# Haemolytic Properties of Cereal 5-*n*-Alk(en)ylresorcinols

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The haemolytic activity of 5-*n*-alk(en)ylresorcinols is temperature dependent and correlated to their transition temperatures. The values of the parameters describing the 5-*n*-alk(en)ylresorcinol-induced red blood cell lysis indicate strong affinity of the compounds to the membrane and their high lytic capacity. The affinity of the compounds for the membrane decrease with the increasing quantity of the molecules incorporated into the erythrocyte membrane and is much higher for saturated resorcinols than unsaturated ones. The amount of 5-*n*-alk(en)ylresorcinol molecules bound to the membrane at a hundred percent lysis is about eighty times and eleven times (for alkyl and alkenyl derivatives respectively) higher than at zero percent lysis. Estimated free energy of erythrocyte lysis was similar for alkyl and alkenyl derivatives of resorcinol provided the preparation of the resorcinolic suspensions above their transition temperatures.

## Introduction

The 5-*n*-alk(en)ylresorcinols (1,3-dihydroxy-5-alk(en)ylbenzenes) with an odd number of carbon atoms in the aliphatic chain (13 to 27) occur in cereal grains and other graminaceous plants [1–4]. The cereal 5-*n*-alk(en)ylresorcinols consist mainly of saturated homologs with the variety-dependent composition [5–7], however significant amounts of homologs with unsaturated aliphatic chain were shown in rye grains [2, 7–9].

The role of 5-*n*-alk(en)ylresorcinols in the deleterious effect of rye on animals showed previously [2, 10, 11] is questionable [12–14]. Their meaning in the cereal biology and biochemistry is not known however the participation of 5-*n*-alk(en)ylresorcinols in grain antimicrobial resistance is proposed [3, 15].

The 5-*n*-alkyl- and 5-*n*-alkenyl-resorcinols isolated from rye (*Secale cereale* L.) grains showed strong interaction with proteins [16], ability for alteration of liposomal and erythrocyte membrane permeability [17] and also changes in bilayer structure [18, 19]. Previously it was found that the 5-*n*-alkenylresorcinols displayed strong haemolytic activity [17].

In this paper the further evaluation of haemolytic properties of 5-*n*-alk(en)ylresorcinols is presented which could allow the anticipation of their possible antibiotic and/or antinutritive activity.

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## Experimental

### *Materials*

The 5-*n*-alkylresorcinols and 5-*n*-alkenylresorcinols from rye (*Secale cereale* L.) grains were isolated with the methods described earlier [17, 20]. Erythrocytes were prepared from freshly obtained human blood (B, Rh+) by centrifugation at $1200 \times g$ and four times washing with isotonic buffered saline, pH 7.3.

### *Methods*

The changes of the erythrocyte suspension optical density under the 5-*n*-alk(en)ylresorcinols treatment were followed turbidimetrically at 600 nm in thermostated (±0.1 °C/cuvette/10 ml volume) with continuous stirring. The changes of the optical density were recorded with a pen chart recorder at a chart speed of 5 cm/min. In each experiment 25 µl of erythrocyte suspension (diluted with saline to 50% haematocrit) were injected rapidly into 6 ml of 145 mM NaCl-10 mM Tris-HCl, pH 7.3. After two minutes of preincubation the microliter amounts of ethanolic solutions of 5-*n*-alk(en)ylresorcinols were rapidly injected. The changes of the optical density were followed for two to four minutes.

The determination of the degree of erythrocyte haemolysis under 5-*n*-alk(en)ylresorcinols treatment was made by estimating spectrophotometrically the amount of haemoglobin released from the cells [17]. The suspensions of 5-*n*-alk(en)ylresorcinols in the

samples were prepared by injection of microliter amounts of agent's ethanolic solutions into 5 ml of phosphate buffered saline, pH 7.3, heated up to 40 °C (5-*n*-alkenylresorcinols) or 80 °C (5-*n*-alkylresorcinols). After the cooling of the suspensions to 37 °C, desired amounts or packed red cells were injected and throughly mixed. The incubation of the erythrocyte suspensions with the agents was carried out for 10 min. Each experiment was repeated 3–4 times with the experimental error below 5 percent.

For the description of the main characteristics of the 5-*n*-alk(en)ylresorcinol-induced haemolysis, namely, the affinity of their molecules for the cell membrane and the mean number of molecules which, upon interaction with an erythrocyte, make it undergo haemolysis, were determined. For the evaluation of these parameters the 5-*n*-alk(en)ylresorcinol concentrations providing different haemolysis degree at different concentrations of erythrocytes were studied. For the calculation of mentioned above parameters the following approach was used. This approach is based on the assumption that an individual cell undergoes lysis if and only if it adsorbes agent molecules in excess of a certain amount (not necessarily the same amount for different individual cells). If each cell comes into an adsorption equilibrium with its surroundings, than its uptake of the agent will be a function of the concentration of unadsorbed agent. When the free concentration of agent is $a_x$ where x is a given % haemolysis, the average amount of agent molecules adsorbed per cell has a corresponding value $b_x$. The total amount adsorbed by the cells is then $b_x \cdot N$ where $N$ is the cell count per unit volume of the system. This quantity and the free agent concentration $a_x$ make up the total amount of agent $C^x$ required to produce X% haemolysis:

$$C^x = a_x + b_x \cdot N .$$

This linear relationship between $C^x$ and $N$ has been shown for different types of water soluble detergent molecules [21–23]. The analogy between the transfer of agent molecules from water to the micelle interior and the transfer of agent molecules from water solution to an hydrophobic membrane environment suggests that the free concentration of agent $a_x$ remains constant at any concentration of cells in the system. It means that when the total agent concentration is increased above the value *"a"* it involves an increase in the amount of bound agent [23]. The free energy of transfer of the agent molecules from water to the membrane can be expressed as $\Delta G = RT \ln a$ and it provides a measure of the agent affinity for the membrane, $\Delta G_{aff}$. Since the parameter *"b"* include the total uptake of the agent molecules by the cells in the system, it can be used as a measure of the agent's lytic power [23, 24].

## Results and Discussion

In the previous article strong haemolytic activity of 5-*n*-alkenylresorcinols was shown. Saturated, 5-*n*-alkylresorcinols, in the same conditions exhibited only an increase of membrane permeability for ions and small solutes [17].

The temperature dependence of 5-*n*-alk(en)ylresorcinol-induced red blood cell lysis followed by the changes of erythrocyte suspension optical density (Fig. 1) indicates a significant differences between the derivatives with saturated and unsaturated aliphatic chains. The 5-*n*-alkenylresorcinols induced dramatical increase of the initial velocity of turbidity decrease above 30 °C whereas the 5-*n*-alkylresorcinols, even at three times higher concentration, exhibited lytic activity not till then above 40 °C. These observations are in good agreement to previously observed differences of the 5-*n*-alkyl- and 5-*n*-alkenyl-resorcinols thermal properties which

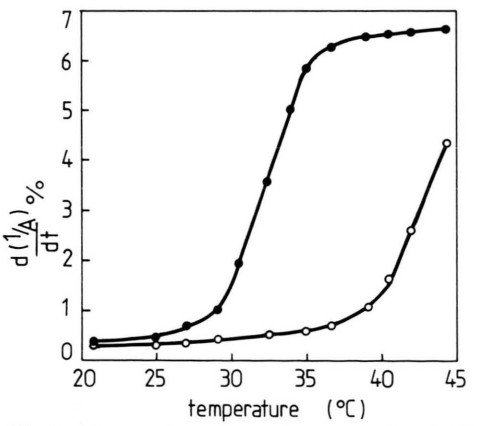


Fig. 1. Temperature dependence of the initial velocity decrease of human erythrocyte suspension turbidity in 145 mM NaCl 10 mM Tris-HCl, pH 7.3, in the presence of 5-*n*-alk(en)ylresorcinols. Concentration of 5-*n*-alkylresorcinols was 15 μM and 5-*n*-alkenylresorcinols – 3 μM. –○–○–○–○–○– 5-*n*-alkylresorcinols; –●–●–●–●–●– 5-*n*-alkenylresorcinols.

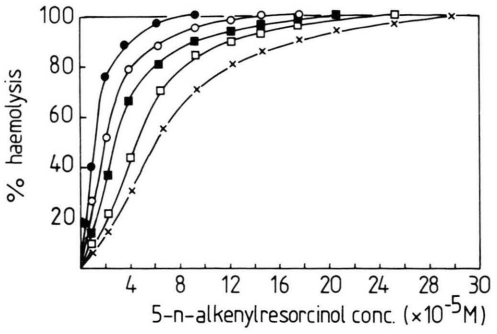


Fig. 2. Haemolysis of human erythrocytes by 5-*n*-alkenylresorcinols in phosphate-buffered isotonic saline, pH 7.3 at various red cell concentrations
–●–●–●–●–●– $0.7 \times 10^{11}$ cell/liter;
–○–○–○–○–○– $2 \times 10^{11}$ cell/liter;
–■–■–■–■–■– $3.2 \times 10^{11}$ cell/liter;
–□–□–□–□–□– $4.6 \times 10^{11}$ cell/liter;
–×–×–×–×–×– $7 \times 10^{11}$ cell/liter.

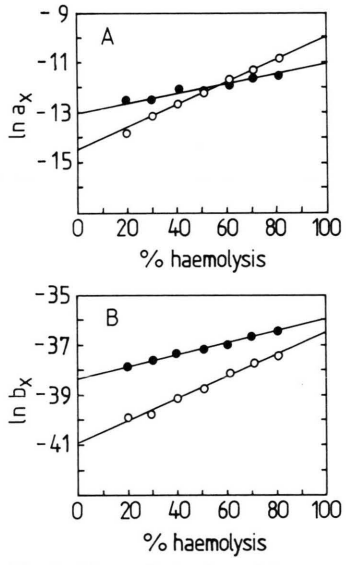


Fig. 3. Plots of the logarithms of parameters $a_x$ (*A*) and $b_x$ (*B*) at various haemolysis degree of human red cells lysed by 5-*n*-alk(en)ylresorcinols.
–○–○–○–○–○– 5-*n*-alkylresorcinols;
–●–●–●–●–●– 5-*n*-alkenylresorcinols.

were dependent on the saturation of the 5-*n*-alk(en)-ylresorcinol aliphatic chain [19]. For these reasons the comparison of saturated and unsaturated derivatives haemolytic activity in further experiments was performed with the use of the 5-*n*-alk(en)ylresorcinolic suspensions prepared above their transition temperatures.

Typical curves showing the degree of red blood cell haemolysis versus 5-*n*-alkenylresorcinol concentration at various concentration of erythrocytes are shown in Fig. 2. Similar curves were obtained for 5-*n*-alkylresorcinols. For the description of the process the $a_x$ and $b_x$ values for different degree of haemolysis were determined from the plots of 5-*n*-alk(en)ylresorcinol concentrations $C^x$ needed for various degree of haemolysis versus cell concentrations. For the determination of $a_0$, $b_0$ and $a_{100}$, $b_{100}$ values the extrapolation of obtained $a_x$ and $b_x$ values to zero and a hundred percent lysis degree using the least squares method from the relations $\ln a_x = f(X\%)$ and $\ln b_x = f(X\%)$ was used (Fig. 3).

The $a_0$, $b_0$ and $a_{100}$, $b_{100}$ values are listed in Table I together with the affinity of 5-*n*-alk(en)ylresorcinols for the human red cell membrane, $\Delta G_{aff}$, values calculated as above (Eqn. 2) from the *a* values. From the data given in Table I it appears that the affinity of 5-*n*-alk(en)ylresorcinols to erythrocyte membrane is high and resembles the activity of some detergents [24]. The high affinity of 5-*n*-alk(en)ylresorcinols showing $\Delta G_{aff}$ values in order of $-8$ kcal/mol can arise from their high lipophility. The low hydrophilic-lipophilic balance value for 5-*n*-alk(en)ylresorcinols (value found from the water-hexadecane partition was about 5.5; A. Kozubek – unpublished) and their ability to form a stable monomolecular layers at the air-water interface [19] also indicate high lipophility of the examinated compounds. The 5-*n*-alk(en)ylresorcinol affinity for the membrane decreases with the increasing quantity of the molecules incorporated

Table I. The affinity for the human red cell membrane (expressed as $a_0$, $a_{100}$ and related $\Delta G_{aff}$) and haemolytic capacity (expressed as $b_0$ and $b_{100}$) of 5-*n*-alk(en)ylresorcinols.

| Compound | $a_0$ [mol/l × $10^{-5}$] | $-\Delta G_{aff}$ [kcal/mol] | $a_{100}$ [mol/l × $10^{-5}$] | $-\Delta G_{aff}$ [kcal/mol] | $b_0$ [mol/cell × $10^{-16}$] | $b_{100}$ [mol/cell × $10^{-16}$] |
|---|---|---|---|---|---|---|
| 5-*n*-alkyl resorcinols | 0.05 | 8.9 | 5.0 | 6.1 | 0.017 | 1.4 |
| 5-*n*-alkenyl resorcinols | 0.19 | 8.1 | 1.9 | 6.7 | 0.23 | 2.5 |

Table II. The amount of agent's molecules per single cell and the area equivalent to one agent's molecule on the erythrocyte surface at zero and a hundred percent haemolysis of human red cells by 5-*n*-alk(en)ylresorcinols.

| Degree of haemolysis [%] | 5-*n*-Alkylresorcinols | | 5-*n*-Alkenylresorcinols | |
|---|---|---|---|---|
| | Amount of agent's molecules per single cell | Area equivalent to one agent's molecules [Å$^2$] | Amount of agent's molecules per single cell | Area equivalent to one agent's molecule [Å$^2$] |
| 0 | $1.03 \times 10^6$ | 15920 | $1.38 \times 10^7$ | 1177 |
| 100 | $8.43 \times 10^7$ | 193 | $1.50 \times 10^8$ | 108 |

into the erythrocyte membrane. The decrease of affinity is much higher in the case of 5-*n*-alkylresorcinols than 5-*n*-alkenylresorcinols. This observation suggests the possible cooperativity in binding of the 5-*n*-alkylresorcinols to the membrane.

The *b* values indicate that the haemolytic capacity of the saturated derivatives is approximately two times higher than unsaturated ones. Taking a value of $1.63 \times 10^{10}$ Å$^2$ for the red cell surface [25], the number of 5-*n*-alk(en)ylresorcinol molecules adsorbed per single cell and the area equivalent to one molecule were calculated (Table II). The obtained values of the area equivalent to one molecule are much higher than obtained for other types of surfactants [22, 24, 25] indicating a stronger membrane perturbing action of 5-*n*-alk(en)ylresorcinols. Such strong action of resorcinol derivatives suggests the possible formation of 5-*n*-alk(en)ylresorcinol-rich domains in the membrane, in the other words unequal affinity of the compounds to the membrane components. The preferential interaction of other surfactants and detergents with some membrane components was already noted [26, 28]. The difference between the quantity of 5-*n*-alkylresorcinol molecules bound to the membrane at a hundred percent lysis is about 80 times higher in comparison to the amount at zero percent lysis. This difference in the case of 5-*n*-alkylresorcinols is only 11 times. Comparing saturated and unsaturated derivatives the quantity of the compounds present in the membrane at the moment of complete break-down differs only twice. These differences could explain observed previously different effect on ions and small solutes release from liposomes between saturated and unsaturated derivatives [17].

According to the model proposed by Haydon and Taylor [29], the point at which the membrane break up, should to a first approximation, be dependent only on a number of adsorbed surfactant molecules. This number is related to the free energy of the interaction of the adsorbed agent with its membrane environment

$$\Delta G_{\text{lys}} = -RT \ln\left(\frac{C_{\text{lys}} - a_0}{N}\right).$$

In this expression $C_{\text{lys}}$ is the concentration of surfactant at which the complete break-down of the cell membrane occurs, $N$ is the cell concentration and $a_0$ is as defined above [24]. The estimated free energy of the membrane break-down under the alkyl and alkenyl derivatives of resorcinol treatment gave the values of $-22.3$ kcal/mol and $-22.0$ kcal/mol respectively ($N = 7.0 \times 10^{11}$ cells/liter). It indicates that the membrane break-down induced by alkyl and alkenyl resorcinol derivatives seems to be dependent on their molecular size and shape which in case if examinated compounds (at given experimental conditions) are similar.

The observed differences in the quantity of compound present in the membrane at the moment of its break-down between 5-*n*-alkenyl and 5-*n*-alkyl resorcinols could be also dependent on their different ability for alteration of the membrane structure. It was shown previously that the saturated derivatives induced the formation of nonlamellar structures – isotropic phases within the lipid bilayer whereas the unsaturated ones induced also the hexagonal ($H_{\text{II}}$) phase [18, 19].

In general obtained results suggest that the detergent-like activity could be the principle of 5-*n*-alk(en)ylresorcinol action on the cell membrane.

It should be mentioned that the presented in this paper results concerned the lytic activity of 5-*n*-alk(en)ylresorcinols at the optimal conditions where the suspensions were made above the transition temperatures. The lytic activity of the examinated

saturated derivatives at 37 °C without prior formation of micellar solutions was practically not detectable [17]. This could explain also the observations obtained from the experiments on the activity of different saturated and unsaturated cardol [30] and urushiol [31] derivatives. This suggests also a biological importance of alkenyl derivatives in the activity of phenolic lipids.

Recent results showed that both 5-*n*-alkyl and 5-*n*-alkenyl resorcinols consist of homologs with the aliphatic chain length of 15 to 25 carbon atoms [9]. The studies of the role of aliphatic chain length on 5-*n*-alkylresorcinols and 5-*n*-alkenylresorcinols activities are now in progress.

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