Vitamin K and Oxidative Phosphorylation

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1. Oxidative phosphorylation was studied in a cell-free preparation of *Mycobacterium phlei* and in rat-liver mitochondria. Phosphorylation was destroyed in both systems by long-wave ultraviolet radiation and restored by the addition of small amounts of [2-Me- ^{14}C , ^3H]phyloquinone. When the radioactive quinones were recovered from the phosphorylating system and chromatographed with carrier phyloquinone and menaquinone-4 in adsorption and partition systems, only the phyloquinone band was labelled, and its isotopic ratio was identical with that of the original [2-Me- ^{14}C , ^3H]phyloquinone. This result does not support the contention that the role of vitamin K in oxidative phosphorylation involves a cyclic mechanism with intermediate formation of a quinone methide. 2. When the [2-Me- ^{14}C , ^3H]phyloquinone was given intravenously to rats and radioactive phyloquinone isolated from their liver mitochondria and microsomes 20 hr. later, its isotopic ratio was unchanged. There was thus no evidence for quinone methide formation *in vivo*. No measurable conversion of phyloquinone into menaquinone-4 was observed. 3. When [^{14}C]menadione was given intraperitoneally to rats whose alimentary tract had been treated with neomycin, conversion into menaquinone-4 was found in the liver mitochondria and microsomes, but there was also some indication that there had been synthesis of phyloquinone.

Since the original observation of Clark, Kirby & Todd (1958) that quinol phosphates could be oxidized, with loss of phosphate, to quinones, several groups of workers have proposed schemes in which natural quinones such as ubiquinone or vitamin K might function in oxidative phosphorylation. Chmielewska (1960) suggested that a 2-methyl-3-polyprenoid quinone could, through an intermediate quinone methide, add phosphoric acid to give a coumaran-5-yl phosphate that might oxidize in the presence of ADP to produce ATP and the original quinone. Vilkas & Lederer (1962) give an alternative mechanism in which there is 1,4-addition of inorganic phosphate to a quinone methide to give an *o*-hydroxychromanylmethyl phosphate: this, by intramolecular transphosphorylation, is considered to give a 5-hydroxymethylchroman-6-yl phosphate, which could subsequently be oxidized in the presence of ADP to complete the cycle. Erickson, Wagner & Folkers (1963) vary the scheme and suggest the oxidation of the intermediate *o*-hydroxychromanylmethyl phosphate to give 'active phosphate' and a further intermediate 5-formylchroman-6-ol, which is reduced to the 5-methylol and subsequently dehydrated to a quinone methide to complete the cycle. All these schemes have in common two

factors: the cyclization of a quinone derivative to a heterocyclic phenol derivative, and the involvement of at least one of the hydrogen atoms of the methyl group *ortho* to the quinone function.

There is some evidence that vitamin K is associated with oxidative phosphorylation. Dallam & Anderson (1957) and Beyer (1959) were able to inhibit oxidation and phosphorylation in rat-liver mitochondria by ultraviolet irradiation and restore both with phyloquinone (vitamin K). The most detailed studies of the problem have been made by Brodie and co-workers, using fractionated extracts of *Mycobacterium phlei*. They have shown that oxidative phosphorylation in this organism can be destroyed by long-wave ultraviolet light and restored by phyloquinone in a highly specific manner (Brodie, 1959; Brodie & Ballantine, 1960*a,b*). Considerable support was given to mechanistic schemes such as those described above by the work of Russell & Brodie (1961), who studied light-inactivated cell-free extracts of *M. phlei* and obtained spectral evidence that chroman-6-yl derivatives of phyloquinone might be enzymically formed during the oxidative phosphorylation process. Although it was later shown by Wagner *et al.* (1963*b*) that the chroman-6-yl derivatives were predominantly of non-enzymic origin (and possibly

artifacts), further attention continues to be paid to this important problem, especially in the chemical studies of Wagner *et al.* (1963a,b) and Erickson *et al.* (1963).

The concept that there is a direct interaction of phosphate with the electron-carrier system is one that has been viewed with reserve (Chance, 1961) and is considered to conflict with the known properties of the respiratory chain (Lehninger, 1960). Since all the mechanistic schemes discussed above require that at least one hydrogen atom be transferred from the 2-methyl group for each cycle of oxidation and phosphate transfer, it should be possible to obtain direct information on the mechanism of vitamin K participation by studying the fate of phyloquinone labelled with ^3H and ^{14}C in the 2-methyl group. Phyloquinone labelled with these two isotopes was synthesized and used to study the restoration of oxidative phosphorylation in the *M. phlei* system and in rat-liver mitochondria. Some experiments were also made to study the fate of the doubly-labelled phyloquinone and [2-Me- ^{14}C]menadione in the living rat.

MATERIALS AND METHODS

Materials

[2-Me- ^{14}C]Menadione. This compound was obtained with a specific activity of 26.3 $\mu\text{C}/\text{mg}$. from The Radiochemical Centre, Amersham, Bucks.

[2-Me- ^3H]Menadione. This compound was prepared from 2-bromomethyl-1,4-naphthaquinol diacetate according to the method of Billeter & Martius (1960) and had a specific activity of 10.4 $\mu\text{C}/\text{mg}$.

[2-Me- ^{14}C , ^3H]Phyloquinone. A suitable mixture of the [^3H]menadione (190 mg.) and [^{14}C]menadione (3.8 mg.) in ether (20 ml.) was shaken with saturated $\text{Na}_2\text{S}_2\text{O}_4$ solution until colourless. The organic layer was washed with water and evaporated. The resulting solid was dissolved in dioxan (5 ml.) and treated with redistilled boron trifluoride etherate (0.1 ml.). Isophytol (0.5 g.) in dioxan (5 ml.) was added over 15 min. to the stirred solution. The mixture was then heated at 50° for 40 min., cooled and diluted with ether. The organic layer was washed successively with water, aq. 2% (w/v) KOH (containing a little $\text{Na}_2\text{S}_2\text{O}_4$) and water, and then dried over MgSO_4 . Evaporation gave an oil (0.7 g.) that was dissolved in ether (20 ml.) and shaken with Ag_2O (0.4 g.) for 1 hr. The yellow solution was decanted from the silver residue and evaporated to give an oil (0.7 g.), which was chromatographed on thin layers of silica gel G (E. Merck A.-G., Darmstadt, Germany) with benzene-light petroleum (1:1, v/v) as developing solvent. The main band was separated and eluted with ether, and gave phyloquinone of specific activity 3.3 μC of $^3\text{H}/\text{mg}$. and 0.17 μC of $^{14}\text{C}/\text{mg}$., and with a radioactive purity of 98%.

Menaquinone-4. MK-4* was prepared from geranyl-linalol by essentially the same route as reported by Isler,

Rüegg, Chopard-dit-Jean, Winterstein & Wiss (1958). The product was purified by thin-layer chromatography on silica gel with benzene-light petroleum (1:1, v/v) as developing solvent.

Suspension of phyloquinone

A suspension of the radioactive phyloquinone was prepared by the phospholipid-micelle method of Fleischer & Klouwen (1961), as used by Fleischer & Brierley (1961) for the solubilization of cholesterol and ubiquinone. The EDTA was omitted from the dialysis during the last 2 days of preparation, as described for the suspension of phyloquinone by Asano, Kaneshiro & Brodie (1965). This preparation was found to be many times more active in restoring oxidative phosphorylation than suspensions made with bacterial supernatants (Brodie & Ballantine, 1960a) or with BRIJ 35 (Wosilait, 1960). The standard preparation contained 45 μg . of phyloquinone/ml. and was checked for stability by chromatographic analysis during the course of the experiments. The chromatograms were scanned for radioactivity, and over 98% of the radioactivity was located in the phyloquinone band. The isotopic ratio remained constant. The suspension could be stored for several months at 4° and was clarified from time to time by centrifuging at 35000g without loss of activity. A control suspension without phyloquinone was prepared and added to control flasks during the incubation procedure.

Radioactive counting

As a result of the use of the carrier technique, it was necessary to count phyloquinone and MK-4 of very low specific activity. This caused difficulties in scintillation counting, since naphthaquinones are powerful quenching agents. The binary counting technique was therefore subject to three errors: (i) the carbon count was spuriously low; (ii) the count in the lower-energy channel, containing the hydrogen and a proportion of the carbon, was too high; (iii) hydrogen pulses were lost below the lower discriminator gate. By introducing a third channel in the upper energies of the carbon channel and using standard amounts of ^3H , ^{14}C and ^3H - ^{14}C mixtures quenched to different degrees, correction curves were obtained by plotting the ratio between the two carbon-channel counts against (i) the efficiency of counting for hydrogen, (ii) the efficiency of counting for carbon and (iii) the net gain in carbon counts in the hydrogen channel. The usefulness of the curves was checked by using known mixtures of ^3H and ^{14}C with various amounts of quenching. The instrument used was the Nuclear Enterprises (G.B.) Ltd. (Edinburgh) scintillation spectrometer NE8305, and the samples were counted in quartz vials with 4 ml. of NE213 scintillator. The binary counting efficiencies were 25% for ^3H and 60% for ^{14}C at a background count of 0.8 count/sec. When ^{14}C was counted alone, the efficiency was 98% with a similar background value.

Bacterial experiments

Preparation of bacterial extracts. The organism used was *M. phlei* A.T.C.C. 354, a culture of which was kindly supplied by Dr A. F. Brodie and grown on the medium described by Brodie & Gray (1956). Colonies were transferred from agar slopes to 100 ml. of medium, which was

* Abbreviation: MK-4, menaquinone-4, i.e. menaquinone with four isoprenoid units in the side chain.

incubated with agitation at 30° for 24 hr. This culture was then added to 700 ml. of medium at pH 7.0. The culture was incubated at 30° with agitation for a further 24 hr. After the purity had been checked, the cells were harvested by centrifugation at 2000g for 10 min., washed four times with water and suspended in 0.1 M-KCl (brought to pH 7.4 with 1 mM-tris buffer) to give a cell concentration of 450 mg. wet wt./ml. The cells were disrupted by shaking 3–5 ml. of this suspension with an equal volume of Ballotini (no. 14; English Glass Co., Leicester) in a stoppered glass tube in a vibrator (after Nossal, 1953) at 50 cyc./sec. at an amplitude of $\frac{1}{8}$ in. for 5 min. at 4°. The glass beads were removed and the supernatant was obtained by centrifuging at 20000g at 0° for 10–15 min.

Irradiation. The extract was dialysed for 1 hr. (Brodie & Gray, 1956) and irradiated for 1 hr. at 4°, as described by Brodie & Ballantine (1960a,b). The Allen long-wave ultraviolet lamp ($\lambda_{\max}$, 360 m μ), which is obtainable in the United Kingdom, was used at 2 cm. from the surface of the extract, which was gently stirred with a magnetic stirrer during irradiation (Anderson & Dallam, 1959). The extract was centrifuged at 56000g for 5 min. to remove a little solid material. The resulting solution normally contained about 10 mg. of protein/ml.

Measurement of activity. The bacterial extracts, before and after irradiation, were assayed for activity in the Warburg respirometer at 30°. Enough extract to contain 20 mg. of protein was mixed with 15 μ moles of MgCl₂, 20 μ moles of glucose, 10 μ moles of KH₂PO₄ (buffered to pH 7.4 with 0.05 M-tris buffer) and 2.5 μ moles of ADP. Phylloquinone was added when required. Saturated KOH solution was added to the centre well. The flasks were equilibrated for 10 min. in air, and 50 μ moles of β -hydroxybutyrate, 25 μ moles of KF and 1 mg. of hexokinase were added from the side arm. Oxygen uptake was measured, generally for 20–30 min.

To those flasks from which the radioactive phylloquinone was subsequently to be extracted was added 0.5 ml. of ethanol containing 200 μ g. of non-radioactive phylloquinone and 200 μ g. of MK-4 to act as carriers for labelled vitamin K compounds. To the remaining flasks 1 ml. of aq. 10% (w/v) trichloroacetic acid was added and the contents of these flasks were used for phosphate determination. A control flask containing trichloroacetic acid added at zero time was used to measure starting phosphate concentration. The precipitated protein was removed by centrifugation and inorganic phosphate was determined by the method of Fiske & Subbarow (1925).

Recovery of quinones. The contents of those flasks to which carrier phylloquinone and MK-4 had been added after incubation were extracted with three 3 ml. portions of diethyl ether. The ethereal extracts were evaporated and dried by azeotropic distillation with benzene-ethanol (1:1, v/v). The lipid was chromatographed on plates (20 cm. $\times$ 10 cm.) coated with thin layers of silica gel containing 20 p.p.m. of sodium fluorescein. The chromatograms were developed with light petroleum-benzene (1:1, v/v) for 20 min.: the phylloquinone and MK-4 separated as bands (R_F 0.3 and 0.2 respectively) that were strongly quenching under ultraviolet light. The bands were eluted into ether, and after evaporation of the solvent they were separately rechromatographed by descending chromatography for 6 hr. in a reversed-phase paper partition system [Whatman no. 4 papers (40 cm. $\times$ 20 cm.) were pre-impregnated with ammoniacal ZnCO₃ solution containing 45 p.p.m. of sodium

fluorescein and then impregnated with liquid paraffin, B.P.; they were developed with aq. 85% (v/v) ethanol] (Green, Marcinkiewicz & Watt, 1955). The compounds were again observed as quenching bands in ultraviolet light and were eluted with ether. The eluates were evaporated with nitrogen in quartz counting vials. The remainder of each chromatogram was cut into pieces and treated similarly. The bands from some chromatograms were eluted with ethanol and their ultraviolet spectra examined and their identity with the original samples of phylloquinone and MK-4 was confirmed.

Rat-liver mitochondrial experiments

Preparation and irradiation of mitochondria. Livers from mature male rats were homogenized at 0–4° in 0.44 M-sucrose in 10 mM-EDTA, pH 7.4, and fractionated as described by Gurban & Christea (1965). The washed mitochondria were suspended in the homogenizing medium to give the equivalent of 167 mg. of liver/ml. The suspension was irradiated for 1 hr. at 0–4°, as above. The mitochondria were removed by centrifugation at 5000g for 20 min. and resuspended in 0.23 M-mannitol–0.07 M-sucrose–0.03 M-tris, pH 7.4, at a concentration equivalent to 1 g. of original liver/ml.

Measurement of activity. Portions (0.5 ml.) of the mitochondrial suspension were assayed for activity at pH 7.4. Each flask contained 30 μ moles of KH₂PO₄, 13.4 μ moles of MgSO₄, 2.0 μ moles of EDTA, 200 μ moles of sucrose, 7.0 μ moles of ATP, 0.04 μ mole of cytochrome *c* and 1.5 μ moles of NAD. At zero time, 20 μ moles of glutamate, 60 μ moles of glucose and 200 μ g. of hexokinase were added from the side arm to give a final volume of 2 ml. The flasks were incubated at 30° for 45 min. and oxygen uptakes were measured. Phosphate determinations and recovery of labelled quinones were carried out as above.

Recovery of quinones. This was carried out as described for the bacterial extracts.

Experiments in vivo in the rat

[2-Me-¹⁴C, ³H]Phylloquinone. Two 5-month-old male rats were given a single intravenous injection of 2 mg. of [2-Me-¹⁴C, ³H]phylloquinone, emulsified with Tween 80 in 0.2 ml. of 0.9% NaCl. After 20 hr., the rats were killed and their livers removed and placed in ice-cold 0.25 M-sucrose. A homogenate was prepared, and the mitochondrial and microsomal fractions were isolated by the method of Hogeboom (1955). After being washed with 0.25 M-sucrose, the mitochondrial and microsomal pellets were extracted under reflux three times with 5 ml. of acetone containing 150 μ g. each of phylloquinone and MK-4. The lipids were chromatographed on thin layers of silica gel with cyclohexane-ether (4:1, v/v) as developing solvent to give a preliminary separation of the quinone fraction, which was detected under ultraviolet light as above and extracted with ether. The quinone fraction was then separated into phylloquinone and MK-4 bands by thin-layer chromatography with light petroleum-benzene (1:1, v/v) as solvent, and the two fractions were further purified by partition paper chromatography and their radioactivities measured as described above.

[2-Me-¹⁴C]Menadione. Two 5-month-old male rats were given an oral dose of 10 mg. of neomycin sulphate in 0.5 ml.

of water and their drinking water was replaced with a solution of neomycin sulphate (1mg./ml. of water). After 24 hr., a further oral dose of 10 mg. of neomycin sulphate was given and, after a further 6 hr., 3.8 mg. of [2-*Me*-¹⁴C]-menadione, emulsified with Tween 80 in 0.5 ml. of water, was given intraperitoneally. The rats were killed 16 hr. later and mitochondria and microsomes were prepared as described above. Phylloquinone and MK-4 were separated by the techniques described above and their radioactivities measured.

RESULTS

Bacterial and rat-liver mitochondrial experiments. The *M. phlei* preparations had good respiratory and phosphorylating activity, although they were somewhat less active than those of Brodie & Ballantine (1960*a,b*), which were prepared by ultrasonic disruption. With both the *M. phlei* and the rat-liver mitochondrial preparations, ultraviolet light destroyed phosphorylation and most of the respiratory activity. Phosphorylation and oxidation were effectively restored in both types of preparation by phylloquinone (Table 1). Restoration of activity was effected with considerably smaller amounts of phylloquinone than were used by Brodie & Ballantine (1960*a,b*), and this can probably be attributed to the superiority of the phospholipid-micelle technique.

Radioactive quinones were recovered from preparations whose activity had been restored by the addition of doubly-labelled phylloquinone, and they were separated by partition chromatography into phylloquinone and MK-4 fractions: 97–98% of the total ¹⁴C was found in the phylloquinone band. The ³H/¹⁴C ratio in the recovered phylloquinone was always identical with that of the original compound.

Experiments in vivo in the rat. When [2-*Me*-¹⁴C,³H]phylloquinone was given intravenously to rats and the radioactive quinone fraction was isolated from their liver mitochondria and microsomes, about 90% of the total radioactivity was in the phylloquinone band. The ³H/¹⁴C ratio was 19.4, which was identical with that of the original phylloquinone. There was no detectable radioactivity in the MK-4 fraction. When [2-*Me*-¹⁴C]-menadione was given intraperitoneally to rats whose alimentary tract had been treated with neomycin and the quinone fraction isolated in a similar way, 41–44% of the total radioactivity was in the MK-4, but the phylloquinone band also contained 15–19% of the total. Bacteriological examination of the contents of the alimentary tract of these rats showed that the neomycin treatment had resulted in the destruction of *Escherichia coli* and all other aerobic organisms apart from a few micrococci and yeasts; the anaerobic flora, consisting chiefly of fusiform bacilli and anaerobic cocci was, however, unaffected.

DISCUSSION

Vitamin K is capable of restoring oxidative phosphorylation in bacterial and rat-liver mitochondrial systems that have been irradiated with ultraviolet light, and it has been suggested that a quinone of the vitamin K type may act as a phosphate carrier by a cyclic mechanism involving conversion of a 2-methyl-3-polyprenoid quinone, via an intermediate quinone methide, into a chroman-6-yl derivative (Russell & Brodie, 1961; Wagner *et al.* 1963*a,b*). It is clear that, in any such process in which a quinone methide is formed, at least one hydrogen atom must be transferred from the 2-methyl group of the quinone for each phosphate group transferred to the phosphate acceptor, subsequently to be replaced by another hydrogen atom from some donor. If the 2-methyl group in the active quinone is labelled with ¹⁴C and ³H the cyclic process can be expected to result in a change in isotopic ratio in some of the quinone molecules taking part in the reaction. Hence vitamin K recovered from the activated systems should show a loss of ³H.

In both systems studied, under the conditions used, as little as 0.02–0.03 μ mole of phylloquinone catalysed the incorporation of about 1 μ mole of inorganic phosphate. If all the molecules of the quinone added to the system were involved in the pathway there was therefore ample phosphorylation to account for a considerable loss of ³H. Even if only one-third of the phosphorylations directly involved the quinone molecules, a significant change in isotopic ratio should be observed. In our experiments, however, the ³H/¹⁴C ratio of the recovered phylloquinone was identical with that of the original phylloquinone.

There are, perhaps, other circumstances that might lead to such a result. If only a small fraction of the added phylloquinone (or perhaps some metabolite of it) became attached to the site at which phosphorylation took place, a situation could exist in which no change in isotopic ratio would be observed in the recovered quinone, since most of it would not be involved in the catalytic cycle. Another possibility is that there is a pronounced 'isotope effect' predisposing towards selection of ¹H transfer and against ³H transfer from the labelled methyl group. Although such an isotope effect is probably unimportant when the hydrogen atoms are stereochemically distinct (cf. the stereochemistry of enzymic hydrogen transfer to nicotinamide nucleotides; Cornforth, Ryback, Popják, Donninger & Schroepfer, 1962), it may be considerable in a methyl group subject to free rotation and where the carbon atom may be attached to hydrogen isotopes of differing mass in the same molecule. Verly, Rachele, du Vigneaud, Eidinoff & Knoll

Table 1. *Effect of phyloquinone on the oxidative phosphorylation of M. phlei and rat-liver mitochondrial preparations and a study of the isotopic ratio in phyloquinone doubly labelled with ^3H and ^{14}C*

Experimental details are given in the text. The radioactivities of ^3H and ^{14}C are given as disintegrations/sec.												
System	Treatment	Phyloquinone added ($\mu\text{g.}$)	Oxygen uptake ($\mu\text{g atoms}$)	Phosphate uptake (μmoles)	P/O ratio	Counting data on phyloquinone						% of initial phyloquinone recovered
						Original phyloquinone			Recovered phyloquinone			
						^3H	^{14}C	$^3\text{H}/^{14}\text{C}$ ratio	^3H	^{14}C	$^3\text{H}/^{14}\text{C}$ ratio	
<i>M. phlei</i> (1)	Irradiated	0	0.97	0.1	0.10	870	—	—	—	—	—	—
	Irradiated	9	4.10	1.5	0.37	870	45	19.4	396	20	19.4	46
	Irradiated	9	2.60	1.2	0.46	870	45	19.4	263	14	19.4	30
<i>M. phlei</i> (2)	None	0	2.63	—	—	—	—	—	—	—	—	—
	Irradiated	0	1.00	0.1	0.10	—	—	—	—	—	—	—
	Irradiated	14	1.70	0.6	0.35	—	—	—	—	—	—	—
<i>M. phlei</i> (3)	Irradiated	14	1.70	—	—	1415	73	19.4	662	34	19.4	47
	None	0	1.73	1.3	0.73	—	—	—	—	—	—	—
	Irradiated	0	0.58	0.0	0.00	—	—	—	—	—	—	—
	Irradiated	14	1.60	0.8	0.47	—	—	—	—	—	—	—
	Irradiated	14	2.60	1.1	0.43	—	—	—	—	—	—	—
	Irradiated	14	2.29	—	—	1415	73	19.4	893	46	19.5	63
Rat-liver mitochondria	Irradiated	14	3.06	—	—	1415	73	19.4	657	34	19.5	46
	None	0	2.4	1.8	0.75	—	—	—	—	—	—	—
	Irradiated	0	0.9	0.2	0.22	—	—	—	—	—	—	—
	Irradiated	14	1.1	0.7	0.64	—	—	—	—	—	—	—
	Irradiated	14	2.6	1.4	0.54	—	—	—	—	—	—	—
	Irradiated	14	—	—	—	1483	76	19.4	1180	61	19.5	80
	Irradiated	14	—	—	—	1483	76	19.4	1184	60	19.6	80

(1952) and du Vigneaud, Rachele & White (1956) distinguish between such 'intramolecularly' labelled molecules and 'intermolecularly' labelled molecules, where one molecule contains ^3H and another only ^1H . Therefore not only may selection occur because of the differing energies between the carbon-tritium and carbon-protium bond, but, in the 'intermolecular' case (the phyloquinone used here was 'intermolecularly' labelled with the two isotopes), metabolic selection may occur, which could also lead to retention of tritiated molecules. Such factors must be considered in the present study, for they may be especially important in experiments such as these that do not involve a search for metabolites of a labelled substrate but rather an examination of a catalytic agent. Therefore, though the results of these experiments provide no evidence for the participation of phyloquinone as a phosphate carrier in phosphorylation, their negative nature does not necessarily preclude this.

It seems unlikely that the restoration of oxidative phosphorylation by phyloquinone in the bacterial and mitochondrial systems involved the formation of a menaquinone, for the carrier MK-4 was never found to contain radioactive material after isolation. When radioactive phyloquinone was given intravenously to rats, and quinones were recovered from their liver mitochondria and microsomes 20 hr. later, again the only radioactive quinone present was phyloquinone itself and no menaquinone was found. This is in agreement with Billeter & Martius (1960), who state that the conversion of phyloquinone into MK-4 hardly occurs in the organs of the rat, this conversion being effected mainly by intestinal microflora (Billeter, Bolliger & Martius, 1964). The phyloquinone recovered from the living rat also had a $^3\text{H}/^{14}\text{C}$ ratio identical with that of the original phyloquinone. This gives further support to the contention that, if vitamin K is concerned with oxidative phosphorylation *in vivo*, the mechanism probably does not involve the intermediate formation of a quinone methide. When [^{14}C]menadione was given intraperitoneally to rats whose alimentary tract had been treated with neomycin, the main radioactive quinone recovered from the mitochondria and microsomes was MK-4, in agreement with Martius & Esser (1958), but we also found appreciable activity in the carrier phyloquinone, which persisted after thin-layer chromatography in two adsorption systems and partition paper chromatography. Although Martius & Esser (1958) did not find phyloquinone, it should be noted that unequivocal separation of phyloquinone from certain menaquinones of greater molecular weight requires a combination of adsorption and partition chromatography.

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