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Hemolysis of erythrocytes and fluorescence polarization changes elicited by peptide toxins, aliphatic alcohols, related glycols and benzylidene derivatives

Valdemar R. Osorio e Castro [2], Edward R. Ashwood [3], Steven G. Wood [1], and Leo P. Vernon [1]

[1] *Department of Chemistry, Brigham Young University, Provo, UT (U.S.A.),* [2] *Department of Chemistry, Pontificia Universidade Catolica, Rio de Janeiro, and Biology Institute, Universidade Estadual do Rio de Janeiro, Rio de Janeiro, (Brasil) and* [3] *Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT (U.S.A.)*

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Hemolysis rates of human erythrocytes induced by C_2 and C_8–C_{14} straight chain 1-alkanols, 1,2-alkanediols and the corresponding benzylidene derivatives (benzaldehyde acetals) have been studied and compared with hemolysis rates obtained by three peptide toxins. The peak of activity occurs at C_{12} for the alkanols and glycols and at C_{10} for the benzylidene derivatives. The most active compound is 1-dodecanol, followed by 1,2-dodecanediol and the C_{10} benzylidene acetal, which show 50% hemolysis at 15, 99 and 151 μM, respectively, at 37°C. A few lysolecithins and longer chain *cis*-unsaturated alcohols were studied for comparison purposes, and were found to be more active than 1-dodecanol. The most active were the 16:0 lysolecithin and *cis*-9-tetradecene-1-ol, which gave 50% hemolysis at concentrations of 2.8 and 5.6 μM respectively. The hemolytic activities of 1-dodecanol, 1,2-dodecanediol and the C_{10} benzylidene acetal were compared to activities of *Pyrularia* thionin and melittin with cow, horse, sheep, pig and human erythrocytes. Whereas the peptide toxins showed clear specificity for human erythrocytes, no selectivity was shown by any of the other compounds tested. Addition of the thionin or *Naja naja kaouthia* cardiotoxin to erythrocyte ghosts caused a slight but reproducible increase in the order of the phospholipid bilayer, as measured with the fluorescent probe NBD-PC. Cardiotoxin gave a greater response than did the P thionin, and extensively iodinated P thionin gave a smaller change than did P thionin. Similar results were obtained with melittin, but this peptide gave a markedly greater response than all other peptides. Addition of dodecanol or the C_{10} benzylidene acetal caused a marked increase in membrane fluidity. All of these data indicate that the organic compounds interact directly with and are incorporated nonspecifically into the membrane lipid bilayer, but the peptide toxins interact specifically with some component on the surface of the membrane, either a protein or specific phospholipid domain, followed by insertion into the membrane and decreasing phospholipid movement.

Introduction

We have recently studied the hemolytic activities of the plant toxic peptide *Pyrularia* thionin and cardiotoxin from cobra venom (*Naja naja kaouthia*) [1,2]. These peptides from widely differing sources bind at the same receptor site on the erythrocyte membrane and appear to act by similar mechanisms. In the case of cardiotoxin, which has been extensively studied, evidence indicates that after binding to the membrane receptor a portion of the molecule is inserted into and perturbs the lipid bilayer of the membrane [3,4]. The hemolytic activities of these peptides, which act through receptors and are species specific [1,2], are compared to general membrane perturbing aliphatic alcohols, related glycols and their benzylidene derivatives of sufficient chain length that they can insert into the erythrocyte membrane and cause hemolysis. Our interest in the benzylidene derivatives stems from the reported antitumor activity of

Abbreviations: P thionin, *Pyrularia* thionin; NBD-PC, 1-palmitoyl-2(6-[7-nitrobenz-2-oxa-1,3-diazol-4-yl)caproyl]phosphatidylcholine; BzC_{10}, the benzylidene cyclic acetal derivative of 1,2-decanediol, or more specifically, 2-phenyl-4-decyl-1,3-dioxolane. Other benzylidene derivatives are abbreviated in a similar manner.

Correspondence: L.P. Vernon, 675 WIDB, Brigham Young University, Provo, UT 84602, U.S.A.

benzylidene derivatives of glucose [5] and ascorbate [6], both of which are water soluble. We compare the most active alkanol, glycol and benzylidene derivative with P thionin, cardiotoxin and melittin for their hemolytic activities with human and animal erythrocytes and also the effect these compounds have on the fluorescence polarization of erythrocyte ghost membranes, using the fluorescence probe NBD-PC. Also included in the fluorescence polarization measurements is P thionin which was iodinated to the extent that it had no hemolytic activity.

Methods

P thionin was prepared as previously described [7]. Melittin was purchased from Sigma Chemical Co. of St. Louis, as were the following: 1-decanol, 1-dodecanol, *trans*-5-decen-1-ol, *cis*-7-dodecen-1-ol, *cis*-7-tetradecen-1-ol, *cis*-9-tetradecen-1-ol, L-α-lysophosphatidylcholine palmitoyl and L-α-lysophosphatidylcholine oleoyl. The various glycols as precursor epoxide compounds as well as benzaldehyde dimethyl acetal were purchased from Aldrich Chemical Co. Benzylidene ascorbate was a gift from Dr. Mutsuyuki Kochi.

Hemolysis of human erythrocytes was carried out essentially as previously described [1]. Venus blood was drawn into vacutainers and used the same day. Five samples were pooled and used for each series of assays after washing three times ($1000 \times g$ for 10 min) in 10 vols. of 150 mM NaCl. The buffy layer of lymphocytes was discarded. For one experiment 0.10 ml of packed red blood cells was diluted to 100 ml final volume with a solution 150 mM in NaCl and 5 mM in sodium phosphate (pH 7.4). 3-ml aliquots were used for each assay (run in triplicate), which consisted of 1 h incubation at 37°C with gentle shaking, following which the cells were sedimented at $150 \times g$ for 15 min and the absorbance of the supernatant fluid determined at 416 nm in a Hewlett Packard 8451 A diode array spectrophotometer. Hemolysis is expressed as a percentage relative to a completely hemolyzed control (erythrocytes incubated in distilled water only) which gave an absorbance reading of 2.2–2.4.

The aliphatic 1,2-glycols and corresponding benzaldehyde acetal derivatives were prepared according to the following generalized procedure. The glycols were formed upon stirring the appropriate epoxide compound with 1.2 equivalents of 80% formic acid overnight, followed by base catalyzed hydrolysis (1 M NaOH for 1 h). They were purified by distillation or crystalization from petroleum ether (C_{12} and C_{14} glycols). The desired benzylidene compounds were prepared by reacting the appropriate glycol with benzaldehyde dimethylacetal (1.2 equivalents.) in toluene at reflux with the aid of a catalytic amount of *p*-toluene sulfonic acid (50 mg). About 1/3 of the toluene was allowed to evaporate during the reaction to remove the methanol. Upon completion of the reaction, as determined by thin-layer chromatography, the reaction mixture toluene layer was washed with saturated $NaHCO_3$ solution. The organic layer was dried, the solvent removed and the resulting benzylidene compounds were purified by short path distillation. The NMR and IR spectra of the products were consistent with the assigned structures.

The benzylidene derivatives described herein are referred to by the general term benzylidene acetals, although rigorously they should be designated as dioxolanes. For example, the derivative prepared by reacting dodecane-1,2-diol with benzaldehyde is 2-phenyl-4-dodecyl-1,3-dioxolane. For simplicity we will refer to this derivative as the benzylidene-C_{12}-acetal, or BzC_{12}.

Membrane fluidity tests were done with erythrocyte ghosts prepared by washing human erythrocytes three times in three vols. of 0.1 M sodium phosphate buffer (pH 7.4) and then four washings in the same buffer at 6.65 mM. Measurements were made with the TDx clinical fluorimeter of Abbott Laboratories of Irving, TX as previously described [8]. The assay mixture of 1.5 ml contained erythrocyte ghosts at $3 \cdot 10^7$ ghosts/ml (from 50 μl of a cell suspension), NBD-PC at 0.65 μM and 1.45 ml of Isoton III buffer, product 8546733 from Coulter Diagnostics, Hialeah, FL. It contained sodium sulfate 9.72 g/l, sodium chloride 4.0 g/l, dimethylolurea 1.0 g/l, and procaine-HCl 0.11 g/l. It was isotonic and pH 7.40. The fluorophore was 1-palmitoyl-2(6[7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl)-phosphatidylcholine (NBD-PC) purchased from Avanti Polar Lipids, Birmingham, AL. Excitation was at 485 and emission at 525–550 nm. Fluorescence polarization was measured at 6–7 min after the NBD-PC was added to the cuvette. Final temperature was 35°C.

P thionin was iodinated with NaI and Iodo-Gen (Pierce) as previously described [9]. Extensive iodination, which leads to iodination of Tyr-13, inactivates P thionin for hemolytic activity.

Results

The hemolytic activities at 25 and 37°C for a series of alkanols and glycols from C_8 to C_{14} are shown in Fig. 1. In both cases the 12-carbon compound showed maximal activity on a molar basis except for the alkanols at 25°C, in which case the 10-carbon alkanol showed the highest activity of the series. The size discrimination for both the alkanols and glycols was quite rigorous, especially at 37°C which shows a rapid drop in activity between the C_{12} and C_{14} compounds. The concentrations of the various alcohols and glycols were 280 μM except for ethanol. In those cases where this concentration gave complete hemolysis, lower concentrations were also tested in order to determine which compound was most active.

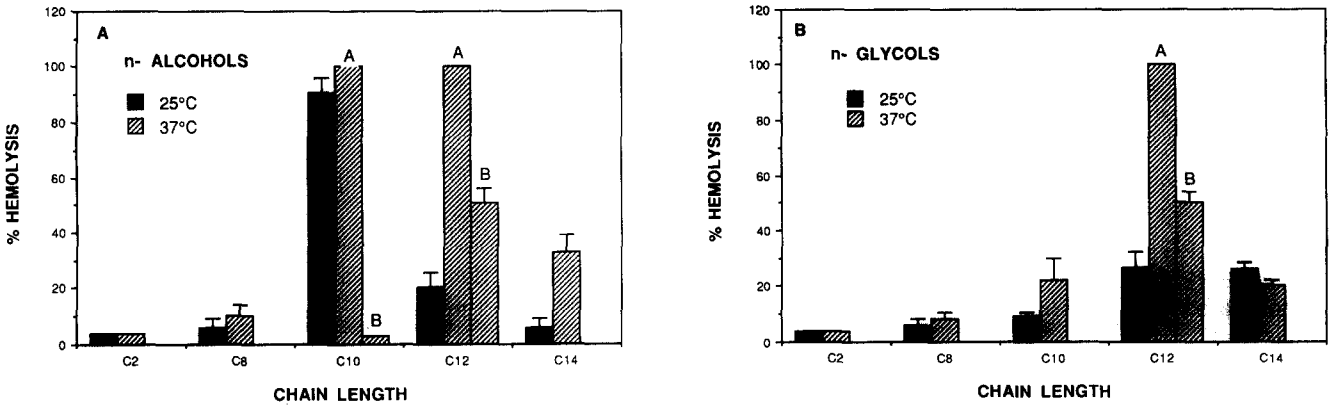

Fig. 1. Effect of aliphatic normal alcohols and glycols on hemolysis of human erythrocytes at 25°C and 37°C. (A) Normal 1-alkanols at 280 μM except for ethanol (110 mM) and B (15.4 μM) (B) Normal 1,2-alkanediols (glycols) at 280 μM except for B (100 μM). Reaction conditions are described in Methods. Mean values of 4–6 determinations are shown by the bars and each determination was made in triplicate.

The activities of the corresponding benzylidene derivatives are shown in Fig. 2A, revealing a similar pattern of size discrimination but displaced to shorter chain length by two carbons. Not surprisingly, the bulky benzylidene group in these compounds affects the insertion of the aliphatic tail into the bilayer. In Fig. 2B the activities of selected related compounds are reported, including decanoic acid, benzyldecyl ether, benzaldehyde and benzylidene ascorbate. None of these compounds is as active as BzC_{10}.

The most active compound reported for Figs. 1 and 2 was 1-dodecanol (lauryl alcohol). For comparison purposes, the dose response for hemolytic activities of a few alkenols and lysolecithins were determined, and the data are shown in Fig. 3. The concentrations which gave 50% hemolysis were calculated for the compounds shown in Fig. 3 along with some additional compounds, and these values (H_{50}) are presented in Table I. These data taken together show that whereas the saturated C_{10} and C_{12} 1-alkanols were more active than the corresponding alkenols, the 14-carbon alkenols were markedly more active than 1-tetradecanol. Of the compounds tested, 16 : 0 lysolecithin (palmitoyl) was the most active, and the 18 : 1 lysolecithin (oleoyl) was only slightly less active. In the case of the alkenols the predominent factors relating to activity are most likely the ease of insertion of the aliphatic tail into the bilayer and the effect this has on the fluidity of the membrane as discussed below. The lysolecithins, which are strong detergents, would be expected to interact not only with the bilayer but also with the ordered polar head groups of the membrane. As expected from the data shown in Figs. 1 and 2, the glycols and their benzylidene derivatives are less active than the alcohols.

It is known that snake cytotoxins and plant thionin toxins show specificity in terms of hemolytic activity with erythrocytes from various animal species. Snake venom cardiotoxin shows differing activity, with he-

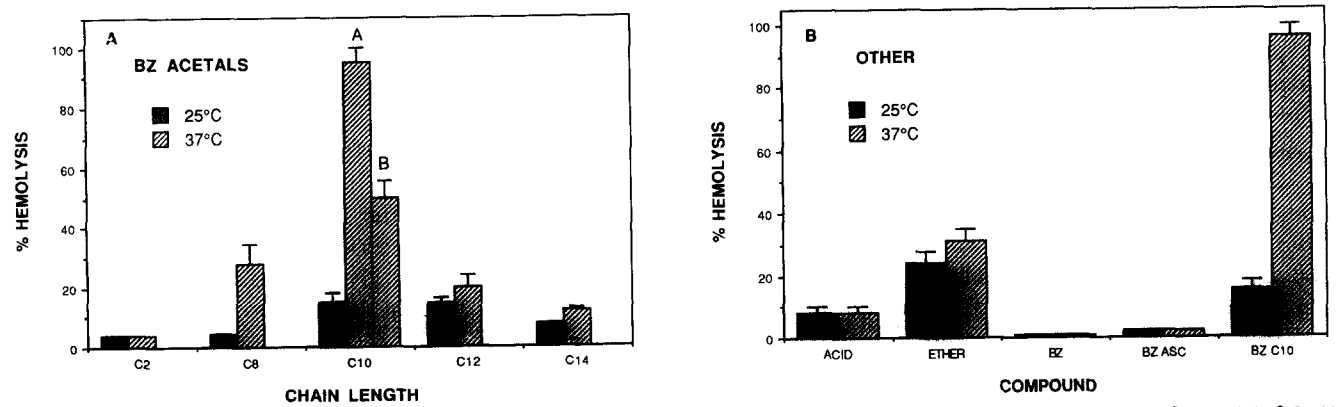

Fig. 2. Effect of benzylidene cyclic acetals (BzC_n) and other organic compounds on hemolysis of human erythrocytes at 25°C and 37°C. (A) Benzylidene cyclic acetals at 280 μM except for B (150 μM). (B) Various related compounds at 280 μM: acid, decanoic acid; ether, benzyldecyl ether; Bz, benzaldehyde; BzAsc, benzylidene ascorbate; BzC_{10}, benzylidene acetal of 1,2-decanediol. All compounds were dissolved in 110 mM ethanol.

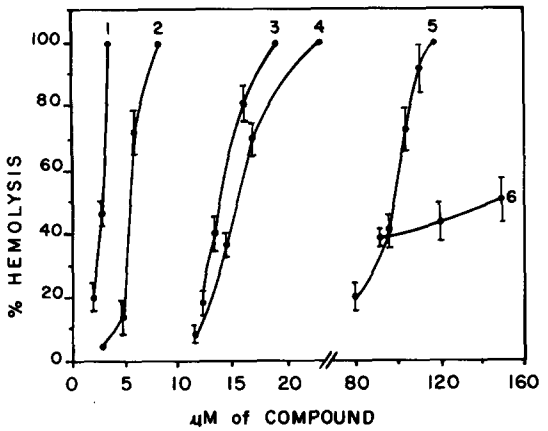

Fig. 3. Dose dependent lysis of human erythrocytes with: (1) 16:0 lysolecithin, (2) *cis*-9-tetradecen-1-ol, (3) *cis*-7-tetradecen-1-ol, (4) 1-dodecanol, (5) 1,2-dodecanediol and (6) BzC_{10}. All compounds were dissolved in ethanol to give a final concentration of 110 mM ethanol. The ethanol control is shown in Fig. 1.

TABLE I

Concentration of aliphatic organic compounds which will produce 50% hemolysis (H_{50}) with human erythrocytes

The experimental conditions were as given for Fig. 3. The values are averages of two experiments (each run in triplicate) and S.E. values are given in parentheses.

Compounds	H_{50} (μM)	
	25 °C	37 °C
16:0 Lysolecithin	3.1 (0.3)	2.8 (0.2)
cis-9-Tetradecene-1-ol	8.9 (0.8)	5.6 (1.2)
18:1 Lysolecithin	6.6 (0.7)	6.6 (0.7)
cis-7-Tetradecen-1-ol	37 (3.0)	14 (1.5)
1-Dodecanol	40 (2.0)	15 (1.0)
cis-7-Dodecen-1-ol	182 (2.0)	53 (2.0)
1,2-Dodecane-diol	290 (10)	99 (3.8)
1-Decanol	195 (15)	130 (3.8)
BzC_{10}	–	151 (22)
1-Tetradecanol	–	304 (20)
trans-5-Decen-1-ol	–	340 (17)

molysis decreasing in the order guinea pig, human, rabbit, horse and sheep erythrocytes [10]. P thionin also shows a clear preference for human erythrocytes followed with only slight activity for rabbit, guinea pig, pig, sheep, horse, cow and mouse erythrocytes [2]. Table II compares the hemolytic activities of the organic compounds involved in this study to the hemolytic activities of melittin and P thionin on five different species of erythrocytes. The erythrocytes from cow, horse, sheep and pig all showed low activity with the peptide toxins melittin and P thionin, and as expected human erythrocytes showed the highest activity. The most active alcohol, glycol and benzylidene acetal species showed similar high levels of hemolysis with all species of erythrocytes. The only unusual feature is the lower activity of BzC_{10} with sheep erythrocytes when compared to the other species. The reason for this difference is not known.

Whereas the organic compounds tested are most likely acting by direct insertion into the membrane bilayer, the mode of action of the peptide toxins is not known for certain. Although they do cause a perturbation of the membrane, since they are hemolytic, it is not known if they bind directly to the membrane or to a protein receptor. The effects of the three toxin peptides and dodecanol on membrane fluidity were measured using the fluorescent probe NBD-CP. Fluorescence from this probe originates from the head group of the phospholipid, and the polarization data reflect the rotational ability of this part of the molecule. Earlier data show

TABLE II

Hemolysis activities of selected aliphatic compounds, melittin and Pyrularia thionin on animal and human erythrocytes at 37 °C

The reported values are averages of three separate experiments (each in triplicate), and the S.E. is reported in parentheses.

Compound (μM)	% Hemolysis				
	cow	horse	sheep	pig	human
1-dodecanol					
36	82 (7.0)	96 (2.0)	96 (0.7)	91 (10)	100
1,2-Dodecanediol					
73	25 (2.0)	30 (3.5)	31 (2.8)	23 (1.5)	20 (1.5)
106	100	100	100	100	80 (3.0)
BzC_{10}					
150	35 (4.0)	36 (4.5)	20 (2.5)	45 (5.0)	50 (5.0)
280	100	100	51 (5.0)	100	100
Melittin					
0.06	4 (1.0)	11 (1.0)	2.5 (0.7)	3.3 (0.3)	43 (3.3)
P thionin					
1.9	1.9 (0.1)	1.4 (0.1)	3.0 (0.3)	3.3 (0.3)	40 (4.0)
19	1.4 (0.1)	1.4 (0.1)	2.9 (0.3)	10 (0.8)	57 (3.6)

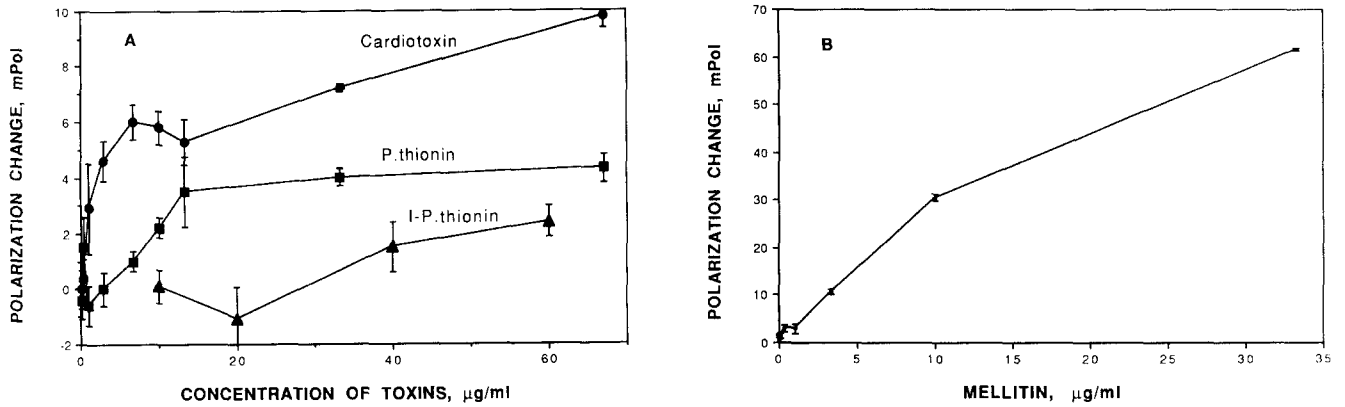

Fig. 4. Fluorescence polarization changes expressed in millipols (mPol) [8] for the probe NBD-PC inserted into human erythrocyte ghost membranes after treatment with the toxin peptides (A) P thionin, cardiotoxin and (B) melittin. Each point, with S.E., represents the average of four determinations. (A) also shows data for P thionin which was iodinated to the extent that it (I-P thionin) showed no hemolytic activity with erythrocytes [9]. The assay conditions are given in Methods. The fluorescence polarization values observed in the absence of peptides were in the range 170–180 mPol, as shown in Fig. 5.

that in terms of fluidity measurements, data obtained with NBD-PC are comparable to the fluorescent probe DPH, which measures movement in the hydrocarbon chains of the phospholipid bilayer [8]. There was a clear difference in the effect of these agents on the membrane fluidity. Figs. 4A and 4B show that P thionin, cardiotoxin and melittin all caused an increase in fluorescence polarization (decrease in membrane fluidity), increasing in that order. The observed changes with P thionin and cardiotoxin were relatively small, but were reproducible and significant. The increased polarization caused by melittin was much greater. Fig. 4A also shows the effect iodinated P thionin has on the fluorescence polarization. The sample used in these experiments was iodinated to the extent that it was inactive in the hemolysis assay, as shown previously [8]. The changes observed with the iodinated P thionin were markedly lower than for the native P thionin, which

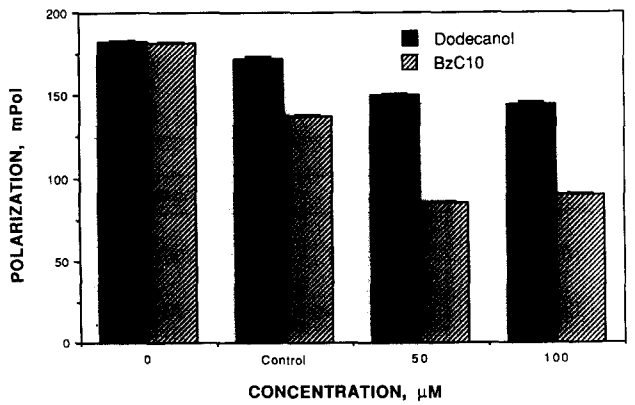

Fig. 5. Fluorescence polarization for the probe NBD-PC inserted into human erythrocyte ghost membranes alter treatment with increasing concentrations of dodecanol and BzC_{10}. The compounds were dissolved in 0.87 M ethanol (dodecanol) or 1.74 M ethanol (BzC_{10}). The polarizations observed upon addition of the ethanol solutions alone are shown as the controls.

relates the changes in fluorescence polarization with the ability of the peptide to produce hemolysis. Dodecanol which is assumed to act by insertion into the membrane bilayer, acted in opposite manner and caused a marked decrease in polarization (increase in fluidity) as expected. These data are shown in Fig. 5. Since the dodecanol was dissolved in an alcohol solution, the effect of ethanol alone is also shown.

Discussion

Whereas the peptide toxins melittin and P thionin do show species specificity for erythrocytes in terms of hemolytic activity, such is not the case with the alcohols, glycols and benzylidene compounds we have studied. The mode of action of P thionin involves an initial specific binding to some component of the membrane, either a protein or specific phopholipid domain [1,2]. We believe it is the latter. A similar situation has been proposed for cardiotoxin [3,4] and melittin [11]. The hemolytic mechanism appears to involve binding to a receptor and insertion of a portion of the cardiotoxin [4] and melittin molecules into the bilayer [11], leading to an increased ordering of phospholipids in the bilayer. The binding to a receptor would account for the observed species specificity for these toxins.

The organic compounds tested do not show any species specificity except for the BzC_{10}, which for some unknown reason is less active with sheep erythrocytes. These data indicate the interaction of the alcohols and related compounds with the membrane is more general in nature, involving a direct insertion into the phospholipid bilayer of the membrane, leading to a decrease in phospholipid order (increase in fluidity) as shown in Fig. 5 above. Because of the low alcohol concentrations which are effective it is not likely that the added alcohols, glycols and benzylidene compounds are forming a new lipid phase in the bilayer [12].

There is ample evidence that long chain alkanes, alkanols and alkanoic acids decrease the extent of ordered gel state in phospholipid bilayers [13,14], which increases the fluidity of the membrane [14], facilitates membrane fusion [15] and increases membrane permeability [16]. Such compounds also induce the formation of the hexagonal H_{II} phase in natural and synthetic membranes [13,14].

Paterson et al. [17] studied *n*-alkanols with chain lengths of 2 to 8 carbons, and showed that increasing the chain length caused corresponding increases in erythrocyte hemolysis and in phospholipid anisotropy. A corresponding decrease in resistance in black lipid membranes was observed. Alkanes and alkanols from C_6 to C_{12} induce H_{II} phase formation in egg yolk phosphatidylethanolamine artificial membranes [14]. Ahkong et al. [13] studied the fusion of erythrocyte membranes as affected by 30 fat soluble compounds and reported that C_{10} to C_{14} alkanoic acids and unsaturated longer chain acids decreased the amount of ordered bilayer (increased the H_{II} phase) and led to an increase in fusion. Another study of $C_{14}$and C_{16} alcohols was conducted by Pringle and Miller [18], who reported that saturated alcohols elevated the transition phase temperature, the *trans*-unsaturated alcohols gave a smaller elevation and the *cis*-unsaturated alcohols decreased the phase transition temperature. Alcohols with fewer carbons were not studied.

The effect of normal alcohols up to 1-dodecanol on the phase transition temperature in synthetic phospholipid bilayers was reported by Lee [19]. Increasing chain length, up to octanol, uniformly decreased the phase transition temperature, which indicates an increasing fluidity. Above 8 carbons the results were mixed and depended upon the nature of the phospholipid bilayer. With 1-dodecanol the phase transition temperature was raised for the artificial lipid bilayers dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine, but decreased the phase transition temperature for dipalmitoylphosphatidylethanolamine. Normal alcohols with chains longer than 12 carbons all increase the phase transition temperature, and a chain length of 12-carbons is the cutoff point for these two opposing effects [20–22]. In addition to the use of straight chain aliphatic compounds, a method for increasing the fluidity of membranes involves the addition of benzyl alcohol. This has been done with rat liver plasma membranes [23] using lipsomes to bind photosensitizers [24] and with erythrocyte membranes as measured by fluorescence anisotropy. Increasing the fluidity of the erythrocyte membrane in this manner causes hemolysis when the benzyl alcohol concentration is above 300 mM [25].

Our results agree with the data obtained from physical measurements of fluidity and phase transition temperatures in that maximal hemolytic activity is obtained with 1-dodecanol. As expected, the glycols resembled the corresponding alcohol in their effect on hemolysis. The fact that the benzylidene compounds show maximal activity with the C_{10} compound indicates that the rigid benzene ring joined to the saturated aliphatic chain via the cyclic acetal allows the lower chain length acetals to insert further into the hydrophobic core of the bilayer. In contrast, the alkanoic acids would retain the polar carboxyl group outside the hydrophobic core, and it would be located in the polar head group region of the phospholipids. The hemolytic effect of saturated fatty acids increases when the chain length is above 12-carbons [26] since only the aliphatic tail inserts into the bilayer. The difference in chain length for maximal hemolytic activity at the two temperatures employed (C_{12} at 37 °C and C_{10} at 25 °C) is of interest. One explanation for this effect relates to the sharp decrease in hemolytic activity at longer chain lengths, probably related to the incursion of the tip of the aliphatic chain of the longer alkanols into the hydrophobic region of the inner membrane leaflet. Such an incursion would take place more readily at the higher temperature.

The data on fluorescence polarization emphasize the difference between the long chain aliphatic compounds and the peptides in terms of how they interact with erythrocyte ghost membranes. The extent of the fluorescence polarization increase caused by the three peptides follows what is known about the ability of these peptides to insert into the membrane bilayer. Melittin, which shows the greatest polarization increase, is well known to insert readily into the bilayer [10]. Its insertion into unilamellar dimyristoylphosphatidylcholine induced an increase in the order parameter of the acyl chains as shown by the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene [27]. There are no data available concerning P thionin insertion, but there is ample evidence that cardiotoxin does insert into negatively charged bilayer membranes [28–32]. Several studies indicate that the first of three hydrophobic loops does insert into phospholipid bilayers [30–32], and would be directly involved in the phospholipid changes induced by cardiotoxin. Once the cardiotoxin molecule is bound to the negatively charged surface of the membrane, insertion of loop 1 and perhaps other portions of the toxin, would lead to rearrangement of the phospholipid membrane structure [33] and formation of phospholipid clusters and lateral phase separation between negative and neutral phospholipids [34].

Since P thionin and cardiotoxin bind to the same site on erythrocyte membranes [2] and have the same general effect on these cells [1,2], we can assume that P thionin binds in a similar fashion and causes similar perturbations in the phospholipid order in the bilayer. For both cardiotoxin and P thionin, the fluorescence polarization increases can be related to the ordering effect these peptides have on the membrane of erythro-

cyte ghosts. This agrees with the binding of cytochrome *c* and apocytochrome *c* to lipid bilayers, as reviewed by Marsh and Watts [35]. In low salt cytochrome *c* increases the effective order parameter, indicating a generalized decrease in chain motion throughout the bilayer [36,37]. With phosphatidylcholine plus cardiolipin mixtures, cytochrome *c* induces a phase separation [38–40]. This was not observed, however, with phosphatidylserine plus phosphatidylcholine bilayers [41]. All the data indicate that the cytochrome *c* binds electrostatically to the surface of negatively charged phospholipid bilayers, slightly penetrates into the bilayer and causes a general increase in order parameter of the lipid bilayer.

The mechanisms by which the peptide toxins and long chain aliphatic compounds induce hemolysis are not known, but are quite different. The increase in membrane phospholipid fluidity seen with the addition of the long chain aliphatic compounds would be expected to cause hemolysis, as reported in the literature for similar compounds which cause an increase in the hexagonal H_{II} phase. However, some fluidizing agents protect against osmotic lysis. The peptides obviously have a different membrane response, causing an increase in order parameter of the bilayer, which relates to the insertion of a portion of the peptide molecule into the bilayer. This could lead to some weakening of protein interaction in the cytoskeleton, but we have no direct information on this.

Acknowledgements

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