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A COMPARISON OF THE TOXICOLOGY OF TRIETHYLENE GLYCOL DINITRATE AND PROPYLENE GLYCOL DINITRATE

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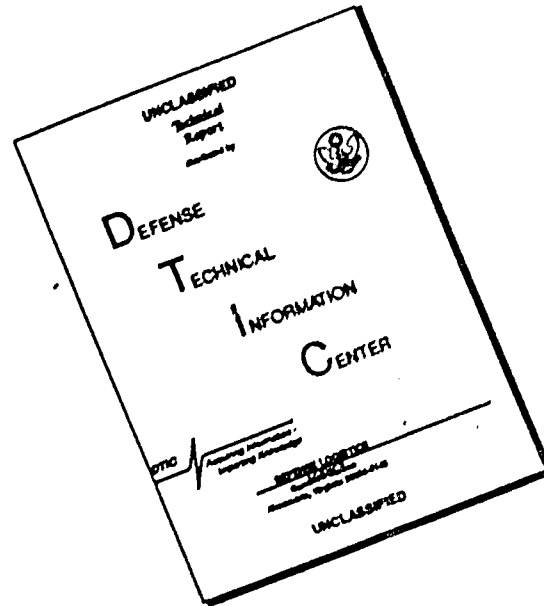
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A Comparison of the Toxicology of Triethylene Glycol Dinitrate and Propylene Glycol Dinitrate

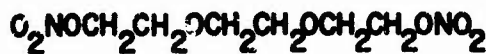
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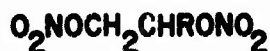
In most species the LD₅₀ of triethylene glycol dinitrate (TEGDN) is two to five times as high as that of propylene glycol dinitrate (PGDN). Both compounds produce methemoglobinemia and hypotension in rat. However, TEGDN-poisoned rats tremor violently and expire, apparently in respiratory arrest, after acute convulsive episodes. PGDN-poisoned rats are lethargic and do not convulse. *In vitro* TEGDN at concentrations above 1.5mM blocks nerve-stimulated contraction of a phrenic nerve-diaphragm preparation, and this interference with nerve-muscle communication appears to be the cause of the tremoring. Chronic daily dermal applications to rabbits of 21 mmoles/kg of both dinitrates cause death in 2 to 3 weeks. PGDN-treated rabbits gain weight normally, while those treated with TEGDN lose 20 to 30% of their body weight. Guinea pigs receiving as little as 100 mg/kg of TEGDN ip daily have decreased food intake and retarded weight gain. The toxic responses to these two dinitrates are therefore sufficiently different to preclude simple comparisons in hazard evaluations.

Introduction

ETHYLENE GLYCOL DINITRATE (EDGN), a major constituent of anti-freeze dynamite, has been implicated as the cause of sudden, fatal circulatory collapse among workers in plants manufacturing this



TEGDN



R=H, EDGN; R=CH₃, PGDN

Figure 1. The structures of several organic dinitrates.

The opinions expressed herein are those of the authors and do not necessarily reflect the view of the Navy Department or the naval service at large. These experiments were conducted according to the principles enunciated in "Guide for Laboratory Animal Facilities and Care" prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council, Washington, D.C.

explosive mixture.¹ Propylene glycol dinitrate (PGDN) has been suggested as a replacement for EDGN in these mixtures.² However, exposure to either EDGN or PGDN may cause severe, debilitating headaches, vasodilation, hypotension, and methemoglobin (met-Hb) formation.¹⁻³

Both EDGN and PGDN are derived by nitration from the corresponding vicinal diol. To our knowledge there are no reports in the literature describing the acute toxicity of organic dinitrates derived from nonvicinal diols. Triethylene glycol dinitrate (TEGDN) is such a compound (Figure 1). We describe in this paper the acute toxicity of TEGDN in several animal species, and compare it with the acute toxicity of PGDN. By several of the criteria studied, the toxicology of TEGDN was found to differ considerably from that of PGDN.

Materials and Methods

Dinitrates

The dinitrates used in these experiments are unstable in the pure state, so small amounts of a physiologically and biochemi-

cally inert diluent of low volatility were added.⁴ The compound used as the diluent has no overt toxic effect at fifty times the maximum dose used in this study. All doses reported herein are based on the dinitrate portion of the mixtures.

Animals

The animals used in toxicity studies were NMRI:O(SD) Sprague-Dawley-derived rats (250 to 300 gm); FTD:Hartley-derived guinea pigs (250 to 300 gm); New Zealand albino rabbits (2.3 to 3.7 kg); and NIH:NMRI mice (20 to 32 gm). Phrenic nerve-diaphragm preparations were excised from Long Evans rats weighing approximately 200 gm. Males were used unless otherwise specified. In mice and rats intravenous injections were made into either the dorsal tail vein or the cannulated femoral vein; in rabbits they were made into the marginal ear vein. Compounds administered subcutaneously were injected into the scruff of the neck.

For LD₅₀ determinations a series of doses in geometric proportion were given to animals in groups of six. From the resultant mortality within 24 hours, the LD₅₀ and its confidence interval were calculated by the method of Wiel.⁵

Blood pressures were determined by means of a pressure transducer implanted in the femoral artery of rats anesthetized with 50 mg/kg sodium pentobarbital ip.

Analyses

In instances where animals were sacrificed

by an overdose of pentobarbital, blood was drawn from the portal vein into syringes containing heparin. The blood was centrifuged at 5000 rpm for 10 minutes, and the supernatant was carefully decanted and recentrifuged at 15000 rpm for 15 minutes. This second supernatant was used as the routine plasma preparation in all experiments.

Plasma alkaline phosphatase, AP (EC 3.1.3.1), was determined by the method of Morgenstern *et al.*,⁶ while plasma activities of aspartate aminotransferase, AsT (EC 2.6.1.1.); creatine kinase, CK (EC 2.7.3.2); and lactic dehydrogenase, LD (EC 1.1.1.27); were assayed with Calbiochem reagent kits. The LD isoenzyme distributions in tissue homogenates and in plasma were determined by using an apparatus described by Nerenberg *et al.*,⁷ and staining for the bands of LD activity was accomplished with *p*-iodotetrazolium dye. The met-Hb levels, determined in whole blood by the method of Evelyn and Malloy,⁸ were calculated as percentage of the total hemoglobin converted.

Phrenic nerve-diaphragm preparations, prepared as described by Bülbring,⁹ were mounted and then immersed in a glucose-fortified Krebs-Henseleit solution¹⁰ containing various concentrations of the dissolved dinitrate. These preparations could be worked by applying a stimulus either to the nerve or directly to the muscle.

Results

The 24-hour LD₅₀ of TEGDN and PGDN were determined in several species by various routes of administration (Table I).

TABLE I
The 24-Hour LD₅₀ of TEGDN and PGDN in Various Animal Species

Species	Sex	Route of Administration	LD ₅₀ and 95% Confidence Limits (mg/kg)	
			TEGDN	PGDN
Mouse	M	ip	945 (792-1126)	1047 (778-1410)
Guinea Pig	M	ip	700 (586-835)	402 (322-502)
Rat	M	ip	796 (785-807)	479 (226-803)
Rat	M	po	1000 (733-1365)	250 (173-361)
Rat	M	sc	2520 (1720-3660)	530 (328-856)

These data show that in most species TEGDN is significantly less toxic than PGDN in these acute situations. The only exception was the mouse, for which the ip LD₅₀ of PGDN was greater than that of TEGDN.

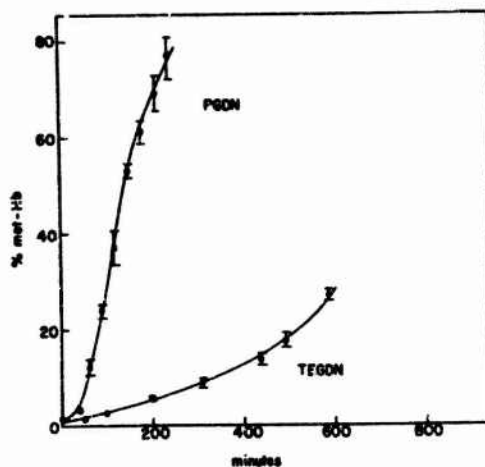


Figure 2. The effect of approximately equimolar doses of TEGDN and PGDN on the appearance of met-Hb in the rat *in vivo*. Four anesthetized fensale rats weighing 250 to 300 gm were injected with 12.4 mmoles/kg PGDN sc, and at intervals 0.1 ml of blood was withdrawn from the femoral vein for met-Hb analysis. Each point on the upper line represents the mean and standard error for met-Hb in these four rats. The lower line represents data obtained under similar experimental conditions, except that 13.0 mmoles/kg TEGDN was administered instead of PGDN.

Both these compounds produce met-Hb *in vivo*; Figure 2 shows the time courses of met-Hb formation in anesthetized rats following subcutaneous injection of approximately equimolar doses of PGDN and of TEGDN. As Andersen and Smith¹¹ found in studies on the mechanism of this oxidation *in vitro*, PGDN produces met-Hb much faster than does TEGDN.

Not only was there a considerable difference in the rates at which these compounds produce met-Hb, but the toxic signs caused by TEGDN differed considerably from those caused by PGDN² or by EGDN.¹² Rodents treated with lethal doses of PGDN, as reported previously by Clark and Litchfield,² are ataxic and lethargic and exhibit respiratory depression. Rodents similarly treated with TEGDN, in addition to the above signs, tremored violently and were hyperreactive to auditory and tactile stimuli. At death, TEGDN-treated rats had only half as much met-Hb (30 to 40%) as rats fatally poisoned with PGDN (70 to 85%).

A number of experiments were performed to determine which organ systems were affected by TEGDN. In the first of these, rats were injected with ip LD₅₀'s of either TEGDN or PGDN, and survivors were sacrificed in groups of three to six at 24-hour intervals. TEGDN treatment caused large,

TABLE II
Changes in Plasma Enzyme Activities in Rats Surviving Intraperitoneal Injection of a LD₅₀ of TEGDN or PGDN

Time (hours)	n	Enzyme Activity ^a				
		AsT	LD	CK	AP	
0	8	43 ± 3	42 ± 3	90 ± 15	83 ± 7	
24	TEGDN	5	205 ± 58	96 ± 53	318 ± 100	124 ± 8
	PGDN	4	53 ± 8	43 ± 8	101 ± 21	94 ± 20
48	TEGDN	4	261 ± 104	134 ± 38	375 ± 91	144 ± 12
	PGDN	4	47 ± 3	42 ± 2	205 ± 115	112 ± 12
72	TEGDN	4	90 ± 26	90 ± 30	268 ± 44	95 ± 15
	PGDN	4	35 ± 2	32 ± 11	140 ± 28	68 ± 8
96	TEGDN	3	41 ± 11	56 ± 12	246 ± 160	73 ± 26
	PGDN	6	36 ± 3	22 ± 4	135 ± 20	81 ± 9
168	TEGDN	3	26 ± 6	48 ± 20	101 ± 3	49 ± 14
	PGDN	3	24 ± 3	29 ± 3	130 ± 50	128 ± 5

^aResults are given as mean ± standard error, and values are in international units per liter at 30°C.

long-lasting increases in all the measured plasma enzyme activities (Table II), while PGDN treatment produced only moderate increases in CK and A^P. The LD isoenzyme distributions were determined in these plasma samples, as well as in homogenates of rat brain and of rat spinal cord. Although at 24 hours there is an increase in all five isoenzymes in the plasma of the TEGDN-treated animals, at 48 and 72 hours the increase is predominantly observed in isoenzymes 1 and 2, producing a pattern very similar to that of the spinal cord homogenate but unlike that of the whole brain homogenate.¹³

The importance of met-Hb in causing death from these dinitrates was estimated by pretreating fasted rats with 5 mg/kg of methylene blue, a dye which increases the efficiency of the met-Hb reductase system by acting as an intermediary between the reductase and the pyridine nucleotide couple.¹⁴ Rats were fasted overnight and then pretreated with either the dye or saline. Two hours later the animals received sc injections of the dinitrate. Following 2056 mg/kg PGDN the time to death in minutes ($\bar{x} \pm$ S.E. (*n*)) was 197 ± 10 (6) in the absence of methylene blue and 421 ± 50 (5) in the presence of the dye. With 4600 mg/kg TEGDN the corresponding times were 597 ± 125 (5) and 1582 ± 235 (5), respectively. In both cases these increases in the time to death were statistically significant ($p < 0.01$).

Since TEGDN-poisoning causes tremors, in contrast to clonic-tonic-type convulsions, we felt that TEGDN might have an effect on the peripheral nervous system. To test this we investigated the effect of TEGDN and PGDN on the *in vitro* phrenic nerve-diaphragm preparation.⁹ With greater than 1.5 mM TEGDN in the Krebs-Henseleit bathing solution the diaphragm did not contract when a stimulus was applied to the phrenic nerve, but it did contract when stimulated directly. However, with concentrations of PGDN above 4.0 mM both nerve- and muscle-

stimulated contractions of the diaphragm were abolished. All these effects were readily reversible, since washing the preparation with fresh buffer rapidly restored the contraction to its original strength.

In contrast to these previous studies which were concerned with the effects of large doses of TEGDN, a hypotensive response was observed at much lower levels. Upon an iv injection of 2.4 mg/kg TEGDN into an anesthetized rat, the mean blood pressure fell from 90 to 49 mm Hg immediately and slowly recovered over the next 15 minutes until it reached 96 mm. An hour later the rat received 0.8 mg/kg iv and the pressure fell from 86 to 59 mm Hg, recovering to 84 mm within 2 minutes.

Two groups of eleven rabbits received daily dermal application of 21 mmole/kg TEGDN or PGDN for 3 weeks. Nine of the eleven rabbits treated with TEGDN died, and the mean time to death was 17 days; six of eleven rabbits treated with PGDN died, and the mean time to death was 16 days.

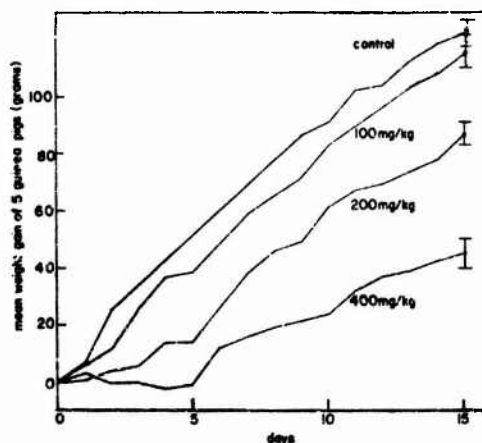


Figure 3. Mean weight gain of guinea pigs receiving daily ip injections of various doses of TEGDN. Four groups of five guinea pigs each were used. The groups received 0, 100, 200, and 400 mg/kg TEGDN daily ip. The animals weighed between 250 and 300 gm at the beginning of the experiment; the results are shown as the mean weight increase in grams for the five animals. The bars at the end of the curves are the standard errors for these determinations. The dose given to each group is indicated in the figure.

Differences in toxic signs were again noted for these two dinitrates. PGDN-treated rabbits gained approximately 10% of their original body weight during the 3 weeks and appeared normal until just prior to death. TEGDN-treated rabbits lost 20% of their starting body weight and appeared strikingly emaciated.

In an attempt to determine the cause of this diminished weight gain during extended TEGDN treatment, a feeding study was conducted in which TEGDN was administered ip to guinea pigs in groups of five at 0, 100, 200, and 400 mg/kg daily for 15 days. Figure 3 shows the mean weight gain for each group of animals, and Figure 4 shows the mean daily intake of dry chow. The results from the 100-mg/kg group have been omitted from the latter figure. The intake for the 100-mg/kg group was essentially identical with, but just below, the intake of the 200-mg/kg group on the first 2 days, and from the fourth day on it was essentially the same as in the controls.

Discussion

The physiological responses to TEGDN administration in experimental animals are not entirely unlike those observed after ad-

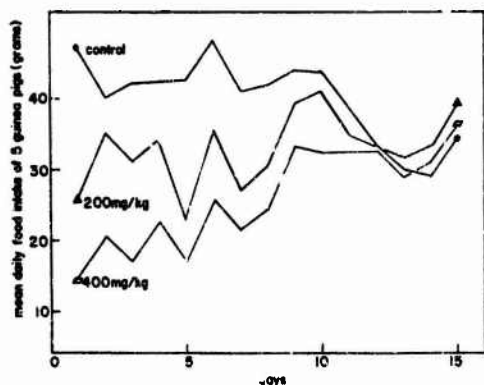


Figure 4. Mean food intake for guinea pigs receiving daily ip injections of various doses of TEGDN. These data, collected on the animals described in the legend to Figure 3, represent the mean daily intake of dry chow. The dose given to each group is indicated in the figure. The data for the group that received 100 mg/kg are not included but are discussed in the text.

ministration of PGDN.² TEGDN in small doses causes hypotension; since the mechanism of headache production by organic polynitrates is proposed to be the nonspecific relaxation of vascular smooth muscle in the meninges,¹⁵ TEGDN should be capable of producing headaches. TEGDN also causes met-Hb formation. Clark and Litchfield² noted previously that PGDN injected sc in lethal doses in the rat gave almost complete conversion of hemoglobin to met-Hb and suggested that methemoglobinemia may well be the cause of death in these rats. We have also observed this with PGDN. However, TEGDN in lethal doses gave at most 40 to 50% conversion, which should not of itself have been lethal. Pretreatment with methylene blue did protect against lethal doses of TEGDN, indicating that methemoglobinemia does, however, play some role in the deaths caused by TEGDN.

The severe tremoring, which was observed in rats receiving TEGDN, the plasma LD isoenzyme patterns, which 48 and 72 hours after the injection of an ip LD₅₀ of TEGDN resembled the isoenzyme pattern of a spinal cord homogenate, and the large elevation in CK activity after TEGDN administration (Table II), all suggest a neurological toxicity for this dinitrate. These nervous system disturbances are most likely mediated by the intact dinitrate, since tremoring was observed when there was only 5% met-Hb, indicating that only very little denitration of the TEGDN had occurred.¹¹ Furthermore, the rat phrenic nerve-diaphragm experiments provide evidence that *in vitro* intact TEGDN at 1.5 mM does interfere with normal neurological function.

Andersen and Koppenhaver have determined the plasma concentration of TEGDN following ip administration of 1200 mg/kg of this compound.¹⁶ At the first sign of tremoring the concentration in mM \pm S.E. (*n*) was 0.84 ± 0.10 (5), and at death it was 1.23 ± 0.09 (6), concentrations very similar to those that *in vitro* blocked phrenic nerve-stimulated contractions of the dia-

phragm. These rats would then be expected to suffer from extreme respiratory distress. Such respiratory depression would be compounded by the increased met-Hb levels and by the increased O_2 requirement of the animals due to exertion during the severe tremors. This combination of factors, which appears to be the cause of death upon TEGDN poisoning, offers some explanation for the protection against the toxic effects of TEGDN by methylene blue pretreatment. Methylene blue, by decreasing the levels of met-Hb, permits better O_2 transport in these rats already suffering from respiratory depression due to the direct effects of the dinitrate on nervous transmission.

It should be noted that the tremors themselves may also arise from the interactions of TEGDN in the vicinity of the neuromuscular junction. Decanethonium, a drug which also blocks neuromuscular transmission¹⁷ and the structure of which is in certain aspects similar to that of TEGDN,¹⁶ also causes TEGDN-like tremors in rats before they expire in respiratory arrest. (Although PGDN did interfere with the normal behavior of the phrenic nerve-diaphragm preparation *in vitro*, no convulsive behavior has been observed in rats receiving up to 1200 mg/kg ip.)

The neurological signs following TEGDN administration are observed at doses greater than the LD_{50} of PGDN. Thus, TEGDN must be considered less acutely toxic than PGDN. In the mouse, however, this is not true. For this species, the ip LD_{50} of PGDN is 1047 (778 to 1410) mg/kg, which is more than double the ip LD_{50} of PGDN in the rat or the guinea pig. This decreased toxicity is probably due to the very active met-Hb reductase system of the mouse,¹⁸ offering better protection against methemoglobinemia.

The reaction between dinitrates and hemoglobin apparently proceeds through a step in which the heme is oxidized and the ester is converted to the mononitrate and nitrite.¹¹ Since intact TEGDN is apparently a neuro-

toxin, hemoglobin and the met-Hb reductase system together constitute a mechanism for the detoxification of TEGDN. In this regard TEGDN may prove considerably more toxic to humans than it is to rodents, since the rate at which human hemoglobin is oxidized by TEGDN is only one-seventh the rate at which rat hemoglobin is oxidized.¹¹

In the chronic studies with the guinea pigs, the cause of the decreased weight gain (Figure 3) appeared to be a dose-related decrease in food intake (Figure 4). Interestingly, the guinea pigs seemed to develop a tolerance to the dinitrate, and their food intake returned to normal after a length of time which also appeared to be dose-related (Figure 4). Once the food intake of the four groups was nearly equivalent, the weight gain was approximately independent of the dose of TEGDN (that is, the slopes of the lines in Figure 2 at the end of the curves are nearly identical). The development of tolerance to the side effects of organic nitrates used medicinally for the treatment of angina is, of course, well known.¹⁹

In summary, the nonvicinal dinitrate TEGDN causes several toxic effects in experimental animals, such as met-Hb formation and hypotension, which are also caused by EGDN¹² and PGDN,² and several other toxic effects, such as the emaciation in the chronically treated rabbits and the nervous system toxicity, which are not observed with the vicinal dinitrates. In acute intoxication PGDN causes death by methemoglobinemia.² With TEGDN, however, death is apparently caused by a combination of respiratory depression, tremoring, and methemoglobinemia. Assessing the relative safety of TEGDN versus these more commonly used dinitrates is difficult, then, as we must deal with materials whose toxic effects are quite different in spite of their chemical similarities.

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