Comparative Toxicity of Prop(en)yl Disulfides Derived from Alliaceae: Possible Involvement of 1-Propenyl Disulfides in Onion-Induced Hemolytic Anemia

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Hemolytic anemia following ingestion of onions has been reported in a number of animal species. This toxic effect has been attributed to dipropyl disulfide, which, together with 1-propenyl and 2-propenyl disulfides, may be formed from the *S*-alk(en)ylcysteine sulfoxides present in these vegetables. In the present study, the toxic effects of dipropyl disulfide have been compared with those of di(1-propenyl) disulfide and di(2-propenyl) disulfide in rats. Both unsaturated compounds were more powerful hemolytic agents than the saturated disulfide, with di(1-propenyl) disulfide being particularly toxic. The comparatively high level of *S*-(1-propenyl)cysteine sulfoxide in onions suggests that 1-propenyl disulfides may make a significant contribution to the hemolytic action of these vegetables. Apart from their natural formation in onions, some 1-propenyl disulfides are approved as artificial flavors for food use. In view of the potent hemolytic activity of di(1-propenyl) disulfide (and, by implication, other disulfides of this type) extension of such use should be viewed with caution.

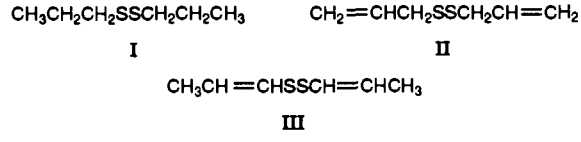
INTRODUCTION

Onions, garlic, leeks, shallots, and other plants of the *Allium* family have been cultivated for food since earliest times (Fenwick and Hanley, 1985a), being particularly valued for their pronounced and characteristic sensory properties. Consumption of these plants has also been considered to be beneficial to health, and they have been recommended as cures or preventatives for a wide variety of illnesses (Abdullah et al., 1988). Some of the reported beneficial effects of onions have been validated in controlled experiments (Carson, 1987), and while others are apocryphal, there is no evidence that consumption of onions by man is in any way harmful.

Humans are not, however, the only species to enjoy the taste of such plants. Farm animals eat them with relish, often to the total exclusion of other feed offered; in this situation, consumption of onions is certainly harmful, since it leads to a life-threatening hemolytic anemia. This phenomenon was first reported in onion-fed cattle in 1909 (Goldsmith, 1909); hemolysis has subsequently been described in cattle, horses, and sheep fed cull onions (Thorp and Harshfield, 1939; Koger, 1956; Kirk and Bulgin, 1979; Gill and Sergeant, 1981) and in animals fed onion-containing vegetable waste (Hutchison, 1977) or grazed on pastures containing wild onions (Pierce et al., 1972). Onion-induced hemolysis has also been reported in cats and dogs (Gruhzit, 1931a; Kalser et al., 1951; Kobayashi, 1981; Harvey and Rackear, 1985; Yamoto and Maede, 1992). Although these animals do not readily accept a diet containing onions, they appear to be particularly vulnerable to the toxic effects of the latter, with a number of clinical cases of hemolytic anemia being recorded in pets fed quite small amounts of these vegetables (Spice, 1976; Stallbaumer, 1981; Kobayashi, 1981; Smith and Ellison, 1986). In experimental studies, hemolysis has been induced in chickens (Baldissera Nordio, 1952a), rabbits (Baldissera Nordio, 1952b), rats (Majori and Squeri, 1954), and guinea pigs (Majori and Squeri, 1954) fed on onions or dosed with onion juice. These species are much less susceptible than domestic animals, however, and large quantities of onions or juice are required. In all species that have been examined, the hemolysis is associated with Heinz body production within the erythrocytes (Pierce et al., 1972; Hutchison, 1977; Kobayashi, 1981; Harvey and Rackear, 1985). The precipitation of these granules of oxidatively denatured hemoglobin indicates a role for oxidative damage in the toxic mechanism (Harley and Mauer, 1961).

Plants of the *Allium* family contain methyl- and prop(en)ylcysteine sulfoxides. When the plant tissue is disrupted, these substances are hydrolyzed to thiosulfinates, which are the primary flavor compounds of these vegetables (Carson, 1987; Fenwick and Hanley, 1985b; Lancaster and Boland, 1990; Block, 1992). On heating, thiosulfinates decompose to a mixture of compounds, among which mono-, di-, and trisulfides predominate (Fenwick and Hanley, 1985b; Carson, 1987; Block, 1990, 1993), and many compounds of this nature have been detected in, for example, steam-distilled onion oil (Boelens et al., 1971). Although the metabolism of thiosulfinates *in vivo* has not been studied in detail, there is evidence that similar decomposition pathways may be followed; disulfides have been detected in animals after ingestion of *Allium* species or *S*-alk(en)ylcysteine sulfoxides (Smith, 1978; Earl and Smith, 1982; Harris et al., 1986). In view of the known hemolytic activity of certain disulfides [for a review, see Munday (1989)], it is possible that such substances, formed from the naturally occurring sulfur compounds in onion, are involved in the toxic action of these vegetables.

Prop(en)ylcysteine sulfoxides predominate in Alliaceae (Lancaster and Boland, 1990), and the proportion of prop(en)yl thiosulfinates in comminuted samples is correspondingly high (Block et al., 1992). On decomposition, these would yield, among other compounds, prop(en)yl disulfides, as typified by the symmetrical derivatives dipropyl disulfide (**I**), di(2-propenyl) disulfide (**II**), and di(1-propenyl) disulfide (**III**). In early studies, dipropyl



I II

III

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Table 1. Splenic Darkening, Relative Splenic Weights, and Hematology of Rats Dosed with Prop(en)yl Disulfides

disulfide	dose level (μmol kg^{-1} day^{-1})	splenic darkening[a]	splenic wt (g/100 g of body wt)	packed cell vol (%)	hemoglobin level (g/L)	Heinz bodies (ΔOD_{700})
none (control)		0.0 ± 0.0	0.24 ± 0.01	41.0 ± 0.8	139 ± 3	0.027 + 0.003
dipropyl	250	0.0 ± 0.0	0.24 ± 0.01	41.4 ± 0.9	140 ± 3	0.024 ± 0.004
di(2-propenyl)	250	0.0 ± 0.0	0.25 ± 0.01	39.4 ± 0.5	133 ± 2	0.029 ± 0.002
di(1-propenyl)	250	5.0 ± 0.0***	0.62 ± 0.05***	27.9 ± 1.0***	103 ± 4***	0.703 ± 0.049***
dipropyl	1000	0.1 ± 0.1	0.26 ± 0.02	38.8 ± 1.0	133 ± 4	0.029 ± 0.004
di(2-propenyl)	1000	1.7 ± 0.3***	0.31 ± 0.01***	36.1 ± 0.4***	117 ± 2***	0.024 ± 0.005
dipropyl	5000	5.0 ± 0.0***	0.51 ± 0.04***	27.6 ± 0.7***	111 ± 3***	0.747 ± 0.065***

[a] Splenic darkening was scored on an arbitrary scale of 0–5, as indicated under Materials and Methods. Values shown are means ± SEM; those marked with asterisks differ significantly (Student's t-test) from the control: ***, $P < 0.001$.

disulfide was shown to cause hemolysis in dogs (Gruhzit, 1931b; Williams et al., 1941), and it has generally been accepted that this is the substance responsible for the toxic effect of onions [see, for example, Fenwick and Hanley (1986)]. The toxic effects of the propenyl derivatives have not, however, been investigated.

In the experiments described in the present paper, the hemolytic activity of di(1-propenyl) and di(2-propenyl) disulfide has been determined in rats and compared with that of dipropyl disulfide.

MATERIALS AND METHODS

Chemicals. Dipropyl disulfide was synthesized according to the method of Drabowicz and Mikolajczyk (1980), di(2-propenyl) disulfide according to the method of Hase and Peräkylä (1982), and di(1-propenyl) disulfide according to the method of Wijers et al. (1969). The identity of these compounds was confirmed by infrared spectroscopy and high-resolution mass spectrometry.

Animals and Dosing. Female rats (10–11 weeks old) from the Ruakura colony of Sprague-Dawley-derived animals were randomly allocated to treatment groups. The animals were housed in solid-bottom cages containing bedding of wood shavings and were allowed food (NRM Feeds Ltd., Hamilton) and water *ad libitum*. The environment was maintained at 21–24 °C, with an artificially controlled light/dark cycle.

The test compounds were administered, as solutions in peanut oil, to groups of six rats by oral intubation on 6 consecutive days. A control group of 12 rats received peanut oil alone.

On the seventh day of the experiment, the rats were anesthetized with halothane and killed by exsanguination, blood being taken into EDTA-containing tubes from the posterior vena cava. At necropsy, splenic darkening was assessed visually on an arbitrary scale of 0–5. Splenic weights were recorded and expressed as a percentage of body weight. The spleen, kidneys, and a portion of the liver of each animal were fixed in 4% buffered formaldehyde, and paraffin wax sections were stained with hematoxylin and eosin and by Perls' Prussian Blue reaction for examination by light microscopy. Sections were examined without reference to treatment group. Splenic sinusoidal engorgement, focal hepatic erythropoiesis, and iron deposition in the spleen, liver, and kidney (as reflected by the intensity of Prussian Blue staining) were scored on an arbitrary scale of 0–5, 0 indicating absence of the specified histological change and 5 its presence to a severe degree.

The packed cell volume of each blood sample was determined by the microhematocrit technique, while the hemoglobin level was assessed using the cyanmethemoglobin method (Evelyn and Malloy, 1938). A quantitative assessment of intraerythrocytic Heinz bodies was made by the turbidometric method of Winterbourn (1979), in which the number and size of Heinz bodies in the cells are reflected by the magnitude of the decrease in optical density of red cell lysates at 700 nm following centrifugation.

RESULTS

All rats remained in good health throughout the experimental period, and no clinical signs of toxicity were observed.

At necropsy, pronounced splenic enlargement and darkening was recorded in animals dosed with di(1-propenyl) disulfide at 250 μmol kg^{-1} day^{-1} and with dipropyl disulfide at 5000 μmol kg^{-1} day^{-1}. Marked decreases in blood packed cell volumes and hemoglobin levels, associated with Heinz body formation, were observed in these rats (Table 1). Some splenic enlargement and darkening, associated with comparatively small decreases in packed cell volume and hemoglobin level, were seen in rats receiving di(2-propenyl) disulfide at 1000 μmol kg^{-1} day^{-1}, although no changes were recorded in animals dosed with this substance at 250 μmol kg^{-1} day^{-1} or with the dipropyl derivative at 250 or 1000 μmol kg^{-1} day^{-1} (Table 1).

Histological examination of sections of spleen, liver, and kidney revealed no degenerative changes in any of the animals of the present experiment. Pronounced splenic sinusoidal engorgement was observed, however, in rats dosed with di(1-propenyl) disulfide and with dipropyl disulfide at the highest dose level. Foci of erythropoietic activity were also identified in the livers of these animals, together with marked splenic, hepatic, and renal deposition of iron. Increased iron levels were also recorded in rats receiving di(2-propenyl) disulfide at 1000 μmol kg^{-1} day^{-1}, although no histological changes were observed in animals dosed with this substance at 250 μmol kg^{-1} day^{-1} or with dipropyl disulfide at 250 or 1000 μmol kg^{-1} day^{-1} (Table 2).

DISCUSSION

The hemolytic activity of dipropyl disulfide observed in the present investigation is consistent with earlier findings (Gruhzit, 1931b; Williams et al., 1941). At 5000 μmol kg^{-1} day^{-1}, this substance caused severe hemolytic anemia in rats, as reflected by marked decreases in blood packed cell volumes and hemoglobin levels. The histological and organ weight changes observed in these animals are also consistent with a specific toxic effect upon erythrocytes. No degenerative changes were recorded in spleen, liver, or kidney, but destruction of red blood cells is indicated by deposition of iron in these organs, this metal being released from erythrocytes following phagocytosis (Munday et al., 1990). The foci of erythropoietic activity observed in the liver and the sinusoidal engorgement of the spleen, associated with enlargement and darkening of this organ, reflect compensatory erythropoiesis following destruction of red blood cells (Munday et al., 1990).

The oxidative nature of the hemolysis induced by this substance, as indicated by Heinz body formation, is consistent with the mechanism of toxicity proposed for substances of this type, in which intraerythrocytic redox cycling of disulfides leads to formation of "active oxygen" species (Munday, 1989). By thiol–disulfide exchange with

Table 2. Splenic, Hepatic, and Renal Histology of Rats Dosed with Prop(en)yl Disulfides

disulfide	dose level (μmol kg^{-1} day^{-1})	spleen[a] sinusoidal engorgement	spleen[a] iron deposition	liver[a] erythropoietic foci	liver[a] iron deposition	kidney[a] iron deposition
none (control)		0.0 ± 0.0	1.0 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
dipropyl	250	0.0 ± 0.0	0.9 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
di(2-propenyl)	250	0.0 ± 0.0	0.8 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	0.2 ± 0.1
di(1-propenyl)	250	3.5 ± 0.4***	2.7 ± 0.3***	1.1 ± 0.2***	1.2 ± 0.2***	0.9 ± 0.2***
dipropyl	1000	0.0 ± 0.0	1.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0
di(2-propenyl)	1000	0.0 ± 0.0	1.4 ± 0.2*	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1*
dipropyl	5000	2.8 ± 0.3***	2.0 ± 0.2***	0.8 ± 0.3**	1.1 ± 0.2***	1.3 ± 0.2***

[a] Splenic sinusoidal engorgement and hepatic focal erythropoiesis were assessed in sections stained with hematoxylin and eosin, iron deposition in sections stained by Perls' Prussian Blue reaction. These parameters were scored on an arbitrary scale of 0–5, as indicated under Materials and Methods. Values shown are means ± SEM; those marked with asterisks differ significantly (Student's t-test) from the control: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

glutathione, the disulfide is reduced to the corresponding thiol (reaction 1). Although thiols are generally recognized

$$2GSH + RSSR \rightleftharpoons GSSG + 2RSH \quad (1)$$

$$RSH \rightleftharpoons RS^- + H^+ \quad (2)$$

$$(Hb)Fe^{III}O_2^{\bullet -} + RS^- + 2H^+ \rightarrow (Hb)Fe^{III} + RS^\bullet + H_2O_2 \quad (3)$$

$$RS^\bullet + RS^- \rightleftharpoons (RSSR)^{\bullet -} \quad (4)$$

$$(RSSR)^{\bullet -} + O_2 \rightarrow RSSR + O_2^{\bullet -} \quad (5)$$

$$RSH + O_2^{\bullet -} + H^+ \rightarrow RS^\bullet + H_2O_2 \quad (6)$$

as antioxidants, they act as pro-oxidants after one-electron oxidation to the thiyl radical (Munday, 1994). In the erythrocyte, such oxidation is mediated by oxyhemoglobin (reaction 3), and hydrogen peroxide formed in this reaction, together with more active oxygen species formed in radical chain reactions of the thiyl radical (reactions 4–6), is held responsible for initiating the cellular damage which leads ultimately to hemolysis (Munday, 1989).

Di(2-propenyl) disulfide was found to be a stronger hemolytic agent than dipropyl disulfide, causing toxic change at 1000 μmol kg^{-1} day^{-1}, a dose at which the saturated compound was without effect. By far the most toxic member of the group, however, was di(1-propenyl) disulfide, which, at 250 μmol kg^{-1} day^{-1}, produced hemolytic anemia as severe as that provoked by dipropyl disulfide at 5000 μmol kg^{-1} day^{-1}.

The comparative hemolytic activity of the prop(en)yl disulfides can be explained in terms of the relative rates of the reactions leading to active oxygen production. Reactions 3 and 4 involve the thiolate anion; their rates will therefore be critically dependent upon the pK_a of the thiol. The pK_a of propanethiol is 10.8 (Yabroff, 1940). This substance will not be extensively ionized at physiological pH, and reactions 3 and 4 will therefore be slow. Faster reactions are anticipated with 2-propenethiol, in which the inductive effect of the double bond lowers the pK_a to 9.9 (Kreevoy et al., 1960). The lowest pK_a of the series is, however, to be expected with 1-propenethiol, since the anion will be stabilized by charge delocalization. Furthermore, the radical of this compound will similarly be resonance-stabilized, thus facilitating electron transfer from the thiolate anion to metal (reaction 3). In this respect, 1-propenethiol resembles aromatic thiols which rapidly form active oxygen species through hemoglobin-initiated redox cycling and which are powerful hemolytic agents (Munday, 1985).

As discussed previously, alk(en)ylcysteine sulfoxides may be metabolized to disulfides *in vivo*, raising the possibility that such compounds are the agents responsible for the hemolytic action of onions. Dipropyl disulfide was previously suggested (Gruhzit, 1931b; Williams et al., 1941) to be the causative agent, but in view of the high toxicity of di(1-propenyl) disulfide and the preponderance of *S*-(1-propenyl)cysteine sulfoxide in onions (Lancaster and Kelly, 1983), 1-propenyl derivatives are more likely to be responsible.

A notable feature of onion-induced hemolysis is its species selectivity. The vulnerability of farm animals may simply reflect the appetite of these animals for onions; sheep are reported to eat as much as 9 kg in a single day (Kirk and Bulgin, 1979). Dogs and cats, however, appear to be genuinely sensitive to onion toxicity, with very much lower amounts being required to cause an effect in these animals than in, for example, rats. This may be attributable to the comparatively weak defenses of the erythrocytes of dogs and cats against oxidative stress, and these animals are sensitive to hemolysis induced by other oxidizing agents (Fertman and Fertman, 1955; Harvey and Kaneko, 1977). In general, human erythrocytes are resistant to oxidative damage. Within particular ethnic groups, however, many individuals are susceptible to such damage due to hereditary deficiencies of erythrocytic antioxidant enzymes, notably glucose-6-phosphate dehydrogenase (Keller, 1971). Although hemolysis occurs in such individuals following exposure to many other oxidant compounds, no cases of onion toxicity have been reported. It is possible that the comparatively small amounts of onions normally consumed by humans may provide insufficient amounts of toxic material for a clinically appreciable effect. A detailed prospective study of the possible effects of onions in man would be valuable, however, particularly in view of the growing interest in the potential therapeutic benefits of plants of the *Allium* family (Abdullah et al., 1988; Carson, 1987).

Also of some concern is that 1-propenyl propyl disulfide and methyl propenyl disulfide are permitted food additives (Hall and Oser, 1970; Oser and Ford, 1978). By use of such compounds, the natural ratio of propyl to 1-propenyl in onion-containing foodstuffs may be altered and hemolytic potential thereby increased. Again, the levels permitted to be used are small, but a possible impact on sensitive individuals or those with an unusually high intake of such foodstuffs should be considered.

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