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Effects of dehydroabietic acid on the physical state of cytoskeletal proteins and the lipid bilayer of erythrocyte membranes

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Abstract

Dehydroabietic acid (DHAA) is a major aquatic toxic resin acid usually found in unbleached pulp mill effluents. This compound has been reported to accumulate in the red cells of rainbow trout and to cause hemolysis. To elucidate further understanding to the mechanism of action of this resin, the interaction of DHAA with human erythrocyte membranes has been monitored by electron paramagnetic resonance techniques of spin labeling. Results presented in this paper indicate that DHAA, in a concentration-dependent manner, significantly altered both the motion and order of the lipid bilayer and the physical state of cytoskeletal proteins, while DHAA had no effect on isolated lipids. It is proposed that the increase in the 'fluidity' of the lipid bilayer induced by DHAA is a secondary effect of primary changes in the physical state of the cytoskeletal proteins of the membrane, and that the latter effect is critically associated with the toxicity of DHAA.

Key words: Dehydroabietic acid; EPR; Resin acid; Erythrocyte; Aquatic contamination; Hemolysis; (Pulp and paper mill); (Trout)

1. Introduction

There are two major types of small molecular weight toxicants in pulp and paper mill effluents, the chlorophenolics and the resin acids. Of the resin acids, DHAA is the most commonly found in the environment at high concentrations [1].

Resin acids are a major class of aquatic toxic compounds which are discharged in the effluent of pulp and paper mills [1–4]. Dehydroabietic acid (DHAA, see Fig. 1 for structure) is the most prevalent of these acids in unbleached pulp mill effluents [3,4]. Recently, concern over potential exposure of marine life to these effluents and the corresponding potential hazards to the environment resulted in extensive research. Resin acids have been shown to be lethal to fish at concentrations around 1 g/l (≈ 4 μM) while at sublethal concentrations these resin acids have been found to affect respiration and energy metabolism [6,7] as well as cause liver dysfunction in fish [6–9]. DHAA and other resin acids are degraded in biological treatment systems and receiving waters [1]. However, significant toxicity in aquatic life is seen after continuous discharge or accidental spills of these chemicals.

One of the most predominant problems observed in fish exposed to DHAA is jaundice, which is associated with an accumulation of bilirubin in the plasma. This is thought to be a result of an alteration of membrane function which in red cells causes hemolysis [10]. A decrease in cellular ATP accompanied by an impaired ability to regulate intracellular ions and hemolysis of rainbow trout red cells, due to DHAA exposure, has been shown in vitro [10–12]. The increased production of bilirubin from hemoglobin produced from the lysed red cells could lead to jaundice [10]. There is evidence of DHAA bioaccumulation in the plasma: very high plasma concentrations (> 200 mg/l) of the resin acid have been seen in rainbow trout exposed to sublethal concentrations of DHAA [13]. It has been suggested that one reason for bilirubin and DHAA accumulation in the plasma is due to competition for the membrane-bound anion transport system, since bilirubin, DHAA,

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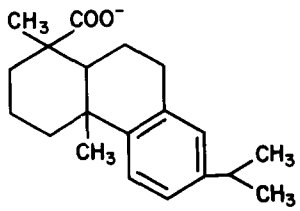

Fig. 1. Structure of dehydroabietic acid.

and their glucuronides are all anions at physiological pH [14,15].

It is possible that the hemolytic property of resin acids is due to detergent-like properties of these amphiphilic compounds. The DHAA molecules could dissolve in the lipid bilayer, increasing its permeability and eventually contribute to, if not cause, the breakdown of the cell [11].

The mechanisms of the toxic action of resin acids are not known. Since many amphiphilic compounds affect the function and integrity of cell membranes [16–18], it seems possible that DHAA, also an amphiphilic compound [19], might act in a similar manner. The present study was undertaken to further understand how DHAA, and possibly other resin acids, interact with cell membranes. Given the known toxicity of DHAA on fish erythrocytes, red blood cells were chosen as the system to investigate this interaction. Our study employs the monitoring of cytoskeletal protein–protein interactions and lipid bilayer fluidity with specific paramagnetic spin probes via electron paramagnetic resonance (EPR).

2. Materials and methods

Dehydroabietic acid (DHAA) (> 99% purity determined by gas chromatography) was obtained from Helix Biotech (Richmond, B.C., Canada). The protein specific spin label, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (MAL-6), and the lipid bilayer-specific spin label, 5-doxylstearic acid (5-NS), were obtained from Aldrich (Milwaukee, WI).

Human blood was obtained from healthy volunteers by venipuncture into heparinized tubes, immediately placed on ice, and processed within 30 min of collection. The intact red blood cells (RBCs) were resuspended in ice-cold phosphate-saline buffer (PBS; 150 mM NaCl and 5 mM sodium phosphate, pH 8.0) and washed three times at $600 \times g$ for 10 min at 4°C. After each wash, the supernatant and buffy coat were carefully removed using a mechanical aspirator. The erythrocyte membranes (ghosts) were obtained by hypotonic lysis in ice-cold 5P8 (5 mM sodium phosphate, pH 8.0), employing 1 volume of RBCs to 20 volumes of 5P8 for 30 min incubation. The membranes were centrifuged at $27\,000 \times g$ for 10 min at 4°C and the supernatant removed. This process was repeated until the ghosts were free of residual hemoglobin. Protein content was estimated by the method of Lowry et al. [20].

The cytoskeletal proteins were selectively and covalently spin labeled with MAL-6 as previously described [21]. Immunological studies of MAL-6-labeled ghost membranes have suggested that most of the spin label is bound to spectrin, the major cytoskeletal protein [22].

Dehydroabietic acid was dissolved in 100% ethanol to yield solutions with concentrations of 25 mM and 100 mM. The pH of each solution was adjusted to a value of 7.4 with NaOH. MAL-6 labeled ghosts were incubated with appropriate amounts of DHAA (final concentrations of 0–1 mM) for 30 min at room temperature prior to EPR analysis. In all experiments, the final concentration of protein content was 2 mg/ml. Control samples contained 1% ethanol, the maximum concentration used in any sample.

For the extraction of lipids, the method of Folch-Pi et al. [23] was used. In brief, 1 volume of erythrocyte membranes (having a protein concentration of 3 mg/ml) was mixed with 20 volumes of a solution containing chloroform and methanol in the ratio of two to one in a 30 ml glass corex tube. Then a solution of 0.76% (w/v) sodium chloride was added to the mixture in the ratio of one part to five parts of the total volume (4.2 ml). The membranes were centrifuged at $500 \times g$ for 3 min at 4°C. A gradient was formed in which a layer of methanol was observed at the top of the glass tube. A disc of proteins separated the methanol top layer from the bottom layer which contained chloroform and lipids. Methanol and the protein disc were carefully removed by aspiration. Lipids were extracted by evaporating the chloroform using a rotary evaporator. The lipids were resuspended in 1 ml 5P8 buffer for further analysis.

Erythrocyte membranes or isolated lipids were labeled with a lipid-specific spin label as previously described [21]. The ghosts were incubated, without addition of DHAA, in the spin-label containing tubes for 30 min at room temperature, followed by addition of the appropriate DHAA aliquots with subsequent incubation for another 30 min at room temperature prior to acquiring EPR spectra.

All EPR spectra were recorded on a Bruker ESP-300 EPR spectrometer equipped with computer acquisition and analysis capabilities. The instrument was located in a climate-controlled room of constant temperature (20 ± 1°C) and humidity. A modulation amplitude of 0.32 G at 100 kHz, a time constant of 0.128 s and a 100 G sweep width were used to record the EPR spectra.

3. Results and discussion

Spectrin, the major component of the cytoskeleton in the erythrocyte membrane, is a flexible rod shaped protein with a combined molecular weight of approx. 485 000 daltons. It is composed of two similar but non-identical subunits, α- and β-, which form heterodimers by aligning in an anti-parallel side-to-side orientation [24]. Head-to-head association of two heterodimers forms tetramers, the major form of spectrin in the erythrocyte membrane [24]. Increased dimeric spectrin is associated with decreased cytoskeletal protein–protein interactions [24]. Spectrin, and other cytoskeletal proteins, have been shown to attach to transmembrane proteins as well as play a role in maintaining cellular shape, strength, flexibility and transport [24].

The protein-specific spin-label MAL-6 is known to covalently bind to cysteine sulfhydryl (SH) groups of cytoskeletal membrane proteins. The majority of this incorporation (70–90%) has been shown to be on spectrin and the cytoplasmic pole of the anion transport protein, band 3 [21]. The usefulness of MAL-6 as a probe for monitoring cytoskeletal protein–protein interactions and spectrin aggregation has been shown by us [21] and others who confirm the specificity of its binding sites [22,25]. The concentration of MAL-6 used (40 μM) is well below the 1 mM concentration of SH-specific agents required to disrupt the tetrameric-dimeric equilibrium of spectrin [26].

A typical EPR spectrum of MAL-6 labeled proteins is shown in Fig. 2 where the $M_I = +1$ low-field lines of the spectrum are presented. This spectrum reflects at least two classes of membrane protein SH-binding sites for MAL-6, strongly immobilized sites (S sites) and weakly immobilized sites (W sites). The S sites could be thought of as being in narrow pockets of the protein, restricting motion of the spin label and causing line broadening. Kahana et al. suggest that S sites are predominantely located in hydrophobic regions of spectrin [24]. Another binding site for the spin label,

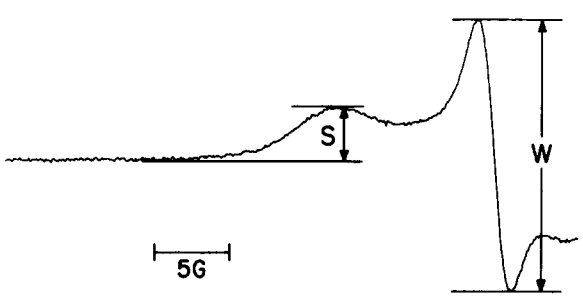

Fig. 2. A typical EPR spectrum of the $M_I = +1$ low-field lines of MAL-6 labeled erythrocyte membranes. The spectral amplitudes of the spin label covalently bound to strongly- and weakly-immobilized sites are indicated by S and W, respectively.

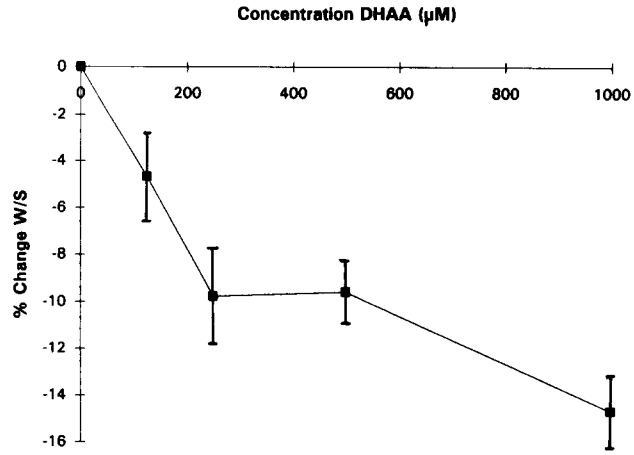

Fig. 3. Change in the relative W/S ratio of MAL-6 covalently bound to cytoskeletal proteins with increasing concentrations of DHAA. $n = 11$ for each point on the curve.

the W sites, could be in a position on the protein which does not restrict greatly the motion of the probe. This would result in relatively faster motion of the probe and, therefore narrower resonance lines.

The relevant EPR parameter measured is the W/S ratio (the ratio of the EPR spectral amplitude of the low field weakly immobilized component to that of the strongly immobilized component). Changes in the W/S ratio have been shown to be a highly sensitive and convenient monitor of membrane-protein conformational changes as well as segmental motion of the protein reaction sites [21,28–33]. Hemin, which increases amounts of dimeric spectrin [30], increased amounts of dimeric spectrin [32], and the specific proteolysis of the linkage protein, ankyrin, which links spectrin in the cytoskeletal network to the major transmembrane protein (band 3) [33], caused decreased membrane cytoskeletal protein–protein interactions and increased the W/S ratio [31–33]. In contrast, spermine, and the potential Alzheimer's disease therapeutic agents tacrine and velnacrine, known to increase cytoskeletal protein–protein interactions, decreased the W/S ratio [29,30].

Fig. 3 shows that DHAA reduced the W/S ratio in a concentration dependent manner ($0.001 < P < 0.05$), implying that DHAA altered the physical state of the cytoskeletal proteins. This change in the W/S ratio can be explained as a conversion of W sites to S sites, as no change in linewidth was observed, and suggests that this resin acid increased membrane cytoskeletal protein–protein interactions.

Unlike the isotropic motion exhibited by non-oriented spin probes like MAL-6, the lipid bilayer specific spin probe 5-NS exhibits anisotropic motion. 5-NS is intercalated into the lipid bilayer with its fatty acyl chain embedded in the hydrophobic bilayer and its polar headgroup oriented near the polar headgroups of

the lipid molecules at the hydrophilic surface of the bilayer. It is conceptualized that the polar headgroup of 5-NS is held rather firmly in place by the headgroups of the bilayer lipids, while the hydrophobic tail is free to undergo rapid anisotropic motion in the interior of the bilayer [21,34]. Since the nitroxide group (the EPR active portion) is covalently bound to the alkyl chain of the probe, the motion of the nitroxide group reflects the motion in the adjacent segment of the molecule. Therefore, 5-NS is a useful probe for monitoring changes in the local environment near the bilayer surface.

A typical spectrum of 5-NS intercalated into the lipid bilayer of the erythrocyte membrane is shown in Fig. 4. Determination of the motion of the 5-NS probe can be accomplished by measuring the half-width at half-height (HWHH) of the low-field ($M_I = +1$) line of the 5-NS spectrum. Low-field line broadening is a result of decreased lipid order and increased lipid motion and is analogous to chemical exchange phenomena [21,34]. In a highly fluid membrane, the nitroxide would be able to rapidly switch between two extremes in orientation. This increase in uncertainty of any one orientation at a given time leads to an increase in line width of the low-field line ($M_I = +1$). The technique of lipid-specific spin labeling has been widely used by our laboratory and others to study the motion and order of the lipid bilayer of erythrocyte and synaptosomal membranes [21,34–36].

We investigated whether DHAA induces alterations in the order of the lipid bilayer employing the lipid-specific spin label (5-NS). Fig. 5 suggests that the resin acid has an effect on the order and motion of the lipid bilayer in a concentration dependent manner ($P < 0.05$ in all cases). The changes induced by DHAA in the conformation of the physical state of the cytoskeletal network (Fig. 3) might be associated with the observed decrease in the order and increase in the motion of lipids in of the bilayer (Fig. 5). To test this idea, EPR studies were performed on the isolated lipids of the erythrocyte membranes. Different concentrations of DHAA (ranging from 0 to 1 mM) caused no significant changes in half-HWHH relative to controls (data not shown). Although not conclusive, these results are consistent with the idea that the effects observed in the physical state of the lipid bilayer (Fig. 5) may be secondary effects caused by alterations in the conformation of membrane proteins, i.e., the results may suggest that DHAA has an indirect effect on the physical state of the lipid bilayer.

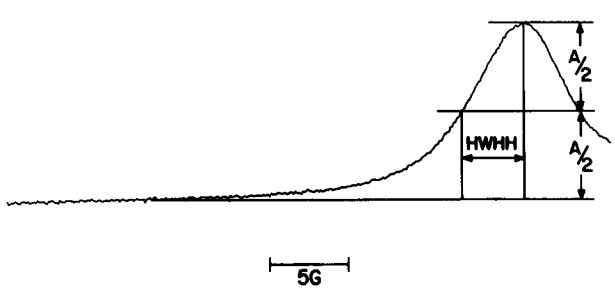

Fig. 4. A typical EPR spectrum of the 5-NS lipid specific spin-probe intercalated in the bilayer of erythrocyte membranes. This spectrum shows the half-width at half-height parameter HWHH.

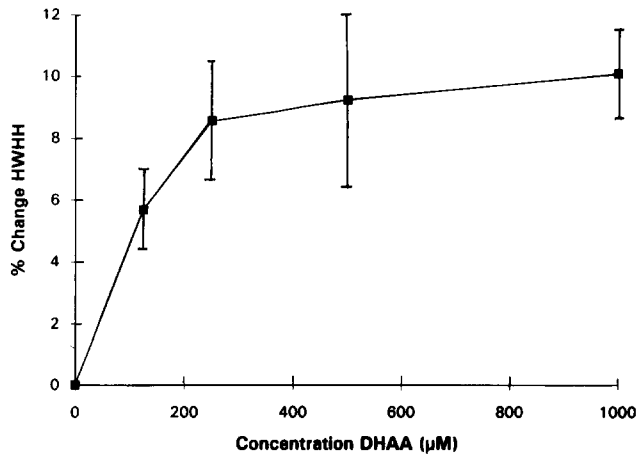

Fig. 5. Change in the relative HWHH with increasing concentrations of DHAA. $n = 4–6$ for each point on the curve.

It has been shown that the concentration of DHAA required for the hemolysis of human erythrocytes is in the same range as for fish erythrocytes [11]. It is still in question, however, whether the hemolysis is due to a 'direct detergent' effect or the alteration of other membrane-connected events. Sublytic concentrations of DHAA are known to induce a shape transformation of the erythrocyte to echinocytes [37]. Intercalation of an amphiphile into the lipid bilayer could affect the ordering and/or packing of the hydrocarbon chains of the phospholipids in the bilayer. The membrane could become more permeable to potassium and other small ions [38,39], and others report that DHAA does increase the permeability of the membrane [11,37]. Our suggestion from the current study that DHAA-induced cytoskeletal protein–protein interactions caused decreased lipid order and increased lipid motion (i.e., increased 'fluidity') of the lipid bilayer allowing more motion of the hydrophobic portion of the spin-probe is in agreement with these findings. The increase in permeability to the electrolytes, due to alterations in the physical state of the membrane, could account for the ATP depletion which is also observed in the trout erythrocytes.

There are many proteins responsible for the transport of molecules across the erythrocyte membrane. Among these are band 3 and Na^+,K^+-ATPase, responsible for the transport of anions (including phosphate ions) and the active influx of potassium, respectively [40]. It is suggested that these two proteins are sensitive to alterations in the dynamics of the lipid bilayer

[41,42] and/or the physical state of cytoskeletal proteins [26]. It is conceivable that the alterations produced by DHAA in cytoskeletal proteins and secondarily in the lipid order, as shown by this work, could affect the function of these two proteins. These effects could be relevant to cells other than the erythrocyte, such as hepatocytes, which, in addition to hemolysis, could aid in the development of jaundice observed in fish.

Continued investigation of the interaction of DHAA with membranes from several cell types should be able to address these questions, and EPR spin labeling methods offer a means of doing so.

Acknowledgements

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