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PERMEABILITY CHANGES OF ERYTHROCYTES AND LIPOSOMES BY 5-(*n*-ALK(EN)YL) RESORCINOLS FROM RYE

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Summary

5-(*n*-Alk(en)yl) resorcinols can induce potassium release from liposomes and erythrocytes. The results suggest that 5-(*n*-pentyl)resorcinol can induce a specific permeability to protons as well as to potassium and other small molecules. The highest permeability changes were found in the presence of 5-(*n*-pentadecyl)resorcinol and alkenyl resorcinols. Orcin and resorcin were without effect. The size of permeant as investigated by turbidity measurements indicated that Ca^{2+} and Mg^{2+} cannot pass through the alkyl resorcinol-modified membrane but can pass through the alkenyl resorcinol-modified membrane. It was observed that alkenyl resorcinol at a concentration of 15 μM induced not only potassium release but also lysis of erythrocytes.

Introduction

Resorcinols (1,3-dihydroxy-5-(*n*-alkyl) benzenes) with an odd number of carbon atoms in the aliphatic chain are found in various plant species [1—14]. In *Anacardiaceae*, *Gymnospermae*, *Proteaceae* and *Myrisinaceae* [1—9], resorcinols with mainly aliphatic chains up to 19 carbon atoms and a varying degree of unsaturation (0—3 double bonds) are predominant whereas resorcinols with longer aliphatic chains [15—18] carbon atoms) were shown in common cereals of *Gramineae* [10—12]. In rye, the composition of the alkyl chains was determined [16] and the presence of alkenyl side chains noted [11,17]. The amount of these compounds in cereals varies from 3000 ppm of dry weight in rye to less than 300 ppm in oat and corn (Refs. 13—15, and Kozubek, A. and Bialkowska, S., unpublished results).

Like the very well known antiseptic activity of 4-hexylresorcinol [18], 5-(*n*-alk(en)yl) resorcinols are toxic, as was demonstrated for homologs with up to 15 carbons in the chain. They have a very strong vesicant activity; thus 5-(*n*-pentadecyl))resorcinol blisters the skin [6]. The hypersensitivity to poisonous ivy (*Rhus toxicodendron radicans*) is connected with the presence of alkyl resorcinols and their degree of unsaturation [19]. They induce contact sensitivity in guinea-pigs [20], acceleration of histamine release from skin [21,22], irritation and serious corrosion of the mucous membrane of the stomach [23] and they have a paralytic effect on isolated rabbit intestines and contractive action on isolated uterus [23]. The growth of heart tissue of chicken embryo is inhibited by addition of *n*-alk(en)yl resorcinols at a concentration of 1 : 3000 (v/v) in the culture medium [24]. The presence of alkyl resorcinols inhibits blastogenesis of cultured human erythrocytes [25], causes errors in thymus lymphocyte chromosomes [26] and induces formation of rosettes of cells [27]. After oral administration of alkyl resorcinols to rats and chicken, the practical absence of gram-positive bacteria in cecum was shown [28]. The rabbits fed with resorcinols showed loss of weight, anorexia, lethargy and diarrhoea [29]. The 5-(*n*-alkyl) resorcinols from rye (having a chain length of 15—31 carbon atoms) were deleterious when fed to young rats, swine [30,11] and chickens [31].

The mechanism of 5-(*n*-alk(en)yl) resorcinol activity has been unknown until now. The amphiphatic character of *n*-alk(en)yl resorcinols makes an action on membranes very likely; these compounds might disturb membrane organization and induce permeability changes. The presence of a weak acidic group corresponds to that of known couplers like dinitrophenol and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP).

In this paper, the effect of 5-(*n*-alk(en)yl) resorcinols on the proton and potassium permeability of liposomes and erythrocytes as well as the haemoglobin release from erythrocytes is studied.

Materials and Methods

Egg phosphatidylcholine was prepared by using the method of Pangborn [32] and phosphatidic acid was prepared from egg phosphatidylcholine according to the method of de Gier et al. [33]; 5-(*n*-methyl)resorcinol (orcinol) and 5-(*n*-pentadecyl)resorcinol were obtained from Aldrich (Milwaukee, WI, U.S.A.) and further purified by column chromatography on silica as described before [34]. 5-(*n*-Pentyl)resorcinol (olivetol) was obtained from ICN and K&K Laboratories (Plainview, NY, U.S.A.). The total alk(en)yl resorcinols from rye were extracted with acetone and purified by preparative thin-layer chromatography (TLC) [34]. The alkyl resorcinols (saturated) and alkenyl resorcinols (unsaturated) were fractionated by pentane extraction and preparative TLC on silica, and on $AgNO_3$-impregnated silica gel [34]. Other chemicals used were of analytical grade.

Preparation of liposomes

K^+ release from liposomes. Liposomes were formed from 15 μmol of egg phosphatidylcholine containing 4 mol% phosphatidic acid in 1 ml of 150 mM

dipotassium succinate by shaking on a vortex mixer for 30 s. The untrapped potassium was removed by dialysis (three times) for 60 min against a cold isotonic solution containing 225 mM $MgSO_4$, 10 mM Tris-H_2SO_4 buffer (pH 7.3) at 30°C. To 10 ml of buffer, 0.1 ml of liposome suspension and ethanolic solutions of alk(en)yl resorcinols and valinomycin in microlitre amounts were added. Changes in K^+ were monitored with a potasium-sensitive glass electrode as described before [35]. The amount of K^+ trapped was determined by addition of 0.1 ml of 10% Triton X-100 in buffer.

Swelling experiments. Liposomes were prepared by dispersing 15 μmol of egg lecithin and 0.6 μmol of phosphatidic acid with or without 3 μmol of alk(en)yl resorcinols in 1 ml of 150 mM KCl, 100 mM $MgCl_2$, 100 mM $CaCl_2$, 300 mM glucose or 300 mM erythritol, respectively, each buffered with 10 mM Tris-HCl (pH 7.3).

Swelling experiments were performed at 37°C (20 μl liposomes were added) to 5 ml of vigorously stirred and buffered medium in a thermostatically controlled cuvette. Changes in the absorbance were recorded at 450 nm (Vitraton, type MPS). In order to avoid changes in turbidity due to external addition of the alk(en)yl resorcinols, 20 mol% was preincorporated in the liposomal membrane.

Erythrocyte permeability. Daily, fresh rabbit blood was collected in acidic citrate/dextrose and centrifuged for 5 min at 3000 rev./min. The erythrocytes were washed three times with 0.15 M NaCl and three times with 10 mM Tris-HCl buffer (pH 7.0) containing 100 mM $CaCl_2$ by suspending and centrifuging for 5 min at 3000 rev./min. The erythrocytes were suspended in the same buffer at a haematocrit of 50%.

The measurements were performed at 37°C. To 10 ml of 100 mM $CaCl_2$, 10 mM Tris-HCl buffer (pH 7.0), followed by 50 μl erythrocyte suspension (haematocrit 50%) and 1—20 μl of ethanolic solutions of alk(en)yl resorcinols were added. Potassium leakage was measured with a potassium-sensitive glass electrode. The total amount of potassium in the erythrocyte suspension was determined after addition of 0.1 ml of 10% Triton X-100 in buffer. The extent of haemolysis was determined spectrophotometrically at 540 nm in the supernatant after centrifugation for 5 min at 3000 rev./min. 100% haemolysis was estimated after suspending the erythrocytes in distilled water. Control measurements in the absence of the resorcinols were performed. All experiments were repeated three times; the deviation was normally not more than 5%.

Results

In order to measure possible uncoupler activity of 5-(*n*-alk(en)yl) resorcinols, their effect on the valinomycin-mediated potassium leakage from liposomes was measured. Liposomes were prepared in a solution of potassium succinate. Due to impermeability of the anion, the valinomycin-induced release of potassium is limited in the absence of an uncoupler [35]. Fig. 1 (curve 6) shows that in the presence of FCCP alone, no potassium is released but that after the subsequent addition of valinomycin, 60% of the trapped potassium is released. Of the resorcinols, only pentylresorcinol showed a stimulation of the valinomycin activity (Expt. 3). The other alk(en)yl resor-

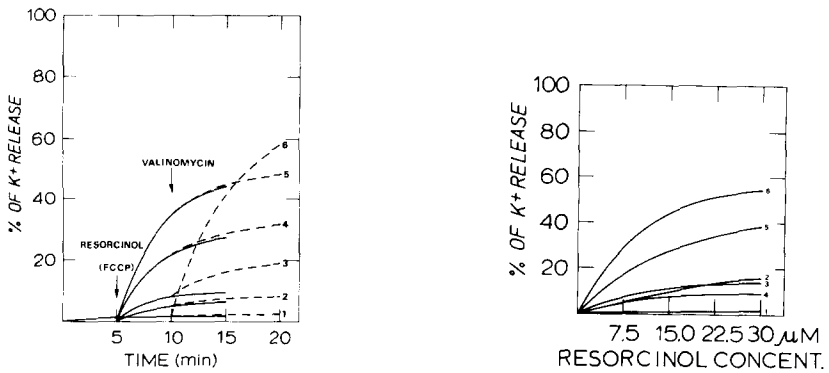

Fig. 1. Effect of 5-(*n*-alk(en)yl) resorcinols on the valinomycin-induced K^+ release from egg phosphatidylcholine liposomes. A comparison with the K^+ release by FCCP and valinomycin. (1) 5-(*n*-Methyl) resorcinol; (2) 5-(*n*-alkyl) resorcinols from rye; (3) 5-(*n*-pentyl)resorcinol; (4) 5-(*n*-pentadecyl)resorcinol; (5) 5-(*n*-alkenyl) resorcinols from rye; (6) FCCP. The final concentrations of 5-n-alkyl resorcinols was 15 μM, of valinomycin 2.5 ng · ml^{-1} and of FCCP 20 ng · ml^{-1}.

Fig. 2. Concentration dependence of the 5-(*n*-alk(en)yl) resorcinol-induced K^+ release after 10 min from egg phosphatidylcholine liposomes. (1) 5-(*n*-Methyl)resorcinol; (2) 5-(*n*-pentyl)resorcinol; (3) total 5-(*n*-alk(en)yl) resorcinols from rye; (4) 5-(*n*-alkyl) resorcinols from rye; (5) 5-(*n*-pentadecyl)resorcinols; (6) 5-(*n*-alkenyl) resorcinols from rye.

cinols had no uncoupler activity. However, all the alk(en)yl resorcinols, except 5-methylresorcinol, caused a strong potassium release in the absence of valinomycin (Fig. 1, curves 1—5). Their activity increased in the order: 5-(*n*-alkyl) resorcinols (saturated resorcinols from rye) < 5-(*n*-pentyl)resorcinol < 5-(*n*-pentadecyl)resorcinol < 5-(*n*-alkenyl) resorcinols (unsaturated resorcinols from rye). The latter caused more than 40% release of trapped potassium in 10 min. The concentration dependence of the alk(en)yl resorcinol-induced potassium release is shown in Fig. 2. The order of activity is essentially the same as that in Fig. 1. The total mixture of 5-(*n*-alk(en)yl) resorcinols from rye obtained from acetone extracts, containing about 25% alkenyl resorcinols, showed a slightly higher potassium release than the alkyl resorcinols.

In order to establish whether alk(en)yl resorcinols are more effective towards natural membranes and if the permeability increase is selective for small solutes, the release of potassium and haemoglobin from erythrocytes was studied. 5-Methylresorcinol had no effect on the potassium and haemoglobin release from erythrocytes (Fig. 3A). In the presence of 5-pentylresorcinol, up to 10% potassium release and about 5% haemolysis in 60 min could be observed (Fig. 3B). However, in the presence of 50 μM 5-(*n*-pentadecyl)resorcinol, a complete release of potassium can be observed after 10 min. The haemoglobin release was not more than 15% (Fig. 3C). Also, the alkyl resorcinols from rye (saturated fraction) show a preferential release of potassium. At a concentration of 50 μM, 40% of the potassium and only 10% of the haemoglobin are released after 10 min (Fig. 3D). On the other hand, the alkenyl resorcinols from rye (unsaturated fraction) are very lytic. At 50 μM there is a complete release of potassium and haemoglobin within 10 min (Fig. 3E). The aceton extract from rye containing the total alk(en)yl resorcinols shows a stronger effect on erythrocytes than on liposomes. 50 μM gives

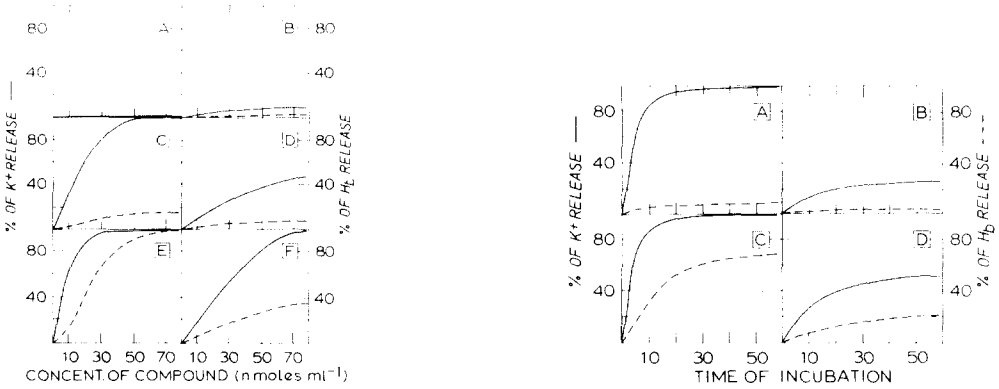

Fig. 3. Percent of K^+ and haemoglobin (H_b) release after 10 min from rabbit erythrocytes induced by different concentrations of 5-(*n*-alk(en)yl) resorcinols. Experiments were performed in 100 mM $CaCl_2$, 10 mM Tris buffer (pH 7.0) at 37°C. The resorcinols were added from ethanolic solutions (1—20 µl). (A) 5-(*n*-Methyl)resorcinol; (B) 5-(*n*-pentyl)resorcinol; (C) 5-(*n*-pentadecyl)resorcinol; (D) 5-(*n*-alkyl) resorcinols from rye; (E) 5-(*n*-alkenyl) resorcinols from rye; (F) total 5-(*n*-alk(en)yl) resorcinols from rye.

Fig. 4. Time dependence of K^+ and haemoglobin release from rabbit erythrocytes induced by 5-(*n*-alk(en)yl) resorcinols at a concentration of 15 µM. Experiments were performed in 100 mM $CaCl_2$, 10 mM Tris-HCl buffer (pH 7.0) at 37°C. (A) 5-(*n*-Pentadecyl)resorcinol; (B) 5-(*n*-alkyl) resorcinols from rye; (C) 5-(*n*-alkenyl) resorcinols from rye; (D) total 5-(*n*-alk(en)yl) resorcinols from rye.

a complete release of potassium but only 30% haemoglobin release (Fig. 3F). At 15 µM, 5-(*n*-pentadecyl)resorcinol gives a very fast and complete release of potassium in 10 min but even after 60 min the haemoglobin release is not more than 10% (Fig. 4A). In the case of 5-(*n*-alkyl) resorcinols from rye there is a selective increase for small solutes such as potassium; practically no release of haemoglobin was found under these conditions (Fig. 4B). In contrast, the alkenyl resorcinols show a strong lytic activity. All the potassium and 70% of the haemoglobin are released (Fig. 4C). The total alk(en)yl resorcinol fraction from rye shows a 50% release of potassium and a 20% release of haemoglobin in 60 min probably due to the presence of alkenyl resorcinols (Fig. 4D).

In order to establish if the permeability changes are restricted to a certain size of permeant, we investigated the change in turbidity (indicating swelling

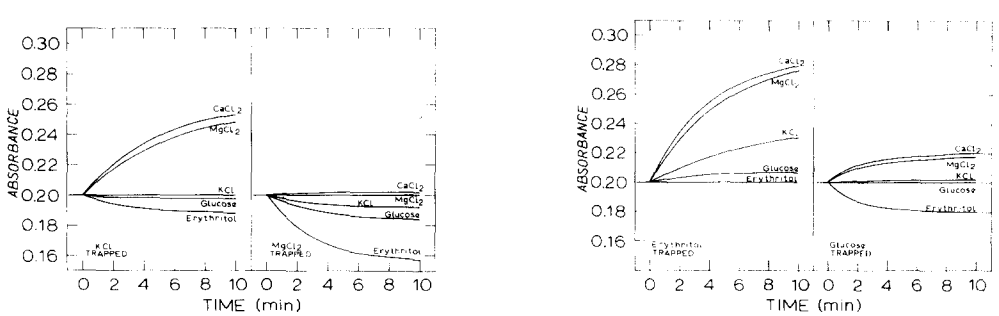

Fig. 5. Effect of 5-(*n*-alkyl) resorcinols from rye on the turbidity of egg phosphatidylcholine liposomes at 37°C. The alkyl resorcinols were incorporated in the liposomal membrane at a concentration of 20 mol%. For experimental details see Materials and Methods.

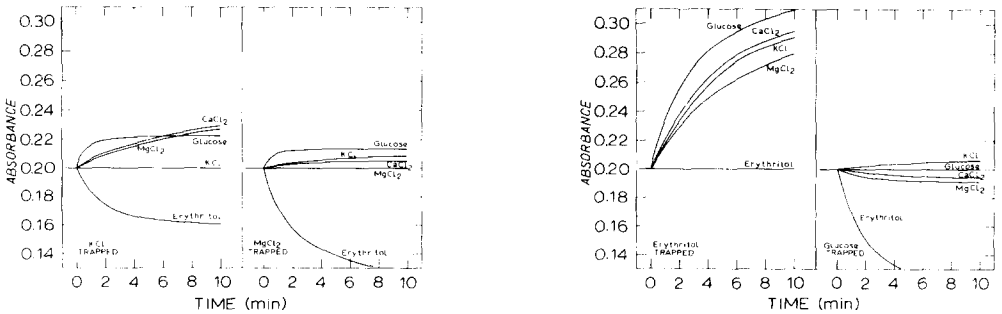

Fig. 6. Effect of 5-(*n*-alkenyl) resorcinols from rye on the turbidity of egg phosphatidylcholine liposomes at 37°C. The alkenyl resorcinols were incorporated in the liposomal membrane at a concentration of 20 mol%. For experimental details see Materials and Methods.

or shrinking) of liposomes containing preincorporated resorcinols injected in isotonic solutions of different solutes.

Fig. 5 shows the effects of 5-(*n*-alkyl) resorcinols from rye on the permeability of liposomes which trapped erythritol, glucose, KCl and $MgCl_2$ and were suspended in erythritol, glucose, KCl, $MgCl_2$ and $CaCl_2$, respectively. Shrinking was observed in isotonic solutions of $CaCl_2$ and $MgCl_2$. Apparently, Ca^{2+} and Mg^{2+} cannot pass through the alkyl resorcinol-modified membrane. Swelling is observed in isotonic solutions of erythritol when glucose, KCl or $MgCl_2$ is trapped inside. This means that the membrane became highly permeable for erythritol. A smaller permeability increase was found for glucose and KCl. In the case of alkenyl resorcinols (Fig. 6), the increase in permeability for erythritol is very high, whereas the permeability change for glucose, KCl, $CaCl_2$ and $MgCl_2$ is very similar.

Discussion

In this paper, the effects of different 5-(*n*-alk(en)yl) resorcinols on the permeability of liposomes and erythrocytes were studied. The phenolic groups of resorcinols correspond to the weak acidic groups of dinitrophenol and FCCP which have a known uncoupler activity [40—42]. Only 5-(*n*-pentyl)-resorcinol induces an increase in proton permeability. 5-(*n*-Methyl)resorcinol is probably too water-soluble whereas long-chain resorcinols may be restricted in their transbilayer movement to increase the proton permeability. Valinomycin can release the trapped potassium only in the presence of an uncoupler. On the other hand, the external addition of only resorcinols can release most of the trapped potassium. This permeability change can be due to a disruption of the membrane, due to a permeability change which is not restricted to potassium only, but which also increases the permeability for normally impermeable anions. An incomplete release of potassium from liposomes even at high concentrations of resorcinols shows that due to their poor water solubility, their action is restricted to the outer shells. This was shown to be the case with polyene antibiotics [36]. The experiments with erythrocytes show that some alk(en)yl resorcinols can indeed induce a permeability change for small solutes. Pentadecylresorcinols showed a complete release of K^+ but only a

very small release of heamoglobin. Also, the alkyl resorcinols from rye, where the chain length varies from 15 to 29 carbon atoms [16], show this selectivity. The alkenyl resorcinols from rye, and to some extent the total alk(en)yl resorcinols from rye which contain 25% unsaturated species, show lytic properties. Although there is primarily a fast release of K^+, haemoglobin also is released. The turbidity measurements show that the saturated resorcinols increase the permeability for K^+, erythritol and glucose but not for Ca^{2+} and $MgCl_2$. In the presence of unsaturated resorcinols the divalent cations also leak.

The results obtained on the action of 5-(*n*-alk(en)yl) resorcinols from rye on liposomes and erythrocytes might also be of biological significance and may explain the possible role of such compounds in cereal grains and their toxic activity. Rye and wheat differ from each other not only in the amount of alk(en)yl resorcinols [13–15] but also in unsaturation. In rye grains, about 5-times higher concentrations of alkenyl resorcinols were found than in wheat [17]. The average chain length of rye alk(en)yl resorcinols is shorter than that in wheat [37,38]. The results presented in this paper show that the shorter chain (pentadecyl) and unsaturated chain derivatives show the strongest action on membranes. The relatively high concentrations of alk(en)yl resorcinols in rye grains could be one of the factors responsible for the toxicity of rye for animals and may cause also changes in the intestinal flora of chickens fed with rye [39]. On the other hand, alk(en)yl resorcinols could also have some antibiotic function for the grain [12].

Further experiments have to be performed to elucidate which structural changes of the membrane are induced by resorcinols.

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