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THESIS

THE METABOLISM OF VITAMIN K, PARTICULARLY VITAMIN K_1

The distribution, fate, and excretion of vitamin K, administered as a colloidal suspension to albino rats, was investigated with the aid of 2-methyl-C^{14}-3-phytyl-1,4-naphthoquinone (radioactive vitamin K_1) and 2-methyl-C^{14}-1,4-naphthoquinone (radioactive vitamin K_3), in particular as affected by route of administration, dose, age, sex, and pregnancy. Radioactivity was found in all tissues following oral, intramuscular, and intravenous administration of radioactive vitamin K_1. The liver accumulated the largest amounts of radioactivity and retained it for six days. The concentration of C^{14} in tissue gave a logarithmic relation with the dose of vitamin K_1. The deposition of C^{14} in the livers and spleens of young rats was less than for older animals. No differences were observed in the tissue deposition of C^{14} in male or female rats. When radioactive K_3 was injected intravenously no marked concentration of C^{14} occurred in the livers. Pregnancy did not influence the deposition of C^{14} in maternal tissues, and furthermore, the levels of C^{14} in fetal tissues were of the same order of magnitude regardless of whether radioactive vitamin K_1 or vitamin K_3 was administered.

Isotope dilution tests were used to ascertain the amount of injected radioactive vitamin K_1 that was present in rat tissues in unchanged form. Most of the radioactive vitimin K_1 in liver and skeletal muscle was present as such, whereas the small intestine, placenta, fetal tissue, feces and bile contained C^{14} predominantly in a form other than the unchanged vitamin. The urine contained no unchanged radioactive vitamin K_1.

Attempts were made to identify a urinary metabolite of vitamin K_3 by comparing its chromatographic behavior with phosphate and sulfate esters of vitamin K_3. Since the radioactive metabolite did not coincide with fluorescent spots produced by vitamin K_3, or the diphosphate, monophosphate, disulfate or monosulfate esters of vitamin K_3, it was concluded that this compound was not identical with any of these substances. Ion exchange, column chromatography, and paper chromatography were used in unsuccessful attempts to isolate the metabolic products of vitamin K_1 from bile and urine.

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1. Tuba, J. and Taylor, J. D. On rat serum lipase. III. The effect of total food consumption and of dietary hydrogenated vegetable fat. *Canadian Journal of Medical Sciences* 30:26, 1952.
2. Taylor, J. D., Madsen, N. B. and Tuba, J. The effects of some dietary derived lipids and fat soluble vitamins on rat serum tributyrinase and alkaline phosphatase. *Canadian Journal of Medical Sciences* 30:308, 1952.
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4. Taylor, J. D.; Millar, G. J.; Jaques, L. B. and Spinks, J. W. T. The distribution of administered vitamin K_1-C^{14} in rats. *Canadian Journal of Biochemistry and Physiology* 34:1143, 1956.

PAPERS PRESENTED

1. Taylor, J. D. and Millar, G. J. Blood and tissue levels of C^{14}-vitamin K_1. Proceedings of the Western Regional Group Division of Medical Research, National Research Council of Canada. Tenth annual scientific meeting. Banff, Alberta. January 25-28, 1956.
2. Taylor, J. D. and Millar, G. J. A comparison of the concentration of C^{14} in the tissues of pregnant and nonpregnant female rats following the intravenous administration of vitamin K_1-C^{14} and viamin K_3-C^{14}. Proceedings of the Western Regional Group Division of Medical Research, National Research Council of Canada. Eleventh annual scientific meeting. Banff, Alberta. January 30 - February 2, 1957.

THE METABOLISM OF
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INTRODUCTION

A. Observations leading to the initiation of vitamin K research.

Several excellent reviews have been published on all aspects of vitamin K including its chemistry and its biological importance (2) (16) (20) (36) (52) (89). Since another exhaustive tabulation of the literature would be of very little value the following paragraphs have been limited to a review of pertinent observations and recent work which appears to be related to the metabolism of vitamin K, particularly vitamin K_1.

The discovery of vitamin K_1 presents an interesting example of the evolution of a new biochemical concept. McFarlane, Graham and Richardson (63) of the Ontario Agricultural College, Guelph, Ontario, Canada, observed that a synthetic diet which contained all of the then known essential dietary constituents would not permit optimal health in chicks. They presented an excellent description of the hypocoaguability of the blood of two groups of chicks that were fed on diets containing either white fish meal or meat meal that had been extracted with ether. Holst and Halbrook (45) and Dam (18) also reported that they had observed the disease. Holst and Halbrook assumed that the disease was scurvy and fed cabbage as a source of ascorbic acid. They found that the cabbage corrected the bleeding tendency.

Dam (19) supplemented the diet with lemon juice as a source of ascorbic acid and he observed that lemon juice did not correct the bleeding tendency. Dam and Schönheyder (27) proved that the disease was not scurvy by administering pure ascorbic acid to chicks; furthermore, Dam (17) demonstrated that some seeds and cereals contained a factor that would prevent the bleeding tendency and suggested the term vitamin K (Koagulations-vitamin in the German and Scandinavian languages). It is now known that both cabbage and lemon juice contain ascorbic acid but that cabbage also contains significant amounts of vitamin K_1, whereas lemon juice is a very poor source.

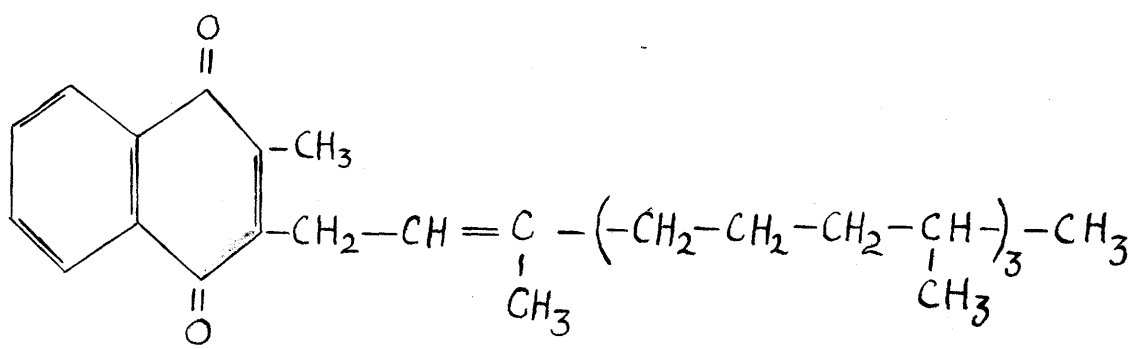
Natural sources of vitamin K_1.
B.

Subsequent experimentation showed that plants are the principal source of vitamin K_1, and that the vitamin is to be found in the green leaf and all the other chlorophyll-containing parts. Most fruits are poor sources but tomatoes are an exception. Cereals, beans and ripe peas contain only a small amount of the vitamin, and the vitamin K_1 content of carrots and potatoes is practically zero. (16) (22). Hog liver contains the equivalent of about 50 µgm. of vitamin K_1 per gram of dry weight (16) (28). It has never been determined whether this vitamin K is present as vitamin K_1 or some other form of the vitamin. Cows' milk and human milk contain the equivalent of 3 µgm. of vitamin K_1 per 100 ml. These values have been calculated from the original data (16) on the basis that 1 Dam Glavind unit is equal to 0.086 µgm. vitamin K_1 (16).

Isolation, purification and synthesis of vitamin K_1.

C.

The chemical properties of vitamin K_1 in concentrates from alfalfa were studied by Dam and co-workers (17) (24) (28) and by Almquist and his group (3). In 1939 Dam, Geiger, Glavind, Karrer, Karrer, Rothschild, and Solomin reported the isolation of vitamin K as a light yellow oil after concentration of an alfalfa extract followed by chromatography on zinc carbonate and permutit (21). McKee, Binkely, MacCorquodale, Thayer and Doisy (65) also announced the chromatographic isolation of the pure vitamin from alfalfa and they designated it vitamin K_1 to distinguish it from vitamin K_2 which they isolated from putrified fish meal (64). These workers reported the ultraviolet absorption spectra of the pure vitamins, identified them as substituted naphthoquinones and verified the suggested structure of vitamin K_1 by synthesis (62). The chemical structure that was assigned (62) to vitamin K_1 was:



Toxicity of vitamin K_1.

D.

Molitor and Robinson (73) reported that no lethal effect could be produced by acute oral or parenteral administration of 25 gm./kg. of vitamin K_1 to rats, mice, or chicks, and that 2 gm./kg. fed to young rats for 30 days produced no symptoms indicating toxicity. Dam, Prange, and Søndergaard (25) administered 100 mgm./kg. of vitamin K_1 intravenously as a colloidal suspension in Tween 80 to rats and chicks without toxic manifestations. This lack of toxicity does not apply to 2-methyl-1,4-naphthoquinone which has an L.D.50 of 500 mgm./kg. when given orally to mice and 350 mgm./kg. fed to rats produced a marked fall of the erythrocyte count (73). Shimkin (91) in a study of the acute and chronic toxicity of six naphthoquinones with vitamin K activity reported that the compounds were respiratory depressants. Synkavite (2-methyl-1,4-naphthoquinone diphosphate tetrasodium salt) has been reported to produce hemolytic anaemia in infants (1) (12).

Metabolism of vitamin K_1.
E.

The metabolism and metabolites of vitamin K_1 are unknown. Hoskin, Spinks and Jaques have demonstrated both glucuronide and sulfate conjugates of 2-methyl-C^{14}-1, 4-naphthquinone in the urine of rats (47). Vitamin K_1 may be conjugated in a similar manner. Moreover, the methyl group on carbon 2 might be oxidized to an alcohol, since Francis, Landquist, Levi, Silk, and Thorp (39) have found 2-hydroxymethyl-3-methylquinoxaline 1:4 dioxide to be a metabolite of 2:3-dimethylquinoxaline 1:4 dioxide in sheep. Finally, the phytyl side chain may be degraded or oxidized to an alcohol, aldehyde, or ketone. Fieser, Chang, Dauben, Heidelberger, Heyman, and Seligman (35) have demonstrated penultimate oxidation (ω-1), hydroxylation, demethylation, oxidation (ω-2), and terminal (ω), oxidation of the alkyl side chains of a series of 2-hydroxy-3-alkyl-1,4-naphthoquinones. Simon, Eisengart, Sundheim and Milhorat (92) isolated two metabolites of vitamin E from the urine of rabbits and humans. The alkyl side chain of vitamin E had been oxidized to give 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethylbenzoquinone and its γ-lactone. The latest views on vitamin A metabolism (58) include scission of the unsaturated side chain although, like vitamin K_1, the active metabolites have not yet been isolated and identified.

In several recent papers Martius and Nitz-Litzow suggest that vitamin K_1 may be involved in oxidative phosphorylation (68) (69) (70) (71). Oxidative phosphorylation was decreased in liver mitochondria from vitamin K deficient chicks (69), while the addition of vitamin K_1 *in vitro* to mitochondria from vitamin K deficient chicks caused an increase in the P/O ratio for the oxidation of β-hydroxybutyrate (70). It is difficult at present to evaluate the significance of these observations or to correlate the *in vitro* effects of vitamin K_1 with the *in vivo* actions of the vitamin although the following observations are of interest. Bouman and Slater (10) reported that a freeze-dried preparation of heart sarcosomal fragments (mitochondria) contained less than 0.01 μ mole vitamin K/gm. of protein. Green, Søndergaard, and Dam (43) observed that the vitamin K activity of 100 gm. of fresh beef liver was equivalent to that of 71 μgm. of vitamin K_3 when determined by chick assay. The highest concentration of vitamin K/gm. of nitrogen was in the mitochondrial fraction but this accounted for only 25 percent of the total vitamin K content of the liver. Attempts to isolate the vitamin by reverse phase partition paper chromatography from a petroleum ether-ethanol extract or by its reaction with aluminum oxide were not successful. Fieser suggested that vitamin K_3 might

be converted to vitamin K_1 in the tissues of vertebrates (36). Recently Martius (67) and others (50) (86) have used this theory to explain the superiority of vitamin K_1 as an antidote in anticoagulant intoxication and the observation that whereas vitamin K_1 increases the oxidative phosphorylation by mitochondria from vitamin K deficient chicks vitamin K_3 inhibits phosphorylation without affecting oxidation (68). Martius (67) extracted a radioactive substance from heart sarcosomes following the feeding of 2-methyl-C^{14}-1,4-naphthohydroquinone diacetate to K deficient chicks. This substance had a distribution coefficient of 1:18.2 between 95 percent methanol and n-heptane. The 2-methyl-C^{14}-1,4-naphthohydroquinone diacetate had a coefficient of 1:0.12, that of vitamin K_3 was 1:0.035 and vitamin K_1 gave 1:18.1. Martius concluded that vitamin K_3 diacetate was transformed to vitamin K_1 or a substance with similar solubility characteristics.

Methods of developing a vitamin K deficiency in rats.

F.

Dam and Glavind (23) pointed out that some rats on a vitamin K-free diet become just as ill as chicks, whereas other rats appear to receive adequate vitamin K of non-dietary origin. It has been established that some bacteria synthesize vitamin K (4) (11) (40) and therefore it was suggested that the non-dietary vitamin K was provided by bacterial synthesis in the caecum and large bowel (5). Various procedures have been employed in an attempt to disrupt the bacterial synthesis of vitamin K or to prevent its absorption from the gastrointestinal tract. Sulfaquinoxaline, fed at a level of 400 mgm./kg. per day, produced a prolonged prothrombin time in 6 out of 10 rats (90). Sulfaguanidine and sulfasuxidine have a similar effect (8). Black, Overman, Elvehjem, and Link (8) suggested that liver damage produced by the administration of sulfonamides was the cause of the deranged coagulation mechanism. Matoth (72) demonstrated that the prothrombin level was not affected by sulfaguanidine, succinylsulfathiazole and orally administered streptomycin to infants that already had a prolonged prothrombin time due to chronic diarrhea. If the sulfonamides had caused liver damage one would have expected an even greater prolongation in the prothrombin time. Vitamin A, administered in massive doses,

also produces a hypoprothrombinemia in rats, and Quick and Stefanini suggested that this was due to its toxic effects on the microflora of the intestine (81). Luckey and co-workers (60) have pointed out that the importance of the microflora in vitamin K synthesis may have been overemphasized. These workers have obtained much useful information on vitamin metabolism through the use of "germfree" animals which they define as animals living in the complete absence of all demonstrable living microorganism (59). Their experiments indicate that "germfree" chicks, maintained on a vitamin K-free diet developed prolonged whole blood clotting times and then recovered spontaneously from the vitamin K deficiency before conventional control birds on the same diet had developed a prolonged blood clotting time. Gustafsson (44) reported that germfree rats had blood and prothrombin clotting times that were very long. Reyniers (82) stated that germfree rats did not develop a vitamin K deficiency readily; however, Luckey et al (61) found that germfree rats on a low protein diet that contained 2 mgm./kg. of 2-methyl-1,4-naphthoquinone developed prolonged blood and prothrombin clotting times and died from hemorrhage. Vitamin E supplement prevented death and partially corrected the prolonged prothrombin times. The livers of the germfree rats were found to be normal on pathological examination, whereas conventional rats on the same low protein diet had necrotic livers.

The incorporation of 20% mineral oil into the diet (31), cecectomy (97), biliary fistula (41), biliary duct occlusion in vitamin K depleted rats (93), and lymph drainage (66) are all techniques that have been employed with a view to preventing the absorption of vitamin K from the intestine. Mineral oil is not absorbed from the gastrointestinal tract to any appreciable extent and presumably serves as a solvent that removes fat soluble vitamins by way of the feces. Contrary to the generally accepted belief, bile may not be essential for the absorption of vitamin K_1 from the intestine since Fisher et al (37) have shown an increase in factor VII and prothrombin in a cholecystnephrostomized dog following a single oral dose of 100 mgm. of vitamin K_1. However, bile increases the rate of absorption. Most of these methods are inefficient. No more than 60% of the rats develop a prolonged prothrombin time and usually the rat plasma must be diluted 1:8 v/v with saline if a prolonged prothrombin time is to be observed.

Warfarin (3- (α-phenyl-β-acetylethyl)-4-hydroxy-coumarin) produces a pronounced hypoprothrombinemia in rats that can be corrected by the administration of vitamin K_1 (85). It is not possible to state at present exactly what relationship exists between vitamin K_1 and Warfarin. The derangement is not, however, identical with a dietary vitamin K deficiency since both vitamin K_1 and 2-methyl-1, 4-naphthoquinone are effective on an equimolar basis in vitamin K deficiency whereas 2-methyl-1,4-naphthoquinone is relatively ineffective as an antidote in anticoagulant intoxication (9).

C. Hypoprothrombinemia of the newborn.

An interesting approach to the study of the metabolism of vitamin K is afforded by the phenomena associated with the prolonged prothrombin time demonstrable in the plasma of the newborn. The prothrombin values of the newborn infant are usually less than half of adult values and appear to be uninfluenced by the antepartum administration of vitamin K to the mother (55) (77). An even greater prolongation of the prothrombin time of the infant appears between the second and fourth day of life (80) (75). Owen and Hurn have shown that the postpartum prolongation of the prothrombin time is due to a decrease in both factor VII and prothrombin, but not in factor V (76). This secondary prolongation in prothrombin time is influenced by the antepartum administration of vitamin K analogs to the mother (77) or postpartum administration of vitamin K_1 or Synkavite to the infant (30).

The present problem.

H.

Our objectives were to study the distribution, transport, fate, and excretion of 2-methyl-C^{14}-3-phytyl-1,4-naphthoquinone (referred to hereafter as vitamin K_1-C^{14}) in albino rats. Factors affecting the tissue distribution such as the route of administration, diet, pregnancy, age, and sex have been investigated. A comparison was made of the tissue deposition and excretion of C^{14} following the intravenous administration of 2-methyl-C^{14}-1,4-naphthoquinone (referred to hereafter as vitamin K_3-C^{14}) and vitamin K_1-C^{14} to rats. Isotope dilution tests were employed to ascertain the amount of vitamin K_1-C^{14} that was present in the liver, skeletel muscle, spleen, bile, feces, and urine in unchanged form. Furthermore, attempts were made to isolate the metabolites of vitamin K_1-C^{14} from urine and bile by ion exchange, column chromatography on Celite and alumina, and by paper chromatography.

2. METHODS

A. Assay of C^{14}

A scaler (Berkeley Scientific model 2105), a printing timer (Nuclear Instrument and Chemical Corporation, model C-111), an automatic sample changer (Nuclear, model C-110), and a time delay switch (R.W. Cramer Co. Inc., model FTEC 15 M) were arranged to function as a single automatic unit. Dr. G.J. Millar designed and supervised the modification in the electrical circuits of this equipment. All measurements were made with a windowless gas flow counter (Atomic Energy of Canada Limited, Ottawa, Canada) that was mounted on the automatic sample changer. The "Q-gas" was purchased from Matheson Co. Inc., East Rutherford, New Jersey, U.S.A. and was composed of 98.7% of helium and 1.3% isobutane. Planchets of 1.0 in. diameter by 1/16 in. depth were pressed in a lucite hand press from sheet aluminum 0.002 in. thick. The sheet aluminum was obtained from Aluminum Rolling Mills, Montreal, Quebec. The principal advantage of these aluminum pans was that they were cheap and could be discarded after use.

Rats containing administered C^{14} were anesthetized lightly with ether and killed by decapitation. The wet tissues were removed from the rat's body with forceps and placed on a tared aluminum pan. They were dried for 48 hours at 85°C. in an oven. The dry tissues were cooled and weighed on the aluminum pan and the weight of the dry tissue obtained by difference. The tissue was then pulverized and ground in a porcelain mortar to a fine powder of about 100 mesh.

250 mgm. or less of the dry powder was spread over the entire surface of a tared aluminum planchet and tamped slightly with a spatula, care being taken to obtain a uniform smooth surface. The mounted sample was then placed in a desiccator over calcium chloride until it was assayed. Each sample was counted for 4000 counts and weighed immediately after assay to eliminate the error due to adsorbtion of water. Urine samples of 0.1 ml. volume were placed on tared aluminum planchets and 0.5 ml. of distilled water was added to spread the sample uniformly over the entire area of the planchet. The water was evaporated first at room temperature and then over calcium chloride in a desiccator. Urine samples were weighed and corrected for self-absorption.

Vitamin K_3-C^{14} was converted to the diacetate and assayed for C^{14} as described by Hoskin (46). It is very important to convert this compound into a nonvolatile derivative such as the diacetate since vitamin K_3 itself sublimes and will contaminate the counting chamber. Vitamin K_1-C^{14} was plated directly as 5 µl. of a 0.1% w/v solution of vitamin K_1-C^{14} in 5% v/v Tween 80. 0.5 ml. of water was added to each planchet to spread the sample uniformly over the surface and the sample was dried as described above.

The grinding and plating of powdered tissues requires practice. The following test can be employed to determine if satisfactory results are being obtained. A dried rat liver containing C^{14} is ground, and three samples are plated on aluminum planchets. The three samples are counted (4000 counts) and the counting is repeated. When the agreement between the calculated specific activities is $\pm 5\%$, the plating procedure can be regarded as satisfactory for the assay of C^{14} in biological material. The main sources of error are dust, insufficient grinding which exposes an irregular and variable surface, and moisture. Removal of all water vapor is particularly important when windowless gas flow counters are employed, since the composition of the counting gas determines the characteristics of the counting chamber.

It was evident from the start of our work that we would be measuring radioactivity in tissues and fluids of both very high and very low specific activity. A calibration curve to correct for absorption losses was constructed as follows:-

Dried, powdered, C^{14}-containing rat liver was plated on eleven tared aluminum planchets in amounts of approximately 1, 2, 4, 10, 25, 50, 75, 100, 125, 250, and 500 mgm.. Each sample was assayed twice (10,000 counts) and the mean values were plotted and the best fitting line calculated. The value for 1 mgm. was chosen as unity and a table of correction factors was calculated in such a manner that

$$\frac{\text{correction factor} \times \text{counts/min.}}{\text{weight of the sample (mgm.)}} = \text{counts /min./mgm./5.1 cm}^2.$$

Since all our planchets were of equal surface area, this unit was disregarded and henceforth specific activity will be expressed in counts /min./mgm.. All counts recorded were less than 2×10^3 counts /min. and therefore no correction for resolving time was employed. A correction for radioactive decay was not required since C^{14} has a half life of approximately 5×10^3 years. No correction was employed for geometry because the geometry of the sample with respect to the gas flow counter was kept constant and absolute disintegration rates were not required. A C^{14} standard was used to correct for changes in the efficiency of the counting

chamber and automatic recording equipment. This standard (Nuclear R-20) assayed 565 counts/min. in our gas flow counter.

When radioactive vitamin K_1-C^{14} has been administered to a rat the extent of metabolism of the vitamin at any given time is unknown and therefore it is convenient to express the radioactivity of the tissue in terms of the equivalent amount of vitamin K_1-C^{14} in the tissue. The vitamin K_1-C^{14} equivalent was calculated from the specific activity of the dry tissue and the specific activity of the injected vitamin as follows:-

Vitamin K_1-C^{14} equivalent (µgm./gm.) =

$$\frac{\text{Specific activity of dry tissue (counts/min/mgm.)}}{\text{Specific activity of vitamin } K_1\text{-}C^{14} \text{ (counts/min/mgm.)}} \times \frac{10^6 \text{ µgm.}}{1 \text{ gm.}}$$

This manner of expressing the concentration of radioactivity in tissues has the added advantage of permitting a direct comparison when the specific activities of the vitamin K_1-C^{14} preparations differed from one another.

Experimental Animals:
B.

Albino rats were used throughout this investigation. These animals were fed ad libitum a stock laboratory diet of Quaker calf meal pellets supplemented with oats unless indicated otherwise. They were housed in groups of from one to ten rats in screen wire cages until injected with either radioactive vitamin K_3-C^{14} or vitamin K_1-C^{14}. They were then housed individually, or on one occasion in groups of four, in circular wire metabolism cages supported on glass funnels. The metabolism cages had a 4 mesh floor and a 16 mesh subfloor and in addition a loosely fitting ground glass stopper was placed in the funnel to keep the urine and feces separate. Usually the rats were fed prior to injection and then deprived of food and water until they were killed: however, if the experiment exceeded 24 hours the rats were given distilled water to drink and calf meal pellets to eat ad libitum.

Although all of the rats used were albino they were obtained from several sources. The majority of the rats were supplied by Mr. Halstead, a breeder in Saskatoon. This stock was later bought by Mrs. Brown and is descended from rats bought from Wistar Institute, Philadelphia, in 1953. Some of the other rats were obtained from the Suffield Experimental Station at Ralston, Alberta, and E.G. Steinhilber and Company, Oshkosh, Wisconsin, and still others were bred in the departmental animal quarters and are of unknown lineage.

Bile was collected by cannulation of the common bile duct of rats as described by D'Amour and Blood (29) except that the cannulae used were eight inch lengths of P.E. 50 polyethylene tubing (Clay Adams Co.) The bile was collected in sterile 10 ml. graduated cylinders. The carotid arteries of four rats were cannulated with 4 inch lengths of P.E. 50 polyethylene tubing in order to collect blood for the fractionation of plasma proteins by the method of Cohn et al. (14).

The vitamin K_1-C^{14} was administered as a transparent colloidal suspension in "Tween 80" (6) (25), by intravenous injection into the jugular or tail vein, using light ether anesthesia, or by intramuscular injections into the left hind leg, or orally by force feeding with a polyethylene stomach tube. When vitamin K_3-C^{14} was administered to rats an equal weight of corn oil was added to solubilize the vitamin K_3-C^{14} before preparation of the aqueous suspension. The animals were killed by decapitation after a period of time varying from 5 hours to 12 days.

Reagents:
C.

(1) Vitamin K_1-C^{14}, vitamin K_1, vitamin K_3-C^{14} and vitamin K_3.

Two lots of vitamin K_1-C^{14} were synthesized by Dr. R. Woods of the Department of Chemistry, University of Saskatchewan. The first lot of vitamin K_1-C^{14} was synthesized using a method developed by Dr. Otto Isler of F. Hoffman-LaRoche, A.G., Basle, (48), (49) with modifications suited to small scale synthesis (56). This involves the reaction between isophytol (3,7,11,15 - tetramethyl-1-hexadecen-3-ol) and 2-methyl-C^{14}-1,4-naphthoquinone with boron trifluoride as catalyst. The second lot of vitamin K_1 was synthesized by the same method except that phytyl alcohol 3,7,11,15-tetramethyl-2-hexadecen-1-ol) was used instead of isophytol.

Isler and Doebel (49) showed that vitamin K_1 prepared from isophytyl alcohol was a racemic mixture whereas vitamin K_1 prepared from phytyl alcohol was an optically pure compound. Both vitamin K_1 preparations had identical ultraviolet and infra red absorption spectra and there were no differences in the products arising from oxidative degradation.

The vitamin K_3-C^{14} used was also synthesized by Dr. Woods according to the method of Fieser (33) (34). The nonradioactive vitamin K_1 that was used as a comparative standard and as a carrier in isotope dilution tests was obtained in 1 gram ampules from Merck and Company. 10 grams of 2-methyl-1,4-naphthoquinone was purchased from Eastman Kodak Company.

(2) Irreverre Sullivan colorimetric assay of vitamin K_1.

Colorimetric assays for vitamin K_1 were carried out as described by Irreverre and Sullivan (51) except that 1 ml. of benzyltrimethylammonium hydroxide was substituted for the 2% sodium alcoholate. The absorption at 540 mμ was read in 10 x 75 mm. Coleman cuvettes in a spectrophotometer (Coleman junior model 6A.). Vitamin K_1 yields a blue color while vitamin K_3 gives a pink color. The blue color is not stable. It reaches maximum intensity 3 minutes after the reagents have been mixed, after which it fades. The benzyltrimethylammonium hydroxide reagent consisted of a 30% solution in methyl alcohol (Chemical Development Corp., Danvers, Mass., U.S.A.). Sodium diethyldithiocarbonate was obtained from Fisher Scientific Company.

(3) Colorimetric assay of vitamin K_3.

The Craven reaction (15) as modified by Richert (83) was used for the assay of 2-methyl-1,4-naphthoquinone. 2-methyl-1,4-naphthoquinones with a substituent in the 1, 3 or 4 position do not give this reaction. A stable blue color develops in the presence of 2-methyl-1,4-naphthoquinone. The ethylcyanacetate was obtained from Brickman and Co., Montreal.

(4) Diphosphate and disulfate esters of 2-methyl-1,4-naphthoquinone.

10 gm. of Synkavite (2-methyl-1,4-naphthoquinone diphosphate tetrasodium salt) was a gift from F. Hoffman LaRoche Company, Nutley, N.J. The 2-methyl-1,4-naphthoquinone disulfate disodium salt was synthesized by Dr. R. Woods according to the method of Fieser (34).

(5) Ultra-violet absorption spectra.

Ultra-violet absorption spectra were determined in silica cells of 10 mm. light path, using the spectrophotometer (Beckman, model D.U.). Spectro grade n-hexane (British Drug Houses) was used as a solvent for purified vitamin K_1 preparations.

(6) Solvents, chromatographic adsorbants and ion exchange resins.

Petroleum ether (Skelly F, B.P. 40°C-60°C, Skelly Oils) was used for the chromatography of vitamin K_1 on Folin's Permutit (Fisher Scientific Company). All the other solvents that were employed were reagent grade chemicals with the exception of chloroform and diethyl ether which were U.S.P. grade. The ether was made peroxide free by extraction with acidic ferrous sulfate followed by water and dried over calcium chloride.

Other chromatographic adsorbants used were activated alumina (Alco, 100 mesh), zinc carbonate, reagent grade powder (Fisher Scientific Company), and Celite 545 (Johns Manville Co.). The amberlite resins employed were 1R-45, 1RC-50, 1R4B, and 1R120, experimental grade (Rohm and Haas). Dowex 1, 10% cross link, 100-200 mesh was obtained from Dow Chemical Company.

(7) Papers and solvents for paper chromatography, and reverse phase partition paper chromatography.

Whatman No. 1 filter paper was used exclusively. Sheets, 18 in. x 22 in. were cut into 9 in. x 11 in. rectangles. A solvent system that proved very useful for the chromatography of naphthohydroquinone esters, urine and bile was butanol: methanol 3:1 saturated with 0.01 N. acetic acid (46). Vitamin K_1, vitamin K_3 and extracts of liver were chromatographed, using reversed phase partition paper chromatography, on siliconed Whatman No. 1 filter paper, according to the method described by Green and Dam (42). Rectangles of Whatman No. 1 filter paper were dipped in a 5% solution of Dow Corning silicone No. 1107 in chloroform and allowed to air dry. Ethyl alcohol: acetic acid: water (30:1:9) was employed as the solvent system.

(8) Hydrolytic agents used to hydrolyse 2-methyl-1,4-naphthohydroquinone esters.

Ceric sulfate reagent was used to hydrolyse naphthohydroquinone esters according to the method described by Richert (84).

β-glucuronidase (Sigma Chemical Company) was employed to test for the presence of β-D-glucopyranuronide conjugates of vitamin K_1-C^{14} or its metabolic products. Mylase P (Wallerstein Laboratories) was used to test for the presence of ethereal sulfate conjugates of vitamin K_1-C^{14} or its metabolic products. It was also used to hydrolyse 2-methyl-1,4-naphthohydroquinone disulfate. Wheat germ acid phosphatase (Nutritional Biochemical Co.) was used as a hydrolytic agent for 2-methyl-1,4-naphthohydroquinone diphosphate. The exact conditions used for each hydrolysis are presented along with the results.

3. RESULTS:

A. Purification of radioactive vitamin K_1-C^{14}

(1) The detection of an impurity in vitamin K_1-C^{14}

Dr. R. Woods provided a second lot of radioactive vitamin K_1-C^{14} in December 1955. This vitamin K_1-C^{14}, lot No. 2, had a specific activity of approximately 1×10^6 counts/min/mgm. whereas vitamin K_1-C^{14}, lot No. 1, had a specific activity of approximately 3×10^5 counts/min./mgm.. We had just succeeded in acquiring the technique of reverse phase partition chromatography on siliconed Whatman No. 1 filter paper as described by Green and Dam. The vitamin K_1-C^{14}, lot No. 2, gave two radioactive spots, Rf 0.20 and Rf 0.85, only one of which, Rf 0.20, corresponded with the added carrier vitamin K_1. Colorimetric estimation of vitamin K_1 by the method of Irreverre and Sullivan (see methods) showed that the preparation was 74% pure and the ethylcyanoacetate test (83) indicated that the impurity was not vitamin K_3-C^{14}. The sample of vitamin K_1-C^{14}, lot No. 1, was assayed next and it was found to be 82% pure and no vitamin K_3-C^{14} could be detected. Dr. Woods had reported that lot No. 1 of vitamin K_1-C^{14} contained less than 5% vitamin K_3-C^{14} as assayed by isotope dilution tests.

No vitamin K_1 was available at the time of synthesis for isotope dilution tests. An ultraviolet absorption spectrum in absolute ethanol was performed by Dr. Woods on the vitamin K_1-C^{14} lot No. 1, and was in good agreement with the literature (32).

(2) Separation of vitamin K_1 and vitamin K_3 by chromatography on zinc carbonate.

Ten mgm. of vitamin K_1 and 10 mgm. of vitamin K_3 were dissolved in Skelly F and chromatographed on a 10 cm. x 1 cm. column of zinc carbonate wet with Skelly F. The vitamin K_1 could be eluted with Skelly F plus 10% benzene but the vitamin K_3 was strongly adsorbed and changed. The vitamin K_3 band could be eluted with diethyl ether but it had an orange color and gave a negative ethylcyanoacetate test.

(3) Chromatography of vitamin K_1 and vitamin K_3 on Folin's Permutit.

A 1 cm. x 15 cm. column of Folin's Permutit was moistened with Skelly F and 10 mgm. of vitamin K_1, plus 10 mgm. of vitamin K_3 dissolved in 2 ml. of benzene were adsorbed on the Permutit. 100 ml. of Skelly F followed by 250 ml. of Skelly F plus 10% benzene, 250 ml. of Skelly F plus 20% benzene, and 250 ml. of Skelly F plus 30% benzene were percolated through the column and 50 ml. fractions were collected. The separation of vitamin K_1 and vitamin K_3 is shown in Table 1.

Vitamin K_1 can be separated from vitamin K_3 using either method of column chromatography but the Permutit column has the advantages that the vitamin K_3 can be recovered and the rate of flow is more rapid than with zinc carbonate.

(4) Purification of radioactive vitamin K_1-C^{14} by chromatography on Folin's Permutit.

31.2 mgm. of the impure vitamin K_1-C^{14}, lot No. 2, was chromatographed on Folin's Permutit as described above. Three radioactive peaks were recovered. The first peak was radioactive vitamin K_1-C^{14}, approximately 20.8 mgm.; the second was present in very small amounts, approximately 3.0 mgm. The third peak could be eluted with diethyl ether. It was a red oil weighing approximately 7.2 mgm. Neither of the last two substances gave a positive Irreverre-Sullivan reaction. The red oil was not only present in our preparations of vitamin K_1-C^{14} but was also recovered from two samples of Merck's vitamin K_1. This red oil might be a product arising on storage because one ampule of Merck's vitamin K_1 was free from detectable impurity.

(5) Synthesis of 2-methyl-3-phytyl-1,4-naphthohydroquinone diacetate.

Although the synthesis of this compound is reported in the literature (7) we felt that our exact method should be recorded since considerable difficulty was experienced in obtaining the first crystalline product.

Merck's vitamin K_1, 50 mgm., was refluxed for thirty minutes with 25 mgm. of fused sodium acetate and 50 mgm. of zinc dust in 1 ml. of acetic anhydride and 0.25 ml. of glacial acetic acid. The mixture was cooled, 25 ml. of distilled water was added and the mixture was warmed at 70° for 1 hour. After cooling, the oil that separated was extracted with two 25 ml. portions of peroxide-free diethyl ether. The ether was pooled and extracted three times with 10 ml. of distilled water and once with 25 ml. of 0.01 N sodium bicarbonate and then dried over 1 gm. of anhydrous sodium sulfate. The ether was evaporated *in vacuo* at the water pump and the resulting oil was placed in the deep freeze at -5°C. After about two weeks crystals were present. Once the first crystals were obtained it was possible to crystallize the vitamin K_1 diacetate from methanol containing 10% ethanol at -5°C. The best yield obtained was 18% calculated on the basis of the vitamin K_1 used. After two crystallizations from the above solvent this material had a melting point of 55-56°C uncorrected (literature 62°C-63°C. (11)).

(6) Biological (anti-Warfarin) activity of vitamin K_1-C^{14}.

It occurred to us that a synthetic vitamin K_1 preparation might give all the chemical tests for vitamin K_1 described above and still fail to possess biological activity. Vitamin K_1 has been reported to correct the prolonged one-stage prothrombin times resulting from the administration of Warfarin (3-(α-phenyl-β-acetylethyl)-4-hydroxycoumarin). Warfarin was chosen because rats respond very readily to this drug.

Eight rats were placed in individual cages and six were fed 5 mgm/kg of Warfarin in 50 gm./kg of oats. Two rats were maintained on the stock laboratory ration of calf meal pellets and served as controls. Prothrombin times were determined by the method of Schwager and Jaques (87) on blood obtained by venipuncture of the tail vein. Immediately after the initial prothrombin times were determined, the two normal controls and two of the Warfarin treated rats were given an intravenous injection of 1 ml. of 5% Tween 80. Two of the Warfarin treated rats were given 1 mgm. of Merck's vitamin K_1 in 5% Tween 80 and the remaining two rats were given 0.2 mgm. of vitamin K_1-C^{14}, lot No. 1, in 5% Tween 80.

It is evident from the results of this experiment

(table 2) that the vitamin K_1-C^{14} does possess biological activity in the sense that it restored the prothrombin times of two rats from greater than 180 seconds to near normal values when the vitamin dosage was 1 mgm./kg.. Merck's vitamin K_1 was tested first and when a response was obtained with 5 mgm./kg. of vitamin K_1 we decided to inject a smaller dose of vitamin K_1-C^{14} in order to conserve our supply.

Isler and Doebel (49) have reported that vitamin K_1 synthesized from isophytyl alcohol had a biological activity identical with vitamin K_1 synthesized from phytyl alcohol when tested in vitamin K deficient chicks and rabbits rendered hypoprothrombinemic with dicoumarol. (3,3'-methylenebis (4-hydroxycoumarin).

(7) Ultra-violet absorption spectrum of vitamin K_1-C^{14}.

An ultra-violet absorption spectrum in n-hexane on a sample of vitamin K_1-C^{14}, lot No. 1, purified by chromatography on Folin's Permutit showed five absorption peaks at 243, 247, 260, 268 and 325 mμ. The values reported in the literature (32) are 243, 249, 261, 270, 325 mμ. Accurate measurements of the absorption spectrum of vitamin K_1 are difficult to obtain since it has a complex spectrum and is destroyed by light. We obtained an $E^{1\%}_{1cm.}$ at 249mμ = 533 (literature $E^{1\%}_{1cm.}$ at 249 mμ = 540 (32)).

(8) Isotope dilution test to ascertain the radiochemical purity of vitamin K_1-C^{14}.

The purified vitamin K_1-C^{14} recovered after chromatography on Folin's Permutit had a specific activity of 1.05×10^6 counts/min./mgm. 0.75 mgm. was added to 108.0 mgm. of nonradioactive vitamin K_1 in Skelly F. The mixture was chromatographed on permutit and the yellow solution obtained was evaporated in vacuo and dissolved in n-hexane. The mixed vitamin K_1 had a specific activity of 7.06×10^3 counts/min./mgm. From this we calculated that 0.72 mgm. of vitamin K_1-C^{14} had been added to the nonradioactive vitamin K_1 carrier. Therefore the purity of the preparation after chromatography was 96%. We were unsuccessful in an attempt to synthesize the diacetate of this sample of vitamin K_1.

B. Tissue deposition and excretion of C^{14} in rats following the administration of vitamin K_1-C^{14}.

(1) Test for the presence of CO_2 in expired air.

To check the possibility that the radioactive methyl group of the vitamin K_1-C^{14} might be metabolized to CO_2, a 156 gram female rat was given a parenteral injection of 6.9 mgm./Kg. of radioactive vitamin K_1-C^{14}, lot No. 1, specific activity 3.0×10^5 counts/min./mgm. The expired air was passed through sodium hydroxide for 5 hours and the carbon dioxide was precipitated as barium carbonate, which was washed, dried, weighed and the C^{14} activity assayed in the manner described above. The barium carbonate was found to contain no significant amount of radioactivity; e.g., specific activity 0.097 ± 0.087 (S.D.) counts/min./mgm. As little as 0.05% of the injected dose could have been detected.

(2) C^{14} in blood, erythrocytes, buffy coat, plasma, and plasma protein fractions.

A dose of 3 mgm./Kg. of vitamin K_1-C^{14}, lot No. 2, specific activity 1.33×10^5 counts/min./mgm., was administered orally to each of four adult male rats. Blood was collected from the carotid arteries into acid citrate-dextrose anticoagulant mixture† in plastic test tubes. Two

†referred to hereafter as A.C.D.

rats were bled one hour after the vitamin was given and the other pair were bled after sixteen hours. The blood from each pair of rats was pooled and fractionated by the method of Cohn et al (14). One hour after the oral ingestion of the vitamin K_1-C^{14}, 69% of the activity present in the blood was in the plasma. The results of this experiment are presented in Table 3. The fraction that contained fibrinogen (I+III-3) had a very high specific activity compared to the other fractions. Fraction III-0, the β-lipoprotein-containing fraction had no significant activity. The specific activity of the washed erythrocytes had decreased very little after 16 hours whereas the specific activity of the buffy coat had increased approximately sevenfold. The level of C^{14} in the 16 hour plasma was only 25% of the radioactivity present in the whole blood. The level of radioactivity had decreased in all the plasma fractions but the greatest decrease was in the fibrinogen containing fraction (I+III-3). The albumin containing fractions IV, V, and VI carried most of the radioactivity in the plasma after 16 hours. This shift might be due to the metabolism of the vitamin K_1-C^{14}.

On analysis of the blood from a rat five hours after intravenous administration of vitamin K_1-C^{14}, specific activity 3.0×10^5 counts/min./mgm., the plasma contained about 64% of the total activity present in the whole blood. On a dry weight basis the buffy coat had a specific activity

of 3.78 ± 0.102 counts/min./mgm. compared with 0.63 ±0.83 counts/min./mgm. for the erythrocytes. The erythrocytes, however, because of their greater weight, had a greater vitamin K_1 equivalent (counts /min./100 ml. of blood) as presented in Table 3.

(3) C^{14} in the blood, liver, spleen and bone marrow.

A comparison of the specific activities in the liver, spleen, bone marrow and blood 5 hours after the administration of radioactive vitamin K_1-C^{14} is presented in Table 4. The spleen had the highest specific activity, 316.0 counts/min./mgm.; the liver followed with 86.6 counts/min./mgm., and the bone marrow and whole blood were nearly of equal value. The blood was 18.2 (or 20.4) counts/min./mgm. and the bone marrow was 20.4 counts/min./mgm. The citrated plasma had an activity of 494 counts/min./0.1 ml. while the whole blood was 359 counts/min./0.1 ml. Since the hematocrit was 32.5% it can be calculated that 334 counts/min. would be present in the plasma of 0.1 ml. of whole blood. Therefore, 5 hours after intravenous administration 93.5% of the activity in the blood is found associated with the plasma. The rat was a young one (160 grams) and the entire bone shafts were filled with red marrow. The sample for plating was scraped from the ends of the femur with aid of a micro spatula. The observation, that all parts of the

reticuloendothelial system do not concentrate vitamin K_1 to the same extent, could be accounted for on the basis of differences in the cytology and chemical composition of the liver, spleen and bone marrow. The amount of vitamin K required for optimal "prothrombin" synthesis in the rat is not known accurately. Quick estimates 0.5 micrograms per kilogram body weight /day for adult dogs and ten times this amount for pups (79). If the requirements of the rat are of the same order of magnitude, as little as 1.3×10^{-4} of the dose administered (6.25 mg/Kg.) might suffice for daily "prothrombin" synthesis. If all the activity in the bone marrow at 20.4 counts/min./mgm. were present in an active form about 20 mg. of bone marrow (dry) would contain enough of the injected vitamin K_1-C^{14}, specific activity 9.5×10^5 counts/min./mgm. to meet one day's requirements.

(4) Estimation of C^{14} distribution and total percent recovery.

A preliminary investigation of the distribution of radioactive vitamin K_1 was conducted as follows:
A 310 gram female rat was given an intravenous injection of 0.32 mgm./Kg. of vitamin K_1-C^{14}, specific activity 3.0×10^5 counts/min./mgm. Nine hours later the liver, lung, kidney, spleen, heart, large bowel and contents, small intestine and contents, stomach and contents, and the urine were assayed for C^{14} content. The rest of the animal was minced in a meat chopper, and treated as above. The results presented in Table 5 show the distribution of radioactivity in this rat. All the radioactivity injected was accounted for. The liver appears to concentrate vitamin K_1 even at doses of less than 1 mgm./Kg. of body weight. The lung and the spleen also accumulated some radioactivity. It is also apparent that activity was present in the gastrointestinal tract in fairly high concentration. The small intestine and its contents showed a higher specific activity than the caecum, large bowel and contents although both contained considerable activity. The caecum, large bowel, and contents contained a greater percentage of the injected dose because of their larger bulk.

(5) Distribution of C^{14} in tissues following oral, intramuscular, and intravenous doses of vitamin K_1-C^{14}.

In order to determine whether the route of administration affected the deposition of vitamin K_1 in the tissues of rats, three female rats weighing 144, 155 and 160 grams were injected with 1 mgm. of radioactive vitamin K_1-C^{14}, specific activity 3.0×10^5 counts/min./mgm. One rat received the colloidal suspension by stomach tube, a second animal was given an intramuscular injection and the remaining rat received an intrajugular injection. After 24 hours the animals were killed and the tissues were assayed for C^{14}. Table 6 shows a comparison of the tissue levels of radioactivity found in these animals. At a dose of 6-7 mgm./Kg. it would appear that there is very little difference in the vitamin K_1-C^{14} equivalent deposited in the liver when administered by the various routes. The vitamin K_1-C^{14} equivalent of the blood and heart of the rat given an intravenous injection was lower than those of either of the other animals. On the other hand the spleen of the animal given vitamin K_1 intravenously had a higher vitamin K_1-C^{14} equivalent than the other animals. It should be pointed out that a large amount of the vitamin K_1 given intramuscularly remained at the site of injection.

(6) Retention of C^{14} in tissues following intravenous and intramuscular administration of vitamin K_1-C^{14}.

In order to study the duration of the retention of radioactivity in the tissues, six female rats were given 5.2 mgm. of vitamin K_1-C^{14} in 5% Tween 80. Three of these animals were given intravenous injections and three were given intramuscular injections. The animals were killed at one, three, and six days. The urine and feces were collected and pooled and the cumulative fecal and urinary excretions were determined. The results of this experiment are shown in Table 7. A progressive decrease of radioactivity in the livers of both groups of rats and an increased fecal and urinary excretion is evident. Radioactivity remained in the livers and spleens of both groups for as long as six days. The cumulative fecal excretions at six days exceeded the urinary excretion by a factor of two to three. The total excretion in 6 days amounted to 52% and 65% of the injected doses. When the radioactive vitamin was given intravenously, the liver and spleen retained appreciable amounts of radioactivity but when the vitamin K_1-C^{14} was injected intramuscularly the spleen retained only a small amount of the radioactivity although the liver levels were similar to those obtained after the intravenous injection.

Approximately 30% of the intramuscular dose remained at the site of injection after 24 hours and about 4% was still present after 144 hours.

(7) The effect of increasing the intravenous dose of vitamin K_1 on the tissue deposition and excretion of C^{14}.

In order to test the effect of the magnitude of the administered dose on the tissue deposition of radioactive vitamin K_1 five young female rats were each given an intrajugular injection of vitamin K_1 in Tween 80. The amount of radioactive vitamin given each rat was 5 mgm./Kg. but the amount of nonradioactive vitamin was varied to give the total amount of vitamin indicated in figure 1 and Table 8. The animals were killed 24 hours after the injection and the radioactivity in the livers, lungs, kidneys, spleens, hearts, blood, skeletal muscle, urine and feces was determined. The amount of radioactivity deposited in all the tissues examined increased with increasing doses of radioactive vitamin K_1 but this increase was most marked in the case of the liver, heart and spleen. Figure 1 shows the calculated vitamin K_1 equivalent in µgm./gm. of dry tissue plotted against the dose of vitamin K_1 in mgm./Kg. (both on log scale). As the

size of the administered dose was increased the percentage of the dose deposited in the liver and the spleen tended to increase while the percentage excreted in the urine and feces tended to decrease.

Figure 2 shows the values for the urinary and fecal excretion of radioactive vitamin K_1 plotted against the dose of vitamin K_1 in mgm./Kg. (both on logarithmic scales). The fecal excretion exceeded the urinary excretion for all the doses used. The large variation in fecal excretion can be accounted for on the basis of correspondingly large variations in the weight of the feces excreted by individual rats.

The data from which figures 1 and 2 were plotted is contained in Table 8 as well as the percent of the injected dose deposited in each organ.

(8) A comparison of the concentration of C^{14} in the tissues of pregnant and nonpregnant female rats following the intravenous administration of vitamin K_1-C^{14} and vitamin K_3-C^{14}.

Female rats weighing 150-200 grams were examined daily by vaginal smear to demonstrate the estrus cycle and a check for sperm in the vagina was made after mating. Eighteen days after the day of conception six pregnant rats were given an intravenous injection of 5 mgm./Kg. of vitamin K_1-C^{14}, specific activity 8.4 x 10^5 counts/min./mgm. and six pregnant rats were given an intravenous injection of 5 mgm./Kg. of vitamin K_3-C^{14}, specific activity 3.3 x 10^5 counts/min./mgm.. Twenty-four hours after the administration the animals were decapitated and their uteri were ligated and removed. The tissues and fluids listed in Table 9 were assayed for C^{14} and the percent of the injected dose and the vitamin K equivalents were calculated as described previously. Twelve nonpregnant female rats were retained as controls. Six of these animals received 5 mgm./Kg. of the vitamin K_1-C^{14} and six received 5 mgm./Kg. of the vitamin K_3-C^{14}. In order to determine the amount of radioactivity present as unchanged vitamin K_1-C^{14} a pregnant rat was given 5 mgm./Kg. of vitamin K_1-C^{14} on the eighteenth day of gestation, killed 24 hours later and isotope dilution tests were performed on the maternal liver and on the placental and fetal tissue.

The data that are presented in Table 9 show that the placenta, fetus, fetal liver, and amnionic fluid did contain C^{14} following the intravenous administration of either vitamin K_1-C^{14} or vitamin K_3-C^{14} and that the vitamin K_1-C^{14} equivalents are of the same order of magnitude as the vitamin K_3-C^{14} equivalents. A comparison of data from animals given vitamin K_1-C^{14} with those given vitamin K_3-C^{14} showed that a marked concentration of C^{14} occurred in the livers and spleens of the animals given vitamin K_1-C^{14} whereas the concentration was much less prominent in the animals given vitamin K_3-C^{14}. It is interesting to note that the concentration of C^{14} in the blood of animals that had received vitamin K_3-C^{14} was greater than the concentration in the blood of the rats that were given vitamin K_1-C^{14}. The same trend was apparent in the skeletal muscle of the two groups but the difference was not statistically significant. The vitamin K_1-C^{14} equivalents for the fetal livers were much lower than those for the maternal livers whereas when vitamin K_3-C^{14} was given the maternal and fetal livers had similar vitamin K equivalents.

Table 10 illustrates another interesting point. When the ratios $\frac{\text{weight of dry tissue (gms.)}}{\text{live weight of the rat (gms.)}}$ were calculated for the livers and spleens of pregnant and non-pregnant rats the following comparisons could be made: the

ratios for the spleens differed significantly whereas the ratios for the livers did not. However, when the weight of the uterus and contents was subtracted from the live weight of each pregnant rat and the ratios recalculated the reverse was found to be true; that is, the ratios for the spleens did not differ whereas a significant difference existed between the ratios for the livers of the two groups of rats.

Since a ratio may be increased either because the numerator was increased, the denominator was decreased or both, it is important to note that the increased ratios presented in Table 10 were due to a marked increase in the dry weight of the livers of the pregnant rats.

(9) The effect of a vitamin K-free, fat-free diet and of a stock laboratory ration on the deposition of C^{14}.

A comparison of the tissue levels of radioactive vitamin K_1 in two groups of rats receiving either a stock laboratory ration or a vitamin K-free, fat-free diet was obtained as follows:- A group of six weanling female rats were maintained on a stock laboratory ration of calf meal pellets for three months. A second group of twelve weanling female rats were given a fat-free, vitamin K-free diet for three months. This diet was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. It was formulated as a modification of diet 674, Wooley and Sebrell (98).

All animals were given water ad libitum; they were fed daily, and housed individually. During the course of the feeding period five of the animals on the synthetic ration died. Autopsy of these animals revealed hemorrhagic lungs. Dam and Glavind pointed out that some rats on a vitamin-free diet become just as ill as chicks. Other rats appear to receive adequate vitamin K from their intestinal flora. After ten weeks some of these rats showed a loss of weight. Just prior to intrajugular injection of 5 mgm./kg. body weight of radioactive vitamin K_1 cardiac punctures were performed to obtain plasma for the determination of

one stage prothrombin times by the method of Quick. No difference was found between the prothrombin times of the two groups of rats. Four rats died as a result of cardiac puncture. There remained five animals on the synthetic ration and four control animals. These were injected with radioactive vitamin K_1-C^{14}, housed in individual metabolism cages, killed by decapitation after 24 hours and the various tissues, feces and urine listed in Table 11 were assayed for C^{14} activity. The vitamin K_1-C^{14} equivalent in the liver and skeletal muscle of the animals on the vitamin K-free, fat-free ration were greater than those for the animals on the stock laboratory ration. The urinary excretion of radioactivity was greatest for the animals on the normal diet.

A marked difference in the color of the liver and kidneys of the two groups of animals was evident during the removal of the tissues. The groups of animals that had been on the synthetic ration had paler mottled livers and the kidneys were pale also. The difference in appearance of the liver and kidneys of the two groups of rats combined with the fact that these animals had rough dry skin about the tail and feet, and furthermore, that they lost weight after a period on the synthetic ration suggest an essential fatty acid deficiency. The exact manner in which such a deficiency could influence the metabolism of vitamin K_1 can only be determined by further investigation.

Further information can be gained if the data in Table 9 and Table 11 are compared. The marked difference in the deposition of C^{14} in the livers of the two groups of normal animals on a stock laboratory ration suggested an uncontrolled variable that was influencing the metabolism of vitamin K_1. It was for this reason that the following experiment was designed.

(10) The effect of age, sex and method of preparation of vitamin K_1-C^{14} on the deposition of C^{14} in the liver, spleen, skeletal muscle, and blood of rats.

Twelve adult rats weighing 195±1.18 (s.e.) gms. were divided into four groups containing three rats per group. Six of the rats were males and six were females. Three of the males and three of the females were given an intravenous injection of 5 mgm./kg. of vitamin K_1-C^{14}, lot No. 1, and two similar subgroups were given an intravenous injection of 5 mgm./kg. of vitamin K_1-C^{14}, lot No.2. Twelve young rats weighing 93.7±0.35 (s.e)gms. were divided into groups and subgroups in the same manner. Twenty-four hours after administration of the vitamin K_1-C^{14} the rats were killed and the tissues were removed and assayed for C^{14}.

The mean percent deposition of C^{14} and the mean vitamin K_1 equivalent are presented in Table 12. An analysis of the data showed that the vitamin K_1 equivalents and the percent of the dose of C^{14} in the livers and spleens of the young animals differed significantly from that of the adult animals. Another observation was a significant difference between the amount of C^{14} deposited in the skeletal muscle of the rats given vitamin K_1-C^{14}, lot No. 1, as compared with the animals given vitamin K_1-C^{14}, lot No. 2. This difference was not demonstrated in any of the other tissues studied. It is of interest to note the observations of Isler and Doebel (49) that vitamin K_1 prepared from isophytyl alcohol is a racemic mixture (e.g., vitamin K_1-C^{14}, lot No. 1) whereas vitamin K_1 prepared from phytyl alcohol is optically uniform. (e.g., vitamin K_1-C^{14}, lot No. 2).

(11) Isolation of vitamin K_1 from rat liver by extraction and reverse phase partition paper chromatography following the intravenous administration of vitamin K_1.

By reverse phase partition paper chromatography we were able to demonstrate the presence of unmetabolized vitamin K_1 in the liver of a 180 gram female rat that had received a massive dose, 100 mgm./kg. of vitamin K_1 in Tween 80, by tail vein. This animal was killed $4\frac{1}{2}$ hours later and the liver was removed, ground with anhydrous sodium sulfate and extracted with peroxide-free ether. The ether was evaporated *in vacuo* and the residue was extracted with absolute ethanol (30). After leaving overnight at -5°C the solution was filtered and the volume was increased to 10.0 ml. The vitamin K_1 content of the liver was estimated colorimetrically by the Irreverre-Sullivan reaction and a recovery of 22.8% of the injected vitamin K_1 was attained. An aliquot of the liver extract containing 25 µgm. of vitamin K_1 was chromatographed simultaneously with control spots of authentic vitamin K_1 and vitamin K_3. After chromatography a spot was present that corresponded to a control spot of vitamin K_1 both in its position on the chromatogram and by its blue fluorescence under ultra-violet light. Green, Søndergaard, and Dam (43) were unable to isolate vitamin K from beef liver. by reverse phase partition

paper chromatograchy although they showed by the chick assay that the liver contained the equivalent of 71 µgm. of menadione /100 gm. of fresh liver.

(12) Isotope dilution tests to ascertain the percent of C^{14} present in tissues as unchanged vitamin K_1-C^{14}.

The following is an example of the procedure and calculation employed in conducting the isotope dilution tests: Approximately 27 grams of wet muscle was removed from a 325 gram male rat that had received, intravenously, 1 mgm. of vitamin K_1-C^{14}, specific activity 1.01×10^6 counts/min./mgm. The animal was killed by decapitation 24 hours after injection. The muscle was chopped, ground and weighed. 2.06 grams of wet muscle had a dry weight of 1.1052 grams and a specific activity of 1.37 counts/min./mgm. (dry). On this basis the remaining 25.4 grams of wet muscle would be equivalent to 13.6 grams of dry muscle containing 1.86×10^4 counts/min. The 25.4 grams of wet muscle was ground with 55 mgm. of vitamin K_1, 50 grams of anhydrous sodium sulfate was added, and the mixture was ground to a powder, and extracted with peroxide-free ether; the ether was removed in vacuo and the residue was extracted with absolute ethanol. The alcoholic extract was refrigerated overnight at -5°C, filtered, extracted with petroleum ether

(B.P.30-60°C) and chromatographed on permutit with petroleum ether, petroleum ether plus 10% benzene, and petroleum ether plus 20% benzene (99). The yellow oil that was obtained weighed 24.0 mgm. and assayed 24.4 mgm. using the Irreverre-Sullivan color reaction. It had a specific activity of 250 counts/min./mgm. The number of milligrams of labelled vitamin K_1-C^{14} present in the muscle before adding carrier was 1.36×10^{-2}mgm., i.e., 1.4% of the injected vitamin K_1-C^{14}. As the total activity in the sample before extraction was 1.86×10^4 counts/min. or 1.9% of the injected activity, it appears that approximately 74% of the activity in the muscle was present as unmetabolised radioactive vitamin K_1-C^{14}.

Isotope dilution tests were carried out on the other tissues in a similar manner and the results are presented in Table 13. It can be seen that the liver of rat No. 1 contained 2.8% of the injected activity of which about 78% was present as unchanged vitamin K_1-C^{14}. The urine contained no unchanged vitamin K_1-C^{14}. The skeletal muscle presents a picture similar to that of the liver of rat No. 1, whereas the small intestine and its contents and the feces are intermediate. Bile was collected by fistula for 16 hours and this contained 26% of the injected activity, but only 1.5% of the C^{14} in the bile was present as unchanged vitamin K_1-C14. Analysis of the vitamin K_1-C^{14} content of the tissues of a

pregnant female rat (rat No. 4) showed that 12% of the activity in the fetal tissue, 59% of the activity in the placental tissue and 120% of the activity in the maternal liver were present in unchanged form. The high value for the maternal liver may be due to a sampling error.

C. Attempts to identify the "third" urinary metabolite of vitamin K_3-C^{14}.

Hoskin (46) demonstrated the presence of three radioactive metabolites in rat urine following the administration of vitamin K_3-C^{14}. He showed that the third metabolite was not hydrolysed by β-glucuronidase but was hydrolysed by 0.2 M hydrochloric acid. An isotope dilution test indicated that vitamin K_3 resulted from the acid hydrolysis.

The radioactive spot of the third urinary metabolite of vitamin K_3-C^{14}, following paper chromatography, was compared with the diphosphate and disulfate esters of naphthohydroquinone and with their products of hydrolysis in attempts to identify this metabolite. In order to determine whether the third urinary metabolite of vitamin K3 was the diphosphate we chromatographed 2-methyl-1,4-naphthohydroquinone diphosphate tetra sodium salt (Synkavite) on Whatman No. 1 paper in butanol:methanol:acetic acid. The diphosphate did not migrate and therefore we concluded that it was not identical with the third urinary metabolite which has an Rf of 0.20 - 0.25. Chromatography in butanol:methanol: 3:1, saturated with 0.01 N acetic acid on Whatman No. 1 paper revealed that the disulfate had an Rf of 0.20 - 0.25. When a 15 ugm. sample of the disulfate of menadione was added to 25 ul. of rat urine containing the third radioactive metabolite of vitamin K_3-C^{14} the fluorescent spot of

the disulfate under ultra-violet light and the peak of the radioactivity did not coincide. It is therefore unlikely that the third urinary metabolite of vitamin K_3-C^{14} is the disulfate of vitamin K_3-C^{14}.

(1) Products of hydrolysis of naphthohydroquinone esters.

The following paragraphs describe attempts to hydrolyse Synkavite with hydrochloric acid, ceric sulfate reagent, alkaline phosphatase, and crystalline wheat germ acid phosphatase. A phosphomolybdate color reaction (54) was used to measure the increased phosphate resulting from the hydrolysis of Synkavite. The disulfate of vitamin K_3 (2-methyl-1, 4-naphthohydroquinone) was subjected to hydrolysis with hydrochloric acid, ceric sulfate reagent and Mylase P. The ethylcyanoacetate color reaction was used to demonstrate the appearance of free vitamin K_3 following hydrolysis. The conditions for each hydrolysis were varied depending on whether the material was to be subjected to paper chromatography, analysis for phosphate, or analysis for vitamin K_3.

(a) Hydrolysis of Synkavite

(i) Hydrolysis with 1.2N hydrochloric acid.

Refluxing 5 gm.% w/v Synkavite with 1.2N hydrochloric acid failed to hydrolyse the phosphate groups as indicated by absence of inorganic phosphate and a negative test for vitamin K_3 following ether extraction and evaporation of the ether in vacuo.

(ii) Oxidative hydrolysis with ceric sulfate reagent.

An attempt was made to hydrolyse 5.4 mgm. of Synkavite in 1 ml. of water by the addition of 1 ml. of 0.1N ceric sulfate in 1N sulfuric acid and 2 ml. of glacial acetic acid (84). The mixture was incubated at 60° for 5 minutes, cooled, diluted with 9 volumes of distilled water, extracted with ether and the ether extract was washed twice with water and evaporated to dryness. The residue was dissolved in 1 ml. of 95% ethyl alcohol and 5 ml. of borate buffer pH 10.5 and 0.1 ml. of ethylyanoacetate acid was added. A stable blue color resulted, indicating the presence of menadione. A control of 2 mgm. of menadione was subjected to the same procedure and it also gave a positive color reaction.

The oxidative hydrolysis of Synkavite with ceric sulfate reagent was used to estimate the amount of menadione in our sample of Synkavite. 51.8 mgm. of Synkavite was dissolved in 2 ml. of water and 10 ml. of ceric sulfate reagent was added, followed by 90 ml. of water. A white precipitate appeared. Two 20 ml. portions of peroxide-free ether were used to extract the vitamin K_3 resulting from hydrolysis. The ether fractions were pooled, washed with 10 ml. of water, evaporated to dryness, and the yellow residue was dissolved in 50 ml. of ethanol and an 0.50 ml. aliquot used for a colorimetric assay of vitamin K_3. A sample of 17.4 mgm. of menadione was treated in the same manner and served as a standard. The Synkavite contained 28.4% vitamin K_3.

An analysis for phosphate was performed after digesting a sample of Synkavite with 60% perchloric acid (54). This sample of Synkavite contained 31.1% phosphate. Drying 47.3 mgm. of Synkavite over phosphorous pentoxide at 94°C, and under reduced pressure revealed that the Synkavite contained 28.6% water. These figures correspond to the decahydrate, e.g., C_{11}, H_8, O_8, P_2, Na_4,.$10H_2O$.

(iii) Hydrolysis with "serum alkaline phosphatase".

Synkavite, 0.2 gm.% w/v was incubated at 37°C. and pH 9.4 for 70 minutes using rat serum as a source of alkaline phosphatase. The results presented in Table 14 indicate that Synkavite contains no orthophosphate (tube 3) and that approximately 38% of the total phosphate ester linkages were hydrolysed (calculated on the basis of Synkavite containing 31.6% esterified phosphate). A 20 µl. aliquot from the test and each of the control tubes was spotted on Whatman No. 1 paper and chromatographed by the ascending technique in butanol:methanol:acetic acid solvent. The test (tube No. 1) gave 3 spots at Rf. 0.0, 0.25 and 0.87. The control tubes (No. 2, No. 3, and No. 4) gave only one spot at Rf. 0.0. The spots were located by their blue fluorescence under ultra-violet light. A control spot of Synkavite had an Rf. of 0.0 and vitamin K_3 had an Rf. of 0.86. A red color developed overnight in tube No. 1 and therefore wheat germ acid phosphatase was used in all subsequent experiments because the free naphthoquinones are destroyed in alkaline media.

(iv) Hydrolysis with wheat germ acid phosphatase.

One spot, at Rf. 0.25 resulted when 0.20 gm.% w/v of Synkavite was hydrolysed at 25°C. for 27 hours by 0.1% w/v wheat germ acid phosphatase and then chromatographed as described above. Control spots of Synkavite, vitamin K_3, and vitamin K_1 were in their correct positions at Rf. 0.0, 0.87, and 0.92 respectively.

The results of an experiment designed to test the extent of hydrolysis of Synkavite with wheat germ acid phosphatase are presented in Table 15. A 90 mgm.% w/v solution of Synkavite in citrate buffer at pH 5.0 was incubated for 2 hours at 37°C. with acid phosphatase in a final concentration of 0.017 gm.% w/v. Aliquots were removed at various times and the inorganic phosphate assayed. The test tube contained 1.68 mgm. of phosphate whereas 0.60 mgm. of phosphate resulted from the hydrolysis of the Synkavite; therefore the extent of hydrolysis was 35.8%.

Since the fluorescent material at Rf. 0.25 had approximately the same Rf. value as the unknown urinary metabolite of vitamin K_3-C^{14} it appeared that the two might have been identical. On adding a sample of acid phosphatase-hydrolysed Synkavite to urine from a rat that had received vitamin K_3-C^{14} the radioactivity for "compound 3" did not

correspond to the fluorescent spot at Rf. 0.25. It is unlikely that "compound 3" is identical with the product resulting from the hydrolysis of Synkavite with wheat germ acid phosphatase.

(b) Hydrolysis of 2-methyl-1,4-naphthohydroquinone disulfate.

(i) Hydrolysis with 2N hydrochloric acid.

Hoskin (46) observed that the monosulfate of vitamin K_1-C^{14} was readily hydrolysed by dilute hydrochloric acid. Similarly the disulfate may be readily hydrolysed by warming at 60°C for 1 hour in 2N hydrochloric acid.

(ii) Oxidative hydrolysis with ceric sulfate reagent.

The disulfate of vitamin K_3 can be hydrolysed in exactly the same manner as the diphosphate using ceric sulfate reagent. This hydrolysis takes place as soon as the reagents are mixed and requires no heating.

(iii) Hydrolysis with Mylase P.

Hoskin (46) reported that Mylase P, a sulfatase of fungal origin, would not hydrolyse the monosulfate of vitamin K_3-C^{14}. This same enzyme will hydrolyse the disulfate of vitamin K_3 when it is incubated at pH 6.0 and 37°C for six hours. We have been able to confirm the observation that Mylase P does not hydrolyse the disulfate to free vitamin K_3, since no vitamin K_3 results from the hydrolysis, but the monosulfate can be detected by its fluorescence under ultra-violet light following paper chromatography of the enzyme reaction mixture. Complete hydrolysis of the disulfate did not take place even for incubation periods of greater than six hours. When chromatographed by ascending technique on Whatman No. 1 paper using butanol-methanol: acetic acid solvent the disulfate of vitamin K_3 had an Rf. of 0.22 and the monosulfate had an Rf. of 0.60. When the disulfate of vitamin K_3 was added to urine from a rat that had received vitamin K_3-C^{14} the radioactivity for "compound 3" did not correspond to the fluorescent spot. Therefore "compound 3" is probably not identical with the disulfate of vitamin K_3.

D. Hydrolysis of radioactive metabolites of vitamin K_1-C^{14} in urine and bile from rats given vitamin K_1-C^{14} intravenously.

Essentially the same technique was used throughout all these experiments. Although only traces of the metabolites of vitamin K_1-C^{14} can be extracted from rat urine and bile with ether or other organic solvents such as ethyl acetate, chloroform or butyl alcohol a significant increase in the ether extractable radioactivity could be detected following various hydrolytic procedures. The urine was collected from rats given vitamin K_1-C^{14}, pooled, and stored in the frozen state until used. Bile was obtained by a biliary fistula consisting of a polyethylene catheter inserted into the common bile duct as described under methods.

(1) Hydrolysis with hydrochloric acid.

A 0.1 ml. aliquot of urine containing 2.1×10^3 counts/min. was added to 0.9 ml. of 1.2N hydrochloric acid and heated for 30 minutes at 70°C. The tube was cooled and 1.5 ml. of peroxide-free ether was added. The tube was stoppered, shaken, centrifuged and 0.5 ml. of the ether extract was plated for counting. 31% of the C^{14} was found in the ether phase after 30 minutes hydrolysis at 70°C. When the hydrochloric acid was replaced with distilled water 28% of the radioactivity was ether extractable. In another experiment 30 ml. of urine was increased to 150 ml. with distilled water and the pH was adjusted to 1.3 by the

addition of 2.0 ml. of concentrated hydrochloric acid. The urine was extracted five times with 50 ml. of peroxide-free diethyl ether and the pooled ether extracts were concentrated to 110 ml. by distillation. Approximately 31% of the radioactivity was extracted from the acidified urine. The ether was then extracted 5 times with 30 ml. of 0.02 M. sodium bicarbonate and only 4.7% of the radioactivity present in the original urine was found in the bicarbonate extracted ether. It is of interest to note that vitamin K_1 cannot be extracted from ether with bicarbonate. The bicarbonate extracts were pooled and acidified with 1 ml. of concentrated hydrochloric acid and extracted 5 times with ether. The pooled ether extracts were concentrated to a volume of 95 ml. and contained 13% of the radioactivity present in the original urine.

It would appear that the ether extractable material was not vitamin K_1 and may have been acidic in nature since it was extracted from acid solution with diethyl ether and was readily removed from the ether with sodium bicarbonate. This was not, however, an efficient method for recovering the radioactivity from the urine of rats that had received vitamin K_1-C^{14}.

(2) Oxidative hydrolysis with ceric sulfate.

0.9 ml. of ceric sulfate reagent was added to 0.1 ml. of urine containing metabolites of vitamin K_1-C^{14}. The mixture was divided into two parts and incubated for 30 minutes at 22°C and 70°C. After cooling to room temperature 1.5 ml. of peroxide-free ether was added; the tubes were stoppered, shaken and centrifuged and 0.5 ml. of the ether extract was plated. Control tubes containing distilled water instead of the ceric sulfate reagent were treated in the same manner. Treatment with the ceric sulfate reagent made possible an increase in the ether extractable C^{14} from 27% and 28% in the control tubes to 33% and 40% in the tests incubated at 22°C and 70°C respectively.

Ceric sulfate reagent was also employed for the following experiment: 3.0 ml. of urine were added to 9.0 ml. of ceric sulfate reagent, 3.0 mgm. of vitamin K_1 (Hoffman LaRoche, Konakion) was added and the solution was warmed at 60°C for 10 minutes. After cooling to room temperature it was extracted twice with 5 ml. of peroxide-free ether. The ether extract was evaporated to 3.0 ml. in vacuo and subjected to reverse phase partition paper chromatography. The vitamin K_1 had an Rf. of 0.22 whereas the radioactivity had an Rf. of 0.90. It therefore appears unlikely that the material resulting from oxidative hydrolysis with ceric sulfate reagent was vitamin K_1-C^{14}. This experiment also showed

that the vitamin K_1 was not destroyed by the ceric sulfate reagent at 60°C. since the vitamin K_1 that underwent the extraction procedure had the same Rf. value as a control spot of vitamin K_1.

(3) Hydrolysis with β-Glucuronidase

Urine and bile were collected for 24 hours from rats that had received 5 mgm./kgm. of vitamin K_1-C^{14}, lot No. 2. These excretions were stored at -5°C. in a frozen state. 0.1 ml. of urine or bile was added to 0.5 ml. of 0.1M veronal-acetate buffer. The pH of the buffers used were 4.0, 4.8, 5.2, 5.6 and 6.0. 0.2 ml. of 2% w/v β-glucuronidase was added and the tubes were incubated for 2 hours at 37°C.. 0.8 ml. of concentrated hydrochloric acid and 2.4 ml. of 3:1 v/v ether:ethanol was added to each tube. It was often very difficult to break the emulsions formed when hydrolysis was performed with either β-glucuronidase or Mylase P and it was for this reason that the ether:ethanol mixture was substituted for straight ether extraction. The tubes were stoppered, shaken, and the solvent layer separated by centrifugation. An 0.2 ml. aliquot of the solvent layer was plated and assayed for C^{14}. A blank was prepared for each tube by replacing the enzyme solution with distilled

water. The solvent layers were more colored in the tubes containing β-glucuronidase and bile than in the corresponding control tubes. It appears that substances other than metabolites of vitamin K_1-C^{14} were hydrolysed by β-glucuronidase. However, the results presented in Table 16 indicate that the β-glucuronidase preparation did hydrolyse C^{14} containing glucuronide conjugates in both urine and bile. The pH optima appear to differ for the two solutions being 4.8 for urine and 5.6 for bile. The rate of hydrolysis was somewhat slower in bile.

Levvy and coworkers (57) have shown that while β-glucuronidase specifically hydrolyses β-D-glucopyranuronosides in general, some of these compounds are not hydrolysed by the enzyme. Moreover, some glucuronides competitively inhibit the action of the enzyme on other glucuronides. Although the enzyme preparation used may have contained enzymes other than β-glucuronidase the increase in ether-extractable C^{14} following hydrolysis suggests the presence of glucuronide conjugates. The large amounts of non-C^{14}-containing material that were extracted prevented paper chromatography.

(4) Hydrolysis with Mylase P.

The experiments described in the preceding section were repeated except that 0.2 gms% w/v Mylase P solution was substituted for the β-glucuronidase. Although no significant increase in ether extractable C^{14} was detected this does not mean that there were no ethereal sulfate conjugates present. Hoskin (46) demonstrated that the monosulfate of vitamin K_3 was not hydrolysed by Mylase P and we were unable to obtain hydrolysis of both ester linkages of 2-methyl-1,4-naphthohydroquinone disulfate using the same enzyme preparation.

E. Attempts to isolate metabolites of vitamin K_1-C^{14} from rat urine and bile.

(1) Paper chromatography

The paper chromatographic system described by Solvonuk (94) and Hoskin (46) gave good separation of esters of vitamin K_3 such as the disulfate and diphosphate and proved useful for the detection of products resulting from hydrolysis of these materials. This same system was used in attempts to separate the metabolic products of vitamin K_1-C^{14} in rat urine and bile following the administration of vitamin K_1-C^{14}. The large amounts of other substances relative to the quantity of C^{14}-containing metabolites rendered this procedure impracticable. Radioactivity was spread from the point of application to the solvent front but no zones of high concentration could be discerned with any degree of reliability.

(2) Ion exchange resins.

Since approximately 25% of an intravenous dose of 5 mgm./Kg. of vitamin K_1-C^{14} could be recovered in a 24 hour sample of rat bile obtained by bile fistula this appeared to be the most suitable medium from which to isolate metabolites of vitamin K_1-C^{14}. Only 1.5% of the C^{14} in the bile was present as unmetabolized vitamin K_1-C^{14} according to the isotope dilution test reported above. A dose level of 5 mgm./Kg. of vitamin K_1-C^{14} was employed throughout all these experiments and bile was obtained by a fistula consisting of a 0.25 mm. polyethylene tube inserted into the common bile duct. The "specific activity", counts/min./mgm. of dry material, was used as an index of purification and wherever the term "specific activity" is used it is intended as counts/min./mgm. dry weight.

5.0 ml. of bile containing 2.4×10^4 counts/min. was made 0.20 normal with respect to trichloracetic acid and the resulting precipitate was centrifuged to the bottom of the tube. The supernatant contained 62% of the original activity and this was chromatographed on a 25 x 2 cm. column of IR-4B that had been washed twice with 500 ml. of 4% hydrochloric acid and 500 ml. of 4% ammonium hydroxide and then washed with distilled water until neutral. The acid supernatant from the bile was placed on the resin bed and

chromatographed with 100 ml. of distilled water followed by 100 ml. of 0.1M borate pH 9.5 and 100 ml. of 4% ammonium hydroxide. 47% of the original activity passed through the column unadsorbed and the remainder of the activity was never recovered. Thirty ten ml. fractions were collected.

This experiment was repeated except that an aliquot of the bile was used that had been acidified with sodium acetate buffer to pH 4.0. Only 33% of the original activity was recovered and only 2% of the original activity of the bile was eluted with 4% ammonium hydroxide. The problem seemed to be threefold. (1) A portion of the radioactivity did not remain on the resin: (2) Another portion was so firmly bound that it was not exchanged with 4% NH_4OH, and (3) no purification was effected: that is to say, the specific activity of the eluates did not increase.

Chromatography of 20 mgm. of a known compound, the disulfate of vitamin K_3, was attempted on IR_4B (OH) IR-45 (OH) and Dowex 1. This substance could not be removed with 4% NH_4OH, borate buffer pH 10.5, IN sulfuric acid, or IN hydrochloric acid.

90% of the radioactivity in bile could be recovered after ion exchange on IRC-50 (H) or IR-120(H) but the precipitation of proteins interfered with flow of the solvents

through the column. The effluent material was strongly acid and the specific activity had not increased.

(3) Chromatography on activiated alumina.

Both the disulfate and the diphosphate of vitamin K_3 could be adsorbed on activiated alumina (100 mesh, Alco) from acetone:water 4:1 and then be eluted with distilled water. The chromatography of bile on alumina was therefore attempted using gradient elution with increasing water concentration.

5 ml. of bile containing 1.54×10^4 counts/min. was added to 20 cc. of acetone at 5°C. and the precipitate was allowed to settle in the refrigerator at 5°C. The supernatant was removed and the precipitate was washed with 80% acetone. The supernatant and washings contained 83% of the original activity and the precipitate showed a threefold decrease in specific activity. The supernatant was chromatographed on alumina by gradient elution. 500 ml. of water was allowed to drop continuously into a reservoir containing 500 ml. of acetone initially. The column was continuously fed the aqueous acetone mixture from the reservoir and 10 ml. fractions were collected.

The forerun and acetone wash contained 17% of the original activity with a twofold increase in specific activity. A second peak was obtained and one 10 ml. fraction from this peak showed a threefold increase in specific activity. All the radioactivity was recovered but the specific activity became progressively lower and lower. A "color-throw" from the alumina was disturbing. The fraction that showed a threefold increase in specific activity was subjected to paper chromatography and showed four peaks at Rf. 0.15, 0.30, 0.45, and 0.80. It would therefore appear that a purification had been achieved in that the specific activity of the two fractions had been increased but a separation of the radioactive components had not been affected. Washing the alumina with 3% acetic acid in acetone v/v or using butanol: methanol 2:1 v/v as the solvent system did not improve the separation and the specific activity was not increased sufficiently to warrant paper chromatography.

(4) Chromatography on Celite.

Several grades of Celite (diatomaceous earth) were tried. Celite 545 (78) was the most satisfactory because it had the greatest flow rate. A preliminary experiment was conducted by adding 50 cc. of Skelly F and 50 cc. of acetone to a 1 ml. aliquot of bile containing 3.1×10^3 counts/min.

This was chromatographed on Celite 545 and washed with 0.5N. sulfuric acid. Two fractions were obtained, one in Skelly F:acetone 1:1 and a second in Skelly F:acetone 3.5:6.5. This experiment was then repeated with the following modifications. A one ml. aliquot of bile was mixed with 1 gm. of dry acid-washed Celite and the resulting slurry was tamped on top of 9 grams of acid-washed Celite in a 3 cm. diameter column. Chromatography was effected with 100 ml. each of Skelly F:acetone, 1:1, 2:3, 3:7, 4:1, acetone, and finally water. At least 3 major peaks of radioactivity were obtained. The first peak washed through with the Skelly F: acetone, 1:1 v/v forerun, the second peak was contained in fractions of the Skelly F:acetone, 2:3 v/v solvent and the third peak in the Skelly F:acetone, 3:7 v/v fractions. Unfortunately the bile salts and other constituents of rat bile were eluted along with the radioactivity. Subsequent attempts to vary the solvent system did not improve on this initial separation. The eluates from this column chromatography were acid and on paper chromatography in butanol: methanol, 3:1 saturated with water all of the radioactivity was located at the solvent front. The radioactivity could be extracted into 0.1M sodium bicarbonate. Attempts to concentrate this material by vacuum distillation yielded a tar that migrated to the solvent front on paper chromatography.

Other columns that were tried but which failed to give separation of the radioactive metabolites were:

1. Cellulose powder with butanol:methanol:H_2O (3:1:4)
2. Folin's Permutit with ethanol, methanol, benzene, pyridine, H_2O.
3. Anhydrous sodium sulfate with pyridine and ethanol.
4. Alumina:silica 25:75 cracking catalyst with ethanol and water.

Acidification of bile to pH 4 resulted in a precipitate (protein conjugate) with 2.08 times the specific activity of the bile but containing only about 30 percent of the radioactivity in the total bile sample. Precipitation of barium insoluble material resulted in no change in the specific activity of the supernatant. Precipitation with zinc carbonate resulted in a twofold decrease in the specific activity in the supernatant.

4. DISCUSSION

The first tests for the purity of vitamin K_1-C^{14} were performed about eight months after the first lot of vitamin K_1-C^{14} was synthesized and therefore a few experiments were conducted using vitamin K_1-C^{14} of unknown purity. It is of some importance to keep this in mind when evaluating these experiments although it would be difficult to revise the data since neither the exact amount nor the tissue distribution of the impurity is known. However, when purified vitamin K_1-C^{14} was used the general picture for the tissue distribution did not differ greatly from that obtained with the impure material.

When radioactive vitamin K_1-C^{14}, labelled in the methyl group, was administered intravenously to a rat no significant amount of the radioactivity appeared in the expired air. It appears, therefore, that the 2-methyl-C^{14} group was not removed and by inference the aromatic nucleus was not completely metabolized. A fractionation of rat blood into plasma, erythrocytes, and buffy coat indicated that radioactivity could be detected in all the fractions. Fractionation of A.C.D. plasma by the method of Cohn et al (14) revealed that the globulin fractions were associated with most of the radioactivity one hour after the administration of vitamin K_1-C^{14} and that the albumin fractions

had the highest concentration after sixteen hours. Solvonuk et al (95) obtained similar results for vitamin K_3-C^{14} given intramuscularly to mice. The difference in the radioactivity between the albumin and globulin fractions may be due to the metabolism of the vitamin K_1-C^{14}.

A comparison of the oral, intramuscular, and intravenous routes of administration showed that the vitamin was rapidly distributed throughout the body regardless of the route of administration. When vitamin K_1-C^{14} was given intramuscularly, up to one-third of the dose could still be found at the site of injection after 24 hours. The spleen accumulates more vitamin K_1 after intravenous injection than it does following either oral or intramuscular administration. Since radioactivity appeared in the bile soon after the administration of radioactive vitamin K_1-C^{14} it was difficult to determine the completeness of absorption of the orally administered vitamin. The intravenous route was therefore chosen for a study of the effect of increasing doses of vitamin K_1. This experiment demonstrated that in all the tissues studied the deposition of the vitamin K_1 increased logarithmically as the log of the dose of the vitamin increased.

The isotope dilution test on the bile is of some interest because in the first place it confirms the observation (25) that no vitamin K_1 could be detected in bile using the colorimetric assay of Irreverre and Sullivan while at the same time the presence of C^{14} indicates that a form of the vitamin is present in the bile. Greaves (41) reported that bile itself contained low levels of an antihemorrhagic factor that would alleviate the prolonged prothrombin times of vitamin K deficient chicks. Furthermore, he was able to demonstrate that the active principle could be precipitated with zinc hydroxide and could be demonstrated after removal of the zinc.

Dam et al (26) have suggested that the high concentration of vitamin K_1 in the liver and spleen of the rat is related to phagocytosis by the reticuloendotheliol system. However, the differences obtained between the concentration of C^{14} in the livers and spleens of rats given vitamin K_1-C^{14} suggest that either this phagocytosis did not prevent the metabolism of the vitamin K_3-C^{14} or the vitamin K_3-C^{14} suspension was not phagocytosed to as great an extent as the colloidal suspension of vitamin K_1-C^{14}. Radioactivity does pass from the maternal tissues to the fetal tissues and, furthermore, in the case of vitamin K_1-C^{14}, a portion of the radioactivity in the fetus exists in unchanged form. The low concentration of vitamin K_1 in fetal and placental tissue

probably accounts for the failure of Dam et al to detect it using their extraction procedure and colorimetric estimation (26). Norris and Bennett (74) have presented evidence that the placenta may be a site of prothrombin formation. When vitamin K_1-C^{14} was administered the concentration of radioactivity was significantly greater in the placenta than in either the maternal blood or skeletal muscle. The vitamin K_1-C^{14} equivalent of the placenta was less than that of the maternal liver but significantly greater than the concentration in fetal liver.

Since a ratio may be increased either because the numerator was increased, the denominator was decreased, or both, it is important to note that the increased ratios presented in Table 10 were due to a marked increase in the weight of the livers of the pregnant rats. If one refers to the data presented in Table 9 it can be shown that the percentage of the injected dose of either vitamin K_1-C^{14} or vitamin K_3-C^{14} present in the livers of the pregnant rats did not differ significantly from that in the livers of non-pregnant rats. It might be suggested that if the liver weight of the pregnant rats had not increased we might have expected a relative deficit due to the increased metabolic demands which the uterine contents could present. Also the tissues of the pregnant rats were probably hydrated in comparison to the tissues of non-pregnant rats. If allowance

were made for the excess tissue water of the mothers the difference between the ratios would have been even greater than shown in Table 10.

In 1940 Tocantins (96) suggested that the low prothrombin level of the newborn infant may be due to a low functional capacity of the liver. Dam (20) pointed out that if this were true then the reduced functional capacity must differ from that observed in chloroform poisoning or cirrhosis as the latter do not respond to vitamin K therapy. Since it has recently been shown (77) that the initial hypoprothrombinemia of the newborn does not respond to vitamin K it would appear that this objection is no longer valid. Moreover, Jurgens (53) observed that if the liver poisons carbon tetrachloride or trypan blue were administered 20 hours after dicoumarol and 50 mgm./Kg. of vitamin K_1 was given intravenously 40 hours after the dicoumarol the prothrombin times of rabbits returned practically to normal in 2 to 4 hours.

Unfortunately it was not possible to perform isotope dilution tests with vitamin K_3 because the added vitamin K_3 could not be recovered once it was added to the tissue. This observation is supported by other reports (13) (88).

It has been shown that the C^{14} [illegible] that is excreted in the bile and urine of rats was not present as unchanged vitamin K_1-C^{14}. Furthermore, at least two metabolites of

vitamin K_3-C^{14} exist as water soluble substances, the diglucuronide, and the monosulfate (47). Our experiments indicate that the bulk of the radioactivity in the fetus, 88%, exists in some form other than as unchanged vitamin K_1-C^{14}. We do not know, however, whether the vitamin K_1 crossed the placental barrier in a water soluble form: for example, an ester which was then hydrolysed to vitamin K_1 in the fetus or whether it was transferred to the fetus as vitamin K_1 and metabolized to some other derivative.

When a comparison was made between the data obtained for various experiments it was observed that there was a marked difference in the percent of the injected dose of C^{14} deposited in the livers of rats. The data presented in Table 13, for example, show that the percent of the injected C^{14} was seven times greater for the adult male rat than for the pregnant female rat given a comparable dose of vitamin K_1-C^{14}. The results presented in Table 12 showed, however, that there was no difference between male and female rats but that there was a marked difference in C^{14} concentration in the livers of young and old rats. Although dietary factors can influence the C^{14} levels in the liver as shown by the data in Table 11 this does not account for all of the variation. Two comparable groups were fed a stock laboratory ration of calf meal pellets ad libitum and whereas one group

of four rats had 3.70± 0.90 (S.D.) percent of the injected C^{14} in their livers (Table 11) another group of six rats (Table 12) had 11.8± 2.7 (S.D.) percent. The difference was statistically significant. The first group of rats (Table 11) were bred in the animal quarters of the Department of Physiology from parents of unknown lineage and the other group was obtained from Mrs. Brown. Since the breeding of rats by the department has been discontinued it was not possible to test for a difference in the vitamin K metabolism of the two strains of rats. If two such strains of rats were available they might be useful tools for the study of vitamin K metabolism.

D.D.T. (1,1,1 - trichloro - 2,2 - bis (p-chlorophenyl) ethene) was employed as an insecticide in the animal quarters and since some of the animals (Table 9) were accidentally exposed several times to D.D.T. a comparison was made between the percent C^{14} deposited in the livers of adult female rats exposed to D.D.T. (Table 9) and the adult female rats (Table 12) that were not exposed to D.D.T. The values obtained were 19.3± 3.5 (S.D.) percent for the D.D.T. treated group and 18.9 ± 3.5 (S.D.) percent for the nontreated group. The difference was not significant and therefore we felt that spraying with D.D.T. had not affected the results obtained.

Attempts to isolate the metabolites of vitamin K_1-C^{14} from the urine and bile of rats were unsuccessful. The best procedure tried thus far was chromatography on Celite 545. It appeared that at least three radioactive metabolites were present in the bile of rats that had received vitamin K_1-C^{14}. All attempts at further concentration and purification of these metabolites failed. Hydrolysis of the radioactive components in rat bile with β-glucuronidase indicated the presence of glucuronide conjugates of the metabolites of vitamin K_1-C^{14}. The fact that vitamin K_1-C^{14} was not obtained on ether extraction or urine hydrolysed with ceric sulfate reagent whereas added vitamin K_1 was recovered indicates that the vitamin K_1-C^{14} underwent some molecular transformation or degradation. In the light of results obtained by Simon and co-workers (92) on the metabolism of vitamin E it seems likely that scission of the aliphatic phytyl side chain of vitamin K_1-C^{14} might have taken place. Furthermore, Fieser and co-workers (35) have demonstrated degradation of various side chains on 2-hydroxy - 3 - alkyl - 1,4-naphthoquinones.

It is of interest to note that complete hydrolysis was not obtained when the diphosphate of vitamin K_3 was hydrolysed with acid phosphatase or when vitamin K_3-disulfate was hydrolysed with Mylase P. In both cases the products of hydrolysis seemed to be the corresponding mono-esters and these mono-esters appeared to resist hydrolysis.

One of the most useful techniques that was acquired was the hydrolysis of esters of both vitamin K_1 and vitamin K_3 with ceric sulfate reagent. This reagent readily hydrolyses the sulfates, phosphates,and acetates without destruction of the vitamin K_1 or vitamin K_3 components.

5. CONCLUSIONS

Chromatography on Folin's Permutit and zinc carbonate provided an effective purification of small amounts of vitamin K_1-C^{14}. No difference was observed in behaviour on the column whether the vitamin K_1-C^{14} was synthesized from phytyl alcohol or isophytyl alcohol. Although equal amounts of C^{14} were present in the liver, spleen and blood following administration of the two lots of vitamin K_1-C^{14}, the C^{14} content of the skeletal muscle was about 1.6 times greater when vitamin K_1-C^{14}, lot No. 1, was administered than when lot No. 2 was given. The vitamin K_1-C^{14}, lot No. 1, possessed biological activity in the sense that it reversed the hypoprothrombinemia caused by Warfarin administration.

When vitamin K_1-C^{14}, labelled in the methyl group, was administered intravenously to a rat no significant amount of radioactivity appeared in the expired air. It therefore appears that the naphthoquinone ring structure was not completely metabolized. A fractionation of the blood into plasma, erythrocytes, and buffy coat indicated that radioactivity could be detected in all the fractions. Fractionation of the plasma proteins by the method of Cohn and co-workers (14), following oral administration of the vitamin K_1-C^{14} indicated that the C^{14} was transported primarily in the globulin-containing fractions of the plasma one hour after oral administration of the vitamin and primarily by the albumin fractions after 16 hours.

A comparison of the oral, intramuscular, and intravenous routes showed that the vitamin was rapidly distributed throughout the rat's body regardless of the route of administration. When vitamin K_1-C^{14} was given intramuscularly up to one-third of the dose could still be found at the site of injection after 24 hours. The spleen accumulated more C^{14} after intravenous injection than following either oral or intramuscular administration. Since radioactivity appeared in the bile soon after the administration of radioactive vitamin K_1-C^{14} it was difficult to determine the completeness of absorption of the orally administered vitamin. The intravenous route was chosen to study the effect of increasing the dose of vitamin K_1. This experiment demonstrated that the deposition of vitamin K_1 increased logarithmically as the log of the dose of the vitamin increased.

Large amounts of radioactivity appeared in the fetus after intravenous or intramuscular administration of the vitamin. Over a six-day period more radioactivity was excreted in the feces than in the urine.

Isotope dilution tests on urine and feces following intravenous administration of radioactive vitamin K_1-C^{14} indicated that all of the radioactivity in the urine was present as metabolites of vitamin K_1-C^{14} and only 6.5% of the activity in the feces was present in the form of the unchanged

vitamin. Since only 1.5% of the radioactivity in the bile was unchanged vitamin K_1-C^{14} while 15% of the activity found in the small intestine was present in this form it appears that some reaction, such as hydrolysis of conjugates, had taken place.

It was evident that the liver of the rat was capable of "storing" vitamin K_1 but this accumulation was not restricted to the liver. All the tissues investigated showed an uptake of radioactivity that increased with increasing doses of vitamin K_1-C^{14}. Isotope dilution studies indicated that not all of the activity present in each tissue was present as unmetabolized vitamin K_1-C^{14}. In the liver of a male rat about 22% of the activity was present as some metabolite of vitamin K; in the skeletal muscle about 26% existed in some altered form. The placental tissue from a pregnant rat contained 59% unchanged vitamin K_1-C^{14} whereas the fetal tissue contained only 12%. Therefore it appears that about 88% of the C^{14} in the fetal tissue was present as metabolites of vitamin K_1-C^{14}.

The vitamin K equivalents of the livers of pregnant and non-pregnant rats given vitamin K_1-C^{14} were about 24 times greater than those of rats that had received vitamin K_3-C^{14}. A fivefold difference in the same direction exists between the concentrations in the spleens of the two groups. The

vitamin K equivalents for skeletal muscle, blood, placenta, fetal liver, and fetal tissue were of similar magnitude regardless of whether vitamin K_1 or vitamin K_3 was administered. The results of this experiment indicate that vitamin K_3-C^{14} was not concentrated in the liver of the rat whereas vitamin K_1-C^{14} was. Furthermore, it would appear that both vitamin K_1 and vitamin K_3 can pass the placental barrier of the rat.

The dry weights of the livers of pregnant rats were greater than those of non-pregnant rats and the increase was proportional to the live weight of the pregnant rat. No significant difference could be demonstrated in the percentage of the injected dose of vitamin K_1 or vitamin K_3 deposited in the livers of pregnant or non-pregnant rats.

The synthetic diet that was fed to rats in order to deplete their stores of vitamin K was also deficient in other essential lipid nutrients and therefore the only conclusion that can be drawn from this experiment is that vitamin K metabolism can be influenced by dietary deficiencies.

An attempt was made to reconcile the variability in the data obtained for the percent of the injected C^{14} in the tissues of different groups of rats by designing an experiment to test the effect of age, sex, and the method of preparation of vitamin K_1-C^{14} on the tissue deposition of C^{14}. Although a

significant difference was noted in the deposition of C^{14} in the skeletal muscle depending on the method of preparation of vitamin K_1-C^{14}, no such difference existed in the livers, spleens or blood. The most marked differences appeared in the percent of the C^{14} deposited in the livers and spleens of the old rats as compared with the young animals. Rats weighing approximately 200 grams have about 1½ times as much C^{14} in their livers and spleens as compared with the young animals weighing about 100 grams. The sex of the animals appears to be unimportant. We were also able to show that the accidental ingestion of D.D.T. did not influence the deposition of C^{14} in the livers of experimental animals.

Since all of the variation in the data was not accounted for it was suggested that different strains of rats might metabolize vitamin K_1 at different rates. However, no experiment was designed to test this hypothesis because one strain of rats was no longer available.

Some interesting observations were made during the course of our attempts to isolate and identify the third urinary metabolite of vitamin K_3-C^{14}. This metabolite was shown by paper chromatography to differ from the diphosphate, monophosphate, disulfate and monosulfate of vitamin K_3. The diphosphate of vitamin K_3 was remarkably stable to acid hydrolysis but was easily hydrolysed to vitamin K_3 with ceric sulfate reagent.

Wheat germ acid phosphatase brought about a partial hydrolysis and it was suggested that the main product was a monophosphate. The disulfate of vitamin K_3 was hydrolysed by refluxing in 1.2 N hydrochloric acid but it was even more readily hydrolysed with ceric sulfate reagent since the ester was split spontaneously at room temperature. Mylase P was able to hydrolyse partially the disulfate of vitamin K_3 and when the products of hydrolysis were subjected to paper chromatography the principal product behaved in a manner similar to the monosulfate described by Hoskin (46).

The C^{14}-containing metabolites of vitamin K_1-C^{14} in rat bile were hydrolysed by β-glucuronidase but not by Mylase P. It was concluded that some of these metabolites existed in the form of glucuronide conjugates. However, reverse phase partition paper chromatography of the products of ceric sulfate hydrolysis indicated that conjugates of vitamin K_1-C^{14} as such were not present. It was suggested that degradation of the phytyl side chain had probably taken place.

Attempts to isolate the metabolites of vitamin K_1-C^{14} from rat urine and bile by paper chromatography and ion exchange on IR4B, IR45, and Dowex 1, and column chromatography on Celite 545 and alumina indicated that at least three metabolites of vitamin K_1-C^{14} were present in rat bile.

6. SUMMARY

1. Vitamin K_1-C^{14} can be purified by chromatography on Folin's Permutit and zinc carbonate.

2. Reverse phase partition paper chromatography, the ultra-violet absorption spectrum, the ethylcyanoacetate color reaction and the diethyldithiocarbamate reaction can all be used to advantage to verify the purity of vitamin K_1.

3. The vitamin K_1-C^{14} used possessed biological activity in that it would serve as an antidote in Warfarin intoxication.

4. No radioactive $C^{14}O_2$ was expired following the administration of vitamin K_1-C^{14} and therefore it was concluded the vitamin K_1-C^{14} was not completely metabolized.

5. C^{14} was detected in the formed elements of the blood and in the plasma following oral and intravenous administration of vitamin K_1-C^{14}. Furthermore, a fractionation of the plasma proteins indicated that the C^{14} was transported primarily by the globulin-containing fractions one hour after oral administration and by the albumin fractions after 16 hours.

6. The levels of C^{14} in the blood and bone marrow were much lower than in the liver and spleen of rats.

7. The degree of deposition of C^{14} in the spleen depended on whether the vitamin K_1-C^{14} was administered orally, intramuscularly, or intravenously. The greatest amount of the vitamin was deposited in this organ following intravenous administration. No marked differences in the liver levels were observed. The vitamin K_1-C^{14} equivalents of the blood and the heart of the rat given the intravenous injection were lower than those of the animals given vitamin K_1-C^{14} orally or intramuscularly.

8. Radioactivity was present for at least 6 days in the tissues of rats given vitamin K_1-C^{14} intravenously and intramuscularly.

9. As the dose of vitamin K_1-C^{14} was increased from 5 mgm./kgm. to 82 mgm./kgm. the concentration of C^{14} in the liver, lung, kidney, spleen, heart, blood, muscle, urine, and feces increased.

10. Vitamin K_1-C^{14} was concentrated in the liver of pregnant and non-pregnant female rats whereas vitamin K_3-C^{14} was not.

11. Radioactivity was detected in the placenta, fetus, fetal liver, and amnionic fluid of rats following the administration of both vitamin K_1-C^{14} and vitamin K_3-C^{14}. Furthermore, the C^{14} concentrations of these tissues were of the same order of magnitude regardless of whether vitamin K_1-C^{14} or vitamin K_3-C^{14} was administered.

12. It was observed that following intravenous administration of vitamin K_1-C^{14} twice as much C^{14} was deposited in the livers of rats on a fat-free, vitamin K-free diet as was deposited in the livers of normal control animals on a stock laboratory ration of calf-meal pellets.

13. The livers and spleens of young rats contained much less C^{14} 24 hours after the administration of vitamin K_1-C^{14} than did the livers and spleens of older rats.

14. More radioactivity was deposited in the skeletal muscle of rats given vitamin K_1-C^{14} that was synthesized from isophytyl alcohol than for vitamin K_1-C^{14} synthesized from phytyl alcohol.

15. The sex of the rats did not influence the deposition of C^{14} in the livers, spleens, blood, or skeletal muscle.

16. Accidental exposure to D.D.T. did not influence the deposition of C^{14} in the livers of adult rats.

17. Isotope dilution tests indicated that none of the radioactivity in the urine of a rat given vitamin K_1-C^{14} was present as unchanged vitamin K_1-C^{14}. Only 1.5% of the activity in the bile was present in unchanged form whereas a value of 6.5% was obtained for fecal material.

18. Values of 78% and 120% were obtained for the C^{14} present as unchanged vitamin K_1-C^{14} in the livers of two rats. The percentage of the injected C^{14} present in these two rat livers were 2.8% and 20% respectively. 76% of the C^{14} in skeletal muscle existed as unchanged vitamin K_1-C^{14}.

19. The fetal tissue from a pregnant rat contained 12% of its total C^{14} content as unchanged vitamin K_1-C^{14} whereas 59% was in this form in the placental tissue.

20. The diphosphate and disulfate of vitamin K_3 are readily hydrolysed by ceric sulfate reagent. The diphosphate is stable to acid hydrolysis whereas the disulfate can be hydrolysed by boiling in 1.2 N hydrochloric acid.

21. Both the diphosphate and the disulfate of vitamin K_3 can be hydrolysed with the corresponding enzymes wheat germ acid phosphatase and Mylase P but apparently only one ester linkage was hydrolysed and the monoesters resulted in each case.

22. It was concluded that one or more of the metabolites of vitamin K_1-C^{14} in urine and bile existed as glucuronide conjugates because hydrolysis with the enzyme β-glucuronidase resulted in an increase in ether extractable, C^{14}-containing, material.

23. The C^{14}-containing, ether extractable, material obtained from rat urine following hydrolysis with hydrochloric acid, was readily removed from the ether extract by dilute sodium bicarbonate and could be re-extracted into the ether after destruction of the bicarbonate and acidification of the solution.

24. Reverse phase partition paper chromatography of the ether extractable, C^{14}-containing, material from rat urine following hydrolysis with ceric sulfate reagent indicated that the C^{14}-containing material was probably not vitamin K_1-C^{14} but rather a degradation product of vitamin K_1-C^{14}.

25. Attempts at the isolation of the metabolites of vitamin K_1-C^{14} in urine and bile of rats by paper chromatography, ion exchange, and column chromatography were all unsuccessful.

7. SUGGESTIONS FOR FUTURE WORK.

Since some evidence has accumulated to indicate that the side chain of vitamin K_1 might be oxidized and degraded (35) (39) (96) it seems that the synthesis and administration to rats of vitamin K_1-C^{14} labelled uniformly in the side chain would be worthwhile. Perhaps the biosynthesis of the phytyl alcohol could be accomplished using plant material. An investigation of the synthesis of phytyl alcohol or of vitamin K_1 by plants might provide useful information that could be applied to the possible synthesis of vitamin K in animals.

The metabolism of vitamin K_1 and vitamin K_3 in species other than the rat might provide some useful information on vitamin K metabolism. The effects of operative procedures such as thyroidectomy, and adrenalectomy on the metabolism of vitamin K could be studied. Metabolic studies should be extended to tissue slices, breis, and homogenates.

The ether extractable material, resulting from the ceric sulfate hydrolysis of the vitamin K_1-C14 metabolites in urine, might be isolated and identified.

Vitamin K_1 is more efficient than vitamin K_3 on an equimolar basis, as an antidote in anticoagulant intoxication. At doses of about 5 mgm./kgm. vitamin K_3 is excreted as

glucuronide and sulfate conjugates much more rapidly than vitamin K_1. At lower plasma levels the reactions by which vitamin K_3 might be transformed into a more biologically active compound might predominate. Perhaps the antidotal effect of vitamin K_3 would be greater if it were given over a longer period of time by continuous intravenous infusion to anticoagulant treated animals.

An isotope dilution experiment was conducted to test for the conversion of vitamin K_3 to vitamin K_1. One mgm. of vitamin K_3-C^{14} was administered to a rat and the animal was killed 24 hours later. One half of the liver was assayed for C^{14} whereas the other half was ground with nonradioactive vitamin K_1 and the vitamin K_1 was isolated by the techniques described previously. The yellow oil that was isolated contained C^{14}, suggesting that vitamin K_1-C^{14} was present, but on repeating the purification procedure the specific activity decreased to 20 percent of the original value whereas the purity of the vitamin K_1, as determined by the Irreverre Sullivan colorimetric reaction, increased only 2 percent. This would indicate that the radioactive component in the extract was not vitamin K_1-C^{14}. Vitamin K_3-C^{14} was separated from vitamin K_1 by the solvent extraction and chromatographic procedures so it seems that the vitamin K_3-C^{14} was transformed into some substance with solubility properties very similar to vitamin K_1. The isolation of this substance, its identification and the metabolic pathway involved in its formation would be a subject for study.

Vitamin K has not been isolated from mammalian tissues. It is not known whether the vitamin K in hog liver or beef liver is vitamin K_1 or vitamin K_2 or even whether it is a naphthoquinone. At least 7 kgm. of fresh beef liver would be required for the isolation of the equivalent of 5 mgm. of vitamin K_3, calculated on the basis that beef liver contains the equivalent of 71 µgm. of menadione (43). If the vitamin K in beef liver were not present as a fat soluble material but existed as a more hydrophilic derivative such as a glucuronide the extraction procedures for vitamin K_1 would be of limited value.

Although a difference in C^{14} deposition was found in the livers of rats on a fat-free, vitamin K-free diet compared with rats on a stock laboratory ration it might be questioned whether the effect was due to the lack of vitamin K or to the lack of fats or essential fatty acids. The effects of these dietary factors on vitamin K metabolism have not been investigated.

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9. APPENDIX.

A. TABLES.

TABLE I

Separation of vitamin K_1 from vitamin K_3 on Folin's Permutit.

Solvent on column	Fraction No. (50 ml.)	Color of eluate	Irreverre Sullivan spot test	Vitamin
Skelly F	1	-	negative	
	2	-	negative	
Skelly F + 10% Benzene	3	-	negative	
	4	-	negative	
	5	-	negative	
	6	-	negative	
	7	-	negative	
Skelly F + 20% Benzene	8	yellow	blue	K_1
	9	yellow	blue	K_1
	10	-	faint blue	K_1
	11	-	negative	
	12	-	faint pink	
Skelly F + 30% Benzene	13	yellow	pink	K_3
	14	yellow	pink	K_3
	15	-	faint pink	K_3

TABLE II

Biological (anti-Warfarin) activity of vitamin K_1 and vitamin K_1-C^{14} administered intravenously to Warfarin treated rats.

	2 rats fed calf meal pellets	2 rats fed 2 doses of Warfarin (5 mgm./kg./dose)	2 rats fed 1 dose Warfarin (5 mgm./kg.)	2 rats fed 1 dose Warfarin (5 mgm./kg.)
Initial prothrombin time	25 sec.	>180 sec.	>180 sec.	>180 sec.
	26 sec.	>180 sec.	>180 sec.	>180 sec.
Solution injected	1 ml., .5% Tween 80	1 ml., Merck's vitamin K_1 (5mgm./kg.)	0.2 ml., vitamin K_1-C^{14} Lot #1 (1mgm./kg.)	1 ml., .5% Tween 80
Prothrombin time 2 hours after injection.	26 sec.	25 sec.	57 sec.	> 180 sec.
	24 sec.	30 sec.	70 sec.	> 180 sec.

TABLE III

A comparison of the vitamin K_1-C^{14} equivalent in plasma, erythrocytes, and buffy coat following oral and intravenous administration of vitamin K_1-C^{14} .

	Time interval to collection of blood sample.		
	1 hr.	16 hr.	5 hr.
	Vitamin K_1-C^{14} equivalent, µgm./100 ml. blood		
	Oral	Oral	Intravenous
Plasma	68	28	53
Washed erythrocytes	31	31	27
"Buffy coat"	0.34	2.8	3.3
Plasma fractions:			
1+ III-3	35	0.97	
II	4.0	N.A.	
III-0	N.A.	N.A.	
III-1,2	1.0	N.A.	
IV-1	2.9	0.75	
IV-6+7	1.2	0.50	
V	6.2	3.7	
VI	2.7	3.3	

Note: Oral dose of K_1-C^{14} = 3.0 mgm./kgm. (specific activity 1.33×10^5 counts/min./mgm.).

Intravenous dose K_1-C^{14} = 6.9 mgm./kgm. (specific activity 3.0×10^5 counts/min./mgm.).

N.A. = no significant activity.

TABLE IV

A comparison of the levels of C^{14} in the blood, reticuloendothelial tissues, and other tissues five hours after intravenous administration of vitamin K_1-C^{14}.

Tissue	% of the administered vitamin K_1-C^{14} deposited in the tissue.	Vitamin K_1-C^{14} equivalent µgm./gm. of dry tissue.
Spleen	5.85	333
Liver	17.70	91.3
Bone marrow	4.17+++	21.5
Blood	6.13+	19.2
Lung	0.72	31.5
Kidney	0.56	17.4
Heart	0.25	12.4
Skeletal muscle	8.85++	2.05
GI Tract and contents	15.70	41.5

Dose of vitamin K_1-C^{14} = 6.25 mgm./kg. (one rat).
Specific activity 9.5 x 10^5 counts/min./mgm. (Lot No. 2)

+Calculated on the basis of blood constituting 2% of the dry weight of the rat's body.

++ Calculated on the basis of muscle constituting 27% of the dry weight of the rat's body.

+++ Calculated on the basis of the dry weight of the bone marrow being equal to that of the liver (38).

TABLE V.

Distribution of radioactivity in a rat nine hours after the intravenous administration of vitamin K_1-C^{14}.

Organ	% of injected C^{14} deposited in the organ.	Vitamin K_1-C^{14} equivalent µgm./gm. tissue.
Liver	16.60	6.07
Lung	1.75	4.80
Kidney	0.23	0.37
Spleen	0.93	4.54
Heart	0.22	0.67
Caecum, large bowel and contents	4.30	3.50
Stomach and contents	0.38	0.90
Small intestine and contents	2.20	3.90
Urine	1.02	—
Remainder	79.00	1.13
Total %	107.0	

Weight of rat 310 gm.

Dose of K_1-C^{14} 0.32 mg./kg. (specific activity 3.0×10^5 counts/min./mgm.). (Lot No. 1, not purified).

TABLE VI

Comparison of oral, intramuscular, and intravenous administration of vitamin K_1-C^{14} on the vitamin K_1-C^{14} equivalent and percent of administered activity in rat tissues.

Route of administration of vitamin K_1-C^{14}	Intravenous		Intramuscular		Oral	
Tissue assayed.	Vitamin K_1-C^{14} equivalent µg/g. dry tissue.	% of the injected activity deposited in the tissue.	Vitamin K_1-C^{14} equivalent µg/g. dry tissue.	% of the injected activity deposited in the tissue.	Vitamin K_1-C^{14} equivalent µg/g. dry tissue.	% of the injected activity in the tissue.
Liver	21.0	3.50	15.0	2.70	18.0	2.60
Lung	5.8	0.13	5.6	0.13	10.0	0.21
Kidney	6.1	0.16	3.7	0.11	4.8	0.15
Spleen	35.0	0.59	8.6	0.22	8.4	0.09
Heart	0.9	0.01	3.7	0.47	8.9	0.13
Blood	0.6	0.19 [+]	4.5	1.39 [+]	3.4	0.99 [+]
Muscle	0.4	1.72 [++]	130.0	19.00 [++]	2.5	9.64 [++]

[+] Calculated on the basis of blood constituting 2% of the dry weight of the rat's body.
[++] Calculated on the basis of skeletal muscle constituting 27% of the dry weight of the rat's body.
Site of intramuscular injection.

Dose vitamin K_1-C^{14} 1.0 mgm., (specific activity 3.0×10^5 counts/min./mgm.), Lot No. 1, not purified.

Rats obtained from E.G. Steinhilber and Company, Oshkosh, Wisconsin, U.S.A.

	Liver	70	2.10	69.0	3.90
	Lung	21	0.13	1.0	0.01
	Kidney	26	0.12	1.6	0.01
	Spleen	290	1.35	4.7	0.02
	Heart	60	0.20	2.5	0.01
	Blood	13	0.95 +	N.A.	N.A
	Injection site			480	4.40
	Feces	382	43.00	190	38.0
	Urine		19.00		14.0

+ Calculated on the basis of blood constituting 2% of the dry weight of a rat's body.
The specific activity of the vitamin K_1-C^{14} was 6.15×10^3 counts/min./mgm.
For the intravenous injections and 5.70×10^3 counts/min./mgm. for the intramuscular injections.

Dose was 26 mgm./kg. , Lot No. 1, not purified.

N.A. = Radioactivity not statistically different from background.

Rats obtained from Mr. Halstead, Saskatoon, Canada.

TABLE VII

Comparison of the vitamin K_1-C^{14} equivalent and the percent of injected activity deposited in tissues of rats 24, 72 and 144 hours after intravenous and intramuscular administration of vitamin K_1-C^{14}.

Route of administration of vitamin K_1-C^{14}.		Intravenous		Intramuscular	
Time of sacrifice (hours)	Tissue assayed.	Vitamin K_1-C^{14} equivalent µg/g. dry tissue.	% of the injected activity deposited in the tissue.	Vitamin K_1-C^{14} equivalent µg/g. dry tissue.	% of the injected activity deposited in the tissue.
24	Liver	300	8.20	180	10.00
	Lung	80	.33	90	.72
	Kidney	50	.28	84	.67
	Spleen	2480	9.10	72	.27
	Heart	52	.18	63	.24
	Blood	50	3.60+	63	5.10+
	Injection site			1080	32.00
	Feces	555	5.30	148	5.50
	Urine		9.70		3.70
72	Liver	98	3.30	96	5.00
	Lung	70	.35	96	.49
	Kidney	21	.12	35	.03
	Spleen	210	.90	42	.02
	Heart	85	.21	49	.02
	Blood	24	1.85+	54	3.80+
	Injection site			490	5.60
	Feces	250	12.00	185	26.00
	Urine		17.00		9.70

TABLE VIII

The effect of increasing the intravenous dose of vitamin K_1-C^{14} on the tissue deposition of C^{14}.

Total vitamin K_1 given (mgm.)	5.6		9.3		22.0		39.0		82.0
Specific activity (counts/min./mgm.)	1.8×10^5		8.6×10^4		4.4×10^4		2.4×10^4		1.2×10^4
Weight of rat (gms.)	90		108		90		103		98
-	%	K-E	%	K-E	%	K-E	%	K-E	%	K-E
Liver -	6.70	16	6.40	34	7.30	74	8.50	180	15.0	600
Lung -	0.52	18	.43	26	.21	35	.42	100	.51	240
Kidney -	0.30	9.3	.28	14	.12	14	.20	39	.25	100
Spleen -	0.14	7.6	.47	36	.35	74	.70	150	.81	580
Heart -	0.01	6.5	.34	33	.09	24	.32	130	.33	270
Blood -	1.98[++]	5.6	2.06[++]	9.6	1.74[++]	19	1.54[++]	30	1.98[++]	81
Muscle -	23.0[+]	4.8	29.8[+]	10.0	19.6[+]	16	21.4[+]	30	14.4[+]	45
Urine -	16.0	900[+++]	15.2	1400[+++]	9.20	2100[+++]	10.0	3900[+++]	9.60	7900[+++]
Feces -	38.0	2100	41.5	3900	17.0	3700	37.0	1400	19.0	1600

% = percent of the injected C^{14}. K-E = vitamin K_1-C^{14} equivalent µgm./gm. dry tissue.

[+] Calculated on the basis of muscle comprising 27% of the rat's body weight.

[++] Calculated on the basis of blood comprising 2% of the rat's body weight.

[+++] µgm./kg./24 hours.

Rats raised in the Department of Physiology animal quarters.

Vitamin K_1-C^{14}, lot No. 1, not purified.

TABLE IX

The percentage of the injected dose of vitamin K_1-C^{14} or vitamin K_3-C^{14} and the vitamin K_1-C^{14} or vitamin K_3-C^{14} equivalents in some rat tissues following the intravenous administration of vitamin K_1-C^{14} or vitamin K_3-C^{14} to pregnant and nonpregnant female rats.

	Vitamin K_1-C^{14} administered				Vitamin K_3-C^{14} administered			
	six pregnant rats (mean ± standard error)		six nonpregnant rats (mean ± standard error)		six pregnant rats (mean ± standard error)		six nonpregnant rats (mean ± standard error)	
Tissue	% of the injected dose	Vitamin K_1-C^{14} equivalent µgm./gm. dry tissue	% of the injected dose	Vitamin K_1-C^{14} equivalent µgm./gm. dry tissue	% of the injected dose	Vitamin K_1-C^{14} equivalent µgm./gm. dry tissue	% of the injected dose	Vitamin K_1-C^{14} equivalent µgm./gm. dry tissue
Liver	18.4±1.1	91.8±5.6	19.3±2.7	103±15	0.732±0.073	3.82±0.30	0.795±0.085	4.41±0.36
Spleen	1.43±0.12	91.2±13	1.24±0.15	76.7±4.3	0.200±0.043	17.9±4.6	0.269±0.052	16.8±2.8
Skeletal muscle	6.70±0.57†	1.64±0.16	6.86±0.34†	1.38±0.071	7.46±0.91†	1.73±0.20	8.23 ±1.8†	2.40±0.10
Blood	1.36±0.060††	4.28±0.23	1.12±0.078††	3.04±0.20	2.63±0.32††	8.40±1.30	2.54±0.26††	7.43±0.61
Feces	4.05±0.62	77.1±5.6	2.70±1.2	47.2±19	12.7±1.6	138±17	9.81±2.0	127±42
Urine	1.93±0.25	413±59	2.39±0.72	558±97	13.5±2.2	2230±58	9.33±1.5	2240±24
Placenta	0.293±0.047	7.96±0.19			0.200±0.034	5.26±0.53		
Fetus (-liver)	1.01±0.29	5.84±0.60			0.333±0.064	3.64±0.41		
Fetal Liver	0.0741±0.015	4.44±0.60			0.0571±0.017	2.82±0.23		
Amnionic fluid	0.121±0.053	15.3±1.90			0.115±0.040	20.4±9.6		

† Calculated on the basis of skeletal muscle comprising 27% of the rat's body weight.

†† Calculated on the basis of blood comprising 2% of the rat's body weight.

Rats obtained from Mrs. Brown, Saskatoon, Canada.

TABLE X.

A comparison of the ratios of $\frac{\text{mean dry weight of tissue}}{\text{mean live weight of rat}}$ (± s.d.)

for pregnant, nonpregnant, and pregnant rats less the weight of the uteri and contents.

tissue	twelve nonpregnant rats	twelve pregnant rats	twelve pregnant rats less weight of uteri and contents
Spleen	$(0.953 \pm 0.127) \times 10^{-3}$	$(0.752 \pm 0.214) \times 10^{-3}$	$(0.895 \pm 0.230) \times 10^{-3}$
Liver	$(10.3 \pm 0.715) \times 10^{-3}$	$(10.6 \pm 1.43) \times 10^{-3}$	$(12.2 \pm 1.22) \times 10^{-3}$

TABLE XI.

A comparison of the percent of the injected dose of C^{14} and vitamin K_1-C^{14} equivalents in the tissues of rats on a stock laboratory ration compared with rats on a synthetic fat-free, vitamin K-free ration.

Tissue	% of the administered vitamin K_1-C^{14} deposited in the tissue (mean ± standard deviation)	Vitamin K_1-C^{14} equivalent ugm./gm. of dry tissue (mean ± standard deviation).
Stock laboratory diet (4 rats) weight 128 ± 14 (S.D.) gms.		
Liver	3.70 ± .90	18.1 ± 4.76
Lung	0.20 ± .07	9.02 ± 2.38
Kidney	0.17 ± .03	4.62 ± 0.76
Spleen	0.22 ± .04	13.1 ± 1.37
Heart	0.19 ± .07	10.9 ± 3.56
Blood	0.71 ± .08	2.78 ± 0.61
Muscle	6.22 ± .67	1.80 ± 0.29
Feces	13.7 ± 11.6	85.1 ± 43.4
Urine	8.4 ± 1.20	N.D.
Fat-free, vitamin K-free diet (5 rats) weight 106 _ 7.2 (S.D.) gms.		
Liver	7.50 ± 1.80	25.9 ± 8.16
Lung	0.32 ± 0.02	7.95 ± 1.70
Kidney	0.24 ± 0.03	5.20 ± 1.08
Spleen	0.28 ± 0.09	16.7 ± 6.41
Heart	0.20 ± 0.03	9.80 ± 2.45
Blood	0.71 ± 0.05	3.35 ± 0.866
Muscle	7.16 ± 0.50	2.60 ± 0.252
Feces	11.1 ± 4.44	73.4 ± 4.15
Urine	6.83 ± 1.23	N.D.

Dose of vitamin K_1-C^{14} 5 mgm./kg. Specific activity 2.77×10^5 counts/min./mgm., lot No. 2, not purified.

N.D. = not determined.

Rats raised in the Department of Physiology animal quarters.

TABLE XLI

The effect of age, sex of rats and the method of preparation of vitamin K_1-C^{14} on the deposition of C^{14} in the tissues of rats following the intravenous administration of vitamin K_1-C^{14}.

Vitamin K_1-C^{14} preparation			Liver		Spleen		Muscle		Blood	
			1	2	1	2	1	2	1	2
12 young rats 93.7±4.18(S.D.) gms.	Males	%	9.81	13.9	.404	.456	10.2†	8.39†	1.08††	1.03††
		K-E	53.1	71.2	42.9	48.5	1.88	1.56	2.70	2.58
	Females	%	12.3	11.1	.751	.560	14.8†	8.39†	1.26††	1.13††
		K-E	65.1	62.8	57.7	48.6	2.59	1.58	3.12	2.82
11 adult rats 195±13.0(S.D.) gms.	Males	%	17.1	18.1	1.71	.696	11.8†*	7.33†	1.42††*	1.19††
		K-E	87.6	101	164	69.5	2.18*	1.36	3.44*	2.91
	Females	%	19.1	18.6	.685	.797	13.3†	7.82†	1.38††	1.46††
		K-E	118	108	65.3	71.4	2.54	1.46	3.46	3.67

Notes: % = percent of the injected C^{14}. K-E = vitamin K_1-C^{14} equivalent µgm./gm. dry tissue.

† Calculated on the basis of muscle comprising 27% of the dry weight of the rat's body.

†† Calculated on the basis of blood comprising 2% of the dry weight of the rat's body.

Each value is the mean of 3 determinations except values marked with an* (* = mean of 2 values).

Rats obtained from Mr. Halstead, Saskatoon, Canada.

TABLE XIII

Isotope dilution tests on rat tissues following administration of vitamin K_1-C^{14}.

Tissue	% of injected C^{14} present in the sample.	% of injected vitamin K_1-C^{14} present in the sample.	% of activity in the tissue which was present as vitamin K_1-C^{14}.
Liver (Rat No.1)	2.8	2.2	78
Skeletal muscle † (Rat No.2)	1.9	1.4	74
Small intestine and contents (Rat No.2)	2.5	0.37	15
Feces (Rat No.2)	6.2	0.40	6.5
Urine (Rat No.2)	0.98	0.00	0.0
Bile (Rat No.3)	26.0	0.39	1.5
Maternal liver (Rat No.4)	20.0	24.0	120
Placenta (Rat No.4)	0.41	0.29	59
Fetus (Rat No.4)	1.34	0.16	12

† This corresponds to 12.2% of the injected activity in the total skeletal muscle on the basis of muscle comprising 27% of the dry weight of the rat's body.

Note: Rat No. 1 was a 260 gm. male and was given 1.0 mgm. of K_1-C^{14} intravenously.

Rat No. 2 was a 325 gm. male and was given 1.0 mgm. of K_1-C^{14} intravenously.

Rat No. 3 was a 350 gm. male and was given 1.0 mgm. of K_1-C^{14} intravenously.

Rat No. 4 was a 222 gm. pregnant female and was given 1.1 mgm. of K_1-C^{14} intravenously.

TABLE XLV

Hydrolysis of Synkavite using rat serum as a source of alkaline phosphatase.

Tube No.	Water (ml.)	Rat serum (ml.)	Phosphate standard 5 mgm% (ml.)	0.2% Synkavite in 0.1 M. Veronal, pH 9.4. (ml.)	Time of hydrolysis at 37°C. (min.)	Inorganic Phosphate (mgm.%)
1	0	0.1	0	1.0	70	26
2	0	0.1	0	1.0	0	2.7
3	0.1	0	0	1.0	0	0
4	0	0	0.1	1.0	0	5

TABLE XV.

Extent of hydrolysis of Synkavite with wheat germ acid phosphatase.

Time of hydrolysis at 37°C. (min.)	Inorganic phosphate in tube. (mgm.)	% hydrolysis
0	0	0
15	.161	9.6
30	.291	17.3
45	.378	22.5
60	.452	27.0
75	.542	32.2
90	.592	35.2
105	.600	35.7
120	.600	35.7

TABLE XVI

The hydrolysis of metabolites of vitamin K_1-C^{14} in bile and urine by β-glucuronidase.

	% increase in ether extractable C^{14} following hydrolysis	
pH	Urine	Bile
4.0	16	0
4.4	0	1
4.8	196	3
5.2	108	14
5.6	23	27
6.0	50	24

B. FIGURES

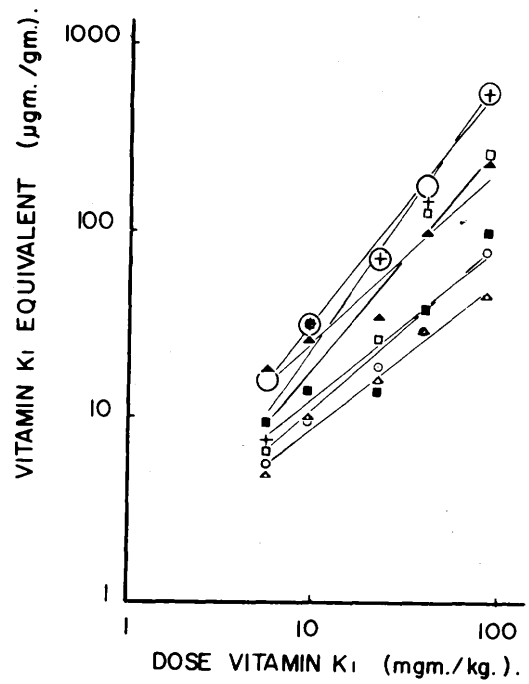

figure I.

Radioactivity of rat tissues following intravenous injection of vitamin K_1-C^{14}.

○——○ liver; ▲——▲ lung; ■——■ kidney; +——+ spleen; □——□ heart; o——o blood; △——△ muscle.

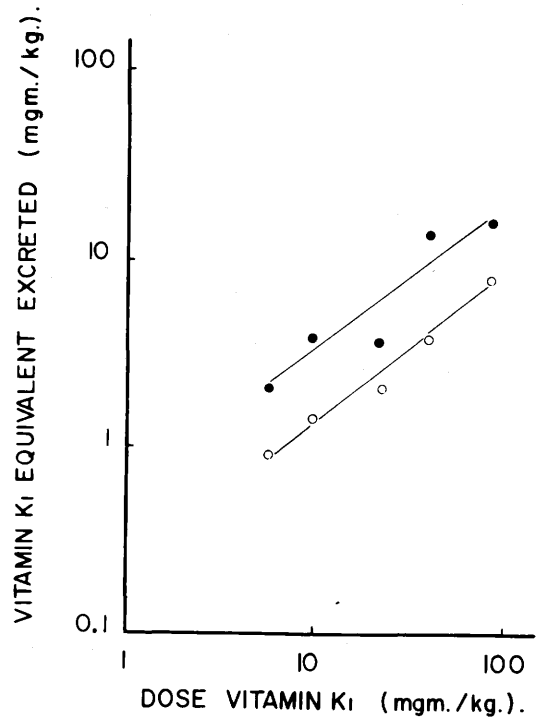

figure 2.

Excretion of radioactivity following intravenous injection of vitamin K_1-C^{14}.

●——● feces; o—— o urine.