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The pH dependence of the hemolytic potency of bile salts

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The membrane damaging potential of dilute solutions of bile salts was evaluated by monitoring continuously the hemolysis of a small sample of red blood cells (RBC) introduced into a defined media containing the bile salts at various pH values. The strength of the hemolytic bile salt was characterized by the rate of the induced hemolysis and by the time that elapsed between the introduction of the RBC sample into the bile salt containing solution and the onset of hemolysis. The potency of the unconjugated bile acids was extremely sensitive to pH, e.g. the rate of hemolysis caused by a 7.5 mM cholate was 1.5%, 20% and 64% per min when the pH of the solution was 7.65, 7.3 and 6.85, respectively. At low pH values the membrane damaging effects of deoxycholate was clearly discerned at micromolar concentration range. The hemolytic potency of glycodeoxycholate was also enhanced significantly by lowering the pH. The taurine-conjugated cholate and deoxycholate were only slightly sensitive to variations in pH. Taurocholate at concentrations that were not hemolytic greatly enhanced the injurious potency of deoxycholate. These results imply that in acidic solutions the presence of bile acids can cause damage to cell membranes. It is suggested that the acidic environment in the proximal duodenum and acidosis developed during hypoxia in the liver are two situations in which the bile salts may constitute a pathogenic factor.

Introduction

Bile salts are naturally occurring detergents that play a major role in the digestion of fats [1]. These compounds are also capable of causing membrane damage expressed eventually in rupture of cells [2–5]. Therefore, bile acids constitute potential, naturally occurring, cytotoxic agents. The ability of bile acids to generate gastric ulcers is well established [6] and their significance in pathogenesis of other diseases has been considered (see, for example, Refs. 7,8). As to their possible role in the pathogenesis of duodenal ulcers there are seemingly conflicting evidence. On one hand, there are experiments that imply a protective effect of bile in experimental cysteamine-induced duodenal ulcers [9]. This protective effect is related most probably to alkalization of the duodenal content by bile and pancreatic secretions. On the other hand, there is evidence that bile diversion prevented the formation of ulcers in the above model [10]. Both groups [9,10], however, agree that inclusion of taurocholate in the drinking water enhanced the formation of the ulcers in the experimental model.

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The study of the membrane damaging effects of bile salts is very complex because they can be affected not only by the nature and concentration of bile acids used but also by a host of other conditions such as pH, calcium concentration, ionic strength, protein content and the like. These factors can modify the membrane damaging effects of the the various bile salts and determine whether a pathologic situation will ensue.

In this study we have employed a method that enables a continuous monitoring of the integrity of red blood cells (RBC) in a very dilute suspension. The method involves measurement of the optical density of RBC suspensions at long wave lengths. Since the volume of the sample of RBC added to the solution is very small, the content of the external solution could be well defined and remain constant throughout the monitoring period. The present study relates to the pH sensitivity of bile acid-induced hemolysis of rat RBC.

Materials and Methods

Rat venous blood was collected into neparin containing test tubes. The RBC were washed three times in isoosmotic cold solution having the following composition: Na 140 mM, K 4 mM, Ca 1 mM, Mg 1 mM, phosphate 1 mM, Cl 148 mM and glucose 5 mM. The pH of the solution was around 7.0. The washed RBCs

were kept at 4° C for up to a week. Before use the RBC were further washed in the above described isoosmotic solution and suspended in the same solution at about 20% (v/v) level. The suspension was kept in an ice cold bath. 5 μl of the RBC suspension were added to a cuvette containing 1 ml of a test solution kept at 25–27° C. The composition of the solution in the cuvette were the same as the above solution except for the addition of 8 mM Tris and a sodium salt of a bile acid. The pH of the solution in the cuvette was adjusted to a predetermined value by the addition of NaOH or HCl. In few experiments, were the pH was lower than 5.0, sodium acetate was used instead of Tris.

Immediately following the addition of the RBC to the test solution the optical density of the cuvette at 700 nm was either monitored continuously or recorded manually at discrete intervals. The results were introduced into files in a computer for analysis and graphic presentation. Control experiments indicated that the optical density of the red blood cells suspension was, to a good approximation, a linear function of the fraction of cells remaining intact. Therefore, the results of the various experiments were expressed as percent of the maximal optical density measured. In many experiments there was some small increase (1–5%) in the optical density during the first few minutes of the experiments. The nature and significance of these initial small changes are still under investigation.

The control experiments were carried out in the following way: a samples of RBC suspension was divided into two equal portions. One was diluted in one stage by the isoosmotic solution described above and the second portion was diluted in two steps first by distilled water to cause complete homolysis of the RBC and then by a hyperosmotic solution that gave the same final volume and osmolarity as with the first portion. The two samples were mixed at various proportions and the optical density of the mixture were read at 700 nm. Fig. 1 represents the results of three such experiments where the OD of the unhemolysed sample varied between 0.4 and 0.7. On the basis of these results we conclude that the optical density of RBC suspension is a fairly good indicator of the extent of hemolysis.

The sodium salts of cholic acid (> 98% purity), deoxycholic acid (> 97% purity), taurocholic acid (> 97% purity) were purchased from Fluka. Taurodeoxycholate was purchased from Sigma and glycodeoxycholate (> 98% purity) from Calbiochem. The free bile acids chenodeoxycholic acid (> 97% purity) and ursodeoxycholic acid (> 99% purity) were purchased from Fluka.

Results

Fig. 2 shows typical time course of changes in optical density of suspensions of RBC exposed to cholate con-

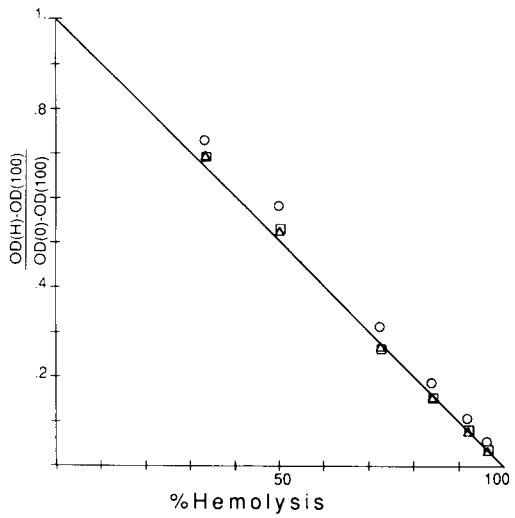

Fig. 1. The relationship between OD at 700 nm, OD(H), and percent of hemolysis in three independent experiments. The percent of hemolysis in a suspension was determined by mixing of two samples that contained the same amount of hemoglobin; one of which contained intact RBC and the other contained completely hemolysed RBC (see text for details). The OD for the zero percent hemolysis, OD(0), of the three experiments shown were 0.4 (△), 0.52 (□) and 0.8 (○). The OD for the completely hemolysed samples, OD(100), was less than 3% of the value for the solution containing the intact RBC. The value of the OD remained constant for up to 15 minutes and did not decrease by more than 2% by 30 min. For longer periods of time shaking of the suspension was necessary to prevent errors resulting from changes in OD due to RBC precipitation.

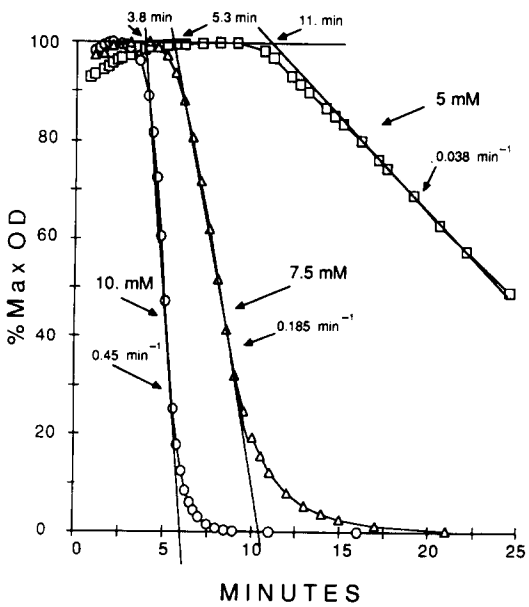

Fig. 2. Time course of hemolysis induced by cholate. The ordinate designate the percent change in optical density (OD) relative to the maximal optical density that occurred 1–5 min after the start of an experiment. The rate of hemolysis, i.e. the fraction of cells rupturing per unit time at the 50% hemolysis level, and the delay time for each curve are indicated. The composition of the external solution was as described in the text. pH 7.3. ○———○, 10 mM; △———△, 7.5 mM and □———□, 5 mM sodium cholate.

TABLE I

The rate and the delay-time of hemolysis induced by bile salts

Rat's red blood cells suspension were added to solutions containing cholate, deoxycholate, chenodeoxycholate or ursodeoxycholate at the indicated concentrations and pH values. ±denote standard deviations and *n* number of experiments.

	Concn. (mM)	pH	Slope (min^{-1})	Delay time (min)	*n*
Cholate	7.5	7.65	0.015	12.5	1
	7.5	7.30	0.202±0.039	7.6 ±1.0	10
	7.5	6.85	0.640±0.027	2.3 ±0.17	3
Deoxycholate	0.8	7.33	0.108±0.045	6.5 ±0.72	5
	0.3	6.35	0.230±0.042	1.77±0.48	5
Chenodeoxycholate	1.0	7.45	0.161±0.023	6.70±0.65	3
	1.0	7.20	0.972±0.20	2.61±0.49	3
Ursodeoxycholate	5.0	7.46	(not-hemolytic upto 25 min)		
	1.0	6.80	(not-hemolytic upto 25 min)		

taining solutions. The characterization of the hemolytic process was evaluated quantitatively by determining the slope of the curves at 50% hemolysis level and by the duration of the delay. The latter was defined as the point of intersection of the slope to the curve at 50% hemolysis level, with the line representing the zero percent hemolysis (see Fig. 2). The rate of hemolysis increased from 3.8% to 45% per min and the delay time decreased from 11 to 3.8 min as the cholate concentration increased from 5 to 10 mM, respectively. Thus, it can be seen that these parameters can be used to quantify the hemolytic potency of membrane damaging agents.

Table I sums up the results of experiments in which the slopes and delays of hemolysis caused by the common unconjugated bile acids at various pH values were determined. It is clear that lowering the pH increases the sensitivity of the cells to the bile acids as expressed by both the increase in the rate hemolysis and by the decrease in the duration of the delay. Thus, the rate of hemolysis induced by 7.5 mM cholate increased 3-fold and the delay time decreased by more than 3-fold, by a decrease of the pH from 7.3 to 6.85. An increase of the pH from 7.3 to 7.65 led to increase in delay time by close to 2-fold and to decrease in the rate of hemolysis by more than 10-fold. A similar increase in the hemo-

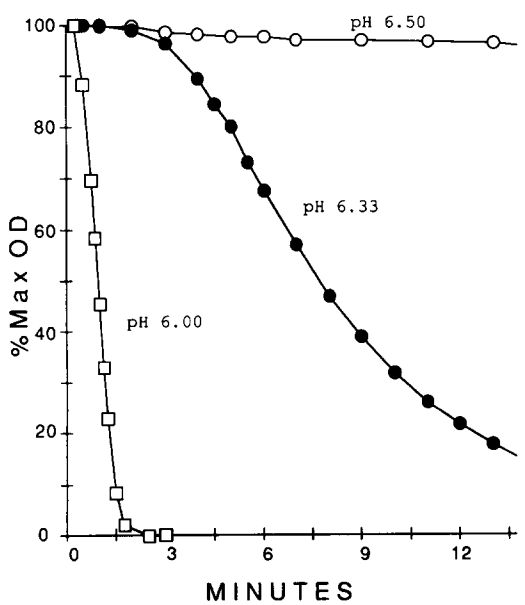

Fig. 3. Time course of hemolysis induced by deoxycholate at various pH values. RBC suspensions were added to solutions containing 0. 3 mM deoxycholate at pH 6.5 (○), 6.33 (●) and 6.00 (□).

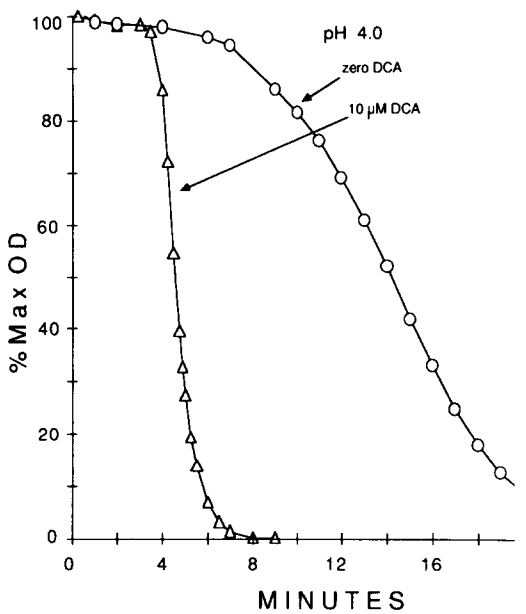

Fig. 4. Time course of hemolysis induced by acidic isoosmotic solutions with and without deoxycholate. RBC suspensions were added to an isoosmotic solution at pH 4.0 with (△) and without (○) 10 μM of deoxycholate present in the solution. The composition of the solution was as indicated in the text. Typical curves representing three experiments with deoxycholate and two experiments without deoxycholate are shown.

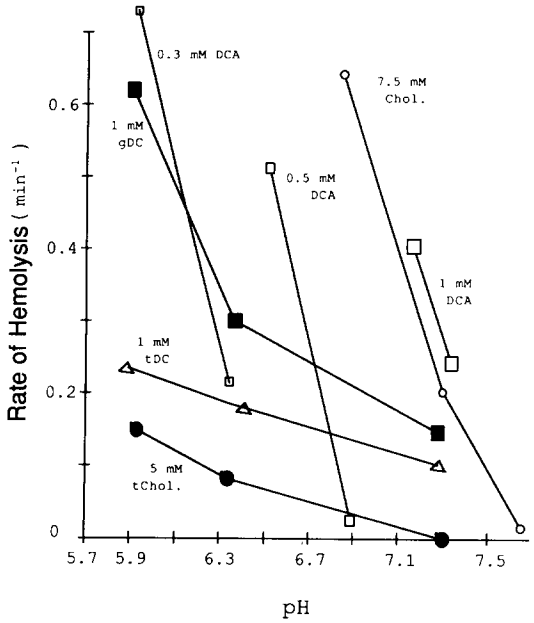

Fig. 5. The slope of the hemolytic curves caused by various bile acids as a function of pH. The points for cholate (7.5 mM) are the same as that presented in Table I. Each of the other points represent the average of at least three determinations. The variability in the values of the slopes did not exceed 20% of the average. Chol., cholate; DCA, deoxycholate; tDC, taurodeoxycholate; gDC, glycodeoxycholate and tChol, taurocholate.

lytic potency of chenodeoxycholate by decrease in pH was observed (Table I). It can be be seen from this table, however, that the hemolytic potency of the deoxycholate and chenodeoxycholate is stronger than that of cholate since the same range of rate of hemolysis and delay times were obtained with much lower concentration with the dihydroxy bile acids. Ursodeoxycholate, on the other hand, did not cause hemolysis even at concentration that were about 5-fold higher than that of the other dihydroxy bile acids. These findings were demonstrated previously by the conventional ways of following hemolysis by other investigators [e.g. 4,5].

Fig. 3 further demonstrates the dramatic effects of lowering the pH on the hemolytic potency of 0.3 mM deoxycholate, e.g. at pH 6.5 there is no evidence of hemolysis whereas at pH 6.0 the hemolysis is complete in less than 2 min. The results shown in Fig. 4 demonstrate that at pH 4 the hemolytic activity of deoxycholate can be clearly discerned at micromolar concentration, even though at such a low pH the acidity itself is also causing membrane damage. Thus, a transient decrease in pH can be detrimental to membranes if concentrations of unconjugated dihydroxy bile acids in the micromolar range are present.

The dependence of the rate of hemolysis caused by various bile acids on pH is shown in Fig. 5. It can be seen that the damage caused by tauroconjugates are the least sensitive to pH, the lysis produced by the glycoconjugate has a moderate sensitivity to pH and that caused by the free bile acids show the highest sensitivity to pH. These results are consistent with the assumption that the protonated neutral form of the bile acids is the more injurious compound to the membrane. Therefore, the tauro-conjugated compounds which have a pK of less than 2 are the least sensitive to pH whereas the unconjugated compounds which have a pK of about 5[11] are the most sensitive to pH.

The general conclusion from Fig. 5 and Table I is that at physiological pH the hemolytic potency of bile acids is in the order of unconjugated bile acids > conjugated bile acids and, with urso-derivative excluded, dihydroxy bile acids > trihydroxy bile acids. This agrees with earlier observations [12,13]. As the pH decreases the discrepancy between conjugated and unconjugated bile acids increases enormously. Due to this huge difference between conjugated and unconjugated bile acids, the results reported in this study do not exclude the possibility that the weak but clear hemolysis observed with conjugated bile acids at low pH values are caused by the presence of small amount (i.e., less than 3%) of free bile acids in the preparation. On the other hand, the hemolytic potency of the unconjugated di- and trihydroxy bile acids increases with decrease in pH in roughly the same degree, so that the difference between those two kinds of bile acids remains constant.

Since under physiological conditions the concentrations of unconjugated bile acids is very low compared to that of the conjugated forms, it was of interest to inquire whether the presence of conjugated bile acids can provide some protection from the injurious effects

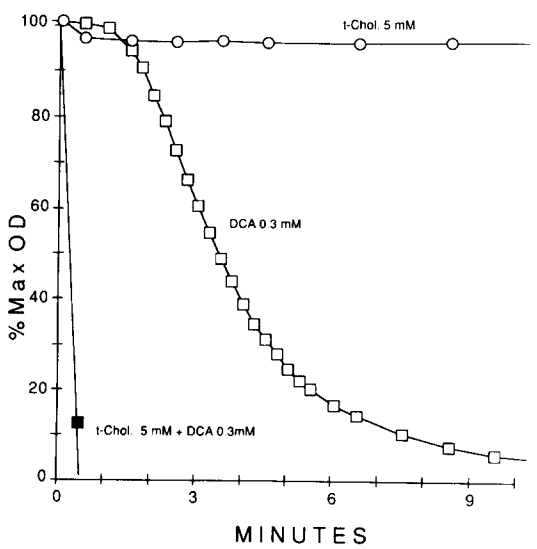

Fig. 6. Time course of hemolysis induced by taurocholate, deoxycholate or the combination of the two bile salts. Except for the bile salts concentrations the composition of the solution was as described in the text. Aliquots of RBC suspensions were added to solutions at pH 6.36 containing 5 mM taurocholate (○), 0.3 mM deoxycholate (□) and 5 mM taurocholate plus 0.3 mM deoxycholate (■).

of the free bile acids. The results shown in Fig. 6 demonstrate that this is not the case. As can be seen from Fig. 6 the presence of taurocholate in concentrations that are not hemolytic (e.g., 5 mM) greatly enhances the hemolytic potency of 0.3 mM deoxycholate. It should be stressed that it is unlikely that the taurocholate effect was only to 'solubilize' deoxycholate in the solution because at the pH of 6.36 the protonated form of deoxycholate should be only about 5% of the anionic form, i.e., about 15 μM which is well below the solubility of the protonated deoxycholic acid which is 28 μM [11].

Discussion

The method of monitoring the hemolytic process used in this presentation is based on the notion that the 'scattering optical density' (OD) is proportional to the concentration of scattering particles at least at low concentrations. The above relationship allows the monitoring of the change in light scattering of RBC suspension at a long wavelength (e.g., 700 nm). At this wavelength the OD measured due to hemoglobin absorption is nil and can be attributed solely to scattering. The notion that the measured OD relative to the initial value represent the fraction of intact remaining cells was validated (see Fig. 1). The method employed is therefore a sensitive and a quantitative way for exploring the membrane damaging effects of a particular compound at constant defined extracellular conditions.

The aim of the present communication is to call attention to the extreme pH sensitivity of cell membranes to damage induced by bile acids. The results suggest that increase in concentration of bile acids and lowering of pH combine to create a hazardous environment for the cells membrane. This conclusion seems to be at variance with the report that the mucosa in the colon is less susceptible to damage cause by deoxycholate at low pH values [14]. The latter finding was attributed to the relative insolubility of bile acids at low pH values. Bile acids have a very steep solubility-pH relationship (see, for example, Ref. 15) and therefore it is plausible that cells that are less susceptible to bile acids, i.e., cells that are injured by higher concentrations of bile acids, will phenomenologically behave as acid-resistant.

The general implication from the results of this study is that the protonated form of the bile acids is the more injurious form. A quantitative analysis of the results shown for the three concentrations of deoxycholate (i.e., 1, 0.5 and 0.3 mM) in Fig. 5 indicate, however, that the absolute concentration of the protonated deoxycholate is not the sole determinant of its membrane damaging potency. Thus, drawing a horizontal line through the lowest point in the 1 mM DCA curve in Fig. 5 cuts the DCA curves of 1.0, 0.5 and 0.3 mM at a pH of 7.34, 6.74 and 6.33, respectively. Assuming a pK of 5 for deoxycholate the corresponding protonated deoxycholate concentrations at these points will be 4.5, 9.0 and 14.0 μM, respectively. Therefore it is concluded that either the anionic form of the bile acids contributes also to its membrane damaging potency or that at this pH range (i.e., about 6.0–7.5) acidosis by itself renders the membrane more resistant to bile acid induced damage.

The pathophysiologic implications of pH changes on the morbidity of cells caused by bile salts is more complex, since the solubility of a particular bile salt can be varied by factors other than the hydrogen ion concentration. Most importantly, the solubility of harmful, more hydrophobic, bile salts, like lithocholate, can be increased by the presence of less injurious trihydroxy bile salts. In this context it is particularly interesting and surprising that the more benign taurocholate augments the susceptibility of the RBC membrane to damage induced by deoxycholate (see Fig. 6). The mechanism by which taurocholate augments the injurious effects of deoxycholate is not clear. It is possible that the initial attachment of deoxycholate to the membrane renders it susceptible to further damage by the taurocholate. Another possibility is that some potent contaminant of taurocholate preparation (about 97% pure) is responsible for the observed phenomenon. In this case it would be of interest to identify it in order to determine whether it may have any pathophysiological significance.

These observations may be of particular relevance to the understanding of the pathogenesis of duodenal ulcer and to damage caused to liver cells under anoxic situations. Both of the above cases raise the possibility that in regions constantly invaded by acid media (proximal portion of the duodenum), or subject to developing hypoxic acidosis, the presence of bile salts, even at relatively low concentrations, may cause cell injury. If mucosal cells in the digestive tract are also sensitive to the combinational presence of bile acids and high hydrogen ion concentration it may explain the prevalence of peptic ulcer in the first part of the duodenum, where such a combination is most likely to occur.

The present study also points to the significance of taurine conjugation process that takes place in the liver in avoiding the dangerous low pH range that could otherwise precipitate cell membrane damage. However, as already pointed out the presence of tauro-cholate does not provide a protection from the damaging effects of the more injurious bile acids if they are present even at low concentrations. In this context it will be worthwhile to look for pathologic situations where unconjugated bile acids, that are normally not found in human bile [16,17], appear in the liver or upper digestive system. Indeed if bacterial overgrowth in the intestine is present, the level of unconjugated bile acids in the serum can exceed 20 μM [18].

The points raised by this paper warrant a study of the pH dependence of membrane damaging effects of natural biles of patients suffering from diseases in which cell membrane damage by bile acids may be a pathogenic factor.

The increased hemolytic potency of bile acids at decreasing pH values corresponds to the general rule that the more hydrophobic species of bile acids are more damaging. Obviously, the protonated bile acid is more hydrophobic than the anionic form. A similar correspondence exists between the rate of transbilayer movement of bile acids and their hydrophobicity, where the 'flip-flop' of protonated bile acids are much more rapid than that of the anionic form [19]. It is tempting to suggest that in order to exert a membrane damage the bile acids has to exist on both sides of the membrane at some critical concentrations. As the pH is lowered, the fraction of protonated permeant form of the bile acid increases and therefore the concentration of bile acids in the interior of the membrane increases more rapidly, i.e., it attains the critical hemolytic level more readily. This possible mechanism for the pH dependence of the membrane damaging potency of bile acids is currently under investigation in our laboratory.

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