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THE HEMOLYTIC ACTIVITY OF CITRAL: EVIDENCE FOR FREE RADICAL PARTICIPATION

I. TAMIR*, A. ABRAMOVICI*, I. MILO-GOLDZWEIG† and R. SEGAL†

*Laboratory of Developmental Pathology, Department of Pathology, Sackler School of Medicine, Tel-Aviv University and Belinson Hospital, Petah-Tiqua, Israel

and

†Department of Natural Products, School of Pharmacy, The Hebrew University, Jerusalem, Israel

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Abstract—In the present investigation the hemolytic properties of citral were examined. Tests with different concentrations of citral showed that hemolysis of rat erythrocytes commenced after a lag period the length of which depended on the concentration of the hemolysin and tended to 100% hemolysis. Comparison of the characteristics of the hemolysis induced by high and low citral concentrations, indicated that two mechanisms are involved—a non specific steroid—terpenoid or glutathione depletion mechanism dominating at high citral concentrations and a free radical mechanism dominating at low citral concentrations. Experiments performed with various free radical scavengers indicate that $^1O_2$ might be involved.

Citral (3, 7 dimethyl – 2, 6 octadienal) I is one of the dominant constituents of lemon oil. Having an agreeable scent and taste it is widely used in the cosmetic and food industry [1, 2]. In recent studies it was observed that this monoterpene possesses important teratogenic effects on chick embryo [3–6]. Morphological evidences [6] were suggestive for altered membrane functions, most probably induced by the $\alpha\beta$-unsaturated carbonyl group of citral or its analogues [7]. Such a reactive group might induce cellular injuries mainly affecting the membrane through a lipid peroxidative mechanism [8].

Since free radicals give rise to lipid peroxidation either by themselves or by secondarily produced oxygen free radicals, this feature might be used as an indicator for their existence [9–13].

One of the methods for testing lipid peroxidation makes use of the red blood cell in which all of the lipid is located in the stroma [9–15]. The free radical oxidation of the unsaturated fatty acids in the membrane ultimately results in the disruption of the cell, i.e. in hemolysis. Moreover, red blood cells are readily available and simple to handle and the hemolysis can be easily observed and its extent evaluated. The present work is an attempt to study the hemolytic properties of citral and some of its analogues under various experimental conditions in order to understand the deleterious effects of citral on biological membranes.

MATERIALS AND METHODS

Materials. All compounds were pure grade chemicals. Citral (I) (*cis* and *trans*) from Fluka A.G. Switzerland, geraniol (II), citronellal (III) and hydroxycitronellal (IV) from Eastman Org. Chem. Rochester, NY. The compounds were dissolved in propylene glycol (propane – 1, 2-diol) 2.5 mg/ml. These solutions were diluted with 6 ml isotonic phosphate buffer pH 7.4 [16] to a concentration of 2.4×10^{-3} M. Aliquotes of these solutions were used. Superoxide dismutase (SOD) 2560 U/mg protein, catalase, dialysed against water, specific activity 1.57×10^6 U/ml; tryptophan; histidine; EDTA (ethylenediaminetetra-acetic acid); and DTPA (diethylenetriaminepenta-acetic acid) were from Sigma Chemical Company, St. Louis, MO, U.S.A.; vitamin E, D-alpha-tocopherylacetate N.F. Type 6–100, from Distillation Product Industry, Division of Eastman Kodak Co. Rochester, NY.

Hemolysis tests. All experiments were performed on fresh, citrated blood drawn from male albino rats of the Hebrew University "Sabra" strain. The erythrocytes were freed from plasma by three washings with cold isotonic saline. A suspension 1% of erythrocytes in phosphate buffer was prepared as described [17]. The test solutions consisted of erythrocytes suspension 2 ml, various quantities of hemolysin solutions and buffer to make up the volume to 4 ml. In the control propylene glycol-buffer (1:6 v/v) was added instead of the hemolysin solution. The components were added in the following order: erythrocyte suspension, buffer, hemolysin solution. The mixtures were incubated in a shaking bath at 37° and aliquotes (0.5 ml) were removed after various periods and centrifuged at 1000 *g* for 10 min. The optical density of the supernatant was determined at 540 nm and the percentage of hemolysis calculated by comparison with a sample in which complete hemolysis was obtained by addition of some grains of digitonin. All the experiments were conducted in day light if not otherwise stated.

Reversibility tests. Hemolysis tests as described above with the varying concentrations of citral were

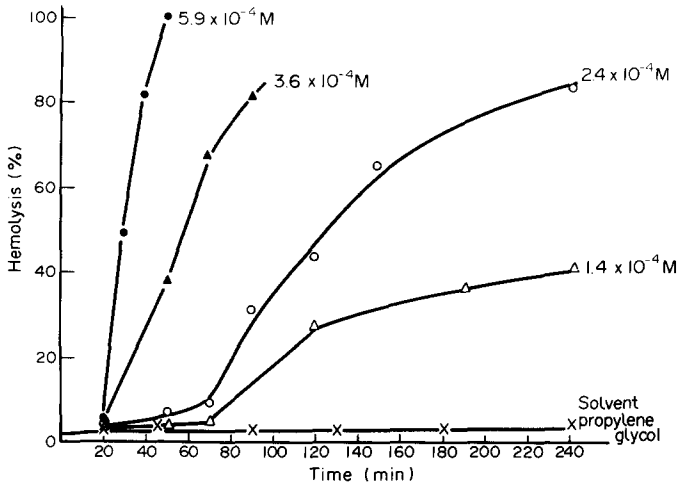


Fig. 1. Time dependence of citral induced hemolysis at various citral concentrations.

performed, incubated for various periods, centrifuged, the supernatant separated and its optical density determined. The precipitated erythrocytes from the incubation mixtures were washed once with cold buffer, then resuspended in buffer to yield the original volume and reincubated at 37°. The supernatant was added to the same quantity of erythrocytes preciptated from the suspension. The course of hemolysis in both was followed.

Effect of inhibitors on citral induced hemolysis. Two sets of experiments were run in parallel: (a) a regular hemolysis test, (b) same incubation mixture as in (a) but including one of the inhibitor solutions in buffer and in case of vitamin E in isopropyl alcohol [17]. The components were added in the following order: erythrocytes, buffer, inhibitor, hemolysin. The percentage of inhibition was determined as described [18].

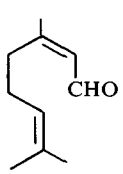
Determinations of percentage hemolysis were reproducible to within 3 percent hemolysis. All hemolytic values recorded represent mean values of four determinations.

RESULTS

The hemolytic activity of the four monoterpenes; citral (I), geraniol (II), citronellal (III) and hydroxycitronellal (IV) was tested. Because of their insolubility in water the compounds were dissolved in a mixture of propylene glycol buffer 1:6 (v/v) which by itself did not give rise to hemolysis. The experiments which were performed at various concentrations of hemolysins showed that only citral was hemolytically active. The maximal concentration tested was 1.2×10^{-3} M at which only citral gave hemolysis (100%).

Time dependence of citral hemolysis. The hemolytic activity of citral as a function of time was tested at different concentrations (Fig. 1). The four curves are characterized by a lag period the length of which depends on the citral concentration. At higher concentrations ($\geq 3.6 \times 10^{-4}$ M) a constant lag of about 10 min was observed. These concentrations will be referred to as "high citral concentrations". Complete hemolysis was obtained only with high concentrations of citral. With lower concentrations partial hemolysis was observed after four hours of incubation. The experiments could not be extended beyond this time period because of pronounced spontaneous hemolysis in the controls. The shape of these curves indicates, however, that they also tend to 100% hemolysis.

Reversibility of citral interaction with erythrocytes. The extent of reversibility depended on the time of incubation before citral was washed off the erythrocytes. When this was done within the first part (about a third) of the lag period, no hemolysis was observed. When the hemolysin was removed after longer periods of incubation, the process continued at a slower rate although no more citral was present in the incubation mixture (Fig. 2). Percentage of hemolysis could not be assessed accurately in these experiments since the number of intact erythrocytes changed during the procedure. The extent of hemolysis was therefore recorded by the optical density of the supernatant. It is noteworthy that the super-



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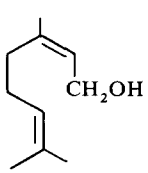

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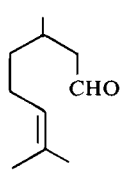

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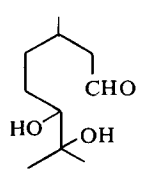

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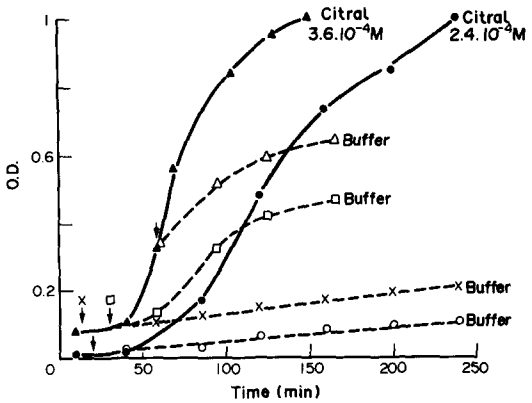

Fig. 2. Effect of citral removal from erythrocytes after different periods of incubation. The arrow indicates the time of hemolysin removal.

natant from the original incubation mixtures, even after very short periods of contact, i.e. 1–2 min did not induce hemolysis in fresh erythrocytes (results not reported).

In view of the similarity between the time dependence curves of hemolysis caused by citral and those obtained by other hemolysins acting by a free radical mechanism, i.e. hydrogen peroxide [19] and polyoxyethylene derived surfactants [20, 21], the influence of factors affecting this type of hemolysis was investigated.

Considering the fact that free radical reactions are often light catalysed, we tested whether citral hemolysis is promoted by visible light, by comparing the extent of hemolysis under normal experimental conditions and in darkness. These experiments were performed at various citral concentrations for different periods of incubation. At low citral concentrations a significant reduction in the hemolytic rates were observed in darkness, although no complete inhibition, was obtained (Figs. 3a and b). However, at high citral concentrations (5.9×10^{-4} M) at rapid progress of hemolysis, there was no difference between the extent of hemolysis either performed in light or darkness conditions (Fig. 3c).

The effect of oxygen on hemolysis induced by citral was tested by comparing the extent of hemolysis under a nitrogen atmosphere, with that obtained under standard experimental conditions. The hemolytic effect was found markedly reduced by anoxic conditions only at the lowest concentration of citral tested (Table 1). At higher concentrations, however, the amount of hemolysis was found identical for both aerobic and anaerobic environments. The data presented were obtained for one period of incubation only, since kinetic studies could not be carried out because of technical reasons.

Effect of inhibitors. The effect of various enzymes, antioxidants and free radical scavengers on citral hemolysis was tested. The hemolysis obtained after incubation of erythrocytes with citral was compared with that obtained by incubation with citral plus inhibitor. The results which are summarized in Tables 2 and 3 and Figs. 4 and 5 show that most of the compounds inhibited citral hemolysis, however, mainly at low citral concentrations, i.e. at slow progress of hemolysis. Mannitol which is a specific $OH^{\cdot}$ scavenger [9, 10] had no effect at low citral concentrations as well.

Hydrogen peroxide as well as superoxide radicals are known to give rise to tissue damage and lipid peroxidation [9, 10]. The possibility that these may be the active hemolysing factors was considered. Catalase and superoxide dismutase were therefore tested as potential inhibitors of citral hemolysis. The

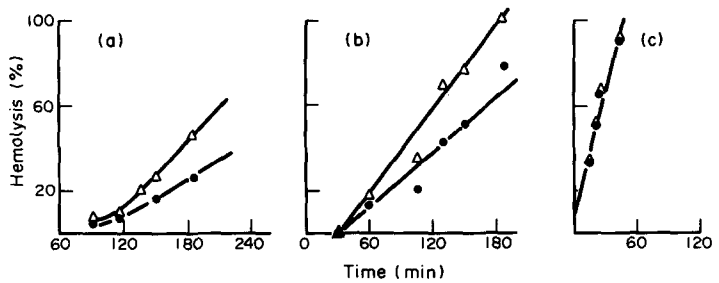

Fig. 3. Citral induced hemolysis in light △ and in darkness●. (a): 2.4×10^{-4} M citral (b): 3.6×10^{-4} M citral (c): 5.9×10^{-4} M citral.

Table 1. The effect of oxygen depletion on hemolysis induced by citral

Citral concentration	Time of incubation (min)	(%) Hemolysis in air	(%) Hemolysis under N_2 atmosphere
1.4×10^{-4}	180	45	16
2.4×10^{-4}	120	35	25
3.4×10^{-4}	110	88	88
5.9×10^{-4}	50	73	73

Table 2. The effect of superoxide dismutase and catalase on citral hemolysis

Inhibitor	Inhibitor concentration	Citral 3.6×10^{-4} M 60′ incubation (%) Hemolysis	(%) Inhibition [18]	Citral 1.3×10^{-4} M 250′ incubation (%) Hemolysis	(%) Inhibition [18]
—	—	55	—	30	—
SOD	137.5 U/ml	39	29	15	50
SOD	68.7 U/ml	44	20	15	50
SOD heat inactivated	137.5 U/ml	72	augmentation	85	augmentation
SOD heat inactivated	68.7 U/ml	72	augmentation	55	augmentation
Catalase	3177 U/ml	55	—	30	—
Catalase	1588 U/ml	50	—	30	—
Bovine serum albumin	0.05 mg/ml	13	76	18	67

results summarized in Table 2 show that catalase does not cause any inhibition thus excluding H_2O_2 as the active hemolysing agent involved. The inhibition obtained by superoxide dismutase is not significant since bovine serum albumin at the same protein concentration as the enzyme gives rise to inhibition as well.

DISCUSSION

Of the four monoterpenes tested for their hemolytic activity, citral was the only one which gave hemolysis and even to a significant extent at relatively low concentrations, i.e. 1×10^{-4} M. It was previously reported that citral produces malformations in chicken embryo [4–6]. This was attributed to the interaction of citral with the lipid constituents of embryonic cell membranes [7]. Since hemolysis is generally due to lesion of the red blood cell membrane the erythrocyte was chosen as a model for investigating the mechanism of citral toxicity.

The time dependence curves of citral hemolysis as documented in Fig. 1 show that in all cases a relatively long lag period was observed before hemolysis started. The length of this period depends on the citral concentration, but in all experiments—even at high hemolysin concentrations—a minimal lag of about 10 min is obtained.

During the first part of the lag period the citral can be removed from the erythrocytes without giving rise to hemolysis (Fig. 2). But after a short contact the process becomes irreversible and exchange of the citral solution with buffer no longer prevents hemolysis. The length of this period depends on the citral concentration. Since the supernatant did not induce hemolysis, even after short periods of contact, we must assume that the citral is rapidly absorbed by the erythrocytes so that its concentration in the supernatant is below the threshold concentration at which hemolysis can be observed. The fact that no hemolysis was obtained in erythrocytes which have been in short contact with citral may indicate that the first phase of citral absorption is reversible and only later becomes irreversible. Since our experiments did not differentiate between both sides of the red blood cell membrane no indication as to the side of attack of the hemolysing agent can be deduced.

Recently it was shown that hemolysis caused by saponins and sapogenins proceeds rapidly and reaches a maximum which depends solely on the hemolysin concentration [16, 21]. However the hemolysis caused by H_2O_2 and polyoxyethylene sur-

Table 3. The effect of inhibitors on citral hemolysis

Inhibitor	Inhibitor concentration	Citral 3.6×10^{-4} M 60′ incubation (%) Hemolysis	(%) Inhibition [18]	Citral 1.3×10^{-6} M 250′ incubation (%) Hemolysis	(%) Inhibition [18]
—	—	55	—	30	—
Mannitol	5×10^{-2}	55	—	30	—
Cystein	1×10^{-2}			6	78
Cystein	5×10^{-3}			13	56
Cystein	2.5×10^{-3}			20	33
Vitamin E	1.3×10^{-5}	41	25	20	33
EDTA	2.5×10^{-4}	24	57	9	70
Histidine	2.5×10^{-2}	27	50	0	100
Histidine	1.25×10^{-2}	38	30	5	84
DTPA	1.5×10^{-3}	4	92		
DTPA	1.0×10^{-3}	11	80		
DTPA	7.5×10^{-4}	17	70		
Copper sulphate	1.0×10^{-6}	70	augmentation	75	augmentation

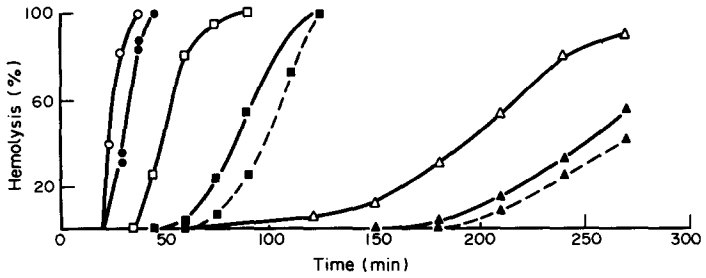

Fig. 4. The rate of citral induced hemolysis in the presence and absence of tryptophan. ○——○ 5.9×10^{-4} M citral; ●——● 5.9×10^{-4} M citral with 6.25×10^{-3} M tryptophan; □——□ 3.6×10^{-4} M citral; ■——■ 3.6×10^{-4} M citral with 6.25×10^{-3} M tryptophan; ■----■ 3.6×10^{-4} M citral with 1.25×10^{-2} M tryptophan; △——△ 2.4×10^{-4} M citral; ▲——▲ 2.4×10^{-4} M citral with 6.25×10^{-3} M tryptophan; ▲----▲ 2.4×10^{-4} M citral with 1.25×10^{-2} M tryptophan.

factants progresses slowly, but always tends to 100% independent of hemolysin concentration [19, 20]. It was suggested that a free radical mechanism is involved in the hemolysis caused by H_2O_2 and the polyoxyethylene surfactants. The similarity of the time dependence curves of hemolysis induced by low citral concentrations (Fig. 1) to the curves observed for the hemolysins acting by a free radical mechanism [17, 21] suggests that this same mechanism is responsible for citral induced hemolysis as well. The suggestion that free radicals are involved in low concentration citral hemolysis was substantiated by the fact that visible light which promotes free radical formation augments this hemolysis (Fig. 3a).

Free radical hemolysis is generally attributed to oxygen-containing free radicals which peroxidize the polyunsaturated fatty acids in the erythrocyte membrane [9, 22–24]. The inhibition of the low concentration citral hemolysis under an N_2 atmosphere (Table 1) and by various antioxidants, i.e. vitamin E, and cysteine (Table 3) supports this assumption.

Various specific inhibitors were tested to identify the active damaging free radical species. Since mannitol, a specific $OH^{\cdot}$ scavenger [9, 10], gave no inhibition, this species had to be eliminated. The fact that histidine (Table 3) and tryptophan (Fig. 4) cause considerable inhibition suggests that $O_2^{\cdot}$ may be involved [9, 10, 25].

Transition metals are known to promote free radical formation [26–31]. The marked inhibition by the chelating agents EDTA and DTPA substantiates the suggestion that free radicals are involved. Since histidine and tryptophan are known to give metal chelates the possibility that their inhibition is due to this capacity must be considered. However, the marked difference in the shapes of the time dependence curves of hemolysis in the presence of the two types of inhibitors (Figs. 4 and 5) greatly reduces this probability.

The augmentation observed for the denaturated superoxide dismutase can be attributed to the liberation of copper from the denaturated enzyme [32]. The addition of copper ions to the incubation mixture (in saline) indeed markedly enhanced citral hemolysis (Table 3).

The measurement of malonaldehyde production by the thiobarbituric acid method [33] is often used as an indicator for estimating the extent of lipid peroxidation. This method could not be applied for our systems, since citral being an aldehyde by itself, gives the same color reaction.

Compounds having $\alpha\beta$-unsaturated carbonyl functions are characterized by their tendency to give free radical reactions [34]. Of the monoterpenes tested citral (I) is the only one in which this function is present. It could therefore be anticipated that free radical hemolysis and considerable teratogenic effects would be demonstrated by this compound only.

Our results indicate that there exists a fundamental difference between the hemolysis induced by high and low citral concentrations. At high concentra-

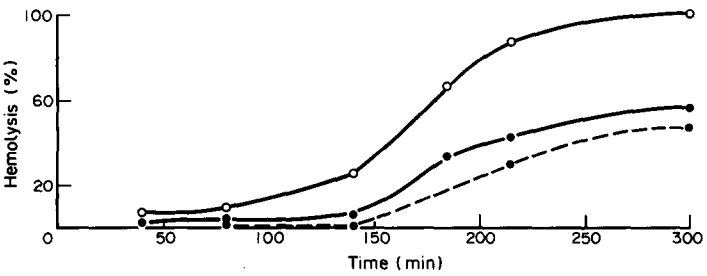

Fig. 5. The rate of citral induced hemolysis in the presence and absence of DTPA. ○——○ 2.4×10^{-4} M citral; ●---● 2.4×10^{-4} M citral with 1.5×10^{-3} M DTPA; ●——● 2.4×10^{-4} M citral with 2.5×10^{-4} M DPTA.

tions, i.e. at a rapid progress of hemolysis or only slight, inhibition was caused by the inhibitors tested, by oxygen depletion and by darkness (Table 1; Figs. 3 and 4). It is therefore suggested that citral hemolysis proceeds by a combination of two mechanisms: (a) an as yet unidentified mechanism, which can be due either to a non-specific steroid–terpenoid interaction with the membrane [20, 35] or to glutathione depletion by a 1,4 addition to the conjugated double bond [8]; (b) a free radical mechanism [17, 19, 20]. The relative contribution of each mechanism depends on the citral concentration applied. At high citral concentrations, the first mechanism predominates; at low concentrations, however, mainly free radicals are involved.

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