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FURTHER STUDY OF THE MECHANISMS OF TOXIC ACTION OF TVOPA

FRANCIS W. WEIR
SANDRA EILTS
DENNIS C. MAYS
DALE H. STEVENS

DEPARTMENT OF PREVENTIVE MEDICINE
OHIO STATE UNIVERSITY
COLUMBUS, OHIO

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The experiments reported herein were conducted according to the "Guide for Laboratory Animal Facilities and Care," 1965 prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences—National Research Council.

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Francis W. Weir Dennis C. Mays
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13. ABSTRACT

This investigation is a continuation of the study and evaluation of toxic effects of the NF compound designated TVOPA emphasizing in vivo and in vitro degradation of the compound and its action at the cellular level. Study of the pharmacologic activity was made in rats, dogs, and cynamolgus monkeys. To some degree, effects were species specific. A very low rate of TVOPA hydrolysis in aqueous solutions was determined in earlier experiments. The assumption that degradation in biological media would not differ significantly from in vitro buffered media was incorrect. Using various blood fractions, our experiments account for the majority of fluoride and cyanide ion metabolites from TVOPA within two hours; however, ionic blood levels were not sufficiently elevated to explain all signs of toxicity. Studies of inhibition of cytochrome c oxidase activity further confirmed that cyanide ions contribute to TVOPA action, but KCN and NaF mixtures were not equal to TVOPA and procedures known to protect against cyanide toxicity were unsuccessful against TVOPA. This investigation supports our earlier contention that TVOPA or early organic degradation products have direct toxic action. Inorganic degradation products influence the delayed effects of TVOPA, but are not responsible for death at high doses.

KEY WORDS:

Toxicology	Rodents	Fluoride
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FOREWORD

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This technical report has been reviewed and is approved.

ANTHONY A. THOMAS, MD
Director
Toxic Hazards Division
Aerospace Medical Research Laboratory

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SECTION I

INTRODUCTION

The investigation described is a continuation of the study of toxic effects of the NF compound designated TVOPA. Previous studies indicated the mechanisms of toxicity of the compound were at least twofold. *In vitro* studies of the hydrolysis of TVOPA indicated that the compound was degraded to inorganic fluoride and cyanide (ref 1). Analyses of blood from dogs after administration of TVOPA confirmed the importance of these ions in toxic activity. However, a more recent study has shown that difluoramino groups on the molecule might be responsible for effects on the central nervous system (ref 2).

Our present study was concerned with further evaluation of the toxic action of TVOPA with emphasis placed on the *in vivo* and *in vitro* degradation of the compound and its action at the cellular level.

SECTION II

METHODS

QUANTITATION OF CYANIDE AND FLUORIDE*

Colorimetric Analysis of Cyanide by Pyridine-Pyrazolone Reagent

A modification of Baar's method (ref 3) was used to analyze cyanide from a basic solution using pyridine-pyrazolone reagent. In the Baar method, sodium dihydrogen phosphate and chloramine T were added to the cyanide solution as a single reagent. This buffered chloramine T solution was unstable so the buffering agent and chloramine T were added separately. The amount of chloramine T was varied until maximum absorbance was obtained after reaction of cyanogen chloride with the pyridine-pyrazolone reagent. Sensitivity was sufficient so that concentration of the colored product by extraction with an organic solvent as described in Baar's work was unnecessary.

A standard curve for the cyanide analysis was prepared as follows: 2 ml aliquots of 0.1 NaOH solutions containing 0 to 0.7 μg CN^-/ml were pipetted into 10 ml volumetric flasks. Then 0.40 ml 1 M potassium dihydrogen phosphate was added to each flask to adjust pH to 7.0. Solutions were cooled to approximately 4°C for 5 minutes in an ice bath. Then 1.0 ml of 0.10% chloramine T was added to each flask, which was covered immediately, mixed, and allowed to react for 2 minutes in the ice bath. At the end of the reaction period, each flask was removed from the bath and 5.7 ml pyridine-pyrazolone reagent was added immediately. This reagent was pipetted directly into the solution without allowing it to touch the neck of the flask. The flasks were filled to volume with distilled water and mixed well. After 30 to 35 minutes the standards were read on a Model DU Beckman spectrophotometer at a wavelength of 625 mμ and slit width of 0.055 mm against a blank set at zero absorbance. A standard curve was prepared by plotting Absorbance against Concentration of CN^- in 0.1 N NaOH.

Biological samples to be analyzed for CN^- were prepared by a microdiffusion separation before the above procedure was performed.

Determination of Fluoride Using the Fluoride Specific Electrode

A method was developed to measure the concentration of fluoride after

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*All reagents and apparatus used in this section are described in the Appendix.

microdiffusion using a fluoride specific electrode and specific ion meter (see Appendix: Apparatus). Standard solutions of 0.20 and 2.00 μg F^-/ml saline were prepared by diluting a stock solution of 5,000 μg fluoride/ml physiological saline. The standards were diluted with equal parts of Total Ionic Strength Adjustment Buffer (TISAB) to adjust the ionic strength and eliminate hydroxide interference (ref 4). The resulting solutions of 0.10 and 1.00 μg F^-/ml saline-TISAB were used to calibrate the specific ion meter. The fluoride specific electrode was immersed in each of these solutions for 10 minute periods until readings became constant. The samples were stirred with a teflon-covered magnetic rod. To test the reproducibility and accuracy of this method, the meter was calibrated at 0.10 and 1.00 μg F^-/ml saline-TISAB and then immersed for 10 munutes in solutions containing 0.10, 0.40, 0.80, 1.00, 1.20, 1.60, and 2.00 μg F^-/ml saline-TISAB. Solutions containing more than 1.00 μg F^-/ml were diluted with additional 1:1 saline-TISAB to bring the readings into the calibrated range of the meter.

Fluoride microdiffusion unknowns were developed by dissolving the absorbent on the diffusion unit lid in 4 ml of a 1:1 solution of saline and TISAB. These solutions were read on the meter. If necessary, additional saline-TISAB was added to bring the reading into the calibrated range of the meter.

SEPARATION OF CYANIDE AND FLUORIDE FROM BLOOD IN THE PRESENCE OF TVOPA

Since TVOPA or its organic degradation products convert to inorganic cyanide and fluoride during microdiffusion from blood, an extraction procedure was developed to remove TVOPA before microdiffusion.

Chloroform, methylene chloride, diethyl ether, ethyl acetate, and carbon tetrachloride were tested as solvents for extraction from blood. Carbon tetrachloride (CCl_4) was selected for TVOPA extraction because it produced a clear separation at the interface and was more dense than blood.

To test the extraction procedure, TVOPA was added to blood, in an amount that would produce approximately 15 μg F^-/ml and 10 μg CN^-/ml if 100% hydrolysis was achieved. Triplicate 2 ml blood samples were removed from the reaction vessel. The samples were extracted 0, 2, or 4 times with CCl_4 prior to microdiffusion and then analyzed for inorganic cyanide and fluoride. The detailed method was as follows:

A 25 ml volumetric flask was partially filled with blood, 0.125 ml of a solution containing 6.1 mg TVOPA/ml polyethylene glycol 400 (PEG-400) added, the flask filled to volume with blood, mixed, and the blood-TVOPA

solution transferred to a polyethylene bottle equipped with a magnetic stirrer. Temperature of the reaction bottle was 25°C. At various times during the reaction period, triplicate 2 ml blood samples were removed and treated by one of the following procedures:

(1) No Extractions: A 2 ml blood sample was pipetted into a 10 ml volumetric flask; 1 drop 2% saponin and 2 drops of CCl_4 were added. The solution was then diluted to volume with distilled water, mixed, and transferred to a polyethylene bottle.

(2) Two Extractions: A 2 ml blood sample was pipetted into a 125 ml glass separatory funnel containing 1 drop 2% saponin and 2 ml water was added. The mixture was extracted twice, once with 40 ml CCl_4 and once with 20 ml CCl_4. Equilibration was aided with a vibrating mixer. The CCl_4 portions were discarded. The aqueous remainder was drained into a 10 ml volumetric flask. The separatory funnel was rinsed 3 times with distilled water, and the washings added to the volumetric flask. The resulting solution was diluted to volume with distilled water, mixed, and transferred to a polyethylene bottle.

(3) Four Extractions: A 2 ml blood sample was extracted as above 4 times, once with 40 ml CCl_4 and 3 times with 20 ml CCl_4. The blood was diluted to 10 ml and transferred to a polyethylene bottle.

All samples were subsequently analyzed in duplicate for cyanide and fluoride as follows:

Cyanide: Edges of a Conway microdiffusion dish were coated with silicone grease, and 3 ml 0.1 N NaOH was added to the center compartment. The dish was tilted slightly. Two ml diluted blood from (1), (2), or (3) above and 1 ml 4% H_2SO_4 were added to the outer compartment of the dish. Care was taken to avoid mixing of the acid and blood before the dish was sealed. The dish was immediately covered with a glass lid, and the contents of the outer compartment were mixed by tilting and revolving the cell for 2 minutes. The sample was incubated for 3 hours $\pm$ 5 minutes at room temperature. After incubation, 2 ml from the center compartment of the dish was pipetted into a 10 ml volumetric flask and analyzed as described above using pyridine-pyrazolone reagent.

Fluoride: 2 ml diluted blood from (1), (2), or (3) above was pipetted into a plastic petri dish and 2 ml silver perchlorate-perchloric acid reagent added. The dish was immediately covered with a prepared lid (see Appendix: Apparatus), then mixed as above for 2 minutes. A weight was placed on the lid to insure a tight seal, and the sample incubated 24 hours at 60°C. Quantitation of fluoride was performed using the fluoride specific electrode described previously.

Blood containing known amounts of cyanide and fluoride was also extracted 0, 2, or 4 times, microdiffused, and analyzed for cyanide and fluoride. Calibration curves were then prepared to determine unknown cyanide and fluoride concentrations.

IN VITRO HYDROLYSIS OF TVOPA AT A CONCENTRATION OF 266 μg/ml IN WHOLE DOG BLOOD, BLOOD FRACTIONS, AND SALINE

The hypothetical concentration of TVOPA in the blood of a dog administered 25 mg/kg TVOPA is 266 μg TVOPA/ml blood if no TVOPA is degraded or lost to other tissues. These calculations were based on 94 ml blood/kg body weight. TVOPA was reacted with whole dog blood, saline, and solutions of serum, plasma, and blood cells reconstituted to volume with saline.

(1) Procedure for Saline, Serum and Plasma: A 0.25 ml aliquot of a solution containing 53.2 mg TVOPA/ml PEG-400 was added to 50 ml saline, reconstituted serum, or reconstituted plasma at 37°C in a polyethylene bottle. The solution was mixed well, placed in a 37°C water bath and stirred with a water-immersible magnetic stirrer. At various times 2 ml aliquots were removed and extracted by the "Two extraction" method above. All samples were then microdiffused and analyzed for cyanide and fluoride as described above.

Calibration curves were prepared by extracting and microdiffusing saline, reconstituted serum, or reconsitituted plasma containing known amounts of cyanide and fluoride.

(2) Procedure for Whole Blood and Reconstituted Blood Cells: A 0.25 ml aliquot of a solution containing 53.2 mg TVOPA/ml PEG-400 was added to whole blood or reconstituted blood cells and extracted using the "Two extraction" method. However, the extracted blood was diluted to 25 ml (rather than 10 ml) with distilled water and 1 ml (rather than 2 ml) portions of this solution were microdiffused. The blood was diluted further because higher levels of cyanide and fluoride were expected. The cyanide-liberating agent was 2 ml 2% H_2SO_4; the fluoride-liberating agent was 2 ml silver perchlorate-perchloric acid reagent. After microdiffusion the samples were analyzed for cyanide and fluoride ions and the concentrations were determined from calibration curves.

An additional reaction of 266 μg TVOPA/ml blood was performed to investigate whether more than 2 extractions might be necessary to remove all extractable TVOPA from the blood at this high concentration. Duplicate blood samples were removed from the reaction container twice during

hydrolysis, extracted 2 or 4 times, microdiffused, and analyzed for cyanide and fluoride.

CYANIDE AND FLUORIDE BLOOD LEVELS IN DOG ADMINISTERED 25 mg/kg TVOPA

A series of eight male mongrel dogs were intraveneously administered 25 mg/kg TVOPA dissolved in PEG-400. All doses were adjusted so that each animal received 0.5 cc of fluid per kilogram of body weight. Two of the animals were anesthetized with 30 mg/kg sodium pentobarbital prior to administration of the compound.

Immediately before, and at various times after administration of TVOPA, blood samples were drawn from the dog into a heparinized syringe. A 2 ml aliquot of blood was pipetted into a glass separatory funnel containing 1 drop of 2% saponin. Two ml saline was added, the mixture extracted twice, microdiffused, and analyzed for cyanide and fluoride as described under "Separation of Cyanide and Fluoride from Blood in the Presence of TVOPA" using the "Two Extraction" method. However, physiological saline was used instead of distilled water throughout the extraction and microdiffusion procedures, including preparation of 4% H_2SO_4.

Calibration curves were prepared by extracting and microdiffusing known blood solutions of 0.5 to 8.0 µg CN^-/ml and 5.0 to 20.0 µg F^-/ml. Reproducibility of the method was determined by repeating the calibration on several days with blood from several dogs.

PHARMACOLOGICAL EFFECTS OF 25 mg/kg TVOPA ON ANESTHETIZED MONKEYS

Four male Cynamolgus monkeys (4 - 5 kg) anesthetized with pentobarbital sodium (30 mg/kg) were prepared for recording of blood pressure using a transducer attached to a cannula in the femoral artery; for recording of respiration using a pneumotachygraph; an electrocardiogram was obtained using standard leads. All measurements were recorded on a Grass Model 5D polygraph. Body temperature was maintained as necessary with a heating pad. A solution of 25 mg/kg TVOPA dissolved in PEG-400 (0.5 cc of fluid per kilogram of body weight) was injected into the right femoral vein by means of a cannula. Venous blood samples were drawn periodically from the left femoral vein for determination of hematocrit and fluoride and cyanide ions using the same procedures and calibration graphs as those reported above.

PHARMACOLOGICAL EFFECTS OF 25 mg/kg TVOPA ON UNANESTHETIZED MONKEYS

Three restrained male Cynamolgus monkeys (4 - 5 kg) were administered 25 mg/kg TVOPA dissolved in PEG-400 by means of a 20 gage flexible Teflon hypodermic needle inserted in the right femoral artery. All solutions were prepared so that each animal received 0.5 cc of fluid per kilogram of body weight.

Baseline observations including gait, degree of alertness, extent of voluntary and stimulated activity, and response to pain were recorded. After these control observations, the solution containing TVOPA was administered and the animals were observed continuously for 4 to 6 hours unless death intervened.

Cyanide and fluoride blood levels were not analyzed in the unanesthetized monkeys.

INVESTIGATION OF THE INFLUENCE OF CYANIDE, FLUORIDE, DIFLUORAMINO COMPOUNDS, AND A NITRILE ON CYTOCHROME C OXIDASE ACTIVITY

Liver cytochrome oxidase activity was determined using a method modified from that of Cooperstein and Lazarow (ref 5). A 10 ml solution of cytochrome c ($\sim 2 \times 10^{-4}$ M) in 0.03 M phosphate buffer (ref 6) was reduced by addition of solid sodium hydrosulfite ($Na_2S_2O_4$). The solution was shaken a few minutes to remove excess hydrosulfite. Hydrogen gas was bubbled slowly through the solution for at least one hour before measurement of cytochrome c oxidase activity. Auto-oxidation then proceeded slowly while hydrogen was continuously bubbled through the solution. Nitrogen gas was bubbled through the reduced cytochrome c solution for 5 minutes prior to measuring cytochrome c oxidase activity to remove excess reducing agent. Spectrophotometric measurements were made with a Beckman DU Spectrophotometer. Details of the method of enzyme assay are given below.

Groups of 10 male Sprague-Dawley rats* (175-250 g) were administered various compounds dissolved in 0.9% saline or PEG-400 according to the schedule in Table 1. Solutions were prepared so that each animal received 0.01 ml fluid per gram of body weight. The time between treatment and decapitation was selected on the basis of prior experiments as the predicted time of first death at each dose.

Animals were decapitated and approximately 1.5 g liver quickly re-

*Harlan Research Animals, Indianapolis, Indiana

TABLE 1

TREATMENT ADMINISTERED TO RATS PRIOR TO DETERMINATION OF CYTOCHROME OXIDASE ACTIVITY

Animal Group	Treatment	Dose (mg/kg)	Solvent	% LD in Rats	Time Between Treatment and Decapitation (min)
I	No Treatment				
II	Saline	90.0			10
III	KCN	7.1	0.9% saline	84	10
IV	NaF + KCN (6:1)	37.8 + 6.3	0.9% saline	84	10
V	NaF	37.8	0.9% saline	<1	10
VI	KCN	6.3	0.9% saline	20	10
VII	Saline	90.0			60
VIII	NaF	52.0	0.9% saline	84	60
IX	PEG-400				30
X	TVOPA	35.0	PEG-400	84	30
XI	1,2-difluoraminopropane (1,2-DP)	68.0	PEG-400	84 in mice*	30
XII	1,2-bis(difluoramino) isobutane (IBA)	83.0	PEG-400	84*	30
XIII	2,2-bis(difluoramino) butane	83.0	PEG-400	**	30
XIV	trimethylacetonitrile	181.2	PEG-400	***	30

*Corn oil solvent

**LD_{84} unknown. Dose chosen was one which would produce the same number of moles of cyanide ion as would the IBA dose if total hydrolysis occurred.

***LD_{84} unknown. Dose was chosen by injecting 4 animals with solutions containing 10 to 100 times as much cyanide as an LD_{84} dose of KCN.

moved. Samples were weighed in a petri dish containing finely divided ice. A 10% weight to volume (W/V) homogenate was prepared by homogenizing the liver in ice cold 0.03 M phosphate buffer (pH 7.4) for 10 to 15 seconds using a motor-driven Teflon tissue homogenizer. A portion of the 10% W/V homogenate was then further diluted with room temperature 0.03 M phosphate buffer to a 0.2% W/V homogenate. In a 1 cm pyrex cuvette, 0.5 ml reduced cytochrome c solution was mixed with 2.4 ml of phosphate buffer at room temperature and the optical density determined at 550 mμ against a water blank in another matched cuvette. After the first determination, 0.10 ml of the 0.2% W/V homogenate (0.2 mg of tissue, wet weight) was added to the cuvette and measurements of the optical denisty were taken every 30 seconds for 5 minutes. Finally, approximately 2 mg of potassium ferricyanide, $K_3Fe(CN)_6$ was added to completely oxidize the cytochrome c and the optical density was again measured. Two ml portions of the 10% W/V homogenate were dried in an oven and weighed to determine the dry weight of tissue used in each cuvette.

Cytochrome oxidase activity was expressed as the first order rate constant, k, derived from the slope of a plot of time against the logarithm of the difference between the optical density of the cytochrome c at a given time and the optical density of the completely oxidized cytochrome c. The rate constant was expressed as milligrams of dry weight of tissue:

$$k = \frac{-2.303 \left[\log (O.D.\ t_2 - O.D.\infty) - \log (O.D.\ t_1 - O.D.\infty)\right]}{(t_2 - t_1) \times \text{mg tissue}}$$

A study was conducted to insure that the rate of oxidation of cytochrome c was proportional to concentration of tissue and therefore to the amount of cytochrome c oxidase present. In 5 untreated animals, the liver homogenates were prepared as described above and the rate constant then determined on solutions containing 0.05, 0.10, and 0.15 ml of 0.2% tissue homogenate according to the above method. The volume of phosphate buffer was adjusted so that the total volume in each cuvette was 3.00 ml.

Daily "no treatment" livers were tested to check cytochrome c activity, and as necessary, saline controls (10 min.) were run daily. This resulted in N = 26 for the "no treatment" group and N = 12 for saline controls.

Results of treatment with the various agents listed in Table 1 were subjected to statistical analysis by the Un-paired T-test (ref 7). Each group was compaired individually with its respective control group, and a p value of .05 was considered to be significant.

MODIFICATION OF THE TOXICITY OF TVOPA WITH VARIOUS DRUGS

To study the possible protective effects of sodium nitrite and sodium

thiosulfate, groups of 10 male Sprague-Dawley rats* (175-250 g) were administered combinations of these compounds dissolved in 0.9% saline at various intervals before and after intraperitoneal injection of 35 mg/kg TVOPA (the LD_{84} dose) dissolved in PEG-400. The doses of sodium nitrite and sodium thiosulfate selected were 5 mg/kg and 200 mg/kg respectively. All solutions were prepared so that each animal received 0.01 cc of fluid per gram of body weight for each injection. The combinations of drugs were administered according to the schedule shown in Table 2.

TABLE 2

SCHEDULE OF DRUG ADMINISTRATION EXPRESSED IN HOURS BEFORE (-) AND AFTER (+) INJECTION OF TVOPA

Animal Group	Sodium Nitrite	Sodium Thiosulfate	Saline
I	+0.5	+0.5	
II	+1.0	+1.0	
III	+1, 2	+1, 2	
IV	-.5, +.5, +1.5	-.5, +.5, +1.5	
V	-.5, +1, +2	+.5, +1, +2	
VI			-.5
VII			+.5

The negative control consisted of administration of each combination of drugs to a group of 10 animals that did not receive TVOPA.

A series of positive control experiments were conducted using the sodium nitrite-sodium thiosulfate treatment against a lethal dose of potassium cyanide. In these experiments, sodium nitrite (5 mg/kg) and sodium thiosulfate (200 mg/kg) was administered either 1 minute after or 15 minutes before administration of 7.5 mg/kg potassium cyanide.

In all experiments, animals were observed continuously for signs of toxicity for 4-8 hour periods immediately following the tests and daily for 7 days.

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*Harlan Research Animals, Indianapolis, Indiana

SECTION III

RESULTS

QUANTITATION OF CYANIDE AND FLUORIDE IONS

Cyanide Analysis by Pyridine-Pyrazolone Reagent

The pyridine-pyrazolone method of analyzing cyanide in a solution of 0.1 N NaOH produced a linear curve of Absorbance vs. Concentration over a range of 0.05-0.70 μg CN^-/ml 0.1 N NaOH. The limit of detection was 0.05 μg CN^-/ml 0.1 N NaOH. Data are presented in Table 3.

TABLE 3

STANDARD CURVE FOR PYRIDINE-PYRAZOLONE ANALYSIS OF CYANIDE IN 0.1 N NaOH

Concentration (μg CN^-/ml 0.1 N NaOH)	Absorbance*
0.05	0.041
0.10	0.093
0.30	0.272
0.50	0.458
0.70	0.642

*Mean of three samples

The reproducibility of the method was shown by the results of the nine analyses given in Table 4. The absorbance was found to be reproducible within ±0.7%.

Epstein (ref 8) states that thiocyanate (CNS^-) interferes with the colorimetric test for cyanide. Thiocyanate was not a problem in our analysis because it does not microdiffuse from blood.

TABLE 4

REPRODUCIBILITY OF CYANIDE DETECTION METHOD

Sample #	Absorbance
1	0.453
2	0.458
3	0.460
4	0.455
5	0.460
6	0.453
7	0.453
8	0.453
9	0.459
Mean	0.456 $\pm$ .003*

*Standard deviation

Fluoride Analysis by Fluroide Specific Electrode

The response of the fluoride specific electrode was determined over the range of 0.10 to 2.00 μg F^-/ml saline-TISAB. This corresponded to actual concentrations of 1 to 20 μg F^-/ml blood in the studies on anesthetized and unanesthetized dogs. The solutions which were initially in the calibrated range of the meter 0.1 to 1.0 μg F^-/ml saline-TISAB gave slightly better reproducibility than those which were diluted in order to be within the calibrated range. See Table 5 for detailed results.

TABLE 5

REPRODUCIBILITY OF FLUORIDE DETECTION
BY THE FLUORIDE SPECIFIC ELECTRODE

Actual Conc. ($\mu g\ F^-$/ml saline-TISAB)	0.10	0.40	0.80	1.00	1.20	1.60	2.00
Detected conc. ($\mu g\ F^-$/ml saline-TISAB)							
#1	0.10	0.39	0.80	1.01	1.16	1.58	2.04
#2	0.10	0.39	0.80	1.02	1.18	1.62	2.00
#3	0.10	0.39	0.80	1.01	1.20	1.60	2.10
#4	0.10	0.40	0.80	1.05	1.24	1.68	2.12
Mean	0.10	0.39±0.0058*	0.80	1.02±0.019	1.20±0.035	1.62±0.043	2.07±0.055
% Average Deviation	±0%	±0.8%	±0%	±1.3%	±2.5%	±1.9%	±2.4%
% F^- detected	100%	98%	100%	102%	100%	101%	104%

*Standard deviation

SEPARATION OF CYANIDE AND FLUORIDE FROM BLOOD IN THE PRESENCE OF TVOPA

Table 6 gives the cyanide and fluoride levels found in the blood after 0, 2, or 4 extractions. Complete conversion to inorganic cyanide and fluoride would be represented by 10.1 μg CN^-/ml blood and 14.7 μg F^-/ml blood.

TABLE 6

COMPARISON OF EXTRACTED AND UNEXTRACTED BLOOD SAMPLES IN AN IN VITRO TVOPA REACTION

Reaction Time (min.)	Blood Cyanide (μg/ml)			Blood Fluoride (μg/ml)		
	0 ext.	2 ext.	4 ext.	0 ext.	2 ext.	4 ext.
10	5.1	0.3	0.3	12.2	3.0	2.9
66	5.1	1.8	1.7	13.4	9.9	9.9
119	4.9	2.6	2.7	14.6	12.0	12.8

The concentrations of cyanide and fluoride in unextracted blood samples were appreciably higher than in samples extracted twice with $CCCl_4$. The concentrations of cyanide and fluoride after 4 extractions were the same, within experimental error, as the concentrations after 2 extractions.

IN VITRO HYDROLYSIS OF 266 μg TVOPA/ml IN WHOLE DOG BLOOD, BLOOD FRACTIONS, AND SALINE

An initial concentration of 266 μg TVOPA/ml would produce 88 μg CN^-/ml and 128 μg F^-/ml with 100% conversion.

Saline: Reaction of 266 μg TVOPA/ml saline showed that only 1.2 μg F^-/ml and less than 0.3 CN^-/ml were present after 3 hours. These values represented less than 1% hydrolysis.

Reconstituted Serum: The cyanide and fluoride results are shown in Table 7. After 120 minutes, 71-75% hydrolysis of TVOPA to the fluoride ion and 5-6% hydrolysis of TVOPA to the cyanide ion were found.

TABLE 7

CYANIDE AND FLUORIDE LEVELS FOR TWO IN VITRO REACTIONS OF 266 µg TVOPA/ml RECONSTITUTED SERUM

Test 1

Reaction Time (min.)	Serum Fluoride µg/ml	% Conversion	Serum Cyanide µg/ml	% Conversion
5	15	12	1	1
60	67	52	3	3
120	91	71	5	6
150	98	77	5	6

Test 2

Reaction Time (min.)	Serum Fluoride µg/ml	% Conversion	Serum Cyanide µg/ml	% Conversion
5	15	12	1	1
60	73	57	3	3
120	96	75	4	5

Reconstituted Plasma: Results for reaction of TVOPA in plasma are shown in Table 8. 59-63% hydrolysis of TVOPA to fluoride and 6-7% hydrolysis of TVOPA to cyanide were found.

TABLE 8

CYANIDE AND FLUORIDE LEVELS FOR TWO IN VITRO REACTIONS OF 266 μg TVOPA/ml RECONSTITUTED PLASMA

Test 1

Reaction Time (min.)	Plasma Fluoride μg/ml	Plasma Fluoride % Conversion	Plasma Cyanide μg/ml	Plasma Cyanide % Conversion
5	13	10	.5	<1
60	58	45	4	5
120	76	59	5	6

Test 2

Reaction Time (min.)	Plasma Fluoride μg/ml	Plasma Fluoride % Conversion	Plasma Cyanide μg/ml	Plasma Cyanide % Conversion
5	17	13	1	1
60	62	48	6	7
120	81	63	6	7
180	88	69	6	7

Blood Cells Reconstituted with Saline: Table 9 shows the cyanide and fluoride levels after reaction of TVOPA in the blood cell fraction; 49-63% hydrolysis to fluoride and 36-49% hydrolysis to cyanide occurred by 120 minutes.

TABLE 9

CYANIDE AND FLUORIDE LEVELS FOR TWO IN VITRO
REACTIONS OF 266 µg TVOPA/ml RECONSTITUTED BLOOD CELLS

Test 1

Reaction Time (min.)	Blood Cell Fluoride µg/ml	% Conversion	Blood Cell Cyanide µg/ml	% Conversion
5	25	20	6	7
60	71	55	32	36
120	80	63	43	49

Test 2

Reaction Time (min.)	Blood Cell Fluoride µg/ml	% Conversion	Blood Cell Cyanide µg/ml	% Conversion
5	17	13	5	6
60	55	43	23	26
120	63	49	32	36
180	67	52	37	42

Whole Blood: At 120 minutes, 72-84% hydrolysis to fluoride and 58-64% hydrolysis to cyanide were found. By 180 minutes, 79-93% hydrolysis to fluoride and 74-78% hydrolysis to cyanide were found. As shown in Table 10, the molar ratio of fluoride to cyanide was approaching 2:1 180 minutes after start of the reaction.

TABLE 10

CYANIDE AND FLUORIDE LEVELS FOR TWO
IN VITRO REACTIONS OF 266 µg TVOPA/ml BLOOD

Test 1

Reaction Time (min.)	Blood Fluoride µg/ml	% Conversion	Blood Cyanide µg/ml	% Conversion	Molar Ratio of F^-; CN^-
5	24	19	6	7	5.5
60	86	67	38	43	3.1
120	107	84	56	64	2.6
180	119	93	69	78	2.4

Test 2

Reaction Time (min.)	Blood Fluoride µg/ml	% Conversion	Blood Cyanide µg/ml	% Conversion	Molar Ratio of F^-; CN^-
5	24	19	12	14	2.7
60	69	54	35	40	2.7
120	92	72	51	58	2.5
180	101	79	65	74	2.1
240	105	82	73	83	2.0

In the third hydrolysis in blood, 2 or 4 extractions of 9- and 95-minute duplicate samples showed that 2 extractions were sufficient to remove all extractable TVOPA from the blood even at this high dose.

CYANIDE AND FLUORIDE BLOOD LEVELS IN DOGS ADMINISTERED 25 mg/kg TVOPA

Results of cyanide and fluoride analysis of blood from one of two anesthetized dogs administered 25 mg/kg TVOPA are presented in Table 11 and Figure 1.

TABLE 11

BLOOD ANALYSIS OF ANESTHETIZED DOG ADMINISTERED 25 mg/kg TVOPA

Time (min.)	Blood Fluoride (μg/ml)	Blood Cyanide (μg/ml)	Molar Ratio of F^-: CN^-
0	<1.0	<0.1	
15	8.0	2.5	4.4
30	11.0	3.9	3.9
45	12.1	4.5	3.7
60	11.9	4.8	3.4
90	11.0	4.9	3.1
120	9.4	4.5	2.9
180	7.3	4.3	2.3

Results of cyanide and fluoride analyses of blood from one of six unanesthetized dogs administered 25 mg/kg TVOPA are presented in Table 12 and Figure 1.

FIGURE 1

CYANIDE AND FLUORIDE LEVELS IN AN ANESTHETIZED
AND AN UNANESTHETIZED DOG ADMINISTERED 25 mg/kg TVOPA

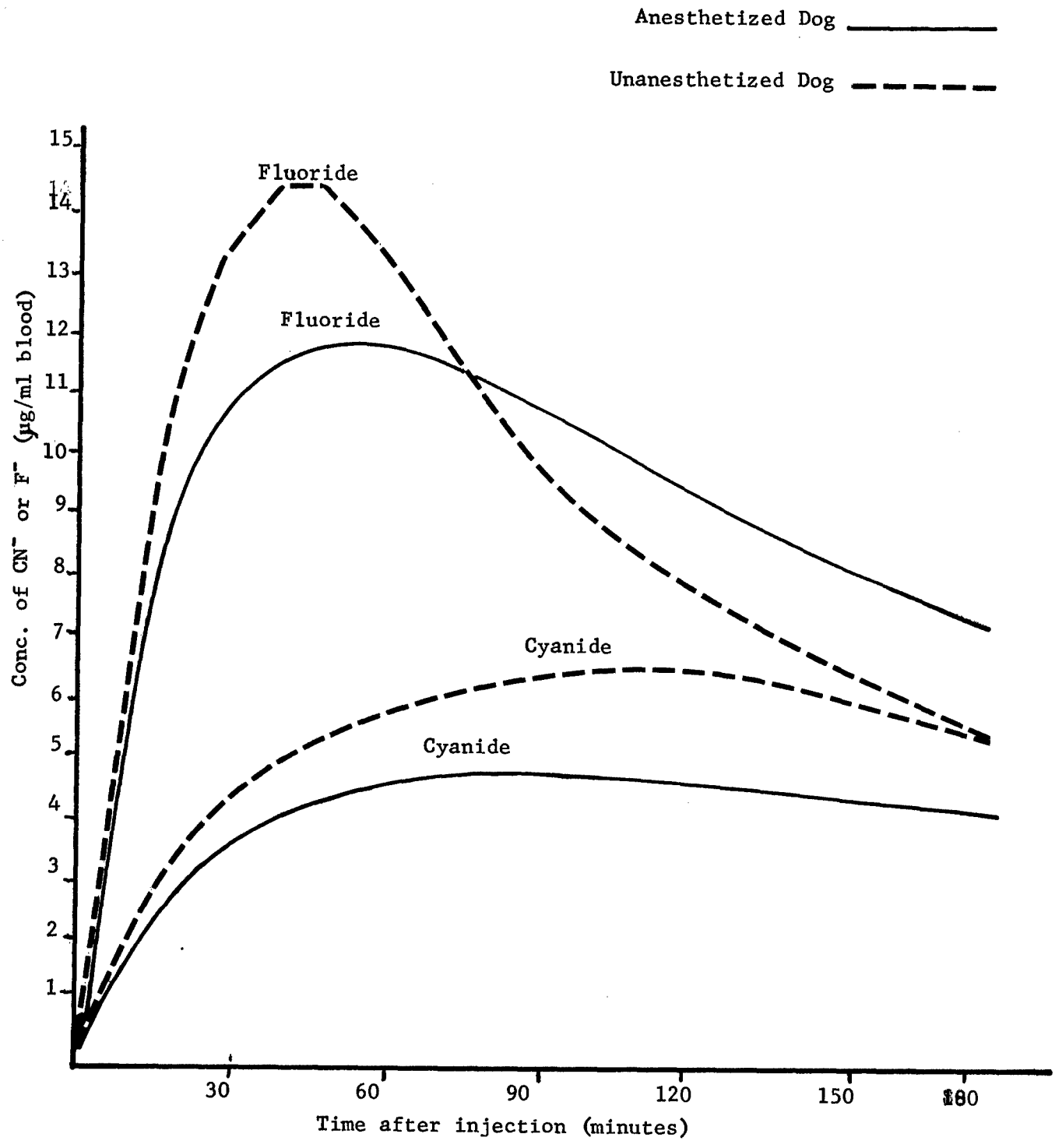

TABLE 12

BLOOD ANALYSIS OF UNANESTHETIZED DOG
ADMINISTERED 25 mg/kg TVOPA

Time (min.)	Blood Fluoride (μg/ml)	Blood Cyanide (μg/ml)	Molar Ratio of F^-:CN^-
15	9.5	2.8	4.6
30	13.6	4.4	4.2
45	14.8	5.4	3.8
60	13.4	5.8	3.2
90	9.8	6.4	2.1
120	7.9	6.6	1.6
180	5.4	5.4	1.4

The amount of TVOPA injected into the dogs could theoretically produce 88 μg CN^-/ml blood and 128 μg F^-/ml if all the TVOPA and/or degradation products remained in the blood for the duration of the experiment. The usual maximum concentrations reached in the dog studies were 4 to 8 μg CN^-/ml blood and 12 to 16 μg F^-/ml blood. Cyanide concentrations usually reached a maximum between 65 and 120 minutes and then decreased. The fluoride concentration generally reached a maximum between 40 and 75 minutes and then decreased.

Calibration data obtained by analyzing standard dog blood solutions containing cyanide and fluoride are shown in Tables 13 and 14. A plot of absorbance versus concentration was nearly linear up to 8 μg CN^-/ml blood, and a plot of detected fluoride concentration versus actual fluoride concentration was nearly linear up to 20 μg F^-/ml blood. The standard deviation and percent recovery at various concentrations of cyanide and fluoride are also given in Tables 13 and 14.

PHARMACOLOGICAL EFFECTS OF 25 mg/kg TVOPA ON ANESTHETIZED MONKEYS

In 4 Cynamolgus monkeys administered 25 mg/kg TVOPA intravenously,

there was a pattern of effects on the blood pressure, heart rate, and respiration typified by changes shown in Figure 2.

In all animals we observed a moderate decrease in blood pressure and heart rate shortly after administration of the compound. Heart rate gradually returned to control values after 30-60 minutes. Blood pressure remained depressed until approximately 90 minutes, whereupon it returned to near control values until death intervened. Respiration rate and depth became greatly elevated shortly after administration of the compound, and remained elevated for 60-75 minutes. This period of hyperpnea was followed by a gradual decline in rate to normal, but a continued elevation in depth of respiration. Between 90-200 minutes all voluntary respiration ceased. Death was apparently the result of this respiratory failure since cardiac activity was still present for several minutes after total cessation of respiration.

The cyanide and fluoride blood analyses for four anesthetized monkeys are shown in Table 15 and Figure 3. The maximum cyanide levels were 4 to 7.2 μg/ml blood. These concentrations were usually reached between 60 and 90 minutes after injection of TVOPA.

The maximum fluoride levels found in three monkeys were 7.8 to 9 μg/ml blood. In one monkey nearly 13 μg F^-/ml was detected. The fluoride maxima were reached after 15 to 60 minutes.

TABLE 13

CYANIDE CALIBRATION DATA FOR DOGS
ADMINISTERED 25 mg/kg TVOPA

Known Conc. ($\mu g\ CN^-$/ml blood)	Absorbance I	II	III	IV	V	VI	VII	Average Absorbance	Standard Deviation	% Recovery
1	0.087	0.085								70
2	0.164	0.164	0.164	0.166	0.165	0.157	0.172	0.165	0.004	67
4	0.275	0.313								60
5			0.362	0.362	0.339	0.333	0.360	0.351	0.014	57
6	0.410	0.407								55
8	0.495	0.541	0.518	0.550	0.524	0.486	0.550	0.523	0.025	53

TABLE 14

FLUORIDE CALIBRATION DATA FOR DOGS
ADMINISTERED 25 mg/kg TVOPA

Known Conc. (μg F^-/ml blood)	Detected Conc. (μg F^-/ml blood) I	II	III	IV	Average Detected Conc. (μg F^-/ml blood)	Standard Deviation	% Recovery*
5	5.1	5.1	5.6	4.6	5.1	0.41	96
10	10.1	9.8	10.5		10.1		98
12.5				10.9		0.35	
15	14.1	13.8					92
20	20.1	19.6	18.8	18.1	19.2	0.88	95

*A concentration of 0.3 μg F^-/ml blood was assigned as the background level.

FIGURE 2 CARDIOVASCULAR AND RESPIRATORY RESPONSES OF AN ANESTHETIZED CYNAMOLOGUS MONKEY FOLLOWING I.V. INJECTION OF 25/mg/kg TVOPA

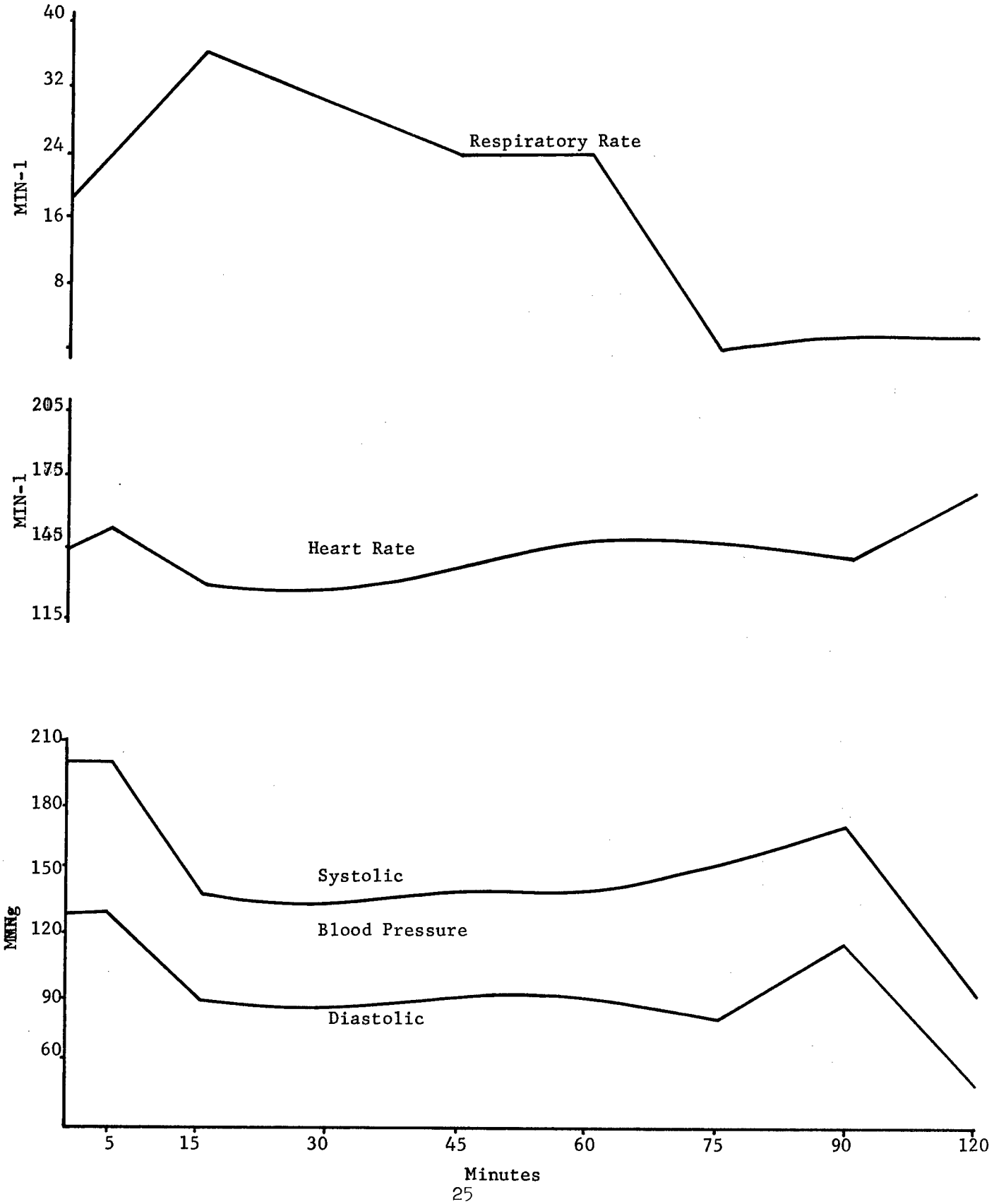

FIGURE 3

CYANIDE AND FLUORIDE LEVELS IN FOUR ANESTHETIZED MONKEYS ADMINISTERED 25 mg/kg TVOPA

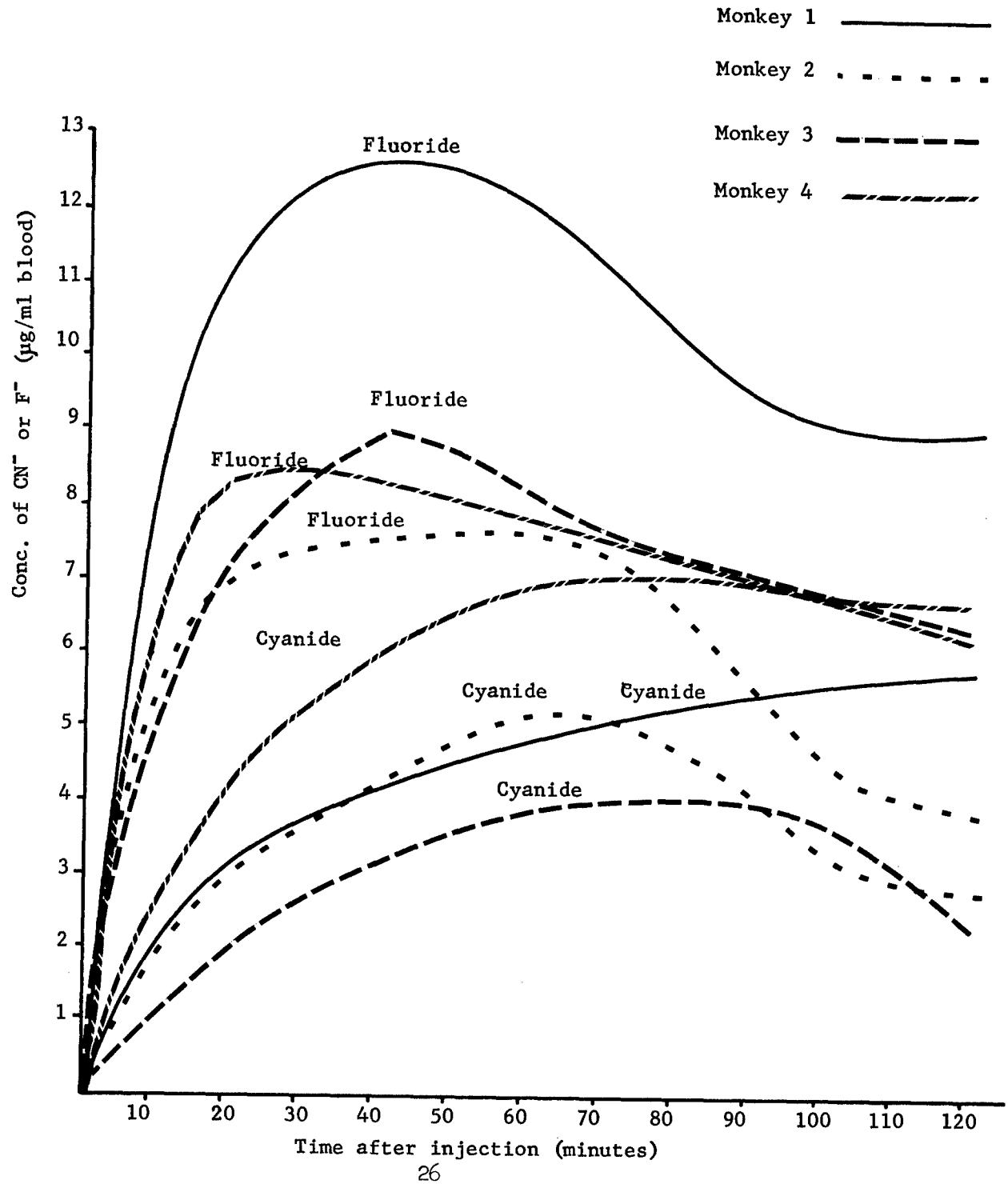

TABLE 15

CYANIDE AND FLUORIDE BLOOD LEVELS IN FOUR ANESTHETIZED MONKEYS ADMINISTERED 25 mg/kg TVOPA

Time (min.)	Monkey 1		Monkey 2	
	Blood Cyanide (µg/ml)	Blood Fluoride (µg/ml)	Blood Cyanide (µg/ml)	Blood Fluoride (µg/ml)
0	0.0	<1.0	0.2	1.7
15	2.9	10.5	2.7	6.7
30	3.8	12.6	3.7	7.5
60	4.8	12.2	5.2	7.7
90	5.5	9.5	4.1	5.6
120	5.8	9.1	2.8	3.9
150			2.6	3.8

Time (min.)	Monkey 3		Monkey 4	
	Blood Cyanide (µg/ml)	Blood Fluoride (µg/ml)	Blood Cyanide (µg/ml)	Blood Fluoride (µg/ml)
0	0.1	1.1	0.0	0.1
15	1.6	6.7	3.5	8.0
30	2.8	8.5	5.3	8.5
60	3.9	8.2	7.0	7.9
90	4.0	7.1	7.0	7.1
120	2.3	6.4	6.6	6.2

PHARMACOLOGICAL EFFECTS OF 25 mg/kg TVOPA ON UNANESTHETIZED MONKEYS

Administration of 25 mg/kg of TVOPA was shown to be fatal to all three animals tested. Intravenous injection of the test compound did not appear immediately painful to any of the animals. The first sign of toxicity noted was a marked increase in the rate and depth of respiration beginning approximately two minutes after injection. In one animal, slight bleeding was noted at the site of the injection after 3 minutes. Between 5 and 10 minutes, respiration became progressively more labored, and the pupils appeared moderately constricted. An increase in vocalization was observed in all three animals.

Between 10 and 20 minutes, the animals appeared sedated, but still responded to physical stimulus. During this period, respiratory sounds during expiration were noted in two of the animals. All three animals

appeared to make full use of accessory respiratory muscles during expiration.

Between 20 and 30 minutes respiration slowed to approximately 4 to 10 breaths per minute and became more labored, marked by maximal inspiration and forceful expiration. The animals appeared heavily sedated and could not be aroused by physical stimulation, however, the blink reflex was still present. Coarse body tremors of approximately 5 second duration were observed repeatedly in all three monkeys. The pupils remained constricted. Pulse rate was elevated to approximately 150 to 175 beats per minute.

Between 30 minutes and time of death, respiration became progressively more labored, cyanosis was evident, and heart rate continued to be elevated until after total cessation of respiration. Deaths occurred between 60 and 75 minutes. The apparent cause of death was respiratory arrest. At no time did any of the animals demonstrate convulsive activity.

INVESTIGATION OF THE INFLUENCE OF CYANIDE, FLUORIDE, DIFLUORAMINO COMPOUNDS, AND A NITRILE ON CYTOCHROME OXIDASE ACTIVITY

The effect on rate constant produced by varying the concentration of tissue was determined in five untreated rats. Table 16 shows that the rate constant k, expressed in terms of mg dry weight of tissue in each cuvette, was found to be constant. Therefore, variation in preparing the 10% homogenate would not cause differences in the rate constant. The rate constant k' in the last column was found by assuming that the dry weight of tissue in the three cuvettes was the same and that only the amount of cytochrome c oxidase in each cuvette was different. In this case, the results show that a slight variation in the amount of cytochrome c oxidase present in each rat would cause a proportionate variation in the rate constant.

TABLE 16

EFFECT OF CONCENTRATION OF TISSUE ON RATE CONSTANT

Cuvette No.	Volume of .2% Homogenate (ml)	k average ($mg^{-1}sec^{-1}$)	k' average* ($mg^{-1}sec^{-1}$)
1	0.05	0.0644	0.0322
2	0.10	0.0651	0.0651
3	0.15	0.0617	0.0925

*Based on dry weight of tissue in cuvette No. 2.

The percentage reduction in cytochrome c oxidase activity produced by KCN, NaF, the difluoramino compounds and the nitrile, was determined by comparison of the rate constant k of the particular treated groups to the rate constant of the respective control group. Groups III to VI, injected with compounds dissolved in .9% saline and sacrificed 10 minutes later, were compared to Group II saline animals. Group VIII, sacrificed 60 minutes after injection of NaF, was compared to Group VII saline animals. Groups X to XIV, injected with compounds dissolved in PEG-400 and sacrificed after 30 minutes, were compared to the Group IX PEG-400 animals.

The cytochrome c oxidase activity of several of the treatment groups was significantly depressed (see Table 17). Animal groups III, IV, and VI, treated with some combination of KCN, were depressed to almost 50% of control activity levels.

The activity of animal groups X, XI, and XII, treated with TVOPA 1,2 difluoraminopropane or 1,2-bis(difluoramino)-isobutane, were also depressed to about 50% of control activity levels.

The activity of PEG-400 treated animals was decreased when compared to untreated animals, but the reduction was not statistically significant.

TABLE 17

EFFECTS OF VARIOUS COMPOUNDS ON RAT LIVER CYTOCHROME C OXIDASE ACTIVITY

Animal Group	Treatment	Dose (mg/kg)	k average ($mg^{-1}sec^{-1}$)	Standard Deviation	% Reduction of Activity
I	no treatment		0.0624	0.0100	--
II	saline (10 min.)	90.0	0.0604	0.0081	--
III*	KCN	7.1	0.0277	0.0067	54
IV*	NaF + KCN (6:1)	37.8+6.3	0.0307	0.0052	49
V	NaF	37.8	0.0677	0.0084	--
VI*	KCN	6.3	0.0251	0.0030	58
VII	saline (60 min.)	90.0	0.0695	0.0097	--
VIII	NaF	52.0	0.0548	0.0073	21
IX	PEG-400	**	0.0496	0.0072	--
X*	TVOPA	35.0	0.0274	0.0067	45
XI*	1,2-difluoramino-propane	68.0	0.0237	0.0034	52
XII*	1,2-bis(difluoramino)-isobutane	83.0	0.0242	0.0042	51
XIII	2,2-bis(difluoramino)-butane	83.0	0.0421	0.0041	15
XIV	trimethylacetonitrile	181.2	0.0530	0.0093	--

*Significant inhibition of cytochrome c oxidase activity at the 95% confidence level by the unpaired t test (ref 7) compared to respective solvent control.

**PEG-400 was used as solvent for groups X, XI, XII, XIII, and XIV in concentrations to provide a 10 ml/kg dose.

MODIFICATION OF THE TOXICITY OF TVOPA WITH VARIOUS DRUGS

Attempts to protect rodents from the toxic effects of TVOPA using various combinations of sodium nitrite and sodium thiosulfate produced no significant change in the final mortality when compared to results obtained from TVOPA alone. Results appear in the following table.

TABLE 18

EFFECT OF SODIUM NITRITE AND/OR SODIUM THIOSULFATE ON THE TOXICITY OF TVOPA IN RATS

Animal Group	Sodium Nitrite	Sodium Thiosulfate	Saline	Mortality No. dead/No. tested
I	+0.5*	+0.5*		10/10
II	+1.0	+1.0		10/10
III	+1, 2	+1, 2		9/10
IV	-.5, +.5, +1.5	-.5, +.5, +1.5		10/10
V	-.5, +1, +2	+.5, +1, +2		9/10
VI			-.5	9/10
VII			+.5	10/10

*Hours before or after administration of TVOPA.

The group of animals which received a combination of drugs without TVOPA (negative control) demonstrated no unusual toxic effects.

A series of positive control experiments conducted using sodium nitrite-sodium thiosulfate treatment against the lethal effects of potassium cyanide produced results shown in Table 19.

TABLE 19

EFFECT OF 5 mg/kg SODIUM NITRITE AND
200 mg/kg SODIUM THIOSULFATE ON THE TOXICITY OF KCN IN RATS

Treatment	Mortality No. dead/No. tested
None (saline 5 min. prior to KCN)	9/10
1 min. after KCN	1/10
15 min. before KCN	9/10

SECTION IV

DISCUSSION

In our experiments with TVOPA, we used PEG-400 as an intermediate diluent. This differs from previously reported experiments in which TVOPA was administered either undiluted or diluted in corn oil. Since neither TVOPA nor corn oil are significantly soluble in water, distribution into aqueous solutions for either the *in vitro* studies or the *in vivo* studies would be limited using these solvents. This decreased distribution probably explains the differences observed in hydrolysis rates and biological activity between the present study and results reported previously (refs 1 and 11). Primary dilution with PEG-400 permitted evaluation of activity not subject to examination by the earlier methods.

Although the eventual use of TVOPA is outside the realm of this discussion, the composition of any formulation containing TVOPA may be a significant factor in predicting the toxic hazard of this compound.

Earlier experiments assigned a very low rate of hydrolysis of TVOPA in aqueous solutions. Less than 5% of the fluoride available from the compound was found in the aqueous phase after 48 hours (ref 11). The assumption was made that the rate of degradation in biological media would not be significantly greater than in the buffered *in vitro* media.

We have conclusively shown that this assumption was invalid. Our experiments using various blood fractions accounts for the majority of the fluoride and cyanide ion metabolites within 2 hours. Comparison of the various solution hydrolysis rates is presented in Table 20.

TABLE 20

SUMMARY COMPARISON OF *IN VITRO* DEGRADATION OF TVOPA IN VARIOUS SOLVENTS AFTER 120 MINUTES OF REACTION

Solvent	$\%F^-$*	$\%CN^-$*
Saline	<1%	<1%
Recon Serum	71-75%	5-6%
Recon Plasma	59-63%	6-7%
Recon Blood Cells	49-63%	36-49%
Whole Blood	72-84%	58-64%

*Calculated from amount theoretically available.

The attenuation in effects observed in dogs under pentobarbital anesthesia compared to conscious animals reported for TVOPA and other compounds of the series (refs 1, 11, and 12) was not apparent in our primate series. We observed no difference in the toxicity of TVOPA between the conscious and anesthetized monkeys. The effects observed in the dogs must be species specific because in a series of unpublished experiments, we were unable to alter the toxicity of TVOPA in pentobarbital anesthetized rats or mice.

The in vitro experiments measuring the rate of degradation in blood as well as the blood fluoride and cyanide levels found in both the dogs and monkeys leave little doubt that these ions play a significant part in the toxicology of TVOPA although the blood levels of these ions are not sufficiently elevated to account for the observed toxicology (ref 11). A summary comparison of blood values and observed effects in dogs is found in Table 21.

The experiments measuring inhibition of cytochrome c oxidase activity further support the contention that the cyanide ion from the degradation of TVOPA is probably a contributing factor in the action of this compound. However, the constellation of effects observed after administration of mixtures of KCN and NaF (ref 2) are not the same as those resulting from administration of TVOPA. Also, attempts to influence the toxic effects of TVOPA using procedures known to protect against the cyanide ion were not successful either in the present experiments or in earlier work (ref 1).

Further, the results reported previously (ref 2) on the activity of TVOPA on the guinea pig ileum and the phrenic nerve-diaphragm cannot be explained by other than the direct action of the compound.

Our present experiments support our earlier contention that the pharmacologic activity of TVOPA results from the action of the intact molecule or early organic degradation products. The inorganic degradation products influence the delayed effects of TVOPA, but are not responsible for death at high doses.

TABLE 21

SUMMARY COMPARISON OF PHARMACOLOGIC ACTIVITY, BLOOD FLUORIDE AND BLOOD CYANIDE IN 6 UNANESTHETIZED DOGS ADMINISTERED 25 mg/kg TVOPA

Time (minutes)	µg F^-/ml*	µg CN^-/ml*	Pharmacological response
Control	<1.0	<0.1	
0-5	2.5 ± 0.56**	0.51 ± 0.19**	Slight increase in rate and depth of respiration, salivation, tongue and gums slightly cyanotic.
5-10	6.6 ± 1.3	1.4 ± 0.47	Respiration labored, coarse body tremors, pulse rate elevated.
10-15	9.7 ± 2.1	2.1 ± 0.66	Piloerection, productive emesis, pupils constricted, rate and depth of respiration increased.
15-30	12.8 ± 3.0	3.1 ± 0.77	Preconvulsive activity, emesis, labored breathing with maximum inspiration.
30-45	15.4 ± 3.4	4.6 ± 1.3	Labored breathing, generalized body tremors, elevated pulse rate.
45-60	16.0 ± 3.5	5.9 ± 1.9	Convulsions, pupils maximally dilated, no response to painful stimulation.
60-90	14.9 ± 3.6	6.5 ± 2.1	Continued hyperpnea.
90-120	12.9 ± 3.8	6.3 ± 1.6	Respiration labored, slow, heart rate elevated, pupils maximally dilated.
120	12.6 ± 4.1	7.3 ± 0.54	Respiratory depression.
120-240	----	----	Respiratory arrest - death.

*Values are averages for each time interval.
**Standard deviation

APPENDIX

Reagents

All chemicals used in this series were reagent grade.

1. Physiological saline = 9 g NaCl/1000 ml distilled water.

2. 0.1 N sodium hydroxide.

3. 2000 µg CN^-/ml saline = 0.5012 g KCN/100 ml saline.

4. 0.50-0.70 µg CN^-/ml 0.1 N NaOH prepared by appropriate dilution of Solution #3 with 0.1 N NaOH.

5. 1 M potassium dihydrogen phosphate, KH_2PO_4 = 136 g KH_2PO_4/1000 ml distilled water.

6. 0.1% chloramine T, 1-$CH_3C_6H_4$ - 4-$SO_2NClNa \cdot 3H_2O$ (Matheson Coleman and Bell).

7. Pyridine-pyrazolone reagent, prepared as follows (ref 3):

   a. 2.400 g 3-methyl-1-phenyl-2-pyrazolin-5-one (Eastman Organic Chemicals, #1397) was dissolved in 1000 ml distilled water in a water bath at 70-80°C. Solution was filtered next day and stored in a dark glass bottle. The solution was stable for at least one month.

   b. 20 mg 3,3'-dimethyl-1,1'-diphenyl-(4,4'-bi-2-pyrazoline)-5,5'-dione (Eastman Organic Chemicals #6969) was dissolved in 20 ml pyridine by stirring with magnetic stirrer for 20-30 minutes. Solution prepared daily.

   c. Pyridine-pyrazolone reagent was prepared by combining five volumes of Solution #7a with one volume of #7b.

8. Total Ionic Strength Adjustment Buffer (TISAB) purchased from Orion Research (Orion 94-09-09) or prepared as follows (ref 4):

   Approximately 500 ml distilled water was placed in a large beaker. 57 ml glacial acetic acid, 58 g NaCl, and 0.30 g sodium citrate were added, then stirred to dissolve. Beaker was placed in cold water bath. 5 M NaOH added until pH was between 5.0 and 5.5. Solution was cooled to room temperature, then poured into 1 liter flask and diluted to volume with distilled water.

9. 5000 μg F^-/ml saline = 1.1050 g NaF/100 ml saline. Stored in polyethylene container.

10. 0.2, 0.8, 1.6, 2.0, 2.4, 3.2, and 4.0 μg F^-/ml saline made by diluting solution #9 with physiological saline.

11. Ion meter standard solutions:

    0.1, 0.4, 0.8, 1.0, 1.2, 1.6, and 2.0 μg F^-/ml saline-TISAB made by diluting solution #10 with an equal volume of TISAB.

12. Saline-TISAB diluent made by mixing equal volumes of 0.9% saline and TISAB.

13. 15,000 μg CN^-/ml saline = 3.7590 g KCN/100 ml saline.

14. 15,000 μg F^-/ml saline = 3.3150 g NaF/100 ml saline. Stored in polyethylene container.

15. Heparinized dog blood.

16. Polyethylene glycol 400 (PEG-400) (U.S.P., Fisher Scientific Company).

17. Cyanide and fluoride blood standards (all blood standards stored in air-tight polyethylene containers):

    a. (10.0 μg CN^- + 25.0 μg F^-)/ml blood prepared by partially filling a 25 ml volumetric flask with dog blood, adding 0.125 ml of 2000 μg CN^-/ml saline, 0.125 ml of 5000 μg F^-/ml saline, and 0.125 ml of PEG-400, then filling to volume with blood and mixing.

    b. 10.0 μg CN^-/ml blood prepared by partially filling a 25 ml volumetric flask with dog blood, adding 0.25 ml of 1000 μg CN^-/ml saline and 0.125 ml PEG-400, then filling to volume with blood and mixing.

    c. 25.0 μg F^-/ml blood prepared by partially filling a 25 ml volumetric flask with dog blood, adding 0.25 ml of 2500 μg F^-/ml saline and 0.125 ml PEG-400, then filling to volume with blood and mixing.

    d. Blood standards containing cyanide (0.5-8.0 μg/ml) and/or fluoride (5.0-20.0 μg/ml) prepared by diluting solutions 17a, b, or c appropriately with blood.

    e. (75 μg CN^- + 150 μg F^-)/ml blood prepared by partially filling a 25 ml volumetric flask with dog blood, adding 0.25 ml of 15,000 μg F^-/ml saline, 0.125 ml of 15,000 μg CN^-/ml saline, and 0.125 ml PEG-400, then filling to volume with blood and mixing.

f. (75 μg CN^- + 120 μg F^-)/ml blood prepared by partially filling a 25 ml volumetric flask with blood, adding 0.20 ml of 15,000 μg F^-/ml saline, 0.125 ml of 15,000 μg CN^-/ml saline, and 0.125 ml PEG-400, then filling to volume with blood and mixing.

g. Blood standards containing cyanide (30.0-60.0 μg/ml) and fluoride (60.0-120.0 μg F^-/ml) prepared by diluting 17e or f appropriately with blood.

18. 2 and 4% H_2SO_4(v/v, diluted with distilled water).

19. 4% H_2SO_4(v/v, diluted with saline).

20. 2% saponin (J.T. Baker purified powder).

21. Carbon tetrachloride.

22. Silicone grease (Dow Corning).

23. Silver perchlorate, $AgClO_4$.

24. 60% perchloric acid, $HClO_4$.

25. Silver perchlorate - perchloric acid reagent (ref 9) - 10 g silver perchlorate was dissolved in 5 ml distilled water, then diluted to 100 ml with perchloric acid. The solution was mixed and transferred to a dark bottle.

26. 1.0 N alcoholic sodium hydroxide (ref 10) - 4 g NaOH was dissolved in 5 ml distilled water, diluted to 100 ml with 95% ethanol, mixed and refrigerated.

27. Cytochrome c (Type III, Sigma Chemical Company, St. Louis, Mo.).

28. Sodium hydrosulfite (sodium dithionite), $Na_2S_2O_4$.

29. 0.03 M phosphate buffer, pH 7.4 - Dilute 4.08 g monobasic potassium phosphate (KH_2PO_4) and 4.26 g dibasic sodium phosphate (Na_2HPO_4) to 1 liter with distilled water.

Apparatus

1. Beckman DU spectrophotometer, 1 cm cells.

2. Magnetic stirrer

3. Orion specific ion meter (Model 401) and Orion combination fluoride electrode (Model 96-09), Orion Research Incorporated, Cambridge, Mass.

4. Teflon-coated stirring bars.

5. 125 ml Pyrex separatory funnels, conditioned with acidic solution of NaF to protect from reaction with fluoride during extraction.

6. Vibrating mixer (Vortex).

7. Conway microdiffusion dishes (Scientific Products #D-1815-1). Lids for the Conway dishes were glass petri dishes.

8. Plastic petri dishes, 50 mm diameter x 12 mm deep (Falcon Plastics, Division of Bioquest).

9. Preparation of lids (ref 10):

   0.05 ml 1 N alcoholic NaOH was pipetted onto petri dish lid and spread into a circular area 3-4 cm in diameter using an L-shaped glass rod. The lids were warmed slightly to facilitate spreading, then dried in an oven at 60°C.

10. Air/water stirrer (Mag-Jet by Bronwell Scientific).

11. Water bath at 37°C.

12. Polyethylene reagent bottles.

13. Teflon tissue grinder (homogenizer) 30 ml chamber volume (Arthur H. Thomas Co., Philadelphia, Pa.).

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