Metabolism of [^{14}C]Menadione*

Raymond Losito,† Charles A. Owen, Jr., and Eunice V. Flock

ABSTRACT: The metabolism of 2-[^{14}C]methyl-1,4-naphthoquinone ([^{14}C]menadione) by the liver has been studied in the isolated perfused rat liver, and the metabolites which were excreted in the bile have been compared with those excreted in the urine by rats with biliary fistulas. The amount of radioactivity present in the blood, bile, and liver at the end of 5 hr of perfusion was 53, 33, and 15% of the dose, respectively. Approximately 93% of the naphthoquinone in this system was metabolized. The major product identified in the bile was the glucuronide of reduced menadione (19% of the ^{14}C dose). The glucuronide was not present in the perfusate, but the sulfate of reduced menadione in the plasma accounted for 9.3% of the ^{14}C dose. This sulfate was not found in the bile. After intravenous or oral administration of menadione, both the glucuronide and the sulfate of reduced menadione were excreted in the urine by normal rats and by rats with biliary fistulas. The glucuronide conjugate was found in greater amounts in the urine after oral than after intravenous administration of menadione. These data suggest that extrahepatic tissues as well as the liver have a mechanism for the conjugation of menadione with glucuronic acid. In contrast, more of the sulfate was excreted in urine after intravenous administration. Menadione and its metabolic products were associated with albumin and prealbumin on electrophoresis. Warfarin, an indirect anticoagulant, did not affect the excretion of metabolites of menadione in the bile.

Although it has been almost four decades since Dam (1929) first observed that the antihemorrhagic compound, vitamin K, was associated with dietary lipids, very little is known about the metabolism of this substance in mammals. Rarely have the metabolites of phylloquinone or the menaquinones in body fluids or urine been investigated. The majority of studies have been made of the metabolism of menadione[1] which lacks the side chain found in the naturally occurring forms of the vitamin. When ^{14}C-labeled menadione was administered to normal rats (Hoskin *et al.*, 1954) or rats with biliary fistulas (Solvonuk *et al.*, 1952), radioactivity was found to appear quickly in the bile and urine. Studies in rabbits, with nonradioactive menadione, also indicated that menadione is quickly excreted in the urine (Richert, 1951). Recently (Losito *et al.*, 1965), it has been found that, after administration of [^{14}C]menadione to dogs, all radioactivity in the bile was in metabolites.

To study further the metabolism of menadione, we added [^{14}C]menadione to an isolated perfused rat liver system and also administered it to rats with biliary fistulas. Radioactive compounds in blood, bile, urine, and liver were separated by chromatography and attempts were made to identify them.

Materials and Methods

Perfusion System. The livers in these experiments came from adult male rats (250–375 g) of the Sprague–Dawley strain maintained on Friskie cubes. The wet weights of the livers ranged from 10 to 13 g. The method used to perfuse these organs was an adaptation of Brauer's technique as described by Flock and Owen (1965). Two livers were used in each experiment, the first being perfused for 40–60 min to remove vasoconstricting substances from the perfusate. After this first liver was removed from the system, the [^{14}C]-menadione (sp act. 37.6 μc/mg), dissolved in ethanol, was added to the perfusing blood and allowed to circulate for 15 min; the dose ranged from 30 to 130 μg. The second liver, which was used to study the metabolism of this compound, was then connected in the system and was usually perfused for 5 hr. In some experiments, a third liver was substituted for the second liver after 3 hr and the perfusion was continued for another 3-hr period.

Samples of perfusate (1 ml) were removed from the perfusion system at 8, 13, 18, 23, 28, 33, 43, 53, 63, 120, 180, 240, and 300 min after the second liver was connected. Bile was collected continuously in fractions representing the first two 30-min periods and the subsequent 1-hr periods. At the end of the perfusion, the liver was homogenized in 80 ml of isotonic saline.

In some experiments, the effect of sodium warfarin on the metabolism of menadione was studied. The

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† Fellow of the Canadian Medical Research Council.

[1] Abbreviations used: menadione, 2-methyl-1,4-naphthoquinone; [^{14}C]menadione, 2-[^{14}C]methyl-1,4-naphthoquinone.

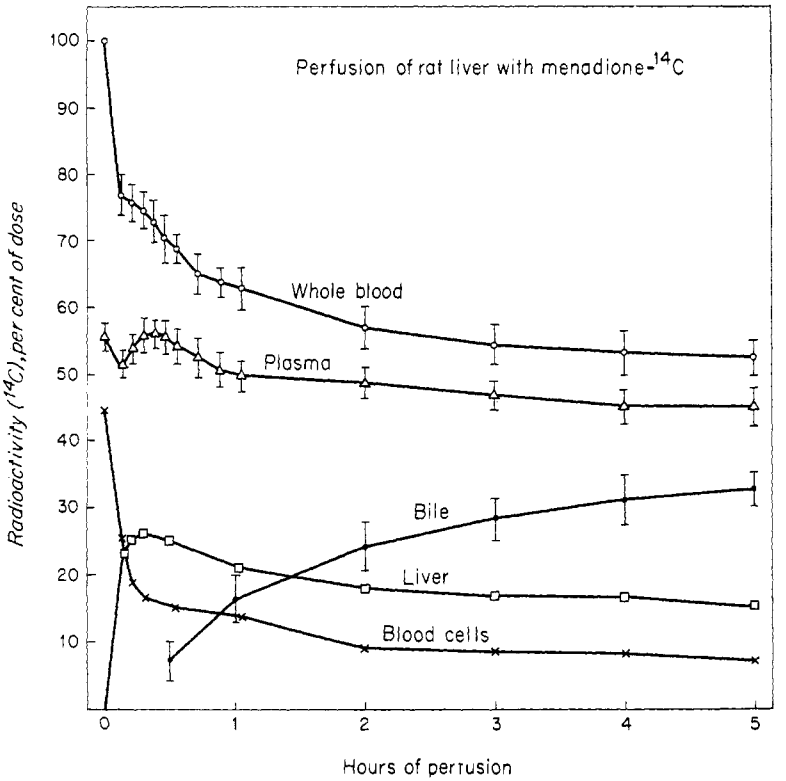

FIGURE 1: Perfusion of isolated rat liver (means of seven perfusions) with [^{14}C]menadione. The radioactivity, as percentage of dose, in whole blood, blood cells, plasma, bile, and liver is plotted against time in hours. Standard errors of the means are given for whole blood, plasma, and bile. The plots for blood cells and liver were calculated indirectly.

anticoagulant was administered to rats before their livers were removed for perfusion (2.5 mg at 48 hr and 1.25 mg at 24 hr before). In two perfusions, 5 mg of sodium warfarin was added directly to the perfusion system with the [^{14}C]menadione.

Normal and Biliary Fistula Rats. Cystostomies were performed in all the rats. [^{14}C]Menadione (150 μg) was administered, either orally or by way of the portal vein, to rats with biliary fistulas. Only the fluids that were excreted within the first 7 hr were studied. To normal (control) rats, 50 μg of [^{14}C]menadione was administered by way of the tail vein. Samples of plasma were obtained from the control rats 1 hr after the dose of [^{14}C]menadione was given.

Measurement of Radioactivity. The radioactivity in whole blood, plasma, bile, urine, and liver homogenates was measured in Kinard's scintillation fluid containing 4% Cab-O-Sil or in a mixture of P25-X and Triton X100 (Packard Instrument Co., Inc.) (Meade and Stiglitz, 1962) in a Packard Tri-Carb liquid scintillation spectrometer, Model 4322. The volume of the various fluids counted was: blood, 5 μl; plasma, 100 μl; bile, 20 μl; and liver homogenate, 200 μl.

Chemical Procedures. The bile was extracted three times with three volumes of peroxide-free diethyl ether to remove any unmetabolized [^{14}C]menadione. The proteins in plasma and in bile were precipitated with four volumes of methanol–acetone (1:1) at $-15°$ for 1 hr. By this method, at least 96% of the radioactivity was recovered in the supernatants of both fluids. The protein-free plasma was concentrated to its original volume and then extracted three times with ethyl ether (ten volumes) to separate the conjugated metabolites from unchanged [^{14}C]menadione.

Aliquots of bile and plasma (or their isolated fractions from paper chromatography) were subjected to hydrolysis by β-glucuronidase (β-D-glucuronide glucuronohydrolase, 3.2.1.31) (Sigma Chemical Co.) (Taurog *et al.*, 1952), arylsulfatase (arylsulfate sulfohydrolase, 3.1.6.1) (Mann Research Laboratory, Inc.) (Cohen and Bates, 1949), and acid and alkaline phosphatases (orthophosphoric monoester phosphohydrolases, 3.1.3.2 and 3.1.3.1) (General Biochemicals) (Linhart and Walter, 1963). The amounts of the enzymes used in 10 ml of the appropriate buffers were: phosphatases, 30 mg; β-glucuronidase, 40 mg; and arylsulfatase, 50 mg. A 3-ml aliquot of the enzyme–buffer solution was used for each hydrolysis. Controls were run in the same buffers without the enzymes. In addition, HCl hydrolysis (final concentration, 6 N) at 80° for 30 min was used. After the enzymatic hydrolysis, the hydrolyzed metabolites were extracted with ethyl ether (three volumes). One-half of the ethereal extract was saved for chromatography. The balance of the ethereal phase was dried, oxidized with chromium trioxide in acetic acid, diluted with distilled water, and

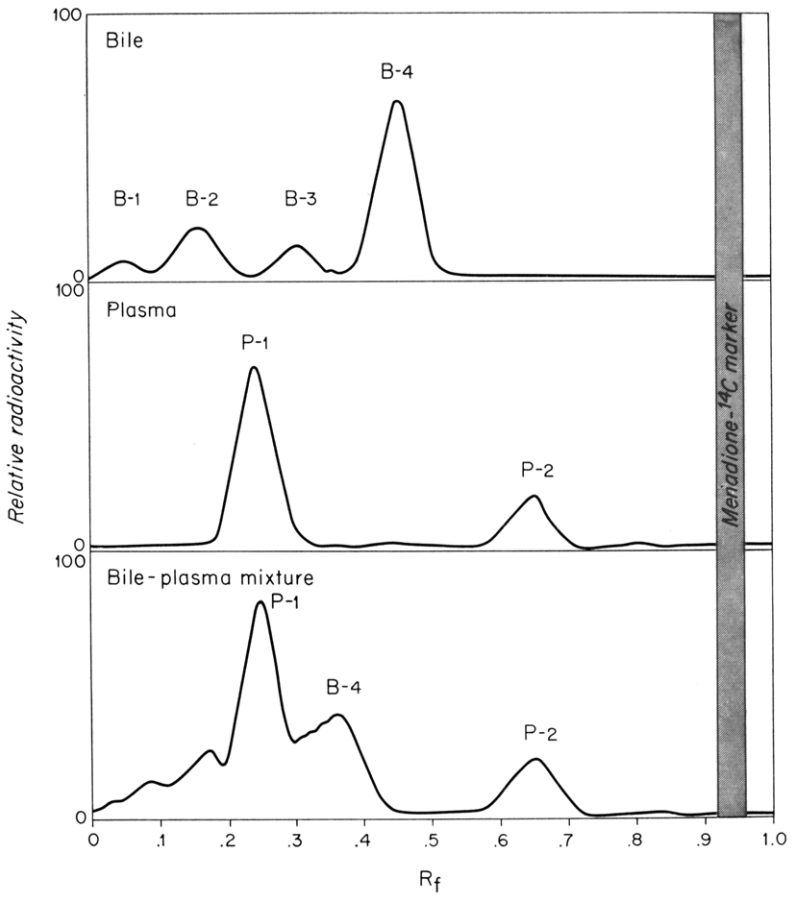

FIGURE 2: Results of paper chromatography of protein-free bile and plasma (after precipitation by methanol–acetone) from perfusion system, shown as relative radioactivity (^{14}C) plotted against R_F.

reextracted with ether to obtain the free menadione. After homogenization the livers were sonified at 20 kcycles/sec (M.S.E. apparatus) for 2 min at 5°, extracted with 2 l. of ethyl ether or petroleum ether (bp 30–60°) and dried.

Chromatography. Bile and plasma (or their isolated components after hydrolysis) were chromatographed on Whatman No. 3MM paper by the descending method for 12–14 hr in butanol–ethanol–water (3:1:1.5). Radioactivity on the paper strips was detected and measured in a Baird-Atomic Scanograter chromatogram scanner. The chromatograms were examined under ultraviolet light for fluorescence or absorbance. Some of the paper strips were stained with ninhydrin. The components containing radioactivity were eluted and dried and then redissolved in *n*-hexane for scanning (Bausch and Lomb Spectronic 505 recording spectrophotometer) between 220 and 350 mμ.

Thin layer plates coated with Bio-Sil A (mesh size, 30–60 μ) (Bio-Rad Laboratories) were also used for part of the chromatographic analysis. The solvent system was benzene–ethyl ether (24:1.5). After development of the thin layer chromatograms, the plates were examined under ultraviolet light. Then the plates were divided into 1-cm lengths and the silicic acid was scraped off directly into counting vials and suspended in the scintillation fluids for counting. Reference compounds included menadione, vitamin K_1, and menaquinones 4–7 and 9.

Electrophoresis. Plasma for paper electrophoresis was obtained from two sources. (1) [^{14}C]Menadione was added directly to rat plasma and allowed to equilibrate for 1 hr, or (2) rats were given [^{14}C]menadione and plasma was obtained 1 hr later. The conditions were: barbital buffer, pH 8.6; ionic strength, 0.5; running time, 16 hr at 4.5 ma. After electrophoresis, the strips were divided into two groups; one group was tested for radioactivity and the other was stained for proteins with the Amidoschwartz reagent.

Results

Perfusion System. The radioactivity in the blood was quickly taken up by the liver (Figure 1), reached a maximum of 25% at 15–20 min, and then gradually decreased to 15% at the end of the perfusion procedure. The radioactivity in whole blood and in blood cells showed an inverse relationship to that in the liver; ^{14}C disappeared rapidly during the first hour of the perfusion and then more slowly. The rate of disappear-

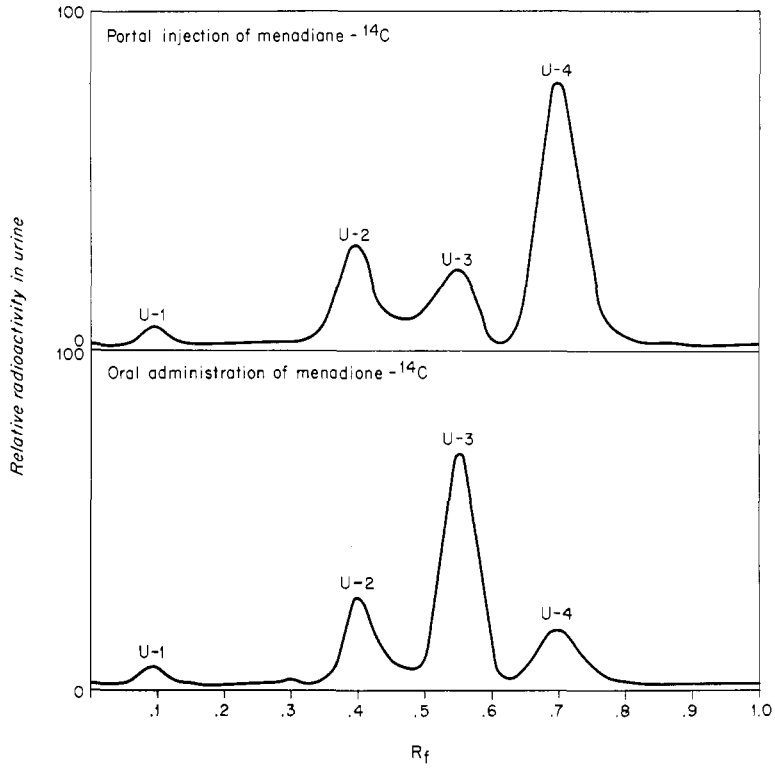

FIGURE 3: Results of paper chromatography of protein-free urine (after precipitation by methanol–acetone), shown as relative radioactivity (^{14}C) plotted against R_F. [^{14}C]Menadione was administered intravenously or orally.

TABLE I: Chemical and Physical Characteristics of [^{14}C]Menadione Metabolites in Bile and Plasma.

	Bile[a]				Plasma[b]	
	B-1	B-2	B-3	B-4	P-1	P-2
Radioactivity (% of ^{14}C in sample)[c]	6.3 ± 0.2	23.3 ± 0.4	13.5 ± 0.5	56.9 ± 0.6	74.9 ± 1.1	25.1 ± 1.1
% of dose	2.1	7.6	4.4	18.6	27.7	9.3
R_F, mean and range	0.07 (0.05–0.08)	0.16 (0.15–0.18)	0.30 (0.29–0.32)	0.46 (0.44–0.49)	0.25 (0.24–0.26)	0.64 (0.62–0.68)
Ultraviolet fluorescence	Purple	Orange-green	Yellow-green	–	Blue	–
Ninhydrin reaction	?	+	Yellow → flesh	–	+	–
Ultraviolet absorption	+	+	+	+	+	+
Hydrolyzed by	HCl	HCl	HCl	HCl, β-glucuronidase	HCl	HCl, arylsulfatase

[a] Determinations (20) of bile from six perfusions. [b] Determinations (24) of plasma from five perfusions. [c] Mean plus or minus standard error.

ance was greater from the cells than from whole blood. The percentage of the dose found in whole blood at the conclusion of the experiment averaged 52.5% for seven perfusions. The ^{14}C in the plasma decreased at first (to 52%), increased (to 56%), and then decreased gradually to 45% at the end of the experiment. In the bile, the ^{14}C appeared within 30 min. The average amount of radioactivity excreted in 5 hr in 13 perfusions

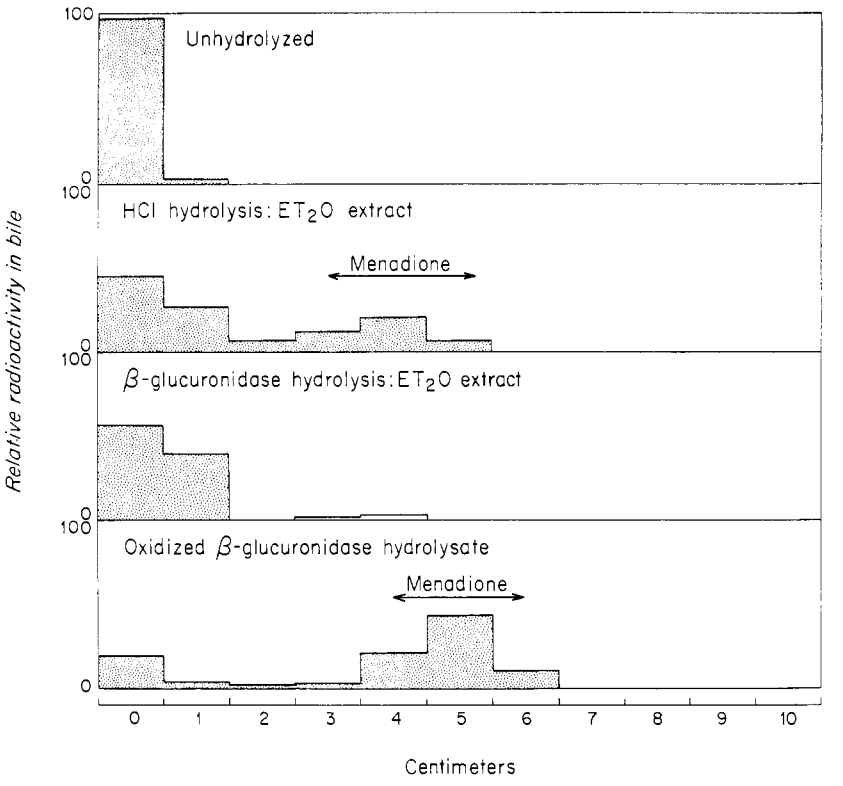

FIGURE 4: Results of thin layer chromatography of whole bile and of ethereal extracts of HCl hydrolysates, β-glucuronidase hydrolysates, and oxidized β-glucuronidase hydrolysates, shown as relative radioactivity (^{14}C) plotted against distance traveled.

was 32.6 ± 2.4% (standard error). The lag for the excretion into the bile is due in part to the dead space of the polyethylene tubing that leads from the common bile duct to the receptacle. In the perfusions that used three livers, the third liver excreted only 5% of the remaining radioactivity present in the system.

Extraction of Bile, Plasma, and Urine. Ethyl ether failed to extract any radioactivity from the bile and urine, but approximately 7.2% of the radioactivity could be extracted from protein-free plasma, all as menadione.

Chromatography and Analysis. It is evident from Figures 2 and 3 that the solvent system used for paper chromatography can separate the metabolites present in bile and plasma (supernatants after precipitation of protein by methanol–acetone). In the bile (Figure 2), there were four main radioactive components (labeled B-1–4, see Table I). The bile from the rats with biliary fistulas did not give as good resolution as the bile from the perfusion system; therefore, the latter bile was studied. The radiochromatograms of plasma showed only two main components (labeled P-1–2). When the plasma and the bile were mixed and then chromatographed, the fastest component of the plasma was still well separated from the rest of the mixture. However, the fastest component of the bile was retarded. Free menadione was not found in the two fluids when they were chromatographed after storage at 5° for less than 1 week. After longer storage (1–2 weeks), the P-2 component of the plasma started to disappear simultaneously with the appearance of menadione (R_F 0.93). Storage for the same period did not affect P-1 or the biliary metabolites.

The largest component of the bile (B-4), which represented 56.9% of the biliary metabolites (18.6% of the dose), was hydrolyzed by β-glucuronidase or HCl (Table I). The remaining biliary metabolites (B-1–3) were hydrolyzed by HCl only; the various enzyme systems failed to cleave these metabolites. Ultraviolet spectroscopy of the components exhibited two absorption bands, one at 225–235 mμ and a smaller one at 240–255 mμ. Only B-2 and B-3 gave a positive ninhydrin reaction.

When the bile was chromatographed on silicic acid, the radioactive material remained at the origin (Figure 4). A similar pattern was obtained with an ethereal extract of the β-glucuronidase hydrolysate but there was some movement of radioactivity into the menadione zone. It was only when the hydrolysate was oxidized or the bile hydrolyzed with HCl that menadione could be well resolved. The menadione reference standard did not have a constant R_F in the thin layer chromatograms but had a range of 0.3–0.5. This was in contrast to paper chromatography, in which its R_F fell within narrow limits (Figure 2).

In the thin layer system, vitamin K_1 and the mena-

quinones traveled close to the front (R_F 0.96). During the course of the experiments, there was never any radioactivity found in the K_1 or menaquinone area in thin layer chromatograms of liver extract as well as bile.

In the plasma of the perfusion system. P-1, which stained intensely with ninhydrin and contained 74.9% of the ^{14}C in plasma or 27.7% of the dose, was hydrolyzed by HCl only. The second plasma metabolite, P-2 (25.1% of ^{14}C in plasma or 9.3% of the dose), was hydrolyzed by arylsulfatase and HCl. The radioactivity was associated with the albumin or prealbumin area in the electrophoretic patterns.

The urine was utilized in these experiments only for confirmation of identification of the glucuronide and sulfate components because these metabolites have been detected in this medium previously (Li *et al.*, 1949; Richert, 1951; Hoskin *et al.*, 1954). The results of paper chromatography of urine from rats given [^{14}C]menadione orally or intravenously are shown in Figure 3. Intravenous injection produced 1.4 times the radioactivity in the urine as compared to the oral route. The urinary components are classified as U-1–4. In some of the chromatograms, U-2 was resolved into two incomplete peaks, indicating more than one metabolite. U-3 and U-4 were hydrolyzed by β-glucuronidase and arylsulfatase, respectively, showing that U-3 is a glucuronide and U-4 is a sulfate conjugate of reduced menadione. The nature of the other metabolites is presently being investigated. Intravenous injection gave rise to greater amounts of sulfate conjugate of reduced menadione in the urine than did oral administration. The reverse was true for the glucuronide. The excretion of ^{14}C-labeled metabolites remained approximately unchanged (31.8% of the dose) when warfarin was added directly to the perfusion system or when livers from rats previously treated with warfarin were used.

Discussion

When [^{14}C]menadione was added to the perfusate of the isolated rat liver perfusion system, it was quickly removed from the blood and metabolized by the liver and the metabolites were excreted into the bile or returned to the blood. The liver may also excrete unaltered menadione into the blood.

In the bile excreted by the perfused liver, the major component, representing close to one-fifth of the original dose, was a glucuronide of 2-methyl-1,4-naphthohydroquinone; this compound was identified on the basis of its hydrolysis by β-glucuronidase. Other enzymes (arylsulfatase and acid and alkaline phosphatases) failed to hydrolyze any of the metabolites excreted into the bile. The exact nature of these products, which were all hydrolyzed by HCl, is not known at the present time. However, menadione can react with thioglycolic acid or denatured proteins (Cornwell and Guyer, 1961) and it may be that these unknown metabolites are thioethers between menadione and sulfhydryl-containing amino acids or peptides (for example, cysteine or glutathione). On chromatograms, spots B-2, B-3, and P-1 stained with ninhydrin, but this may have been due to extraneous substances.

It seems that, after 3 hr of perfusion, the liver was not very efficient in excreting ^{14}C-labeled metabolites into the bile. This was not due to hepatic dysfunction because, when a new liver was substituted in the system at 3 hr, only 5% of the radioactivity was excreted in the next 3-hr period. This small excretion may have been due to unmetabolized [^{14}C]menadione present in the blood. If the new liver had handled the metabolites of [^{14}C]menadione in a manner similar to the preceding one, approximately 28% of the plasmatic radioactivity should have been excreted into the bile.

When the plasma was examined by the same techniques, a different picture was obtained. Neither of the components in the plasma was hydrolyzed by β-glucuronidase but arylsulfatase hydrolyzed the P-2 component. Thus, the blood perfusing the isolated liver contained a sulfate conjugate (sulfate conjugates were not found in the bile) but no glucuronide conjugate. Furthermore, the plasmatic conjugates did not contain phosphate moieties.

It has been shown (Scudi and Buhs, 1942; Canady and Roe, 1956) that the plasma proteins react with menadione when it is added to the blood. Fractionation of pooled blood (Solvonuk *et al.*, 1952) from mice 15 hr after they were given [^{14}C]menadione demonstrated that significant amounts of radioactivity were associated with the blood cells and albumin. In the present investigation, the same distribution of radioactivity in the electrophoretic patterns was obtained whether the plasma was from rats that had been injected 1 hr previously or was equilibrated directly with added [^{14}C]menadione for 1 hr. This shows that the [^{14}C]-menadione, whether metabolized or not, is bound to albumin.

The urine of intact rats contains glucuronide conjugates of reduced menadione (Li *et al.*, 1949; Hoskin *et al.*, 1954; Hart, 1958) as well as sulfate conjugates (Richert, 1951; Hoskin *et al.*, 1954; Hart, 1958). Our studies in which rats had been given [^{14}C]menadione either orally or parenterally, confirmed these earlier observations. However, consideration of these whole-rat experiments in conjunction with the perfusion experiments presents a clearer picture of how menadione is handled by the animal.

The glucuronide of reduced menadione was excreted into the bile by the isolated liver but was not found in the perfusate. Yet, this compound was found in the urine of the biliary fistula rats. Hence, there seem to be extrahepatic mechanisms for conjugating this naphthoquinone with glucuronic acid. Larger amounts of glucuronide were found in the urine of the biliary fistula rats after oral administration of menadione than after parenteral administration. This further supports the concept of conjugation of menadione with glucuronic acid in extrahepatic tissues (possibly intestinal or renal), as has been shown with other substances such as *o*-aminophenol (Hartiala, 1955) and salicylate (Schachter *et al.*, 1959).

The sulfate of reduced menadione showed a different

pattern. It was absent from bile of the isolated liver and of the whole rat but a sulfate conjugate was present in the plasma of the isolated system and in rat urine. This is strong evidence that the kidney must be the main organ for excretion of sulfate conjugates of [^{14}C]-menadione from the body. In contrast to the glucuronide, smaller amounts of sulfate conjugates of reduced menadione were found in the urine after oral administration of menadione than after parenteral administration.

It has been reported that menadione is converted to MK-4 which is assumed to be the biologically active compound of the K vitamins (Martius and Esser, 1959; Martius, 1961). In the fluids we analyzed, neither vitamin K, menaquinones, nor metabolites of these could be identified. This and other reports (Richert, 1951; Hoskin *et al.*, 1954; Hart, 1958; Cornwell and Guyer, 1961) imply that menadione did not add a phytyl or isoprene side chain at the 3 position but reacted only with hydrophilic-promoting moieties. It could be that, although the ethereal glucuronides, sulfates, and other unidentified metabolites are excreted, the vitamin K_1 or the MK's are the actual forms in the tissues, but this was not shown in our investigations. Warfarin did not affect the excretion of metabolites of [^{14}C]menadione into the bile and hence did not enhance or decrease its excretion *via* this route.

Acknowledgment

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References

Canady, W. J., and Roe, J. H. (1956), *J. Biol. Chem. 220*, 571.

Cohen, H., and Bates, R. W. (1949), *Endocrinology 44*, 317.

Cornwell, D. G., and Guyer, K. E. (1961), *Federation Proc. 20*, 452.

Dam, H. (1929), *Biochem. Z. 215*, 475.

Flock, E. V., and Owen, C. A., Jr. (1965), *Am. J. Physiol. 209*, 1039.

Hart, K. T. (1958), *Proc. Soc. Exptl. Biol. Med. 97*, 848.

Hartiala, K. J. (1955), *Ann. Med. Exptl. Biol. Fenniae (Helsinki) 33*, 239.

Hoskin, F. C. G., Spinks, J. W. T., and Jaques, L. B. (1954), *Can. J. Biochem. Physiol. 32*, 240.

Li, L., Elliott, W. H., and Doisy, E. A. (1949), 116th National Meeting of the American Chemical Society, Atlantic City, N. J., Sept. 18–23, pp 66–67c.

Linhart, K., and Walter, K. (1963), *in* Methods of Enzymatic Analysis, Bergmeyer, H. U., Ed., New York, N. Y., Academic, p 779.

Losito, R., Millar, G. J., and Jaques, L. B. (1965), *Biochem. Biophys. Acta. 107*, 123.

Martius, C. (1961), *Am. J. Clin. Nutr. 9*, 97.

Martius, C., and Esser, H. O. (1959), *Biochem. Z. 331*, 1.

Meade, R. C., and Stiglitz, R. A. (1962), *Intern. J. Appl. Radiation Isotopes 13*, 11.

Richert, D. A. (1951), *J. Biol. Chem. 189*, 763.

Schachter, D., Kass, D. J., and Lannon, T. J. (1959), *J. Biol. Chem. 234*, 201.

Scudi, J. V., and Buhs, R. P. (1942), *J. Biol. Chem. 144*, 599.

Solvonuk, P. F., Jaques, L. B., Leddy, J. E., Trevoy, L. W., and Spinks, J. W. T. (1952), *Proc. Soc. Exptl. Biol. Med. 79*, 597.

Taurog, A., Briggs, F. N., and Chaikoff, I. L. (1952), *J. Biol. Chem. 194*, 655.