2,3,5,6-Tetrachloro-4-pyridinol: A New Chemical Structure for Anticoagulant Activity (36114)

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Presently, the orally effective anticoagulant agents can be classified according to chemical structure as the coumarin or indanedione type. Their anticoagulant activity is characterized by a slow onset of action and includes a reduction in the activity of prothrombin, as well as in factors VII, IX, and X. This report describes the anticoagulant activity of the sodium salt of 2,3,5,6-tetrachloro-4-pyridinol (TCP) which constitutes a new structure for this pharmacologic action. In addition, the acute toxicity of the compound, as well as the activity of eight structurally related compounds, was determined.

Materials and Methods. Forty male and 40 female ICR strain mice (Harlan Industries, Cumberland, IN) were used in the determination of the acute intragastric toxicity of TCP. Four groups of 10 male and 4 groups of 10 female mice each received different doses of TCP dissolved in distilled water. Mice were observed at 72 hr post drug for death. This observation period was determined to be appropriate on the basis of previous pilot experiments. The computations for fitting the probit-log dose regression lines determining the LD_{50} and 95% confidence intervals were done according to the method of Litchfield and Wilcoxon (1).

Eighty male, Sprague-Dawley strain rats (Sprague-Dawley Farms, Madison, WI) were used in the investigation of the dose–response–duration relationship for TCP and prothrombin time. All animals had free access to a standard laboratory rodent diet and water throughout the experiment. TCP was dissolved in distilled water and administered intragastrically in doses of 76.6, 60.2, and 43.8 mg/kg. This was equivalent to 70, 55, and 40 mg/kg respectively, of the free acid form of the compound. Each of the doses was administered to four separate groups of rats at different times such that 1-stage prothrombin times could be determined at the conclusion of the experiment, on the same day. An additional group of rats served as controls. In this manner, the mean prothrombin times for the groups reflected the activity of TCP at 24, 48, 96, and 144 hr after administration of compound. All groups contained 6 rats each with the exception of the control group and 76.6 mg/kg-144 hr post drug group contained 7 rats each. Two rats died in the latter group at 3 and 4 days post drug.

Blood was obtained by cardiac puncture after the rats were lightly anesthetized with ether. Platelet-poor plasma was prepared by centrifugation after mixing blood with a 10% volume of 0.1 *M* oxalate solution. Twenty five percent solutions of plasma were prepared for prothrombin time determinations with 0.85% sodium chloride solution and kept at 4° until used. One-stage prothrombin time determinations were performed in duplicate using a Mecrolab clot timer. Two tenths milliliter of Simplastin suspension (General Diagnostics) was placed in the cup and 0.1 ml of diluted plasma was placed on the rotor. One animal in the 76.6 mg/kg-48 hr post drug group showed a prothrombin time greater than 500 sec. This datum was excluded from the statistical analysis. Since peak activity for TCP was observed at 48 hr post drug, regression analysis was performed using log dose of TCP and prothrombin activity at this time. The method used is outlined by Finney (2). Data from the control, 24, and 96 hr post-TCP groups were analyzed using the Tuckey test as outlined by Snedecor (3).

Twenty seven male, Wistar derived rats (Harlan Industries, Cumberland, Indiana)

were used to determine the prothrombin depressant activity of the eight structural analogs of TCP. Nine groups of 3 rats each were formed. Animals in each group received 150 mg/kg/day for 2 days of one of the analogs. Suspensions of the compounds were prepared using 0.5% Methocel HG (The Dow Chemical Company) solution and administered intragastrically. One group receiving an equivalent volume of Methocel solution served as a control. The procedure for determining prothrombin times was the same as that described previously except that the determinations were done manually. A standard curve was constructed from data obtained by determining prothrombin time on a number of dilutions of the pooled plasmas from animals in the control group. Percentage of control prothrombin activity was calculated for the groups receiving drugs, from the standard curve.

Results and Discussion. The intragastric LD_{50} of TCP for male mice at 72 hr post drug was found to be 155 mg/kg with a 95% confidence interval of 130–184 mg/kg. Similarly, the LD_{50} for female mice was found to be 190 mg/kg with a 95% confidence interval of 168–215 mg/kg.

The mean prothrombin time of the control group and of the groups dosed with TCP and tested at different times post drug, can be found in Fig. 1. A significant regression ($p =$

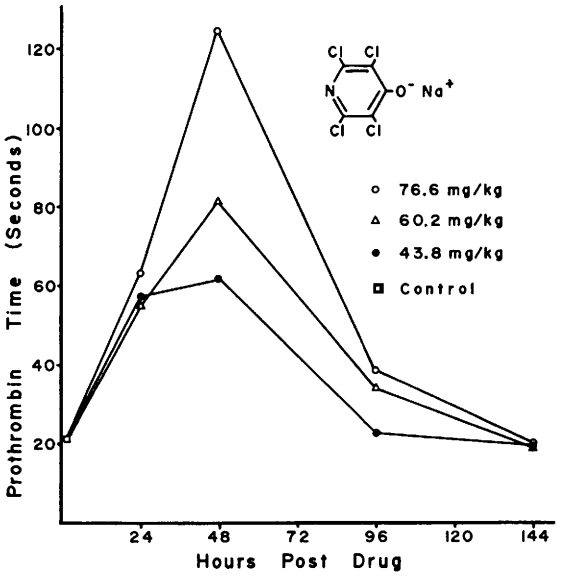


FIG. 1. Effect of different doses of TCP administered intragastrically to rats on 1-stage prothrombin time determined on a 25% dilution of plasma in 0.85% sodium chloride solution at different times post drug.

$<.01$) was found for prothrombin time at 48 hr post drug administration on log dose of TCP. This regression is illustrated in Fig. 2. As shown, at this time post drug, the 76.6 mg/kg group showed a 6-fold increase in prothrombin time. Similarly, the 60.2 mg/kg group showed a 4-fold increase and the 43.8 mg/kg group a 3-fold increase, approximate-

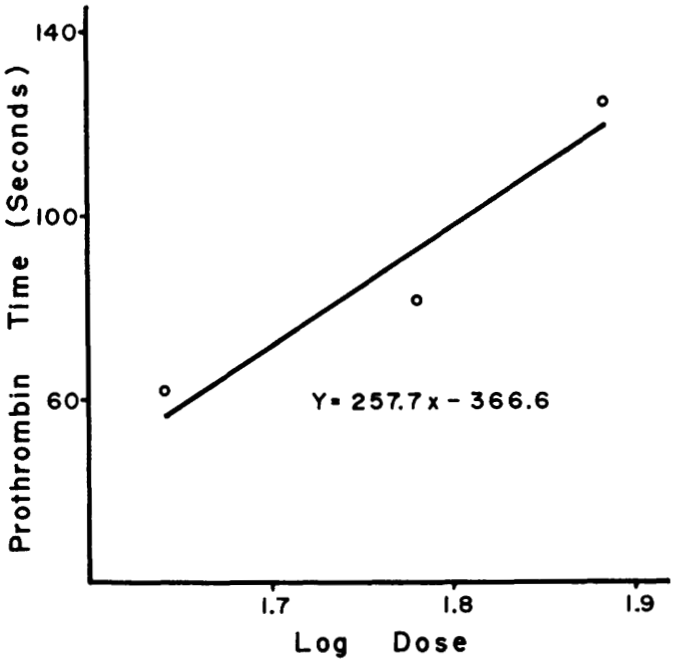
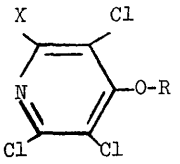


FIG. 2. Regression of 1-stage prothrombin time on log dose of TCP (significant at $p = 0.05$): Prothrombin time was determined for three groups of rats 48 hr following intragastric administration of TCP.

ly. No differences could be found among the mean responses of the 24 hr post-TCP administration groups using the Tuckey test but all three means of the groups were found to be different from control. The means of the groups approximated a 3-fold increase in prothrombin time when compared to control. At 96 hr post-TCP administration, the 76.6 mg/kg group was significantly different ($p = <.05$) from the control and from the 43.8 mg/kg group. At 144 hr post-TCP administration, no differences among the dosed groups and the control group could be found. Two rats from the 76.6 mg/kg-144 hr post-TCP group were found dead at 3 and 4 days. Intramuscular and gastrointestinal bleeding was found in these animals. In addition to these data, which show hypoprothrombinemic activity of TCP following intragastric administration, it has been previously found that the compound is also effective following intraperitoneal and intravenous administration to rats. Furthermore, repeated dosing of TCP at doses of 9 mg/kg/day or greater intragastrically for varying periods of time, has produced deaths in rats with evidence of severe hemorrhage.

Table I shows the structures of the analogs of TCP studied. The activity of the compounds are expressed as the mean percentage of control prothrombin activity ± standard deviation. All compounds were found to increase prothrombin time and are listed in the order of decreasing activity. A similar experiment performed previously at a dose of 50 mg/kg/day for 2 days with the same compounds showed no increases in prothrombin times compared to control. This suggests that none of the compounds are as potent as the parent compound, TCP. Compounds 1 and 2, which are the methyl and ethyl carbamic acid derivatives of TCP, were among the most active of the compounds tested. Compound 4 demonstrates that tetrachloro substitution on the pyridine ring is not essential for activity. Compounds 7 and 8, which are the benzenesulfonic and benzoic acid ester derivatives of TCP, were found to be the least active compounds. The activity of compounds 3, 4, and 6 would indicate that esterification of TCP with an aliphatic acid as

TABLE I. Hypoprothrombinemic Activity of Analogs of TCP.[a]



Compound Number	X	R	Mean % of Control Activity[b] ± Std. Deviation
1	Cl	$-\overset{O}{\overset{\|}{C}}-NH-CH_3$	15.0 ± 3.0
2	Cl	$-\overset{O}{\overset{\|}{C}}-NH-CH_2-CH_3$	20.3 ± 3.8
3	Cl	$-\overset{O}{\overset{\|}{C}}-C-Cl_2-CH_3$	22.7 ± 3.2
4	H	$-\overset{O}{\overset{\|}{C}}-CH_3$	23.3 ± 9.0
5	Cl	$-\overset{O}{\overset{\|}{C}}-S-C_6H_5$	35.7 ± 4.1
6	Cl	$-\overset{O}{\overset{\|}{C}}-(CH_2)_{16}-CH_3$	42.7 ± 4.2
7	Cl	$-SO_2-C_6H_5$	68.0 ± 29.9
8	Cl	$-\overset{O}{\overset{\|}{C}}-C_6H_5$	70.7 ± 44.8

[a] Each compound was administered intragastrically to groups of three rats each at a dose of 150 mg/kg/day for 2 days. One-stage prothrombin times were performed on day 3.

[b] One-stage prothrombin times were determined on a number of dilutions of the pooled plasmas of the control group and a standard curve was constructed. Prothrombin times were determined for animals receiving drugs using a 25% dilution of the plasma in 0.85% sodium chloride solution and converted to percentage of control prothrombin activity using the standard curve.

well as by an aromatic acid, does not destroy activity.

Summary. The LD_{50} and 95% confidence interval for the sodium salt of 2,3,5,6-tetrachloropyridinol (TCP) was found to be 155 (130–184) mg/kg at 72 hr following intragastric administration to male mice. Similarly, the LD_{50} and 95% confidence interval for female mice was found to be 190 (168–215) mg/kg.

The effect of three doses of intragastrically administered TCP was studied in rats. Statistical analysis of the data indicated that a significant increase in 1-stage prothrombin time could be found at 24 hr post drug administration. No difference in the magnitude of effect could be demonstrated for animals treated with 76.6, 60.2, and 43.8 mg/kg of TCP after this amount of time. The peak effect of the compound was found to occur at approximately 48 hr post drug when a significant regression of prothrombin time on log dose could be demonstrated. Evidence for lowered prothrombin activity could be found

at 96 hr but not at 144 hr post-TCP administration. The activity of eight structural analogs of TCP is discussed.

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2. Finney, D. J., "Statistical Method in Biological Assay." Hafner, New York (1952).

3. Snedecor, G. W., "Statistical Methods." Iowa State Univ. Press, Ames (1956).

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