Vitamin K as a Regulator of Benzo(a)pyrene Metabolism, Mutagenesis, and Carcinogenesis

STUDIES WITH RAT MICROSOMES AND TUMORIGENESIS IN MICE

LYONEL G. ISRAELS, GERALD A. WALLS, DIANE J. OLLMANN, ELSA FRIESEN, and ESTHER D. ISRAELS, *The Manitoba Institute of Cell Biology and the Departments of Medicine and Pediatrics, University of Manitoba; The Manitoba Cancer Treatment and Research Foundation, Winnipeg, Manitoba, Canada R3E OV9*

ABSTRACT Vitamin K_3 inhibits the conversion of benzo(a)pyrene to its more polar metabolites in an in vitro rat liver microsomal system. Vitamin K_3 also inhibits benzo(a)pyrene metabolism in rat liver fragments and reduces its mutagenicity in the Ames test. Higher concentrations of vitamin K_3 are required to comparably reduce benzo(a)pyrene metabolism when the microsomal system has been induced with 3-methylcholanthrene. High pressure liquid chromatography analysis of the products of benzo(a)pyrene metabolism shows a uniform reduction of all the metabolic products. When tumors were induced in ICR/Ha female mice by the intraperitoneal injection of benzo(a)pyrene, those mice given vitamin K_3 before or both before and after benzo(a)pyrene had a slower rate of tumor appearance and tumor death rate as compared with those receiving benzo(a)pyrene alone. However, vitamin K_1 increased the rate of tumor death while vitamin K deprivation and warfarin decreased the rate of tumor appearance and death in benzo(a)pyrene-injected mice.

These studies indicate that vitamin K_3 is an inhibitor of aryl hydrocarbon hydroxylase and reduces the carcinogenic and mutagenic metabolites in vitro, and inhibits benzo(a)pyrene tumorigenesis in vivo. That vitamin K_1 enhances the benzo(a)pyrene effect while warfarin and vitamin K deficiency inhibit benzo(a)pyrene tumorigenesis indicates that vitamin K_1, vitamin K deprivation, or possibly blockade of its metabolic cycle also modulates benzo(a)pyrene metabolism in vivo but by a mechanism or at a site different from the vitamin K_3 effect. The vitamin K series should be considered as capable of serving a regulatory function in the metabolism of benzo(a)pyrene and possibly other compounds metabolized through the mixed function oxidase system.

Received for publication 4 October 1982 and in revised form 17 January 1983.

INTRODUCTION

A number of carcinogens occur in the environment as procarcinogens that undergo metabolic activation in vivo to yield products that react with cellular components to bring about the carcinogenic event. The metabolism of these compounds may follow multiple pathways from initial epoxidation to irreversible binding with nuclear and cytoplasmic components and many of these steps are initiated or mediated by the microsomal mixed function oxidases. The mixed function oxidase (MFO)[1] system consisting of multiple forms of cytochrome p450, NADPH-dependent cytochrome p450 reductase, and microsomal membrane components is key to the metabolism of many xenobiotics including chemicals, pesticides, drugs, and dietary components. The process yields water-soluble metabolites and subsequent conjugation products more easily excreted than the parent molecule. However, as some of the intermediates are more metabolically active than the parent compound those not excreted may react with cellular components to produce toxic, mutagenic, teratogenic, or carcinogenic events. Among the potentially carcinogenic polycyclic aromatic hydrocarbons, benzo(a)pyrene (BP) is an extensively studied prototype. The microsomal MFO system me-

[1] *Abbreviations used in this paper:* BHA, butylated hydroxyanisole; BHT, butylated hydroxytolene; BP, benzo(a)pyrene; DMSO, dimethyl sulfoxide; HPLC, high pressure liquid chromatography; MFO, mixed function oxidase.

 J. Clin. Invest. © *The American Society for Clinical Investigation, Inc.* · *0021-9738/83/05/1130/11 $1.00*
Volume 71 May 1983 1130–1140

diates the initial epoxidation of BP as well as further metabolic steps to yield a series of phenols, quinones, diols, and diol epoxides. A number of these products are potent carcinogens (1).

The primary function of vitamin K has been considered to be the mediation of the carboxylation of specific glutamyl residues in precursor proteins to form the functional calcium binding γ-carboxy-glutamyl sites of the active derivatives (2). This produces the physiologically active forms of the vitamin K-dependent coagulation factors as well as other calcium-binding proteins. Carboxylation of glutamic acid residues is coupled to the cycling of vitamin K through its 2,3 epoxide and reduced forms (3). The vitamin K-metabolizing enzymes are also microsomal and require O_2 and a reduced pyridine nucleotide (3). Vitamin K_3 (menadione) has been demonstrated in vitro to inhibit the MFO-mediated oxidative demethylation of aminopyrine (4). We previously demonstrated that in vivo vitamin K_3 modifies the toxic effects of rapeseed meal in chickens (5), a toxicity probably mediated by xenobiotics activated through the MFO system. These observations led us to postulate that vitamin K may have an important in vivo metabolic role as a regulator of the MFO system. This paper explores this idea with particular reference to the modification of BP metabolism in vitro and its tumorigenic effect in vivo in the presence of vitamin K_1, vitamin K_3, warfarin, or vitamin K deprivation.

METHODS

Microsomal metabolism of BP. The microsomal metabolism of BP was measured by the assay system for aryl hydrocarbon hydroxylase (benzo(a)pyrene monoxygenase) as described by De Pierre et al. (6). In this system the metabolism of BP is assayed by its conversion to more polar products by partition of the unreacted BP and its metabolic products between the hexane phase and the alkali-water-ethanol phase of the final extraction procedure.

Liver microsomes were prepared from male Sprague-Dawley rats by the methods of Ernster et al. (7). Where microsomes from induced animals were used the rats were injected intraperitoneally 48 and 24 h earlier with 40 mg/kg 3-methylcholanthrene (3-MC) suspended in corn oil. Fresh microsomes pooled from three livers were incubated with an NADPH-generating system in which a 1-ml reaction mixture contained 0.5 mg of the microsomal protein, 1 mM NADPH+, 6 mM isocitrate, 0.36 U isocitrate dehydrogenase, 5 mM $MgCl_2$, 5 μM $MnCl_2$, and 50mM Tris-HCl pH 7.5. To the reaction mixture the following test substances were added in concentrations from 1 to 500 μM in 20 μl of solvent or suspending medium: vitamin K_3 (Sigma Chemical Co., St. Louis, MO) in acetone, vitamin K_1 in Tween 80 (Abbott Laboratories, North Chicago, IL; vitamin K injectable), vitamin C (L-ascorbic acid, BDH Ltd., Poole, England) in Tris buffer, vitamin E (α-tocopherol, Sigma Chemical Co.), in acetone, butylated hydroxyanisole (BHA) in ethanol (Sigma Chemical Co.), butylated hydroxytolene (BHT) in ethanol (BDH Ltd). In each case the control reaction mixture contained an equivalent amount of the solvent. The tubes were incubated for 5 min at 37°C in a shaker bath and the reaction started by the addition of 80 nmol [^{3}H]BP (Amersham Corp., Arlington Heights, IL) in 25 μl of acetone. The specific activity of [^{3}H]BP was 12.5 μCi/μmol. Microsomal protein was determined by the method of Lowry et al. (8).

The reaction was stopped after 30 min by the addition of 1 ml 0.5 N NaOH in 80% ethanol. The sample was shaken in 3 ml of hexane and centrifuged at 700 *g*. A 25-μl aliquot of the organic phase and a 300-μl aliquot of the aqueous phase were each pipetted into 10 ml of Aquasol and counted. Results are expressed as nanomoles BP metabolized per minute per milligram protein. The reaction mixture was standardized to 0.5 mg of protein and a time of 30 min to give a suitable yield of products within the linear range of the assay.

High pressure liquid chromatography (HPLC). HPLC was used to separate BP metabolites formed in the presence and absence of vitamin K_3. The method is based on that of Selkirk et al. (9). Rat liver microsomes from induced and noninduced animals were prepared and incubated with an NADPH-generating system containing vitamin K_3 in acetone in concentrations of 10–400 μM. Control tubes contained the reaction mixture and solvent but no vitamin K_3. The tubes were incubated at 37°C for 5 min before starting the reaction by the addition of 80 nmol BP in 25 μl acetone. After a further 30 min at 37°C, the reaction was stopped by the addition of 1 ml cold acetone. Tube contents were transferred to 12-ml conical centrifuge tubes containing 2 ml ethyl acetate, extracted for 3 min and centrifuged at 700 *g* for 5 min. 2 ml of the upper phase from each of five tubes with identical contents were pooled and dried over 1.0 g anhydrous $MgSO_4$. After centrifugation at 700 *g* for 10 min 6 ml of the supernatant was removed and stored overnight at 4°C in the dark under vacuum. The extract was dried under nitrogen and the residue resuspended in 0.4 ml methanol. An aliquot of 20μl was injected into a Waters liquid chromatograph (Waters Instruments, Inc., Rochester, MN) equipped with a DuPont Zorbax ODS column (DuPont Instruments, Wilmington, DE), 4.6 mm × 25 cm. BP and its metabolites were eluted using a 60–100% methanol linear gradient over 60 min with a flow rate of 0.8 ml/min. The metabolic peaks were identified by cochromatography of eight metabolites kindly supplied by Dr. David G. Longfellow of the National Cancer Institute, Bethesda, MD.

BP metabolism in liver fragments. BP metabolism in whole liver cells was examined in a rat liver fragment system based on the technique of Chen (10). In this system a total of five rat liver fragments weighing 1–2 mg each were placed on a piece of siliconized lens paper floated onto 2.0 ml of medium 199 in plastic petri dishes (35 mm × 10 mm) and incubated at 37°C in an atmosphere of 5% CO_2. After 1 h vitamin K_3 in 20 μl of acetone was added to test dishes to a final concentration of 5–100 μM; control dishes received 20 μl of acetone. After a further preincubation of 15 min the reaction was started by the addition of 40 nmol of [^{3}H]BP (12.5 μCi/μmol) in 50 μl of acetone and was allowed to proceed for 3 h at 37°C. As it was determined that the production of BP metabolites increased to 3 h and then plateaued the reaction was stopped at that time. The reaction was stopped by transferring the rafts to empty petri dishes on ice and then blotted dry. The fragments of triplicate dishes were pooled and homogenized in 1.0 ml of 50 mM Tris-HCl buffer pH 7.5 then extracted with 3.0 ml of *n*-hexane for 5 min. After centrifugation at 900 *g* for 10 min, 0.5 ml aliquots of the aqueous phase and of the organic phase

were each added to 10 ml of Aquasol for liquid scintillation counting. All calculations were corrected against boiled tissue blanks and expressed as picomoles BP metabolized per milligram wet weight of liver.

Effect of vitamin K_3 on the mutagenicity of BP and aflatoxin B_1 in the Salmonella typhimurium assay system. The mutagenesis plate assay of Ames et al. (11) was used for BP using Salmonella strain TA100. A buffered mixture containing a rat liver homogenate (S-9) prepared from rats previously induced with Aroclor 1254 (Analabs, a unit of Foxboro Analytical, North Haven, CT) was used to mediate the metabolic conversion of the procarcinogen. In the plate assay the following were added in order to 2 ml of molten top agar at 45°C: 0.1 ml of overnight culture of TA100, 0.1 ml dimethyl sulfoxide (DMSO) containing 2.5 or 5 μg of BP, 0.1 ml distilled water containing 2.5–150 μg vitamin K_3 as menadione sodium bisulfite (Sigma Chemical Co.) and 0.5 ml S-9 mix. Following gentle mixing this was poured onto the minimal glucose agar plates. Control plates were prepared using equimolar concentrations of sodium metabisulfite in place of the menadione.

A liquid suspension modification was used to explore the effect of vitamin K_3 on the mutagenicity of aflatoxin B_1. An overnight culture of Salmonella TA 98 was used to inoculate an aliquot of nutrient broth. After 4 h at 37°C 1 ml of the log-phase bacteria was placed in a 5-ml plastic conical tube, centrifuged at 1,500 *g* for 5 min and then washed with phosphate-buffered saline. The cell pellet was resuspended in 1 ml of an incubation mixture that contained 0.5 ml of S-9 mix, 0.5 ml aflatoxin B_1 (0.025 or 0.1 μg) in 3% DMSO diluted with phosphate-buffered saline and 1–20 μg menadione sodium bisulfite in 0.5 ml distilled water. The suspension was incubated at 37°C for 20 min. A 0.1 ml aliquot of the suspension was added to 2 ml of molten top agar at 45°C, mixing by swirling and then poured onto the minimal glucose agar plates. The *Salmonella typhimurium* tester strains were generously supplied by Dr. Bruce N. Ames of the University of California at Berkeley.

BP tumor induction in mice. All studies were in female ICR/Ha mice aged 8 wk obtained from Sprague-Dawley, Madison, WI and maintained on a normal diet of Lab-Blox F6 (Allied Mills, Chicago) except for one group fed a vitamin K-deficient diet prepared according to the formula of Mameesh and Johnson (12). Mice were divided into groups of 24 animals. All mice were weighed and examined at weekly intervals for tumor. Mice with palpable tumor or ascites were followed to death, postmortem examination performed, and tissue taken for histological examination.

RESULTS

The effect of vitamin K_3 on the microsomal metabolism of BP (Fig. 1)

In liver microsomes from noninduced animals BP metabolism is significantly decreased ($P < 0.01$) by vitamin K_3 at 50 μM and largely abolished at a vitamin K_3 concentration of 100 μM. Induced microsomes, which metabolize over twice the amount of BP are significantly inhibited ($P < 0.05$) at a concentration of 100 μM vitamin K_3 and at 400 μM the inhibition is complete. Boiled microsomes produce no significant conversion of BP to its water-soluble metabolites.

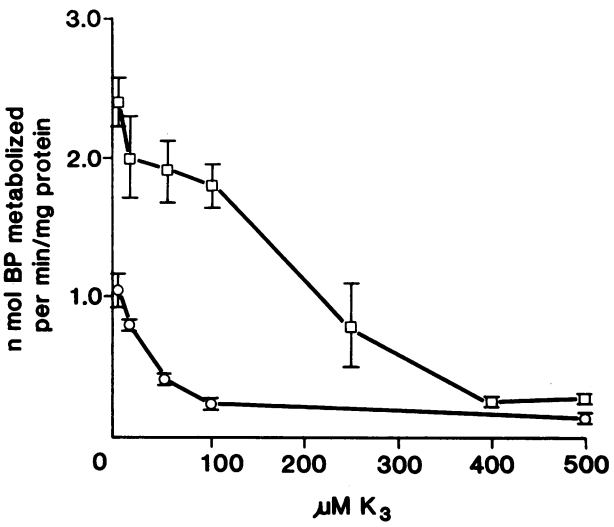

FIGURE 1 The microsomal metabolism of [^{3}H]BP to its epoxidation products in the presence of increasing amounts of vitamin K_3. Microsomal metabolism is expressed as nanomoles BP metabolized per minute per milligram microsomal protein. Microsomes were obtained from livers of rats induced with 3-MC (□) and from noninduced animals (○). Results are means±SE of six experiments.

HPLC patterns of in vitro BP metabolism in microsomes derived from the livers of noninduced and 3-MC-induced rat livers (Fig. 2)

In the noninduced microsomes vitamin K_3, at a concentration of 50 μM, markedly decreased the identified metabolites and at a concentration of 100 μM they essentially disappeared. A similar pattern was seen in the 3-MC-induced microsomes with ~50% reduction of these metabolites at a concentration of 200 μM vitamin K_3 and a further reduction at 400 μM. In both cases there was no selective depletion of one or more identifiable metabolites but all decreased proportionately.

The effects on BP metabolism in the rat liver microsomal system of reducing agents and antioxidants (Fig. 3)

These agents may be inhibitors of mutagenesis or carcinogenesis in various test systems. In this microsomal system vitamin C has no effect and the effects of vitamin E are minimal up to concentrations of 500 μM. Both vitamin K_3 and BHA have a significant effect ($P < 0.01$) at 50 μM. The maximum inhibitory effect on BP metabolism is produced by vitamin K_3 and is significantly greater than BHA ($P < 0.05$) at a concentration of 100 μM. Vitamin K_1 reduces BP metab-

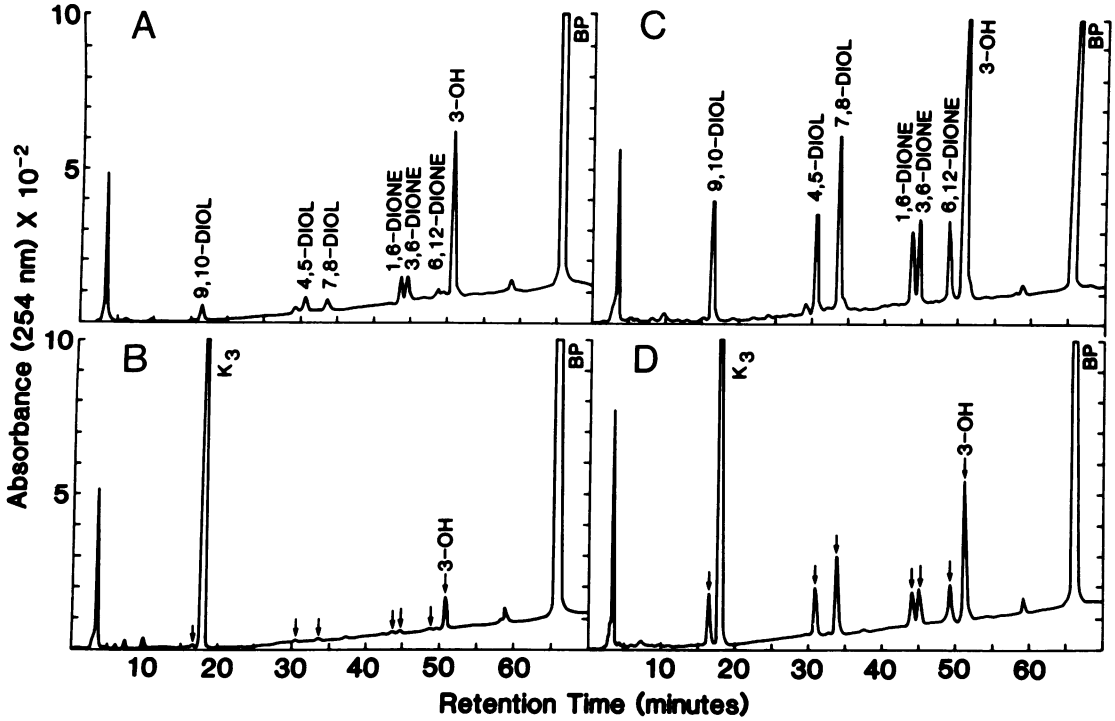

FIGURE 2 HPLC pattern of BP and its metabolites formed by microsomes obtained from noninduced rat liver (A) and from microsomes of rats induced with 3-MC (C). BP metabolism with noninduced microsomes in the presence of 50 μM vitamin K_3 (B) and in the induced microsomes in the presence of 200 μM vitamin K_3 (D). The initial peak at 1–2 min is acetone solvent.

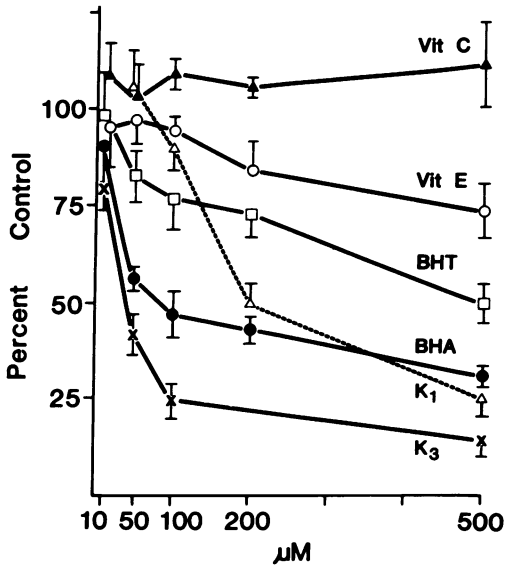

FIGURE 3 The effect of increasing concentrations of vitamins C, E, K_1, K_3, and of the antioxidants BHA and BHT on the microsomal metabolism of BP. The microsomes were obtained from the livers of noninduced rats. Values represent means±SE of three experiments.

olism to 50% of the control at a concentration of 200 μM ($P < 0.01$).

The ability of vitamin K_3 to inhibit BP metabolism in rat liver fragments (Fig. 4)

BP metabolism is significantly inhibited by vitamin K_3 at a concentration of 25 μM ($P < 0.02$) and maximally inhibited in this system at 50 μM of vitamin K_3. There are important differences from the microsomal system as the fragments are portions of liver, not subcellular fractions, and there is no external NADPH-generating system.

Vitamin K_3 reduced the mutagenic effects of BP and aflatoxin B_1 as measured by the Ames assay (Figs. 5 and 6)

The abolition of the mutagenic effect of 2.5 μg and 5.0 μg BP in the plate assay was obtained at a concentration of 150 μg of vitamin K_3 per plate. In the suspension assay abolition of the mutagenic effect of 0.025 μg aflatoxin B_1 was obtained at a vitamin K_3

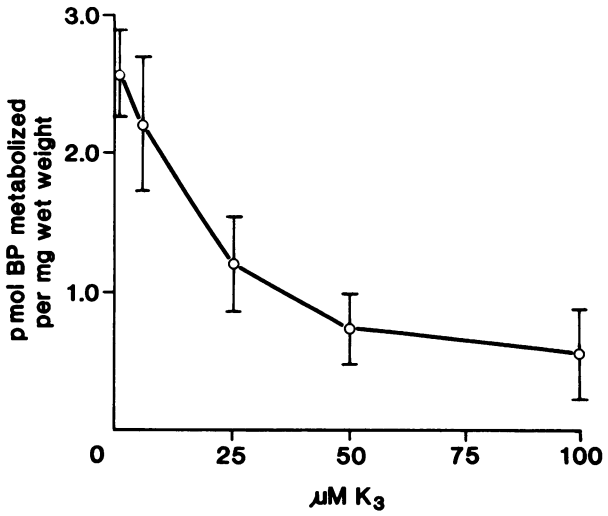

FIGURE 4 The metabolism of [^{3}H]BP in rat liver fragments in the presence of increasing amounts of vitamin K_3 expressed as picomoles of BP metabolized per milligram wet weight of tissue. The results are means±SE of four experiments.

concentration of 15 μg/plate and at 20 μg/plate for the higher concentration of 0.1 μg aflatoxin. No inhibition of bacterial growth of the parent organism nor its mutant was imposed by the menadione metabisulfite alone and sodium metabisulfite in equimolar concentrations did not inhibit mutagenesis.

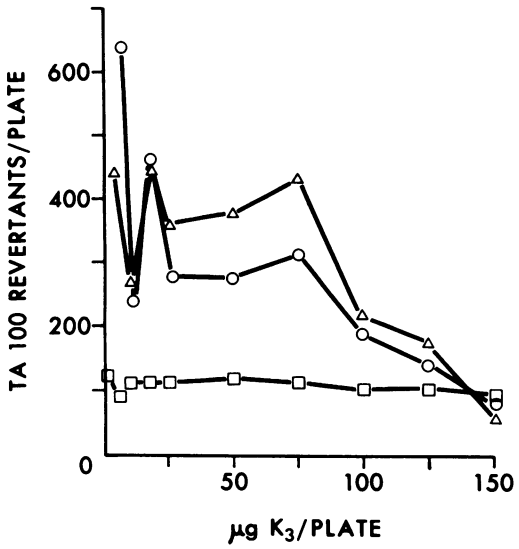

FIGURE 5 Salmonella TA 100 revertants per plate in the presence of increasing concentration of vitamin K_3. The inducing agent is BP 2.5 μg in DMSO (○); BP 5.0 μg in DMSO (△) and DMSO alone (□).

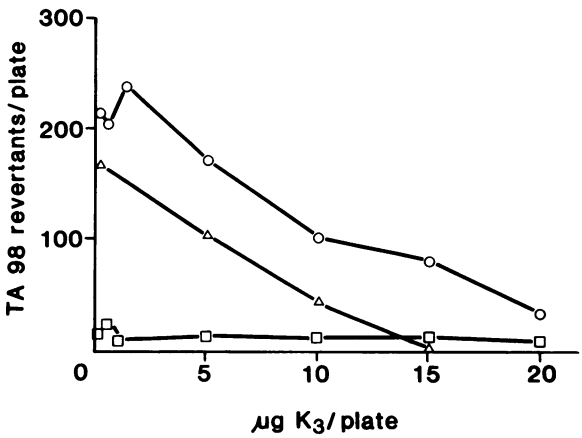

FIGURE 6 Salmonella TA 98 revertants per plate produced by aflatoxin B_1 0.025 μg/plate (△), and 0.1 μg/plate (○) in DMSO; and of DMSO alone (□) in the presence of increasing concentrations of vitamin K_3.

In vivo studies

Group I. Tumors began to appear in BP-injected animals at 75–80 d (Fig. 7). Analysis of the curves for the appearance of tumor by the method of Gehan (13) indicate that those mice that received vitamin K_3 and those on the vitamin K-deficient diet developed tumors at a significantly slower rate ($P < 0.01$) than the BP group. The warfarin-treated mice also developed tumors at a significantly slower rate ($P < 0.05$) than those receiving only BP. There is no difference between the warfarin, vitamin K-deficient, and vitamin K_3-supplemented groups. The tumor deaths in the warfarin, vitamin K-deficient, and vitamin K_3-supplemented groups occur at a significantly slower rate ($P < 0.01$ for all three groups) as compared with the BP control group. There is no difference between the three test groups. Tumors were pleomorphic small cell or fibrosarcomas with numerous giant cells and tended to occur in intraperitoneal, mediastinal, pulmonary, and soft tissue sites. No hepatomas were seen but perivascular invasion of liver by secondary tumor was present.

Group II. Tumors were evident in the BP, BP plus vitamin K_1, and BP plus warfarin groups at 75 d postinjection. The tumors increased rapidly in number and by 110 d mice began to die of tumor (Fig. 8). Analysis of the data on the time of appearance of tumors by the method of Gehan (13) over the entire curve shows a significant difference between the warfarin-BP group and the vitamin K_1-BP ($P < 0.01$) and the BP alone ($P < 0.03$) groups. There is no difference between the BP alone and the BP-vitamin K_1 groups. Analysis of the data for death with tumors over the

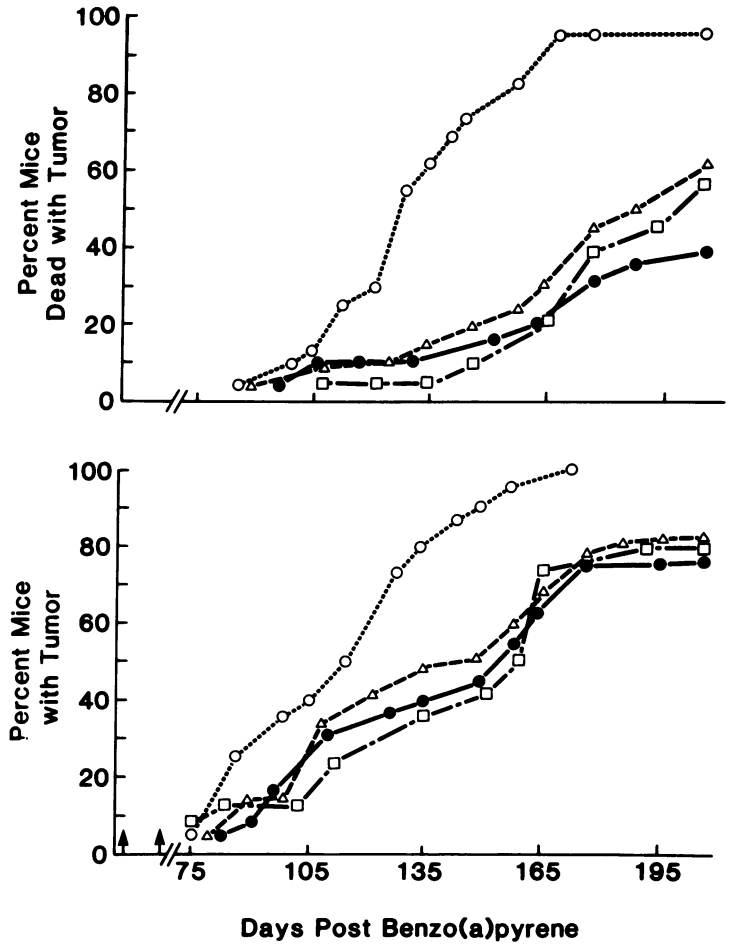

FIGURE 7 Group I. The appearane of tumor and deaths due to tumor in mice following the intraperitoneal injection of BP 3.0 mg/mouse in 0.2 ml of sesame seed oil on days 1 and 14. Time is in days following the first injection. The groups represent BP alone (○); vitamin K_3 as menadione bisulfite 5 mg/kg in 0.1 ml of saline i.p. 3 h before and 24 h after each BP injection (●); warfarin sodium (Coumadin, Endo Laboratories, Inc., Garden City, NY) 10 mg/kg in 0.1 ml of saline i.p. 24 h and again 1 h before each BP injection (△); animals maintained on a vitamin K-deficient diet for 2 wk before the first BP injection until 2 wk following the second (a total of 6 wk) (□).

entire curve shows a significant difference between BP alone, the BP-vitamin K_1 ($P < 0.02$), and BP-warfarin ($P < 0.05$) groups. The difference between BP-warfarin and BP-vitamin K_1 is significant at $P < 0.01$.

Group III. Tumors appeared more slowly in this lower dose BP experiment (Fig. 9). In the vitamin K_1-treated animals tumors appeared earlier and the rate of tumor death was greater than in the BP alone group. Analysis of the data over the entire time curves by the method of Gehan shows a significant difference in both time to tumor appearance ($P < 0.05$) and survival ($P < 0.05$).

DISCUSSION

Benzo(a)pyrene is metabolized by mammalian cells to its epoxides that undergo a series of subsequent reactions to form at least 40 metabolites including phenols, dihydrodiols, diol-epoxides, and conjugation products with glutathione, sulfate, and glucuronic acid. The reaction leading to the production of the more polar compounds is mediated by a microsomal NADPH-dependent monoxygenase system. This enzyme system, which may be assayed by the separation of the more polar products of BP metabolism (6) or

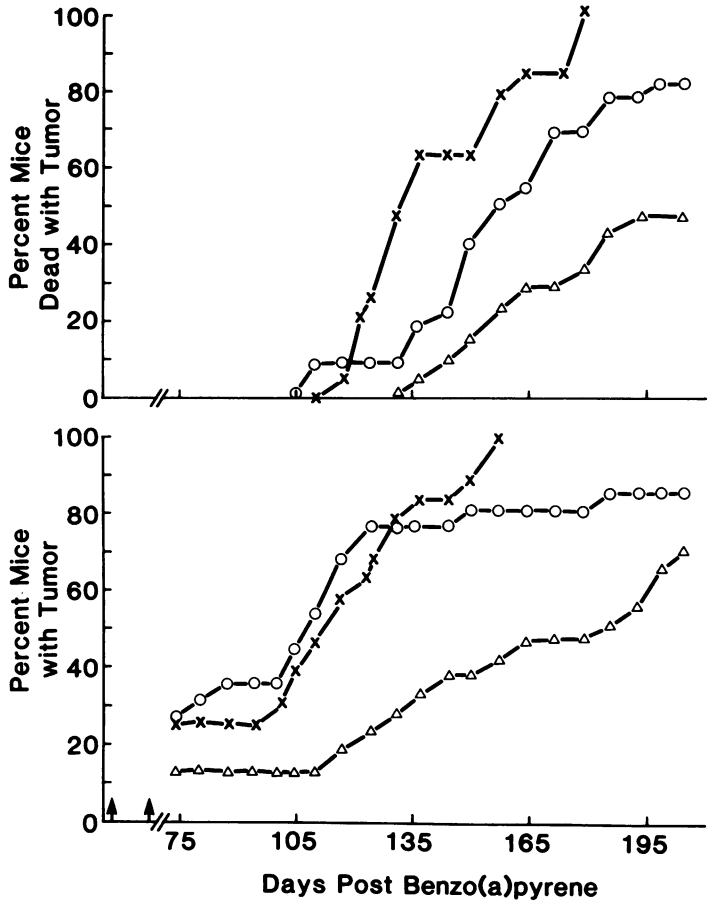

FIGURE 8 Group II. The appearance of tumor and time to tumor death following the i.p. injection of BP 3.0 mg/mouse in sesame seed oil on days 1 and 14. Time is in days following first BP injection. The groups are BP alone (O); vitamin K_1 5 mg/kg in 0.1 ml of sesame oil i.p. 3 h before each BP injection (×); warfarin sodium 10 mg/kg in 0.1 ml of saline 24 h before each BP injection (Δ). No tumors were observed in three other groups given only warfarin, vitamin K_1, or sesame seed oil.

by the formation of the highly fluorescent 3-OH BP (14), has been documented in a number of organs as well as in cells in culture. The absolute and relative quantities of the reaction products are dependent on the in vitro or in vivo conditions and the amount of substrate (15). Inhibition of the MFO system could decrease the first step in the conversion of BP to its epoxides and proportionately all subsequent products. Alternatively, as MFO mediates a series of subsequent metabolic steps the relative production of some metabolites would change should there be any selective MFO inhibition further down the chain.

Vitamin K_3 decreased BP metabolism in microsomes derived from the livers of both induced and noninduced rats (Fig. 1) and reduced the mutagenic products of BP generated by the microsomal S-9 mix in the Ames assay (Fig. 5). Similar results were obtained with aflatoxin B_1 (Fig. 6), which is also converted to its mutagenic epoxide through an NADPH-dependent MFO system (16). The relative effects of equivalent molar concentrations of some reducing agents and antioxidants that may modify mutagenic or carcinogenic effects are shown in Fig. 3. Under the conditions of this assay vitamin K_3 is the most effective inhibitor of BP metabolism. Vitamin K_1 is a relatively poor inhibitor at low concentration, which may relate in part to the permeability of the microsomal components of this system.

In isolated microsomes vitamin K_3 is probably functioning as an electron acceptor and competing for the

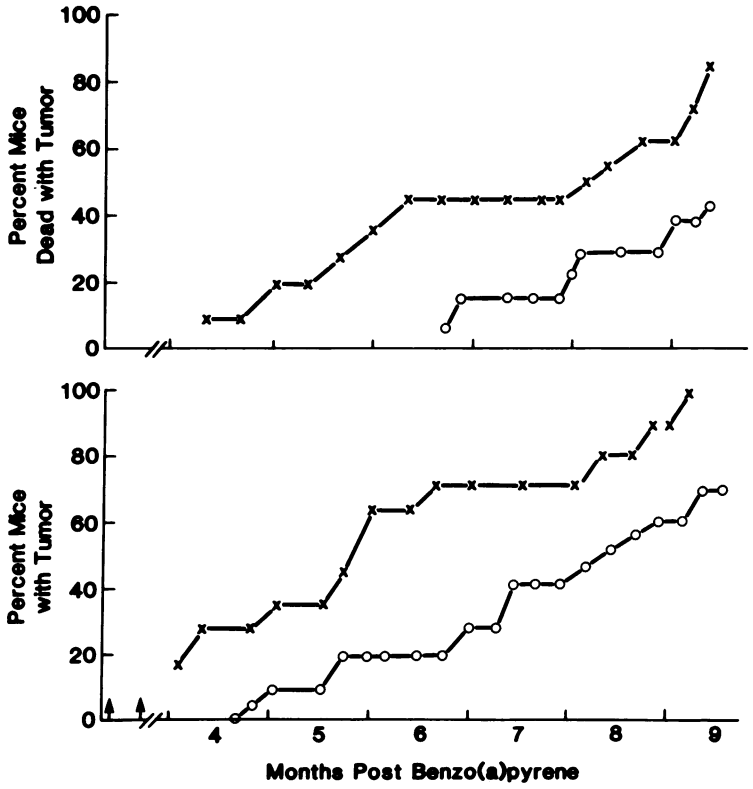

FIGURE 9 Group III. The appearance of tumors and the mortality of mice given a reduced dosage of BP (2.4 mg/mouse) on days 1 and 14. The time is in months following the first intraperitoneal injection of BP. Groups are BP alone (O); vitamin K_1 5 mg/kg in 0.1 sesame oil i.p. on each of 3 d preceding and for 3 d after each BP injection (×).

electron transfer from NADPH in the NADPH-dependent cytochrome p450 system. The HPLC data show that in vitro all the identified metabolic derivatives of BP were reduced equally suggesting that the primary in vitro effect is mediated at the initial epoxidation. Vitamin K_3 has been documented to be an inhibitor of in vitro microsomal oxidative demethylation of aminopyrine and of *p*-chloro-*N*-methylaniline by Wills (17) who suggested that vitamin K_3-mediated NADPH oxidation rendering it unavailable to the electron transport system required for the metabolic oxidation of competing substrates. He demonstrated that although vitamin K_3, vitamin E, and BHT all inhibit lipid peroxidation, only vitamin K_3 strongly inhibited the microsomal oxidative demethylation of aminopyrine in an in vitro system. Vitamin K_3 may alternatively or in addition interact with the sulphydral group on the NADPH reductase to inactivate the enzyme (18, 19). Other examples of vitamin K_3 blockade include the in vitro microsomal metabolism of cortisol (20) and the *N*-oxidation of NN-dimethylaniline (21). In the studies cited in which vitamin K_3 is inhibitory to the MFO system, either NADPH or an NADPH-generating system was included in the reaction mixture. This is similar to our microsomal preparations but not the liver fragment system in which the cells are intact and no external NADPH or generating system is included (Fig. 4).

In vivo vitamin K_3 delayed BP-induced tumorigenesis and tumor death (Fig. 7). The mechanism may be analogous to that observed in the in vitro metabolism of BP. There is indirect evidence that vitamin K_3 may affect metabolic processes in vivo including the hepatic MFO system in chickens (5) and glutathione reduction in human erythrocytes with resultant hemolysis and hyperbilirubinemia (22, 23). Both of these systems are NADPH dependent.

We also observed that vitamin K_1 was a much weaker inhibiter of the microsomal metabolism of BP in vitro and it accelerated BP tumorigenesis and death in vivo. Marked differences have been recognized between the fat-soluble and water-soluble K vitamins in

their metabolic activity that may relate to differences in penetration of and solubility in the microsomal membrane (24). There are also major differences in tissue distribution and clearance between vitamin K_1 and K_3. 24 h after the intravenous injection of vitamin K_3 in rats 20% was excreted in urine and stool with <1% of the injected dose residual in liver and 10% in skeletal muscle (25). In the same study 6% of the injected dose of vitamin K_1 was excreted in urine and stool with 20% remaining in liver and 7% in skeletal muscle 24 h postinjection (25). In man Bjornsson et al. (26) found the turnover time of vitamin K_1 to be ~153 min and estimated the total body pool to be only ~100 μg, which is the same as the hepatic pool calculated by Duello and Matschiner (27). This small hepatic storage pool may reflect a mechanism designed to protect other microsomal systems from high concentrations of natural reactive quinones. In view of the present studies in animals the effect of parenteral vitamin K_1 loading in man, particularly the newborn, requires further consideration.

The liver is the principal site of both BP and vitamin K metabolism. In the liver BP is oxidized to its more polar lipophobic products that may be conjugated for rapid excretion. When [^{3}H]BP is given to rats intravenously 50% is excreted in the bile within 6 h primarily as glutathione, glucuronide, and sulfate conjugates (28). The role of the liver may be even larger for intraperitoneal or orally administered BP with absorption into the portal system. Although the microsomal metabolism of BP as assayed by the activity of aryl hydrocarbon hydroxylase may be ubiquitous its activity in liver exceeds by one to two logs that in other tissues (29). The rate of microsomal metabolism of BP in hamster liver is some 100 to 200 times that in hamster lung, while human liver exceeds that of human lung or kidney by 20- to 50-fold (30).

Although the liver is a major organ of BP metabolism it is not the principal site of tumor formation. Oral administration of BP gives rise primarily to lung adenomas and tumors of the forestomach while subcutaneous or intraperitoneal administration produces primarily sarcomas at or near the site of injection. The mice in our studies developed ascites, intraperitoneal, mediastinal, and subcutaneous sarcomas. There were no hepatomas but perivascular invasion of the liver with secondary tumor was seen.

That in vivo the naturally occurring vitamin K_1 accelerates BP tumorigenesis and death while vitamin K depletion and warfarin are protective suggests that there may be an interrelationship between the MFO activation of BP and the vitamin K system. The primary function of vitamin K is a cofactor for the carboxylation of specific glutamate residues in certain precursor proteins including some coagulation factors. This microsomal system requires O_2, CO_2, and the reduced hydroquinone form of vitamin K. During the carboxylation reaction the hydroquinone is oxidized to its 2,3 epoxide, which is then recycled by an epoxide reductase to its quinone. Subsequent reduction of the vitamin K quinone to regenerate the hydroquinone requires a cytosolic NAD(P)H reductase (DT diaphorase, menadione reductase, EC 1.6.99.2) (31). This enzyme is induced by 3-MC, is dicoumarol sensitive, and may function as a general quinone reductase (32).

It is possible that the reductase phase of the vitamin K cycle might modulate the MFO system by competing for NAD(P)H and DT diaphorase. There is no information as to which metabolic steps of BP metabolism mediated by the MFO would be most vulnerable in vivo. If there is competition for NAD(P)H or one of its reductases the multiple sites of MFO activity and the recycling of the metabolites makes the in vivo situation complex. Changes in the ratio of MFO to epoxide hydratase activity may also significantly alter the metabolic products (1). Following the initial epoxidation of BP, subsequent MFO activity mediates the conversion to phenols, quinones, diols, and diolepoxides. In addition the major DNA binding diol-epoxides I and II are converted to inactive triol metabolites by an NADPH-dependent, MFO-independent step and competition for NADPH at this site may be very significant in BP metabolite detoxification and excretion (33).

The interest in anticoagulants as antitumor agents has revolved around the use of heparin, vitamin K antagonists, and defibrinating venoms as antimetastatic agents. The experimental systems have been primarily assays of tumor growth or metastases following tumor transplants or the intravascular infusion of tumor cells with evidence to suggest that tumor seeding is in part dependent on the formation of a fibrin bed for seeded tumor cells. In the present experiments the effects of warfarin and vitamin K deficiency were not mediated by inhibition of the coagulation mechanism. Warfarin was given at the time of BP injection and many weeks before the appearance of tumor. The vitamin K-deficient animals were restored to a normal diet 2 mo before tumors could be identified. Similarly a reduction in 7,12-dimethylbenz(a)anthracene-induced mammary tumors in rats pretreated with coumarin was also reported by Feuer et al. (34).

The protective effects of vitamin K deficiency and of warfarin on BP-induced tumor formation may be mediated by decreased competition of the vitamin K cycle for NAD(P)H or its reductase allowing more rapid and complete BP metabolism by the MFO system. In the liver this would allow rapid excretion of

the conjugated products. However, it cannot be assumed that vitamin K deficiency and warfarin blockade of the vitamin K cycle produce identical effects on BP metabolism. Lind et al. (35) demonstrated that the dicoumarol-sensitive DT diaphorase also mediates the glucuronidation of 3-OH BP. Thus, dicoumarol or warfarin through its effect on this reductase inhibits both the reduction of vitamin K and the probable rearrangement of 3-OH BP before glucuronidation.

These studies establish a role for the K series of vitamins in the metabolism of BP. It is apparent that vitamin K_1, vitamin K depletion, and warfarin on the one hand and vitamin K_3 on the other probably act primarily at different metabolic sites in BP activation and detoxification. Vitamin K_1 and K_3 are not interchangeable in this context. If it is possible to modify the carcinogenic effect of some procarcinogens or the metabolism of other xenobiotics by vitamins K_1 and K_3 it is important to more fully explore this aspect of vitamin K function.

ACKNOWLEDGMENTS

Statistical analysis of the data was done by Dr. Norma Nelson, Department of Epidemiology and Statistics.

This work was supported by a grant from the National Cancer Institute of Canada. Equipment was purchased by grants from the Kiwanis Club of Winnipeg and the Marnie McDiarmid Foundation.

REFERENCES

1. Gelboin, H. V. 1980. Benzo(a)pyrene metabolism, activation, and carcinogenesis: Role and regulation of mixed-function oxidases and related enzymes. *Physiol. Rev.* **60:** 1107–1166.
2. Esmon, C. T., and J. W. Suttie. 1976. Vitamin K-dependent carboxylase: solubilization and properties. *J. Biol. Chem.* **251:** 6238–6243.
3. Sadowski, J. A., C. T. Esmon, and J. W. Suttie. 1976. Vitamin K-dependent carboxylase: requirements of the rat liver microsomal enzyme system. *J. Biol. Chem.* **251:** 2770–2776.
4. Wills, E. D. 1972. Effects of vitamin K and naphthoquinones on lipid peroxide formation and oxidative demethylation by liver microsomes. *Biochem. Pharmacol.* **21:** 1879–1883.
5. Israels, E. D., A. Papas, L. D. Campbell, and L. G. Israels. 1979. Prevention by menadione of the hepatotoxic effects in chickens fed rapeseed meal. Observations on coagulation factors and cytochrome p450. *Gastroenterology.* **76:** 584–589.
6. De Pierre, J. W., M. S. Moron, K. A. M. Johannesen, and L. Ernster. 1975. A reliable, sensitive, and convenient radioactive assay for benzpyrene monoxygenase. *Anal. Biochem.* **63:** 470–484.
7. Ernster, L., P. Siekwitz, and G. E. Palade. 1962. Enzyme-structure relationships in the endoplasmic reticulum of rat liver. A morphological and biochemical study. *J. Cell Biol.* **15:** 541–562.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
9. Selkirk, J. K., R. G. Croy, and H. V. Gelboin. 1974. Benzo(a)pyrene metabolites: efficient and rapid separation by high-pressure liquid chromatography. *Science (Wash. DC).* **184:** 169–171.
10. Chen, J. M. 1954. The cultivation in fluid medium of organized liver, pancreas, and other tissues of foetal rats. *Exp. Cell Res.* **7:** 518–529.
11. Ames, B. N., J. McCann, and E. Yamasaki. 1975. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. *Mutation Res.* **31:** 347–364.
12. Mameesh, M. S., and B. C. Johnson. 1959. Production of dietary vitamin K deficiency in the rat. *Proc. Soc. Exp. Biol. Med.* **101:** 467–468.
13. Gehan, E. A. 1965. A generalized two sample Wilcoxon test for doubly censored data. *Biometrika.* **52:** 650–653.
14. Nebert, D. W., and H. V. Gelboin. 1969. The in vivo and in vitro induction of aryl hydrocarbon hydroxylase in mammalian cells of different species, tissues, strains, and developmental and hormonal states. *Arch. Biochem. Biophys.* **134:** 76–89.
15. Holder, G. M., H. Yagi, D. M. Jerina, W. Levin, A. Y. H. Lu, and A. H. Conney. 1975. Metabolism of benzo(a)pyrene. Effect of substrate concentration and 3-methylcholanthrene pretreatment on hepatic metabolism by microsomes from rats and mice. *Arch. Biochem. Biophys.* **170:** 557–566.
16. Lin, J. K., K. A. Kennan, E. D. Miller, and J. A. Miller. 1978. Reduced nicotinamide adenine dinucleotide phosphate-dependent formation of 2,3 dihydro-2,3-dihydroxyaflatoxin B_1 from aflatoxin B_1 by hepatic microsomes. *Cancer Res.* **38:** 2424–2428.
17. Wills, E. D. 1969. Lipid peroxide formation in microsomes. *Biochem. J.* **113:** 315–324.
18. Green, A. P., M. Hooper, and A. J. Sweetman. 1974. The interaction of 2-phenylisatogen and menadione with rat liver mitochondrial NADH dehydrogenase. *Biochem. Pharmacol.* **23:** 1569–1576.
19. Young, J. M. 1971. Comparison of the effects of menadione and 2,3-dimethylnaphthoquinone on the energy-coupling reactions of beef heart mitochondria. *Biochem. Pharmacol.* **20:** 163–171.
20. Kupfer, D., and L. Peets. 1968. Inhibition of cortisol metabolism in vitro by 2-methylnaphthoquinone (menadione): a possible interpretation of the potentiation of cortisol activity in vivo. *Experientia (Basel).* **24:** 893–894.
21. Hlavica, P., and M. Kehl. 1977. Studies on the mechanism of hepatic microsomal N-oxide formation. The role of cytochrome p450 and mixed-function amine oxidase in the N-oxidation of NN-dimethylaniline. *Biochem. J.* **164:** 487–496.
22. Zinkham, W. H. 1959. An in vitro abnormality of glutathione metabolism in erythrocytes from normal newborns: mechanism and clinical significance. *Pediatrics.* **23:** 18–32.
23. Asteriadou-Samartzis, E., and S. Leikin. 1958. The relation of vitamin K to hyperbilirubinemia. *Pediatrics.* **21:** 397–402.
24. Yen, C. S., and D. O. Mack. 1980. Solubilized rat liver vitamin K carboxylase demonstrates little selectivity between vitamin K_1 and the menaquinones. *Proc. Soc. Exp. Biol. Med.* **165:** 306–308.

25. Taylor, J. D., G. J. Millar, and R. J. Wood. 1957. A comparison of the concentration of C^{14} in the tissues of pregnant and non-pregnant female rats following the intravenous administration of vitamin K_1-C^{14} and vitamin K_3-C^{14}. *Can. J. Biochem. Physiol.* **35:** 691–697.
26. Bjornsson, T. D., P. J. Meffin, S. E. Swezey, and T. F. Blaschke. 1980. Disposition and turnover of vitamin K_1 in man. *In* Vitamin K Metabolism and Vitamin K-Dependent Proteins. J. W. Suttie, editor. University Park Press, Baltimore. 328–332.
27. Duello, T. J., and J. T. Matschiner. 1972. Characterization of vitamin K from human liver. *J. Nutr.* **102:** 331–336.
28. Boroujerdi, M., H. Kung, A. Wilson, and M. W. Anderson. 1981. Metabolism and DNA binding of benzo(a)pyrene in vivo in the rat. *Cancer Res.* **41:** 951–957.
29. Bridges, J. W. 1980. The role of the drug-metabolizing enzymes. *In* Environmental Chemicals, Enzyme Function, and Human Disease. Ciba Foundation Symposium 76. Excerpta Medica, Amsterdam. 5–17.
30. Prough, R. A., V. W. Patrizi, R. T. Okita, B. S. S. Masters, and S. W. Jakobsson. 1979. Characteristics of benzo(a)pyrene metabolism by kidney, liver, and lung microsomal fractions from rodents and humans. *Cancer Res.* **39:** 1199–1206.
31. Wallin, R., and J. W. Suttie. 1981. Vitamin K-dependent carboxylation and vitamin K epoxidation. Evidence that the warfarin-sensitive microsomal NAD(P)H dehydrogenase reduces vitamin K_1 in these reactions. *Biochem. J.* **194:** 983–988.
32. Ernster, L., L. Danielson, and M. Ljunggren. 1962. DT diaphorase: I Purification from the soluble fraction of rat liver cytoplasm, and properties. *Biochim. Biophys. Acta.* **58:** 171–188.
33. Yang, S. K., and H. V. Gelboin. 1976. Nonenzymatic reduction of benzo(a)pyrene diol-epoxides to trihydroxypentahydrobenzo(a)pyrenes by reduced nicotinamide adenine dinucleotide phosphate. *Cancer Res.* **36:** 4185–4189.
34. Feuer, J., A. Kellen, and K. Kovacs. 1976. Suppression of 7,12-dimethylbenz(a)anthracene-induced breast carcinoma by coumarin in the rat. *Oncology.* **33:** 35–39.
35. Lind, C., H. Vadi, and L. Ernster. 1978. Metabolism of benzo(a)pyrene-3,6-quinone and 3-hydroxybenzo(a)pyrene in liver microsomes from 3-methylcholanthrene-treated rats. *Arch. Biochem. Biophys.* **190:** 97–108.