

Factors affecting the absorption of vitamin K-1 *in vitro*

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SUMMARY Factors which might affect the absorption of vitamin K of dietary origin were investigated using everted small bowel sacs. Increasing the bile salt concentration to 20 mM or the addition of long chain fatty acids, monoolein, or lecithin all resulted in significant ($P < 0.05$) decrease in the absorption rate of the vitamin. The addition of 2.5 mM short and medium chain fatty acids did not change the absorption rate of vitamin K-1 ($P > 0.05$). The absorption rate of vitamin K-1 appears to be modified by the presence of compounds in the incubation medium which either alter the partition of the vitamin between the micelle and the cell membrane or which change the permeation characteristics of the compound through the unstirred water layer or modify the physical characteristics of the cell membrane itself. It is possible that some of the above factors modify the absorption of lipid soluble compounds in general.

Vitamin K-1 (phylloquinone) is the major dietary form of the vitamin. It is absorbed *in vitro* by an energy requiring absorption process which displays saturation kinetics and is inhibited by nitrogen atmosphere, the addition of 2,4-dinitrophenol, or low incubation temperatures (Hollander, 1973). In normal circumstances, absorption of the vitamin would be expected to take place in the presence of fatty acids of varying chain lengths and degree of saturation as well as differing bile acid concentrations. Therefore, the influence of the presence or absence of the above compounds on the intestinal absorption of vitamin K-1 was investigated.

Methods

INCUBATION MATERIALS

Radioactively labelled vitamin K-1 (phylloquinone) had a specific activity of 41.4 $\mu\text{Ci}/\mu\text{mol}$ and was labelled with tritium at the 2-methyl position. Unlabelled vitamin K-1 was purchased from Nutritional Biochemicals Co., Cleveland, Ohio. The purity of the radioactive as well as non-radioactive vitamin preparations was ascertained by thin layer chromatography on silica gel-G developed in 10% methanol in benzene (Doisy and Matschiner,

1970). The compounds were purified by silicic acid column chromatography using increasing concentrations of benzene in hexane if more than 5% impurities were found (Matschiner *et al.*, 1967). Sodium taurocholate (K & K Laboratories, Plain View, N.Y.) was found to have less than 5% impurities by thin layer chromatography (Gregg, 1966). Oleic acid, monoolein, octanoic acid, lecithin, linoleic acid, and butyric acid were purchased from Sigma Chemical Co., St. Louis, Mo.

The micellar incubation solution was prepared in Krebs phosphate buffer, pH 7.4, with dextrose concentration of 200 mg/100 ml (Umbreit *et al.*, 1964). The final micellar solution was 10 mM with respect to sodium taurocholate and contained various concentrations of monoglycerides and fatty acids as indicated. The solution was prepared by ultrasound irradiation for five minutes with a sonicator (Artek Corporation, Farmingdale, N.Y.). Varying concentrations of radioactive as well as non-radioactive compounds were added to the micellar solution after sonication by homogenisation of the compounds with the micellar solution in a tissue homogeniser. The pH of the incubation medium was measured after incubations and was found to vary between 7.30 and 7.39.

INCUBATION METHODS

Male Sprague-Dawley rats weighing between 100 and 180 g were fed regular chow (Check-R-Board,

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Novis, Michigan) *ad libitum* and were not fasted before experimentation. The rats were killed by cervical dislocation and the bowel was rinsed *in situ* with iced 0.9% sodium chloride solution. The entire small bowel was removed and everted. A 10 cm segment immediately distal to the insertion of the common bile duct was designated as the proximal small bowel segment. A second portion designated as the distal segment was the distal 10 cm of the small bowel immediately proximal to the ileocecal valve. Each segment was subdivided with ligatures into five 1 cm everted sacs which were identified with tags indicating their exact origin. Peyer's patches were avoided. The sacs were placed in a Plexiglass incubation chamber with internal dimensions of 2 × 6 × 30 cm containing 50 ml micellar incubation medium which had been aerated with 100% O₂ gas and equilibrated to 37°C before experimentation. The oxygen stream was presaturated with water in order to minimise evaporation during the procedure. Continuous bubbling of oxygen was maintained throughout the experiment. The chamber was positioned in a metabolic water bath (at 37°C) which was agitated at 40 oscillations per minute. The length of all incubation experiments was five minutes. Immediately after incubations, the sacs were rinsed for 60 seconds in a beaker containing 300 ml 1 mM sodium taurocholate solution not containing the vitamin in order to remove some of the incubation solution which had remained adherent to the sac walls. Less than 1% of the total radioactivity present on and within the sac walls was washed by the taurocholate solution within the 60 seconds' rinse. The sacs were then dried at 50°C under 51 cm (20 in.) of mercury vacuum for 24 hours. All calculations pertaining to tissue weight refer to the constant dry weight of the tissue.

RADIOACTIVITY DETERMINATIONS

Tissue radioactivity was determined by total combustion into tritiated water or ¹⁴C labelled CO₂ gas using a sample oxidiser (306 Tri-Carb sample oxidiser, Packard Instruments, Downers Grove, Illinois). Measurements of radioactivity were carried to a counting error of 1% using a Beckman LS 250 liquid scintillation counter with automatic quench calibration at ambient temperature.

SUBSTANTIATION OF RADIOACTIVE PURITY OF ABSORBED VITAMIN

Intestinal tissue was extracted in a 1:1 mixture of acetone ethanol. The extract was spotted on silica gel G thin layer plates developed in chloroform (Doisy and Matschiner, 1970). More than 90% of the radioactivity from the absorbed phylloquinone

migrated with the same Rf values as that of pure phylloquinone standards, indicating that the radioactive label did represent unaltered vitamin K-1.

Results

MORPHOLOGICAL INTACTNESS OF INTESTINAL TISSUE

After incubation, intestinal sacs were placed in 10% formaldehyde solution and processed for light microscopy. Intestinal tissue slides were examined by two individuals who were not aware of the time source of each specimen. No morphological changes were detected during the five minutes of incubation time indicating the lack of visible tissue damage during the experimental procedures.

DETERMINATION OF ADHERENT MUCOSAL FLUID COMPARTMENT

Despite the taurocholate rinse, a certain volume of incubation fluid remained adherent to the sac walls adding radioactive counts to those emitted by the absorbed vitamin. In order to subtract these counts from those emitted by the absorbed vitamin, a non-absorbable marker ¹⁴C-inulin was added to the incubation medium of multiple experiments (Esposito and Czaky, 1974). Assuming an equal distribution of inulin between the bulk aqueous phase and the adherent fluid compartment, the volume of the adherent layer was determined specifically for each segment at each specific time interval, and the amount of vitamin K-1 present in that volume was then subtracted from the apparent total absorption value of the vitamin. The mean ± SE steady state volumes of proximal and distal intestinal adherent fluid compartments were 49.4 ± 0.8 and 46.4 ± 2.3 μl/100 mg of tissue respectively. All experimental results reported in this communication were corrected by subtraction of the adsorbed amount of vitamin K-1 from the total apparent amount of absorption.

REQUIREMENT FOR BILE SALTS IN VITAMIN K-1 ABSORPTION

Clinical observations have suggested that the absence of bile could result in malabsorption of vitamin K (Butt *et al.*, 1938). The influence of bile salt concentration on vitamin K-1 absorption was therefore investigated. Absorption of the vitamin when solubilised in a 10 mM sodium taurocholate micellar solution was examined in six animals. The mean ± SE absorption rates of the vitamin were 26.1 ± 1.4 and 12.7 ± 1.1 nmol/min/100 mg of tissue by the proximal and distal small bowel respectively (Fig. 1). When the bile salt concentration was increased to 20 mM the absorption rate of

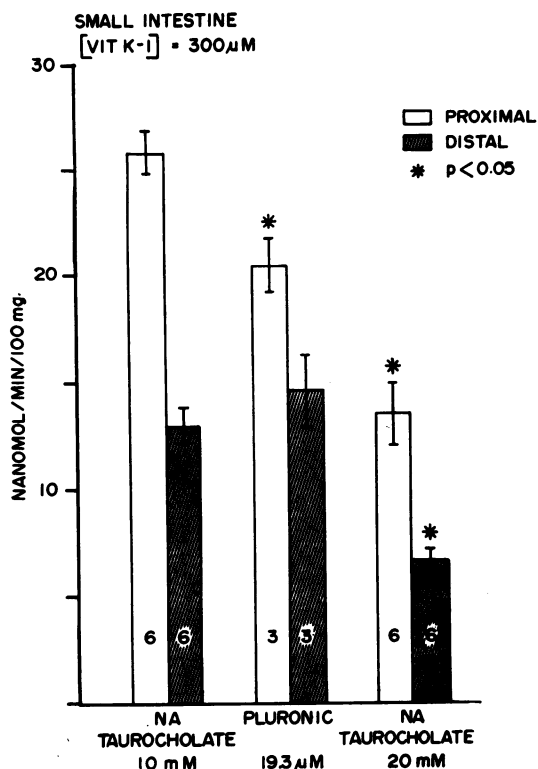


Fig. 1 The effect on the absorption rate of vitamin K-1 by the proximal and distal small bowel of changes in the concentration of bile salts or their complete absence. The number of animals used is indicated by the numeral at the base of the bars. Each bar represents the mean \pm SE calculated from absorption values of 10 different sacs per animal. The statistical significance of the differences between experiments was calculated using Student's *t* test for paired observations.

vitamin K-1 was decreased significantly ($p < 0.02$) to 13.7 ± 1.9 and 6.9 ± 0.8 nmol/min/100 mg of tissue respectively (Fig. 1). The effect of complete removal of sodium taurocholate from the incubation solution was tested by the use of non-ionic solubiliser-pluronic F-68 (polyoxypropylene-polyoxyethylene MW 8350) at $19.3 \mu\text{M}$ concentration. The rate of absorption of the vitamin by the proximal and distal small bowel segments solubilised in pluronic solution was 20.3 ± 1.7 and 14.3 ± 2.3 nmol/min/100 mg of tissue respectively (Fig. 1). The above rates were significantly lower ($p = .03$) only in the proximal small bowel when compared with baseline absorption rates with 10 mM sodium taurocholate solution.

INFLUENCE OF FATTY ACID CHAIN LENGTH AND SATURATION ON VITAMIN K-1 ABSORPTION RATE

The influence of chain length and degree of satura-

tion of fatty acids on the absorption rate of vitamin K-1 by the proximal and distal small bowel was investigated. The absorption rate of the vitamin when solubilised in sodium taurocholate micellar solution not containing fatty acids was 26.11 ± 1.4 and 12.7 ± 1.1 nmol/min/100 mg of proximal and distal small bowel respectively (Fig. 2). When butyric acid (C4:0) and octanoic acid (C8:0) were added in a 2.5 mM concentration to the 10 mM sodium taurocholate solution no significant change in the absorption rate of the vitamin was observed ($p > 0.05$) (Fig. 2). The separate additions of the long chain unsaturated fatty acids oleic (C18:1) and linoleic (C18:2) resulted in a significant ($p < 0.05$) decrease in the absorption rate of vitamin K-1 both by the proximal and distal small bowel segments (Fig. 2). The decrease in the absorption rate of the vitamin was greater with the addition of the polyunsaturated linoleic acid (C18:2) than with the monounsaturated oleic acid (C18:1).

INFLUENCE OF MONOGLYCERIDES AND LECITHIN ON VITAMIN K-1 ABSORPTION

As the intestinal milieu contains monoglycerides and lecithin, their effect on the absorption rate of vitamin K-1 was compared with absorption of the vitamin out of pure sodium taurocholate micellar solution. Monoolein and 3-phosphatidyl choline (lecithin) were added to the 10 mM taurocholate micellar solution in a 2.5 mM concentration in separate groups of experiments. A significant ($p < 0.05$) decrease in the absorption rate of vitamin K-1 both by the proximal and distal small bowel was seen with the addition of either monooleate or lecithin to the incubation medium (Fig. 3).

Discussion

Previous studies of vitamin K-1 absorption (Hollander, 1973) did not define the influence of the various constituents of the intestinal lumen on the absorption of the vitamin. Elucidation of the possible influence of fatty acids, bile salts, monoglycerides, and lecithin on vitamin K-1 absorption is important not only because of the physiological role of the vitamin but also because of the applicability of the concepts derived from such investigation to the general field of absorption of lipid soluble compounds.

The major determinants which could modify the absorption rate of lipid soluble compounds are the chemical preparation of the compound for absorption—that is, pancreatic factors (Simmonds, 1974)—the composition and characteristics of the micelle (Simmonds, 1974), partition of the compound between the micelle and the mono-

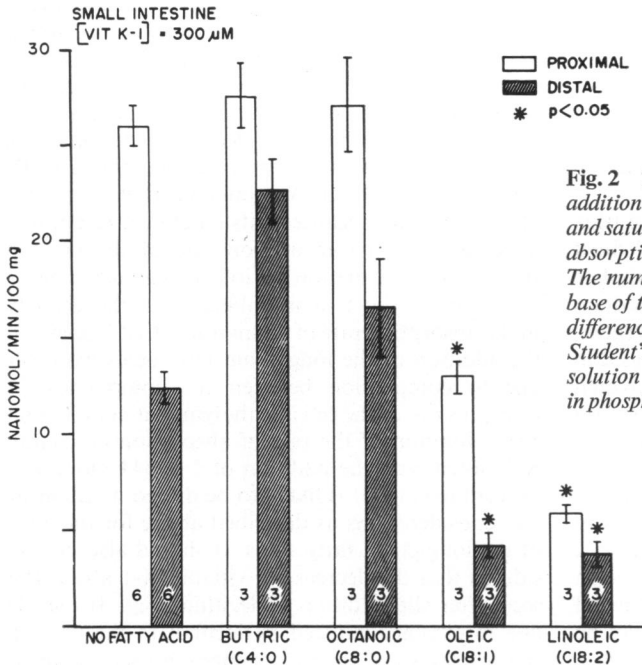


Fig. 2 The effect on the absorption of vitamin K-1 of the addition of 2.5 mM fatty acids of varying chain length and saturation. The results represent the mean \pm SE for absorption of the vitamin by 10 different sacs per animal. The number of animals per experiment is indicated at the base of the bar. The statistical significance of the differences between experiments was calculated using Student's t test for paired observations. The incubation solution was composed of 10 mM sodium taurocholate in phosphate buffer.

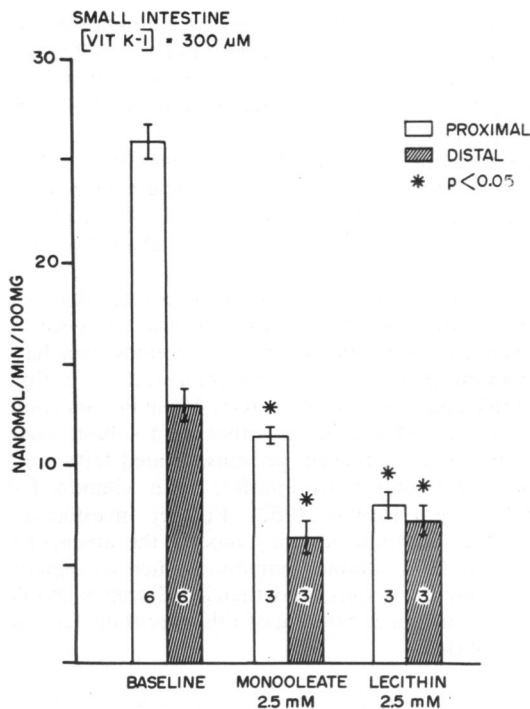


Fig. 3 The effect on the absorption rate of vitamin K-1 of the addition of 2.5 mM monoolein and lecithin to the sodium taurocholate incubation solution. The values plotted are mean \pm SE of 10 sacs per animal. The number of animals per experiment is indicated by the numeral at the base of the bar graph. The statistical significance of the differences between experiments was calculated using Student's t test for paired observations. The incubation solution was composed of 10 mM sodium taurocholate in phosphate buffer.

molecular aqueous solution (Diamond and Katz, 1974), the permeation coefficient of the compound through the unstirred water layer (Westergaard and Dietschy, 1974) the characteristics of the cell membrane itself (Vandenhuevel, 1971), and, finally, factors within the cell which affect the rate of transfer of the compound from the cell into the circulation (Thompson *et al.*, 1969).

In this investigation, pancreatic factors have been circumvented by the use of chemically defined components of triglyceride hydrolysis. The complete removal of bile salts from the incubation medium and the substitution of a non-ionic detergent, pluronic F-68, resulted in only a minimal decrease in the absorption rate of vitamin K-1 by the proximal small bowel and no decrease in the absorption rate by the distal small bowel (Fig. 1). Thus, bile salts appear to have no direct role in the absorptive process of vitamin K-1 except for their micellar solubilising properties. Increasing the concentration of sodium taurocholate up to 20 mM resulted in a significant ($P < 0.05$) decrease in the absorption rate of vitamin K-1 both by the proximal and distal small bowel segments (Fig. 1). The above may be due to changes in the partition of vitamin K-1 between the micelle and the cell membrane or the aqueous monomolecular phase (Simmonds, 1974) in favour of the micelle. A shift of vitamin K-1 into the micelle would be expected to result in diminished absorption of the vitamin due to decreased release of the vitamin from the micelle (Diamond and Wright, 1969). It is also possible that a high concentration of bile salts may in some way modify the characteristics of the cell membrane itself, making it less permeable to lipid soluble compounds such as vitamin K-1 (Harries and Sladen, 1972).

The effect of expansion of the micelle by the addition of fatty acids of varying chain lengths and degree of saturation on the absorption of vitamin K-1 was examined in the next series of experiments. When compared with the absorption rate of the vitamin out of a pure bile salt micelle, the addition of a short chain fatty acid-butyric acid (C4:0) and the medium chain fatty acid-octanoic acid (C8:0) caused an increase in the absorption rate of the vitamin primarily by the distal small bowel (Fig. 2) which was not statistically significant ($P > 0.05$). The addition of the long chain fatty acids-oleic (C18:1) and linoleic (C18:2) significantly ($P < 0.05$) decreased the absorption rate of the vitamin when compared with its rate of absorption out of pure bile salt micelles (Fig. 2). The decrease in the absorption rate observed in the presence of long chain fatty acids could be due to interactions at two major points in the absorptive pathway of vitamin K-1. The long chain fatty acids are known

to change the physical characteristics of the micelle (Simmonds, 1974) which could result in a greater affinity of the micelle for vitamin K-1, hence diminishing its transfer to the absorptive cell membrane (Lee *et al.*, 1971). An alternative explanation is the possibility that the expanded micelle containing long chain fatty acids may diffuse through the unstirred water layer (Wilson and Dietschy, 1974) at a slower rate because of its large size, resulting in a decrease in the absorption rate of the vitamin. Because the incubation period in our experimental procedure is short, it is unlikely that the decrease in the absorption rate of vitamin K-1 that is seen with the addition of the long chain fatty acids would be due to competition between the compounds for transport out of the cell into the lymphatic circulation. The inhibition of the rate of absorption of vitamin K-1 noted with the addition of 2.5 mM monoolein or lecithin (Fig. 3) is likely to be due to mechanisms and considerations as described above for the effect of the long chain fatty acids. It should also be considered that the decrease in vitamin K-1 absorption seen after the addition of lecithin (Fig. 3) may be due to changes induced by lecithin in the physical characteristics of the cell membrane itself (Vandenhuevel, 1971). The observed increased absorption of the vitamin with the addition of short and medium chain fatty acids even though statistically insignificant is still noteworthy. The effect of these fatty acids is most noticeable in the distal bowel segments. The reason for these observations is unknown. The short chain fatty acids are less likely to affect the micellar characteristics than are the long chain fatty acids because of their greater aqueous solubility and independence from micellar solubilisation as a requirement for absorption (Jackson, 1974). Whether the short and medium chain fatty acids change the characteristics of the lipid membrane itself, thereby affecting vitamin K-1 absorption rate, is unknown.

The above experiments demonstrate that the composition of the micelle and the presence or absence of other fat soluble compounds, can have profound effects on the absorption rates of a lipid soluble compound-vitamin K-1. These factors could modify the absorption of other lipid soluble compounds as well. Indeed, polyunsaturated fatty acids have been shown to interfere with vitamin E-1 absorption (Horwitt, 1962). Further investigation into the factors which may modify the absorption of other lipid soluble compounds such as digitalis compounds, steroids, and thiazide diuretics should enlarge our understanding of this important facet of absorption.

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