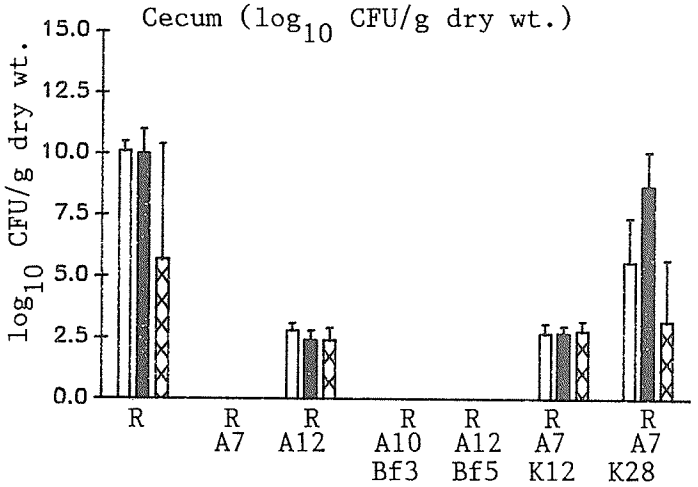
* ND - Not done
a $p \leq 0.05$ as compared to rice, d 7
b $p \leq 0.05$ as compared to rice & CFX/G, d 12
c $p \leq 0.05$ as compared to rice & CFX/G, d 7

Figure 5.20 Restoration of normal PT by feeding killed B. fragilis and by recolonization with MK-producing flora in hypoprothrombinemic rats (results given as mean ± S.D; n=5 rats/group).









6. DISCUSSION

A. Chromatography

Thin layer chromatography is the most widely used method for the determination of menaquinones in bacteria. Preparative TLC followed by reverse phase TLC of extracts remains a useful method for the analysis of menaquinone profiles in bacteria. The sensitivity of the system has been improved upon to the extent that a commercially available "high performance" TLC plate is available which is reputed to be able to detect 10 ng of menaquinone (Collins et al, 1977). Usually, identification of particular menaquinones is done by using authentic standards and comparing Rf values or in other cases mass spectral analysis of the quinone. Often both methods are used.

More recently, HPLC has been used with excellent results for the detection of menaquinone in bacteria (Tamaoka et al, 1983).

In the current study, TLC was largely used to survey bacterial strains for their menaquinone profile, identification of the components being exclusively done by comparing their Rf values with those of standards. A number of strains were analysed by both TLC and HPLC and the minor differences seen related to increased sensitivity of the HPLC method.

The assay of vitamin K_1 and especially menaquinone from biological samples always presents a problem. Because of large quantities of contaminating lipids and the low concentrations of the compound of interest, successive chromatograpic steps prior to HPLC analysis are necessary to obtain a sample of sufficient purity which would be suitable for analysis. Multiple chromatographic steps may introduce errors and decrease recovery of the vitamin, thus reducing the sensi-

tivity of the assay. Also contaminants may mask peaks of interest.

Improvement of sensitivity and selectivity was achieved in the HPLC method in this study by utilising wet chemical post column reduction of the quinone to the hydroquinone followed by detection with fluorescence spectroscopy. Fluorescence detection is inherently more sensitive than UV absorption. Additionally, since there is less naturally fluorescing material in the samples, selectivity is increased since fewer interfering peaks seen with UV detection are observed. Detection was linear well over the range of concentrations of vitamin K_1 and menaquinone encountered in this study. The lower limit of detection was in the nmole range for both forms of vitamin K.

A suitable internal standard could not be found for the menaquinone assays since all the different molecular forms of menaquinone were present in samples. Hence determination of menaquinones was carried out without correction for incomplete recovery during extraction or losses during chromatography. However, it was demonstrated both with human feces and rat cecal contents that recovery rates of both forms of the vitamin from spiked samples were generally in the order of 90%, indicating good agreement between the calculated and added values of the vitamin. Repeated analysis of samples showed good precision in these determinations.

The serum vitamin K_1 assay utilized MK-4 as the internal standard. This compound was chosen because it could not be detected in serum at the sensitivity of the assay. Also, it co-eluted with vitamin K_1 from Sep-Pak® chromatography but was well separated on reverse phase HPLC. The precision of the method was validated by repeated

analysis of spiked serum on the same and on different days. The coefficient of variation was about 10% indicating reasonably good precision.

In all the assays, peak detection was carried out based on comparison of retention times of standards and unknown peaks. The purity of the peaks was not assessed by other means such as UV or mass spectral analysis.

B. Choice and Dosage of Antimicrobial Regimens

Moxalactam has been frequently associated with clinical hypoprothrombinemia and therefore this drug was used with the vitamin K_1 deficient rice diet to determine the mechanism by which it induces coagulopathy. Moxalactam is excreted into the intestinal tract via the bile in sufficient concentrations to affect the intestinal flora (Shimada and Ueda, 1982). At the dosage used in the study, moxalactam concentrations in the cecum, as measured by bioassay were high enough to cause suppression of the menaquinone producing flora such as B.fragilis group organisms.

Cefoxitin and clindamycin were chosen because of their significant in-vitro activity against anaerobes, including B.fragilis group organisms (Brodgen et al, 1979). Used in combination with gentamicin which is active against the gram-negative facultatively anaerobic organisms such as E.coli, it was felt that these antimicrobial combinations would eradicate the menaquinone producing flora in the intestine. Both of these agents did not contain an NMTT side chain that may cause inhibition of the hepatic carboxylase. Furthermore, gentamicin and cefoxitin are very poorly absorbed after oral adminis-

tration. Therefore, should hypoprothrombinemia occur with these regimens, it is unlikely it would be due to the inhibition of the hepatic microsomal carboxylation reaction. There was concern that clindamycin would be sufficiently well absorbed that concentrations in the intestinal lumen would be insufficient to suppress intestinal anaerobes. In humans, 90% of clindamycin is absorbed after oral administration. Hence, it was important in these experiments to use two types of regimens (clindamycin/gentamicin and cefoxitin/gentamicin) to suppress the intestinal microflora. Measurement of antimicrobial concentrations in the cecum showed that both regimens attained sufficient concentrations to suppress or eliminate the indigenous microorganisms. Some absorption of cefoxitin did occur as reflected by mean serum concentrations of 4.3 ug/mL.

C. Menaquinone Profiles of Bacteria

In this study representative members of the microbial genera most frequently recovered from feces were examined by chromatographic methods, (mainly TLC) for their ability to produce menaquinones. Prior to this study only a limited number of strains in the genera Bacteroides, Lactobacillus, Bifidobacterium, Clostridium and Actinomyces had been analysed for menaquinone profiles (Collins and Jones, 1981; Hess et al, 1979; Shah and Collins, 1980). Thus, there was limited knowledge as to the spectrum of menaquinone production by bacteria of clinical importance. Additional organisms tested in this study included K.pneumoniae, Fusobacterium spp., organisms of the B.bivius-B.disiens complex, Eubacterium spp., Peptostreptococcus spp., Peptococcus spp., Veillonella spp., and Arachnia spp. The findings with regard to production of MK-8 by E.coli confirm those of previous

studies (Bishop et al, 1962; Newton et al, 1971; and Wallace and Young, 1977). In addition, the other gram-negative facultative rod tested, K.pneumoniae also contained MK-8. All the Bacteroides fragilis group organisms examined contained MK-10, MK-11, while some organisms also produced minor quantities of MK-9. These findings extend those of Shah and Collins (1980) who also found similar profiles in organisms analysed by TLC, in their study. They examined two strains each of B.fragilis, B.vulgatus, and B.thetaiotaomicron.

Studies of the fecal flora have indicated that Bacteroides fragilis group organisms are the predominant gram-negative obligate anaerobes found in the bowel with counts as high as 10^{11-12} CFU/g dry weight of feces. As well, E.coli counts can be found in the order of 10^{11} CFU/g dry weight feces (Finegold, 1977), but the usual ratio of anaerobic to aerobic is 100 to 1000 to 1. It appears therefore that based on numerical predominance, B.fragilis group and E.coli probably constitute the majority of the menaquinone pool in the intestinal lumen. This supposition is supported by the chromatograms of fecal matter in rat and man showing the contribution of MK-8, MK-9, MK-10 (and probably MK-11) to total menaquinone content in feces.

On the basis of a single strain examined, Veillonella spp. may also produce menaquinones. Additional strains must be examined to confirm this observation.

E.lentum, P.acnes, P.jensenii, and A.propionica all produced a single menaquinone isoprenologue having the same mobility characteristics on reverse phase TLC, indicating that the compound was the same one in all the organisms. With the techniques used in this study precise identification of this menaquinone isoprenologue was not pos-

sible. Migration characteristics on TLC indicate that the menaquinone produced by these organisms may perhaps have a partially saturated side chain. In reverse phase TLC systems, increasing the length of the isoprenoid side chain by addition of an isoprene unit leads to a negative shift in Rf value. Saturation of a double bond in the side chain also leads to a negative shift in Rf value but to a lesser extent than when an isoprene unit is added (Dunphy et al, 1971; Phillips et al, 1969). Thus on reverse phase TLC, MK-9 (H_2) would migrate to a point between MK-9 and MK-10 and MK-9 (H_4) probably between MK-10 and MK-11. The unidentified spot seen in the organisms examined here migrated between MK-10 and MK-11. Furthermore, previous studies have shown that P.acnes, P.arabinosum,and P.shermanii produce MK-9 (H_4) as the major menaquinone (Sone, 1974a; Collins and Jones, 1981). This finding tends to support the argument that the menaquinone recovered from the Propionibacterium spp. as well as the Arachnia spp. and Eubacterium spp. was MK-9 (H_4).

Other genera of the intestinal anaerobes including Bifidobacterium, Lactobacillus, Peptostreptococcus, Clostridium and Fusobacterium did not produce menaquinones, in vitro, in this study. Although peptostreptococci, peptococci and bifidobacteria have previously been reported to produce menaquinones, these analyses were done using a microbiological plate assay system with vitamin K requiring B.melaninogenicus as the indicator organism (Fernandez and Hill, 1975; Gibbons and Engle, 1964). This assay is of limited specificity and does not yield incontrovertible evidence that vitamin K is actually produced by the tested strains. The study of Hess et al (1979), examined Bifidobacterium spp. for menaquinones by TLC with negative results.

Eubacterium is also one of the genera that predominates in the human intestine, occurring at counts up to 10^{12} CFU/g dry weight in some individuals. One of the strains of E.lentum examined in this study contained MK-7 with two others containing an unidentified menaquinone thought to be partially saturated. E.lentum may therefore contribute to the menaquinone reservoir in the gut. It should be pointed out that menaquinones with partially saturated side chains are considered less bioactive than fully saturated ones (Parrish, 1980). This may reduce the contribution of E.lentum in terms of biologic activity of menaquinones in general to the host mammal.

It is generally accepted that lactobacilli lack menaquinones. A single report has described menaquinones in L.casei (MK-n) (Hess et al, 1979) but similar strains tested in this study contained none. The discrepancy could well be due to strain differences.

None of the Clostridium spp. contained menaquinones, a finding that is in agreement with those of other studies (Hess et al, 1979). The lack of menaquinones in this group of organisms is consistent with the lack of respiratory chain in Clostridium species.

D. Hypoprothrombinemia in Neutropenic Patients

Severely ill, febrile neutropenic patients on antimicrobial therapy provide the ideal group in which to study antibiotic associated hypoprothrombinemia. Such subjects are usually on a severely reduced dietary intake because of anorexia, nausea, vomiting, mucositis, and debilitation associated with chemotherapy and/or radiotherapy for malignant diseases. Dietary intake is most often curtailed when the patients are profoundly neutropenic and most

susceptible to infection. In addition, the intake of foods high in vitamin K_1 content such as fresh fruits and green leafy vegetables is restricted in this population because of large numbers of potential pathogens colonizing these foods. It is essential that subjects with depressed immune systems due to chemotherapy and/or radiotherapy be prevented from exposure to potential pathogens (Remington and Schimpff, 1981). As a result, the intake of vitamin K_1 in such a patient population is poor.

The vitamin K deficient state in these patients may also be promoted by several other factors. Chemotherapy may alter absorption of the vitamin in the intestine or the half-life of the vitamin K-dependent factors may decrease, especially during febrile illness, leading to increased daily requirements (Loeliger et al, 1963). Additionally, the turnover time of vitamin K_1 was estimated at 153 minutes, indicating that the total body pool is replaced every 2-1/2 hours (Bjornsson et al, 1980). This suggests that in the absence of adequate vitamin K_1 supplies, rapid depletion of hepatic stores will occur.

In this study, febrile neutropenic patients suffering from leukemia and solid tumors were enrolled in studies to empirically test the efficacy of various broad spectrum antimicrobial regimens. Hypoprothrombinemia occurred in a significantly larger proportion of patients receiving moxalactam in combination with either tobramycin or ticarcillin, compared to those on other regimens such as aztreonam/tobramycin, aztreonam/cloxacillin or tobramycin/ticarcillin. This finding is similar to those of other reports of hypoprothrombinemia in patients using moxalactam (Pakter, et al, 1982; Bang et al, 1982;

Weitekamp and Aber, 1983; Panwalker and Rosenfeld, 1983; Holt et al, 1983; Mathisen et al, 1982; Schentag et al, 1983; Fainstein et al, 1983; Joehl et al, 1983 and MacLennan et al, 1983). Antibiotic-associated hypoprothrombinemia has frequently been seen in hospitalized, debilitated patients on a reduced vitamin K_1 intake (Klippel and Pitsinger, 1968; Ham, 1971, Pineo et al, 1973; Ansell et al, 1977; Reddy and Bailey, 1980; Rymer and Greenlawn, 1980; and Pakter et al, 1982). The mechanism by which hypoprothrombinemia occurs is uncertain. Two possible mechanisms have been suggested. These are: (1) direct inhibition of the post-translational carboxylation of vitamin K-dependent factors by the NMTT leaving group in beta-lactam antibiotics, and (2) deficiency of vitamin K resulting from eradication of menaquinone producing bacteria in the intestine and concommitant loss of the endogenous pool while on a low dietary vitamin K_1 intake.

Because of a steric similarity between NMTT and glutamic acid, the former may act as a competitive inhibitor for the carboxylase enzyme in liver microsomes. It is hypothesized that the NMTT moiety must first be cleaved from the intact antibiotic by bacterial enzymes in the intestine and absorbed into the hepatic circulation reaching the liver where it reaches sufficiently high concentrations to exert its inhibitory effect (Lipsky, 1983). Lipsky (1983) found as low as 1.1 mM NMTT caused 50% inhibition of the carboxylase enzyme in-vitro; however Uotila and Suttie (1983) found that 36.5 mM NMTT was required for the same level of enzyme inhibition. These authors suggested that even though inhibition occurs in vitro, it is, perhaps, unlikely that pharmacologically significant levels of NMTT can be reached, in-vivo, to exert its effect. Patients given four, 3 g doses of moxalactam at

8 hour intervals attained peak levels of 0.3 mM in their plasma (Smith and Sundboom, 1984). Since vitamin K-responsive coagulopathy is also produced by antimicrobial agents that lack the NMTT group, it is more likely that hypoprothrombinemia occurs secondary to the inhibition of menaquinone producing intestinal bacteria. Ampicillin, cloxacillin, cefazolin, cephaloridine, cefoxitin, chloramphenicol and tetracycline (Ham, 1971; Pineo et al, 1973; Klippel and Pitsinger 1968; Colvin and Lloyd, 1977; Reddy and Bailey, 1968; Lerner and Lubin, 1974) have all been associated with hypoprothrombinemia and many of these agents are excreted via biliary secretion into the gut in concentrations which may have a significant impact on the microflora (Mortimer et al, 1969; Ratzan et al, 1978). As well, antimicrobial agents containing the NMTT group such as moxalactam have been shown to be excreted into the bile at levels capable of suppressing the intestinal flora. Shimada and Ueda (1982), demonstrated biliary levels of nearly 70 ug/ml, 4 hours after a 1 g intravenous dose of moxalactam was given to healthy volunteers.

The capacity of the antibiotics to reduce the fecal flora has been described in various studies. Cefoperazone (Mulligan et al, 1982), combinations of ampicilin/sulbactam (Kager et al, 1982) and ampicilin/gentamicin (Bennett et al, 1981), as well as cefmetazole, cefmenoxime, ceftazidime and moxalactam (Sunakawa et al, 1984) all caused decreases or total eradication of coliforms (E.coli, Klebsiella spp.) and anaerobes such as B.fragilis group organisms, and Eubacterium spp. These organisms are the ones we have previously identified as being the major contributors to the endogenous menaquinone pool.

In this study we have demonstrated that patients on moxalactam, in which the highest incidence of hypoprothrombinemia occurred, had almost complete eradication of B.fragilis group organisms. B.fragilis group, as has been previously discussed, produces MK-9, MK-10 and MK-11 and probably constitutes the major source of endogenous menaquinone. The loss of the major menaquinone microflora, correlated well with the reduction of menaquinone concentrations in the feces of these patients. These subjects had reduction of total menaquinone levels in the 90% range, and this was almost exclusively due to loss of MK-9 and MK-10. These data support the hypothesis that hypoprothrombinemia associated with the use of broad spectrum β-lactam antibiotics, is occurring secondary to the eradication of the menaquinone producing microflora and the loss of the endogenous menaquinone stores in the intestines.

E. Hypoprothrombinemia in Healthy Volunteers

To further test the hypothesis that antimicrobial suppression of the intestinal flora reduces the amount of available menaquinone, thus leading to hypoprothrombinemia, a study was designed to use an antimicrobial agent lacking NMTT. Ciprofloxacin is a quinolone antibiotic having some activity against B.fragilis group organisms (MIC_{90} = 8.16 ug/ml with gut concentrations >500 ug/gram feces). Additionally, the study was designed to use a vitamin K_1 deficient diet (O'Reilly, 1971) to remove a confounding variable which could obscure the biologic effect of menaquinones produced by the intestinal bacteria, if it was present.

Removal of this variable would allow the impact of the endogenous menaquinone pool on human vitamin K requirements to be assessed. The

higher doses of ciprofloxacin, (250 mg and 500 mg) reduced but did not eradicate the B.fragilis group organisms in subjects on a vitamin K_1 deficient diet. Volunteers on the 500 mg dose and their regular diet demonstrated no significant reduction of B.fragilis group counts after 7 days of therapy.

From the data generated, a direct correlation between the occurrence of hypoprothrombinemia in volunteers on the vitamin K_1 deficient diet and low serum and fecal vitamin K_1 and fecal menaquinone levels could not be shown. Hypoprothrombinemia was observed only in subjects ingesting the deficient diet and who received sufficiently high dosages of antimicrobial agents to partially suppress the microflora. However, of the four individuals with hypoprothrombinemia, only one (a recipient of 500 mg ciprofloxacin b.i.d.) showed marked reduction of fecal menaquinones, eradication of both E.coli and B.fragilis group organisms and elevation of the prothrombin time in conjunction with low serum vitamin K_1 concentrations. The other subjects with hypoprothrombinemia were observed to have low serum vitamin K_1 concentrations, eradication of E.coli, reduction of B.fragilis group counts but increased fecal menaquinone concentrations. Thus, only in one subject was the data able to support the hypothesis that hypoprothrombinemia occurs secondary to removal of the menaquinone source.

A number of factors may have contributed to the failure to demonstrate a clear cut correlation between hypoprothrombinemia, bacterial suppression and reduction of the menaquinone pool. One of these was the poor choice of drug probe leading to modest reduction of the menaquinone producing flora. A probe which would produce marked changes in the microflora would have been more clear cut. As well, it would

have overridden the effect of the vitamin K_1 deficient rice diet. Secondly, the O'Reilly diet used to remove a confounding variable that would obscure the effect of menaquinone on coagulation homeostasis presented its own, unexpected problem. While the vitamin K_1 deficient diet reduced fecal and serum vitamin K_1 concentrations by day seven, it stimulated menaquinone production in the gut, as shown by findings in the placebo group, where there was an overall increase in menaquinone concentrations in feces. The dietary effect suggested that a change in the metabolic activity of the microflora to adapt to the change in substrate conditions had occurred, since the overall numbers of menaquinone producing bacteria remained constant. Thus, the stimulatory effect of the diet on menaquinone production coupled with the inadequate reduction of the microflora prevented the decrease of the menaquinone pool to levels which would induce hypoprothrombinemia. The wide variations in fecal menaquinone profiles between different persons also posed a problem in statistical analysis. Finally, data obtained from fecal samples may not be reflective of microbial activity elsewhere in the large bowel.

Since ciprofloxacin is not known to have an effect similar to that of NMTT on the liver carboxylase, and since hypoprothrombinemia only occurred in subjects at high antibiotic dose while on vitamin K_1 deficient diet, these findings to some extent validate the hypothesis that bacterially synthesized menaquinones contribute to normal coagulation homeostasis.

The serum vitamin K_1 levels in fasting individuals were for the most part undetectable as were the post-fasting mid-day levels in subjects ingesting the vitamin K_1 deficient diet. Unexpectedly, post-

fasting mid-day levels in subjects on a normal diet were also low, increasing in only one of five subjects. We failed to specify the vitamin K_1 intake on the "normal" diet. Intake of the vitamin could have been low. Furthermore, the vitamin is cleared rapidly from the serum. Measurement of serum vitamin K_1 concentrations as an indicator of the vitamin K_1 deficient state may be unreliable. Shearer et al, (1972), using K_1-^3H delivered intravenously into human volunteers found rapid clearance of the label from the plasma in these subjects. After 2 hours only 10% of the initial label remained. Comparison of fecal concentrations of MK 9 and 10 and B.fragilis group counts in the feces of vitamin K_1 deficient diet subjects, regardless of drug regimen, showed that the MK 9 and 10 concentrations paralleled B.fragilis group counts. Since B.fragilis group organisms produce MK-9, MK-10 and MK-11, and since MK-9 and MK-10 usually account for 80-90% of the total menaquinone concentration in fecal samples, it is likely that this group or organisms account for the majority of the endogenous menaquinone pool.

F. Antibiotic Associated Hypoprothrombinemia in the Rat Model

Development of the animal model to examine the mechanism of antibiotic associated hypoprothrombinemia had several advantages. The diets of the animals could be manipulated to a greater extent than the human studies and their vitamin K_1 intake closely monitored. Analysis of in-situ microflora and menaquinone concentrations at the various levels of the animal intestinal tract was possible. This would serve to identify the area(s) of the intestine containing the major endogenous pool and determine the relative importance of the different

levels of the gut with respect to potential contribution to the animals' vitamin K requirement. The vitamin K state of the animal could be directly determined by examining the livers for both vitamin K_1 and menaquinone concentrations. In the human volunteer study, subjects were placed on a vitamin K_1 deficient rice diet which unexpectedly caused stimulation of the menaquinone levels while the bacterial count remained constant or was marginally reduced with antimicrobial administration. It was clear that the diet induced changes in the metabolic activity of the organisms in the intestine, leading to increased production of menaquinones. It is generally accepted that it is difficult to change the fecal flora of human subjects by changing their diet. In studies of the role of fecal microbiota of human adults in the etiology of colon cancer, the numbers and types of fecal microflora remained remarkably unaffected by dietary changes. However, there were major changes in metabolic activity, especially with bile metabolism induced by the different types of diets (Finegold and Sutter, 1978; Hentges, 1978; Moore et al, 1978). The metabolic changes observed in both rat and man are another example that biochemical changes as well as microbiologic shifts need to be measured in experimental studies.

The effect of several vitamin K_1 deficient diets and the effect of normal rat chow on the menaquinone content of the intestinal tract was studied. Rats received a high carbohydrate vitamin K_1 deficient diet (rice), high protein diet (beef), the glucose-soy protein diet of Mameesh and Johnson or rat chow. The rice and beef diet reduced the animals' vitamin K_1 stores and stimulated increases in cecal and colonic menaquinone concentrations, thus validating the observations

seen in the human volunteer study. Thus, the type of diet plays an important role in regulating the endogenous pool of menaquinone in animals and man by effecting changes in the metabolic activity of the menaquinone producing flora.

It has been suggested that coprophagy plays an important role in making endogenously produced menaquinones (from the intestine) available to rats (Barnes and Fiala, 1959; Mameesh and Johnson, 1960a). It is thought that in order for these animals to utilise menaquinones, feces must be ingested (coprophagy) and absorption does not take place within the colon or cecum. The animals in this study were found to have the same menaquinone types in the liver that were found in the intestinal tract. Additionally, concentrations in the liver seem to correlate with concentrations in the intestine. For instance, higher levels of menaquinone in the cecum of rice and meat fed rats were reflected by higher concentrations in the animals' livers when compared to chow fed rats. Conversely, the loss of the menaquinone pool in the intestine seen in rats placed on antibiotics that destroyed the menaquinone producing flora was associated with a decrease to very low menaquinone concentrations in the liver. Although coprophagy was not prevented by using special tube-shaped coprophagy-preventing cages or tail cups, the cages used in this study were of a coarse wire mesh type with holes large enough to allow fecal material to fall through and be unaccessible to the animals. Coprophagy was also unlikely in these animals since examination of the upper and lower small intestine of animals showed that very low concentrations of menaquinones were present. These data imply that absorption of menaquinone was occurring in situ in those animals on a vitamin K_1 deficient diet and which

had normal prothrombin times (vide infra). If ingestion of menaquinone by coprophagy had occurred higher concentrations of menaquinones should have been found in the upper small intestine. The site of absorption was most likely the cecum, since cecal concentrations were much higher than in all the other sections (small intestine and colon) tested.

The distal small intestine has been shown to be a site of absorption of both vitamin K_1 and menaquinones by passive diffusion (Hollander, 1973; Hollander and Rim, 1976). It was thought that menaquinone producing bacteria and therefore a menaquinone pool may be sufficient at this site to provide for an animal's daily requirement of menaquinones. However, both E.coli and B.fragilis group organisms were inconsistently recovered at high enough numbers, and menaquinone concentrations were much lower than seen in the lower gastrointestinal tract. Since it was not proven that the lower small bowel was the major site of absorption of menaquinone, additional studies are required to determine the role of the ileum in menaquinone absorption in man. Also, the vitamin K status of colectomized humans who develop a fecal type flora in the small bowel needs to be further studied.

The data from this animal study indicates that the cecum was probably the major site of the endogenous pool and that the small intestine was probably less important. It is probable that dietary vitamin K_1 is absorbed via the small intestine while menaquinone is internalised in the cecum and colon under normal conditions. Data supporting the bioavailability of menaquinones was derived from experiments with rats fed the vitamin K_1 deficient rice diet. If menaquinones were not bioavailable and cannot be absorbed directly

from the cecum then these animals should have become hypoprothrombinemic, since their dietary source of the vitamin K_1 was being withheld. However, these rats, did not become hypoprothrombinemic after 7 or 14 days and small bowel concentrations of menaquinone were low or undetected. Their prothrombin times were normal and their serum, cecal and hepatic stores of vitamin K_1 were all negligible. Their hepatic stores of menaquinones were high, indicating that in the face of vitamin K_1 deficiency, bacterially produced menaquinones in the gut had been absorbed and functioned to maintain normal coagulation homeostasis. The meat diet also served to lower body stores of vitamin K_1 but to a lesser extent than the rice diet. However, like the rice diet, meat induced high concentrations of cecal and hepatic menaquinones and normal prothrombin times.

When rats were fed their regular chow diet and given antibiotics, there was no elevation of prothrombin times even though the intestinal menaquinone producing microflora had been eradicated and the intestinal and hepatic menaquinone levels were reduced. Dietary vitamin K_1 intake was sufficient in these rats to maintain normal prothrombin times.

Of note, rats receiving moxalactam and the chow diet did not develop hypoprothrombinemia. Moxalactam is one of the third generation cephalosporin antibiotics often associated with hypoprothrombinemia and bleeding in the clinical setting. It is thought that the NMTT leaving group from the drug (Lipsky, 1983) induces hypoprothrombinemia by inhibition of the hepatic microsomal carboxylase. However, moxalactam failed to produce hypoprothrombinemia when adequate levels of vitamin K_1 were maintained in chow fed rats. Therefore, the NMTT

inhibition of carboxylase was not observed in these experiments. However, it is possible that higher doses of moxalactam could have produced such an effect in the presence of an adequate vitamin K_1 supply. This is unlikely, however, since Lipsky has not been able to produce hypoprothrombinemia in rats on a vitamin K_1 sufficient diet.

The contribution of NMTT to antibiotic induced vitamin K_1 responsive coagulopathy when animals are vitamin K_1 sufficient is still unsettled. Lipsky (1983) obtained 50% inhibition of the carboxylase enzyme at 1.1 mM concentrations of NMTT and subsequently demonstrated elevated prothrombin times in rats with doses of NMTT and moxalactam after 3 days treatment. However, the effect of the vitamin K_1 deficient diet (vide infra) is not accounted for in these studies. Wold et al, (1983), were unable to induce hypoprothrombinemia in rats at doses of moxalactam of 2700 mg/kg. As well, NMTT at 500 mg/kg in rats and at 100 mg/kg in dogs could not produce hypoprothrombinemia in either animal after 31 days of treatment.

Further evidence that NMTT-induced carboxylase inhibition was not a major mechanism in the etiology of hypoprothrombinemia came from the experiments with rats on rice in combination with moxalactam or cefoxitin/gentamicin. Rats developed hypoprothrombinemia whether they received moxalactam which contains the NMTT group or received cefoxitin which lacks it. Cefoxitin also is very poorly absorbed and systemic levels were $\leq$4 mg/L. Both of these agents have a broad spectrum of activity and are effective in reducing B.fragilis group counts. Both drug regimens eradicated or severely suppressed E.coli and B.fragilis group organisms at all levels of the intestine, and reduced cecal and hepatic menaquinone concentrations. In other words,

the animals were deficient of both forms of vitamin K and therefore it is highly likely that hypoprothrombinemia was occurring secondary to removal of the endogenous menaquinone pool and was not due to NMTT inhibition of the microsomal carboxylase.

Clindamycin/gentamicin did not produce hypoprothrombinemia in rats even though the menaquinone producing flora had been reduced. This was probably due to the fact that in these animals hepatic menaquinone levels were still adequate, and probably sufficient to maintain normal prothrombin times. The absorption of clindamycin may have results in a sub-optimal or delayed intra-luminal effect, resulting in a persistence of hepatic and intestinal menaquinone. A timed study would be necessary to show this.

G. Restoration of Normal Prothrombin Times by Feeding B. fragilis and by Recolonization

The rice diet in combination with cefoxitin/gentamicin provides an excellent model to study the contribution of menaquinone to coagulation homeostasis. This diet reduced body stores of vitamin K_1 to levels that essentially leave the animal totally deficient of this form of the vitamin. Thus, the effect of menaquinone can be assessed in terms of importance to mammalian coagulation.

Evidence to support the contention that bacterial menaquinones were active in maintaining normal mammalian coagulation was supplied by the experimental feeding of hypoprothrombinemic rats with B.fragilis.

Animals that were made hypoprothrombinemic using this model, when fed heat killed B.fragilis developed normal prothrombin times. This

was correlated with increased menaquinone levels in both the cecum and small intestine as well as the livers of these animals. Since both sections of the intestine contained similar levels of menaquinone, the primary area of absorption could not be determined as it could have occurred at both sites. Hollander et al, (1977b) demonstrated that absorption of menaquinone can occur in both the small and large intestine of the rat. The livers of the animals fed B.fragilis contained primarily MK-9 and MK-10, the menaquinone isoprenologues produced by this organism, suggesting that they were absorbed in the intestine and taken up by the liver. It also indicates, that these isoprenologues are bioactive in the liver and function as a cofactor for the microsomal carboxylase. This also suggests that it may not be necessary for longer chain menaquinones to be first converted to MK-4 for activity in the hepatic carboxylation reaction and refutes the contention that only MK-4 is the active form of menaquinone in the liver (Martius, 1961). Martius had suggested that long chain menaquinones and even vitamin K_1 must first be dealkylated and then realkylated to MK-4 in order to be bioactive. Additional single menaquinone reconstitution experiments, eg. MK-9 and monitoring of hepatic menaquinone profiles over time would be of interest.

The dosage of killed B.fragilis was perhaps higher than what would be normally available under physiological conditions in the small inestine. This dose was based on the cecal counts of B.fragilis group organisms. A dose response study to determine the minimum level of menaquinone necessary to maintain normal coagulation homeostasis was not done.

Rats on cefoxitin/gentamicin and fed the rice diet became consistently hypoprothrombinemic after 7 days. In all cases the cecal B.fragilis and E.coli counts, menaquinone concentrations, and hepatic menaquinone concentration were reduced to very low levels. Rats that were treated with this regimen beyond 7 days, to 12 days, had more severe hypoprothrombinemia. The cecal vitamin K_1 and menaquinone concentrations were similarly low at day 7 and at day 12, and hepatic vitamin K_1 concentrations were similar. It appears that the rice diet reduced vitamin K_1 body stores in less than one week and the antibiotic regimen eradicated cecal menaquinone producing flora early, causing a rapid decrease of cecal menaquinone concentrations to a very low and constant level. On the other hand, the hepatic menaquinone concentrations appeared to decline further since there was a five fold reduction of hepatic menaquinone concentrations between day seven and day 12. Thus, progressive depletion of hepatic menaquinone levels led to progressively more severe hypoprothrombinemia. Therefore, it is probable that there is a threshold value of menaquinone level in the liver above which normal coagulation homeostasis is maintained. As the menaquinone concentrations drop below this threshold, hypoprothrombinemia results and becomes exacerbated as the pool further declines. Presumably, prothrombin times elevate to a point where clinical bleeding occurs.

The final piece of evidence generated in these studies, supporting the role of menaquinone in mammalian coagulation stems from rats made hypoprothrombinemic on rice combined with cefoxitin/gentamicin and then allowed to recolonize their intestinal tract. Rats that became hypoprothrombinemic demonstrated undetectable counts ($<10^2$

CFU/gram wet weight feces) of B.fragilis and E.coli in their cecum. Removal of the drug suppression while keeping the animals on their rice diet induced recolonization of the intestine with B.fragilis and E.coli after 20 days but not after 5 days. The minimum interval for recolonization and reversal of hypoprothrombinemia was not determined by doing timed studies. However, after 20 days, rats had regained B.fragilis group and E.coli counts close to that of animals untreated by antimicrobials despite ingestion of the vitamin K_1 deficient diet. Their cecal and hepatic menaquinone concentrations were similar to those of animals fed B.fragilis and prothrombin times were also normalized. This indicates that menaquinone produced in the cecum was absorbed in situ. Reconstitution experiments using enemas to the level of the cecum also support this conclusion.

H. Hypoprothrombinemia in Rats on a Synthetic Diet

Diets similar to that of Mameesh and Johnson (1960a,b) are widely used in studies to routinely induce hypoprothrombinemia in rats. Mameesh and Johnson, (1960a,b) first used this diet, consisting largely of glucose, soy protein, salts and oils such as wheat germ and cod liver oil to determine the dietary vitamin K requirements of rats and the role of menaquinone in coagulation. They found that the diet induced mild hypoprothrombinemia in rats and that the condition was further exacerbated by preventing coprophagy. Nutritional hypoprothrombinemia associated with diets such as those of Mameesh and Johnson has generally been explained by the presumption that intestinally synthesized vitamin K was not available for the synthesis of vitamin K-dependent coagulation factors. It was also suggested

that resistance to hypoprothrombinemia was dependent on the degree of coprophagy practised by the animals.

In this study, the rice diet which is also deficient in vitamin K_1 did not induce hypoprothrombinemia in rats. The diverse outcomes seen with different vitamin K_1 deficient diets led us to examine the effects of the Mameesh and Johnson diet on the intestinal flora and the menaquinone pool in the gut, and on hepatic menaquinone concentrations in rats. Of note, most investigators have used a variant of the Mameesh and Johnson diet in their studies. A minor reduction in B.fragilis group counts was observed in the cecum of rats on the Mameesh and Johnson diet. Cecal menaquinone concentrations were similar to those animals receiving rat chow. However, hepatic menaquinone concentrations were extremely low and similar to that seen in rats on rice and cefoxitin/gentamicin for 12 days. Elevation in prothrombin times in these groups was also similar.

It is apparent that even though the Mameesh and Johnson diet only caused minor changes in the menaquinone concentrations and moderate reduction of the flora in the intestine, major changes occurred in the liver. It appears that reduced absorption of the endogenous menaquinone pool could account for the low hepatic menaquinone stores and explain the biologic effect of this diet.

Interference with absorption could be a property of the diet being used or of metabolites produced from the diet but it is uncertain what factor could play such a role. Doisy, (1961), demonstrated that the degree of vitamin K-responsive hypoprothrombinemia was dependent on the level of vitamin A and vitamin A acids in the diet. For instance, rats fed a beef based diet maintained prothrombin levels

that varied inversely with the amount of vitamin A added. It was further determined that minute amounts of the vitamin were sufficient to produce bleeding in these animals.

The type of dietary protein supplied also influenced the degree of hypoprothrombinemia. Soy protein was more effective in reducing prothrombin levels when compared to casein or lactalbumin. Also, increasing the quantity of soy protein in the diet, caused an increase in the severity of prothrombin deficiency. Overall, it appeared that high protein diets increased the vitamin K requirement of male rats (Matschiner and Doisy, 1965). Matschiner and Doisy speculated that the effects of dietary proteins on vitamin K deficiency may be due to changes in the intestinal supply of vitamin K and may also influence intestinal synthesis and absorption of vitamin K. However, there is little experimental evidence to support such a claim, even though our observations also support the general hypothesis.

The precedent of specific diet type affecting levels of lipids in plasma is seen from the study of Sirtori et al (1977). Patients placed on a soybean protein diet consisting of approximately 20% soy protein, reduced cholesterol levels in the plasma of subjects by 21% after 3 weeks. It was unclear by what mechanism the diet was affecting cholesterol levels in the plasma or even its absorption. However, the soy protein in the Mameesh and Johnson diet may be exerting some type of effect on overall lipid absorption in rats fed the diet. Also, if high protein diets do increase the vitamin K requirements of these animals, then perhaps, the levels of menaquinone in the cecum of these animals may be inadequate to supply their needs.

7. SUMMARY AND CONCLUSIONS

The development of vitamin K deficiency and resultant hypoprothrombinemia with the use of antimicrobial agents may be dependent on a number of factors. These include dietary intake of vitamin K_1, vitamin K turnover, status of the menaquinone producing microflora, the endogenous menaquinone pool, and probably menaquinone absorption in the intestine. These studies have provided insights into the interplay between these factors and their relative importance in inducing hypoprothrombinemia and the attendant risk of bleeding in animals. Several conclusions have been drawn. These include:

1. Diets have a major effect upon the intestinal menaquinone pool, and,secondarily, the intrahepatic stores of both vitamin K_1 and menaquinones. Chow fed rats contained large cecal quantities of vitamin K_1 and lower menaquinone levels than meat and rice fed rats. Conversely, meat and rice decreased cecal vitamin K_1 concentrations but increased menaquinone levels.
2. Changes in vitamin K_1 and menaquinone concentrations in the cecum were reflected in changes in hepatic stores of these forms of the vitamin.
3. Type of diet and amount ingested influences the amount of substrates available to intestinal microorganisms. Changes in the menaquinone producing flora, eg. 2 $\log_{10}$ CFU/g dry weight cecal contents, is associated with qualitative and quantitative changes of menaquinone content.
4. Certain diets may affect the bioavailability of the endogenous menaquinone pool. The conditions that affect bioavailability are unresolved.

5. Hypoprothrombinemia is not observed if animals are deprived of vitamin K_1 alone, while the intestinal and hepatic menaquinone reservoir persists. Elimination of both forms of the vitamin is necessary to produce hypoprothrombinemia.

6. Since changes in luminal concentrations of menaquinones are reflected in proportional changes in hepatic content, this indicates that menaquinones produced by bacteria in the gut are absorbed in situ, probably by a passive concentration dependent mechanism, as suggested by Hollander.

7. Antibiotic associated hypoprothrombinemia is not due to inhibition of the hepatic microsomal carboxylation by the NMTT moiety of these agents. Evidence for this is provided by the following: (i) animals on a vitamin K_1 sufficient diet in combination with an NMTT containing agent (moxalactam) do not become hypoprothrombinemic, and (ii) hypoprothrombinemia is produced with equal facility by antibiotics containing (moxalactam) or lacking (cefoxitin) the NMTT moiety during vitamin K deficiency.

8. Hypoprothrombinemia in rats on a vitamin K_1 deficient diet occurs with use of an antibiotic capable of eradicating the menaquinone producing flora and the resultant decline of the endogenous intestinal and hepatic stores of menaquinone.

9. Hypoprothrombinemia that has been induced in animals placed on a vitamin K_1 deficient diet and antibiotics capable of removing the menaquinone producing flora can be reversed by the following: (i) oral ingestion of killed B.fragilis, (ii) recolonization of the gut with menaquinone producing organisms, and lastly, (iii) by rectal enemas of live or dead organisms into the colon and cecum.

10. These data support the hypothesis that menaquinone plays an important role in maintaining normal coagulation homeostasis in mammals.

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9. APPENDICES

Appendix 1: Culture Media

Brain Heart Infusion Broth - Supplemented with yeast extract and hemin

Brain heart infusion broth (dehydrated)	37.0 g
Yeast extract	5.0 g
Cysteine $HCl.H_2O$	0.5 g
Hemin solution	10.0 ml
Water	1,000.0 ml

Autoclave for 15 min. at 121°C

Lactobacillus broth

Peptone	10.0 g
Beef extract	10.0 g
Yeast extrct	5.0 g
Glucose	10.0 g
Tween	1.0 ml
K_2HPO_4	2.0 g
Sodium acetate	5.0 g
Triammonium citrate	2.0 g
$MgSO_4.7H_2O$	0.2 g
$MnSO_4.4H_2O$	0.05 g
Water	1,000.0 ml

Dissolve and autoclave at 121°C for 15 min.

Peptone-Yeast extract - glucose (PYG) broth

Peptone	20.0 g
Yeast	10.0 g
Cysteine $HCl.H_2O$	0.5 g
Resazurin solution	4.0 ml
VPI salt solution	40.0 ml
D-glucose	10.0 g
Water	1,000.0 ml

Hemin Solution

Dissolve 0.05 g hemin in 1.0 ml 1 M NaOH and make up to 100 ml with distilled water. Add 10.0 ml of this solution to 1000 ml of BHI broth.

Appendix 2: Buffers

VPI Salt Solution

$CaCl_2$ (anhydrous)	0.2 g
$MgSO_4$ (anhydrous)	0.2 g
K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
$NaHCO_3$	10.0 g
H_2O	1,000.0 ml

Mix $CaCl_2$ and $MgSO_4$ in 300 ml distilled water. When dissolved, add 500 ml distilled water. Swirl slowly while adding remaining salts and continue mixing until all salts are dissolved. Add 200 ml distilled H_2O. Filter (0.45 um) sterilize. Store at 4°C.