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VITAMIN K-DEPENDENT CARBOXYLASE: INHIBITORY ACTION OF POLYCHLORINATED PHENOLS

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Abstract—The compound 2,3,5,6-tetrachloropyridinol (TCP) is a known inhibitor of the rat liver vitamin K-dependent carboxylase. A series of chlorinated phenols was also assayed for their abilities to inhibit the carboxylase *in vitro*. One compound, 2,3,5,6-tetrachlorophenol, was as potent a carboxylase inhibitor as TCP (I_{50} = 5–10 μM). Four compounds with substituents in the 4 position exhibited I_{50} values 5–20 times greater than the identical structures with hydrogen in the 4 position. Tetrachloroanisol, the methyl ether of tetrachlorophenol, did not inhibit the reaction, and inhibition by 2,5-dichlorophenol, which has a pK_a of 7.2, was pH dependent, suggesting that the anionic form of the phenol is the inhibitor. No other direct structure/function correlations were evident. Previous reports have shown that TCP inhibition of the carboxylase is not competitive versus vitamin K *in vitro*, but that *in vivo* antagonism by TCP can be reversed with vitamin K. Rats given 40 mg/kg TCP had decreased plasma prothrombin levels and increased amounts of liver microsomal prothrombin precursors, whereas rats injected with 1 mg vitamin K 24 hr before the TCP injection had normal levels of both. Vitamin K administration could not overcome completely the effects of 100 mg/kg TCP. Animals injected with TCP had increased levels of vitamin K 2,3-epoxide in the liver, which would be consistent with a partial inhibition of the microsomal vitamin K-epoxide reductase by this anticoagulant.

The liver microsomal vitamin K-dependent carboxylase catalyzes the post-translational conversion of glutamyl to γ-carboxyglutamyl (Gla) residues in precursors of a limited number of proteins including the plasma clotting factors II (prothrombin), VII, IX, and X [1]. The enzyme, which requires a Glu-containing substrate, O_2, reduced vitamin K, and CO_2, also catalyzes the conversion of reduced vitamin K into vitamin K 2,3-epoxide [1]. This epoxide can be recycled to reduced vitamin K via dithiol-dependent microsomal epoxide reductase and quinone reductase activities, and the commonly used coumarin anticoagulants act by inhibiting these enzymes and preventing the recycling of vitamin K [1].

The 2-chloro analog of vitamin K is also a known anticoagulant [2], and the reduced form of the inhibitor has been shown to be competitive against the reduced vitamin site on the enzyme [3]. A second chlorinated compound, 2,3,5,6-tetrachloropyridinol (TCP), has been reported to cause hemorrhage and to lower plasma prothrombin levels in animals [4]. These effects can be reversed by the administration of either phylloquinone (K) [5] or phylloquinone epoxide (KO) [6]. Reversible inhibition of the rat liver microsomal carboxylase by TCP has been shown to be noncompetitive against the Glu site substrate, oxygen, sodium bicarbonate, or reduced vitamin K [7]. Thus, it appears that vitamin K can reverse the anticoagulant effects of TCP *in vivo* but cannot overcome carboxylase inhibition *in vitro*. The data presented here further examine the *in vivo* relationship between TCP and vitamin K and correlate some structural aspects of TCP with its activity as a carboxylase inhibitor.

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METHODS

Chemicals. TCP was a gift from F. N. Marshall (Dow Chemical, Midland, MI). Other compounds were purchased as follows: chlorophenols and other inhibitors assayed, Aldrich (Milwaukee, WI); sodium[^{14}C]bicarbonate, Amersham (Chicago, IL); and Aquasol, New England Nuclear (Boston, MA).

Vitamin K-dependent carboxylase assays. Microsomes were prepared as previously described [8] from the livers of male 250–300 g rats (Holtzman, Madison, WI) that had been given 5 mg/kg warfarin intraperitoneally 12 hr before they were killed. The microsomal pellets were suspended in SIK buffer (0.25 M sucrose, 0.025 M imidazole, 0.5 M KCl, pH 7.2) containing 1.5% Triton X-100 and 1 mM dithiothreitol (DTT) so that 1 mL of suspension was equal to 0.5 g of liver. Incubations contained 0.6 mL of microsomal suspension, 1 mM Boc-Leu-Glu-Glu-OMe, and 30 μCi/mL $NaH^{14}CO_3$ in a total volume of 0.7 mL. Inhibitors were added as aqueous solutions with the pH adjusted to 7.2. Incubations were at 17°, and reactions were started by the addition of 40–80 μg of chemically reduced [9] phylloquinone in 10 μL ethanol. Aliquots of 200 μL were removed every 3 min up to 15 min and added to 0.5 mL of 10% trichloroacetic acid. After low speed centrifugation the supernatant fraction was removed, free $^{14}CO_2$ was removed by bubbling with CO_2, a 0.2-mL aliquot was mixed with 3.8 mL Aquasol, and radioactivity was determined in a liquid scintillation spectrometer. Inhibitory properties of various phenols were expressed as I_{50} values determined by plotting the rate of $^{14}CO_2$ incorporation versus inhibitor concentration for at least five concentrations of inhibitor, and graphically determining the inhibitor concentration corresponding to $\frac{1}{2}$ V_{max}.

Epoxide reductase assay. Unless otherwise noted, vitamin K epoxide reductase activity was assayed at 25° in 0.01 M 3-[tris(hydroxymethyl)-methylamino]-1-propanesulfonic acid (TAPS)/0.25 M sucrose/0.15 M KCl, pH 8.8 (TAPS buffer) containing 1 mM DTT, 40 μM vitamin K epoxide in 1% Emulgen 911, and 0.25 mL liver microsomes solubilized in TAPS buffer containing 0.3% sodium cholate. The 0.5 mL assay mixtures were quenched after 5 min with 1.0 mL isopropanol/hexane (3/2, v/v). The hexane layer was removed, dried under nitrogen, and dissolved in 0.2 mL methanol. Vitamin K was quantitated by HPLC using a Waters (Milford, MA) model 712 WISP automatic injector, M-45 pump, 440 absorbance detector, and 730 data processor. Separation was achieved on a Waters C18 μBondapak 10 μM analytical column run at 2 mL/min in 95% methanol/5% water. External standard quantitation was based on integrated absorbance at 254 nm. Extinction coefficients of 6,170 M^{-1} cm^{-1} at 259 nm for vitamin K epoxide and 18,900 M^{-1} cm^{-1} at 248 nm for vitamin K were used [10].

Liver KO/K levels. Male 150 g rats (Sprague–Dawley, Madison, WI) were fed Purina rat chow for 1 week prior to TCP injection and killed 24 hr after injection. Microsomal pellets were prepared and frozen at −70°. Microsomal pellets made from 2 g liver were thawed at 37° and homogenized in 2 mL of SI buffer (0.25 M sucrose, 0.025 M imidazole, pH 7.2) with a Dounce homogenizer. *trans*-[^{3}H]Phylloquinone (7000 dpm) was added, and the homogenate was mixed on a vortex mixer, allowed to stand for 1 hr at room temperature, then extracted and purified on a silica Sep-pak cartridge as previously described [11]. The Sep-pak eluant was evaporated under N_2 and redissolved in 250 μL hexane. The total sample was injected onto a Waters μPorasil P/N 27477 HPLC column and eluted with 25% CH_2Cl_2 in hexane with a flow rate of 1.5 mL/min. The eluant was monitored at 254 nm and the fraction containing *trans*-phylloquinone and phylloquinone epoxide, as determined by a previous standard injection, was collected and dried under N_2. This fraction was then dissolved in 250 μL methanol, and a portion was used for determination of ^{3}H to assess recovery in the extraction and semi-preparative steps.

To quantify phylloquinone and phylloquinone epoxide, 200 μL of the MeOH solution was injected onto a reverse-phase Zorbax ODS (DuPont, Wilmington, DE) analytical HPLC column (25 cm × 4.6 mm i.d.) and eluted with 15% dichloromethane/84.5% methanol/0.5% aqueous zinc solution (2 M zinc chloride, 2 M acetate buffer, pH 4.7) at a flow rate of 1 mL/min. Phylloquinone and phylloquinone epoxide were detected fluorometrically (Ex = 340 nm, Em = 430 nm) after reduction via a solid-phase post-column zinc reactor [12]. Differences between experimental groups were assessed by ANOVA [13].

Prothrombin assays. Male 125 g rats (Sprague–Dawley, Madison, WI) were fed Purina Rat Chow for 1 week and vitamin K was injected i.p. 24 hr before TCP. Plasma was collected by cardiac puncture and rats were killed 24 hr after the TCP injection. Liver microsomal pellets were prepared and frozen at −70°. Plasma prothrombin was measured using a chromogenic substrate as previously described [14] and expressed as a percentage of the concentration of a pool of normal rat plasma. Microsomes were assayed for prothrombin precursor by activation with *Echis carinatus* venom and measuring liberated thrombin [14].

RESULTS

The inhibitory properties of a number of other polychlorinated phenols were assessed and compared to TCP. The compound 2,3,5,6-tetrachlorophenol (Fig. 1) inhibited carboxylase activity at a concentration similar to that observed for TCP (Table 1). Four other chlorinated phenols differing from these strong inhibitors only by the presence of a chloro, or in one case a hydroxyl, group on position 4 had I_{50} values 5- to 20-fold greater than the corresponding compound with hydrogen in the 4 position. The methyl ether of tetrachlorophenol, 2,3,5,6-tetrachloroanisol (Fig. 1), did not inhibit carboxylation at a concentration of 500 μM; and of the nonchlorinated compounds tested, only 2,4-dinitrophenol caused inhibition at 1 mM.

The polychlorinated phenols exhibiting inhibitory activity have pK_a values in the region of the assay reaction pH, and their inhibitory properties are likely to be dependent on the degree of ionization. Inhibition of carboxylase activity by 800 μM 2,5-dichlorophenol ($pK_a = 7.2$) was measured in solubilized microsomes adjusted to pH 6.9 or 7.7. Carboxylase activity was dependent on pH, and activity at the lower pH was only about 30% of that at pH 7.7. As indicated in Fig. 2, at pH 6.9, 800 μM 2,5-dichlorophenol decreased activity to 54 ± 8% of the control, whereas at pH 7.7, only 14 + 2% of control activity remained.

To verify the report [7] that TCP is not competitive versus vitamin K, inhibition of the carboxylase by 7 μM TCP was measured at various concentrations of reduced vitamin K. The apparent K_m of reduced vitamin K is about 50 μg/mL, and when the activity was measured at four vitamin KH_2 concentrations ranging from 20 to 250 μg/mL, the inhibition caused by 7 μM TCP remained at 72 ± 4%.

The data in Table 2 verify that TCP is an effective anticoagulant *in vivo*. Plasma prothrombin concentrations in rats injected with TCP were markedly lower than those observed in controls. Prior (24 hr) injection of 1 mg vitamin K was sufficient to counteract this effect in rats given 40 mg/kg of TCP. However, rats given 1 mg vitamin K prior to 100 mg/kg TCP still had prothrombin levels that were lower than in controls. Administration of TCP also caused an increase in the level of liver prothrombin precursor, and this effect was still evident in rats administered vitamin K. Administration of 2 mg vitamin K did not overcome the effects of 100 mg/kg of TCP on plasma prothrombin or liver prothrombin precursor (data not shown). In contrast to the effects of TCP, rats given 50 mg/kg of the equally effective *in vitro* inhibitor, 2,3,5,6-tetrachlorophenol, showed no change in plasma prothrombin levels (data not shown).

The ability of vitamin K to at least partially counteract the effects of TCP *in vivo* would be

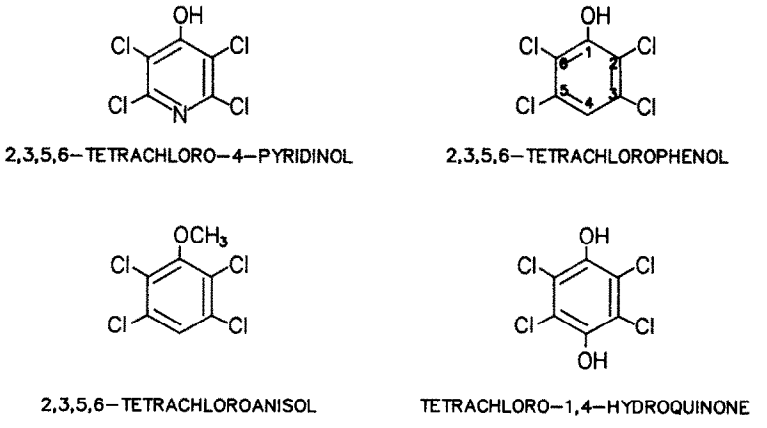

Fig. 1. Structures of some of the compounds studied.

Table 1. Inhibition of the vitamin K-dependent carboxylase by polychlorinated phenols

Compound	I_{50} (μM)
2,3,5,6-Tetrachloropyridinol	5–10
2,3,5,6-Tetrachlorophenol	5–10
2,3,5-Trichlorophenol	20
2,3,5,6-Tetrachloro-1,4-hydroquinone	50
2,3,4,5,6-Pentachlorophenol	190
2,3,4,5-Tetrachlorophenol	100
2,3,6-Trichlorophenol	350
2,6-Dichlorophenol	400
2,5-Dichlorophenol	750
3,5-Dichlorophenol	1000
2,3-Dichlorophenol	1500
2-Chlorophenol	4000
2-Nitrophenol	2000
2,4-Dinitrophenol	1000

The enzyme was assayed at various concentrations of the potential inhibitors and I_{50} values were determined as described in Methods. 2,4,6-Trichlorophenol, 2,3,5,6-tetrachloroanisol, 3-nitrophenol, 4-nitrophenol, benzoic acid, 4-hydroxypyridine, and 2,3,5-trimethylphenol were assayed and found to cause no significant inhibition at 1000 μM.

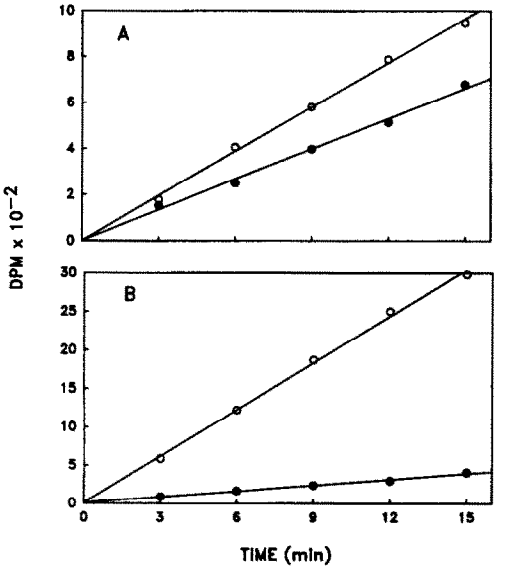

Fig. 2. Effect of pH on inhibition of the vitamin K-dependent carboxylase by 2,5-dichlorophenol. Incorporation of radioactivity into tripeptide is shown in the presence (●—●) or absence (○—○) of 800 μM 2,5-dichlorophenol. (A) pH 6.9; (B) pH 7.7. The data presented are from a single experiment, representative of three separate experiments.

consistent with an effect of the inhibitor on the vitamin K-epoxide reductase. This enzyme is inhibited by warfarin and other 4-hydroxycoumarins, and the *in vivo* anticoagulant effects are readily reversed by vitamin K. It has been reported [7] that 800 μM TCP inhibits the rat liver microsomal vitamin K epoxide reductase system by 55%. In this study, the enzyme was found to be much more sensitive, and TCP inhibited the epoxide reductase with an $I_{50} = 34 \pm 1.4$ μM ($N = 2$). The reaction rate in the presence and absence of inhibitor was linear over the standard assay period, and inhibition by TCP was not competitive versus phylloquinone epoxide. The potent carboxylase inhibitor, 2,3,5,6-tetrachlorophenol, was a poor inhibitor of the epoxide reductase with an $I_{50} > 500$ μM.

If the *in vivo* effects of TCP were due to inhibition of the epoxide reductase and not the carboxylase, liver vitamin K epoxide levels should increase with TCP treatment. Phylloquinone and phylloquinone epoxide levels were measured in liver microsomal fractions from rats injected with 100 mg/kg TCP, and the ratio of the two forms of the vitamin was determined (Fig. 3). Rats receiving TCP had KO/K ratios that were slightly greater ($P < 0.01$) than normal. However, the increase in microsomal KO was not as great as that seen in rats injected with warfarin.

DISCUSSION

These data have demonstrated that the previously reported inhibition of the vitamin K-dependent

Table 2. Effect of tetrachloropyridinol and vitamin K on plasma prothrombin concentration and liver prothrombin

Treatment	Plasma prothrombin (% of control)		Prothrombin precursor (μg/g liver)	
	No K	1 mg K	No K	1 mg K
No TCP	97 ± 7	97	7.1 ± 0.6	6.2 ± 0.8
TCP (40 mg/kg)	47 ± 7	97 ± 19	12.8 ± 1.0	8.2 ± 1.2
TCP (100 mg/kg)	8 ± 1	31 ± 5	13.2 ± 1.1	13.7 ± 0.9

Values are means ± SEM for 3–5 rats/group. Vitamin K, when injected, was given 24 hr prior to TCP. Plasma was obtained and animals were killed for assay of liver prothrombin precursor 24 hr after TCP administration. Plasma prothrombin is expressed as a percentage of the prothrombin concentration of a normal rat plasma pool.

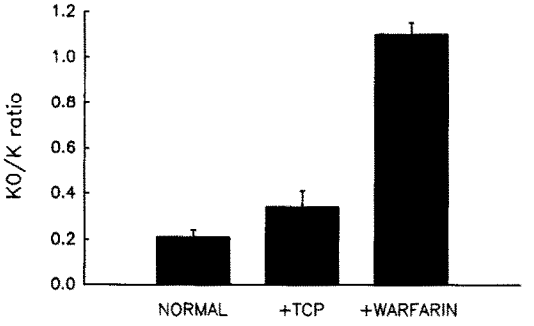

Fig. 3. Effects of TCP and warfarin on liver vitamin K epoxide concentration. Rats were injected with 100 mg/kg TCP or 5 mg/kg warfarin, and the ratios of vitamin K-2,3-epoxide (KO) to vitamin K in liver microsomes were determined 24 hr later as described in Methods. Values are means ± SD, N = 4.

carboxylase by tetrachloropyridinol extends to a large number of other polychlorinated phenols. Of the chlorinated phenols assayed as carboxylase inhibitors, 2,3,5,6-tetrachlorophenol was the most potent. A comparison of the I_{50} of 2,3,5,6-tetrachlorophenol to those of 2,3,4,5,6-pentachlorophenol and 2,3,5,6-tetrachloro-1,4-hydroquinone suggests that the presence of a substituent in the 4 position interferes with inhibition. This is supported by comparisons between 2,3,5-trichlorophenol and 2,3,4,5-tetrachlorophenol, and between 2,6-dichlorophenol and 2,4,6-trichlorophenol. In each case the compound with a substituent at position 4 has a higher I_{50} than the corresponding compound with no substituent in the 4 position. These data have also confirmed the previous [7] observation that the inhibition of the carboxylase by TCP is not competitive versus the reduced vitamin.

The data obtained are consistent with enzyme inhibition by the anionic form of these phenolic compounds. At a fixed concentration of 2,5-dichlorophenol, which has a pK_a of 7.2, several times more carboxylase activity remained at pH 6.9 than at pH 7.7. Further support of inhibition by the anionic form of the phenol is the failure of tetrachloroanisol, which is the methyl ether of tetrachlorophenol, to inhibit carboxylase activity.

Results of the *in vivo* administration of TCP were consistent with those previously reported by Marshall [5]. Administration of TCP caused a decrease in plasma prothrombin concentration and the expected increase in liver prothrombin precursor levels in rats. Prior injection of 1 mg vitamin K to these vitamin K sufficient rats prevented the anticoagulant effect in rats administered 40 mg/kg of TCP. However, in rats administered 100 mg/kg TCP, prior administration of either 1 or 2 mg vitamin K could not prevent the anticoagulant effect of TCP nor did it prevent TCP from elevating microsomal precursor levels. These data suggest that the inhibition is to some extent overcome by increasing tissue vitamin concentration, but that this effect is limited.

Chlorophenols have been used extensively as pesticides, and pentachlorophenol is a fungicide commonly used to treat wood products. It has been known for over 30 years that pentachlorophenol [15] and di- and trichlorophenol [16] are potent uncouplers of mitochondrial oxidative phosphorylation. A number of chlorophenols have been tested and found to also inhibit a microsomal mixed-function oxidase activity [17, 18] involved in drug metabolism by inhibiting a P450-dependent oxygenation step. Since enzymatic oxygenation of reduced vitamin K appears to be a necessary part of the carboxylase mechanism [1], it may be that TCP inhibits the carboxylation by interfering with the reaction between vitamin K and oxygen.

One possible explanation of the ability of vitamin K to partially overcome the anticoagulant effects of TCP is that the *in vivo* effects of TCP involve inhibition of the vitamin K epoxide reductase rather than the carboxylase. In support of this explanation, TCP was shown to inhibit the epoxide reductase *in vitro* at lower concentrations than previous studies [7] had indicated. Furthermore, tetrachlorophenol is as potent a carboxylase inhibitor as TCP but has little effect on the *in vitro* epoxide reductase activity. Administration of tetrachlorophenol was also found to have little effect on plasma prothrombin levels. Finally, the slightly elevated liver KO/K ratio in TCP-injected rats suggests that these rats did have decreased epoxide reductase activity. The increase in the KO/K ratio, however, was small relative to that seen in warfarin-injected rats. A previous report [6] has indicated that vitamin K epoxide is as effective

as vitamin K in reversing the *in vivo* effects of TCP. Although the epoxide administration will not reverse the effect of warfarin anticoagulation, it is likely that the weak inhibition of the epoxide reductase evidenced by TCP allows sufficient conversion of the epoxide to the active form of the vitamin to overcome the partial defect in carboxylation rate.

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