Biochemical Pharmacology, Vol. 30, No. 16, pp. 2287–2292, 1981.
Printed in Great Britain.

0006-2952/81/162287-06 $02.00/0

STUDIES ON THE HAEMOLYTIC ACTION OF AMPHIPHILIC SUBSTANCES *IN VITRO*

INHIBITION BY *O*-(β-HYDROXYETHYL)-RUTOSIDES*

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(*Received* 3 *December* 1980; *accepted* 11 *March* 1981)

Abstract—The water soluble rutin derivative *O*-(β-hydroxyethyl)rutosides (HR)† was found to inhibit the haemolytic action of a variety of amphiphilic substances on human red blood cells, without causing haemolysis by itself. The effect of HR on haemolysis caused by aescin, digitonin, cetyltrimethylammonium bromide, sodium dodecyl sulfate, polidocanol, sodium deoxycholate, chlorpromazine, promethazine, and thiazinamium was investigated *in vitro*. The antihaemolytic potency of HR was determined for each haemolysin and expressed as the AH_{50}, which is the concentration necessary to reduce 50 per cent haemolysis to 25 per cent. The biphasic behavior of the unspecific haemolysins in hypotonic buffer solution was studied in the presence and absence of 2×10^{-2} M HR. Both the stabilizing and the destabilizing part of the curve were shifted to higher concentrations, with the exception of sodium deoxycholate, where only the destabilizing part was affected. Two possible mechanisms are discussed: (1) the unspecific, hydrophobic interaction of HR with the single haemolysins resulting in complex formation, and (2) the protection of the membrane by competition of the inhibitor with the haemolysin for the binding site.

INTRODUCTION

The mechanism of haemolytic action of amphiphilic substances has been studied extensively in recent years [2–10]. Nevertheless, it is still not clear how these compounds alter the cell membrane rendering it permeable for haemoglobin. Most of amphiphilic agents exhibit a biphasic action, i.e. they protect *in vitro* red blood cells against hypotonic haemolysis (first phase: stabilization) but cause haemolysis at higher concentration (second phase: destabilization) [11–13]. Saponins lack the protecting effect and are therefore called specific lysins [11].

One approach to gain more insight into the mechanisms of haemolysis would be the application of substances which inhibit the action of amphiphilic substances. Haemolysis caused by acyl carnitins, lysolecithins and acyl cholins was studied by Cho and Proulx [14] using isolated membrane components as inhibitors. Complex formation has been suggested as a reason for the inhibition of saponin haemolysis by cholesterol [15, 16]. Segal [17] investigated the protective action of glycyrhizin on haemolysis caused by saponins and by the cationic surfactant *N*,*N*-dimethyl-*N*-hexadecyl-*N*-(1,1-dimethyl,2-hydroxyethyl)ammoniumbromide. Furthermore, the use of inhibitors for characterization of substances with haemolytic activity is very common and has been used recently to study the action of the Portugese Man of War toxin [18] and also by Seeger [19] to characterize rubescenslysin a haemolysin from the edible mushroom *Amanita rubescens*. It has been proposed by Felix [20, 21] and Hammersen [22] that *O*-(β-hydroxyethyl)rutosides (HR) which consist of water soluble rutin derivatives (Fig. 1) and are used in the therapy of venous circulation disorders [20] exert their action by an unspecific membrane protection. This has been suggested not only for the endothelium but also for smooth muscles as reported by Felix [20]. Using the erythrocyte as a model we studied the effect of HR

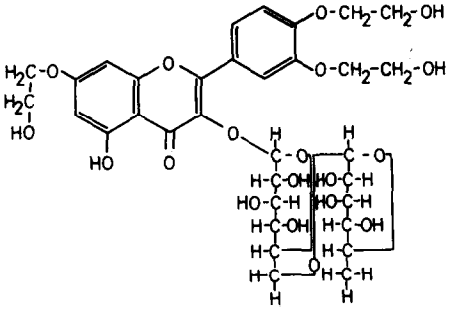

Fig. 1. Structural formula of 3′,4′,7′,tri-*O*-(β-hydroxyethyl)rutoside. The substance which has been used in the described experiments consists of a mixture of mono-, di-, tri-, and tetrahydroxyethyl-substituted rutosides, the depicted compound being the main component (60–65 per cent).

* A preliminary report has been given at the 21st Spring Meeting of the German Pharmacological Society at Mainz, 18–21 March, 1980 [1].

† Abbreviations used: HR, *O*-(β-hydroxyethyl)-rutoside; SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; CTAB, cetyltrimethylammonium bromide; CPZ, chlorpromazine.

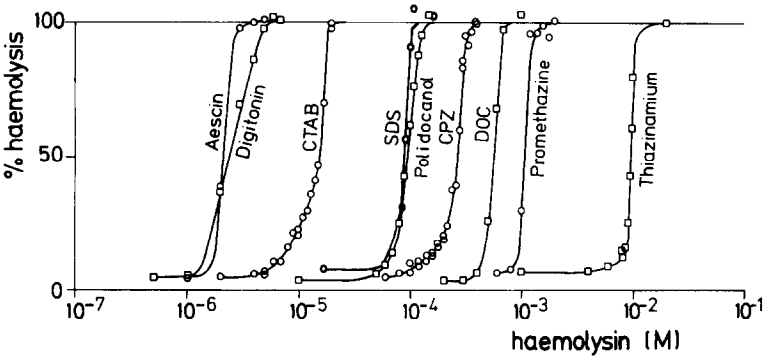

Fig. 2. Haemolytic potency of the haemolysins. Sixty-six microlitres of a erythrocyte stock solution with 5 per cent haematocrit were added to 1 ml of a haemolysin solution in phosphate buffered saline in the indicated concentrations and incubated for 30 min at room temperature. The change in absorbance at 540 nm was measured and per cent haemolysis was calculated by comparison with a blank solution containing distilled water.

on haemolysis caused by positively, negatively, and neutrally charged surfactants, as well as by saponins, and phenothiazine-derivatives, and will discuss the possible mechanism of this reaction.

EXPERIMENTAL SECTION

Materials. HR was kindly supplied by Zyma (München, West Germany); cetyltrimethylammonium bromide was purchased from Sigma (München, West Germany); sodium deoxycholate and digitonin were from E. Merck (Darmstadt, West Germany); sodium dodecyl sulfate from BioRad (München, West Germany); hydroxypolyethoxydodecane (polidocanol) from Kreussler (Wiesbaden-Biebrich, West Germany); aescin was from Madaus (Köln, West Germany); chlorpromazine, promethazine and thiazinamium methyl sulfate were obtained from Bayer (Leverkusen, West Germany). All other used chemicals were reagent grade and bought from E. Merck (Darmstadt, West Germany).

Methods. Erythrocytes were obtained from outdated human blood which had been treated with EDTA to prevent coagulation. Plasma and buffy coat were removed by centrifugation at 2500 g_{max} for 10 min at 4° and the erythrocytes were washed three times with 150 mM NaCl containing 5 mM phosphate pH 7.4 by centrifugation. The packed cells were then diluted with the same buffer to give a stock suspension of 5 per cent haematocrit.

Determination of haemolytic activity was carried out by adding 66 μl of the erythrocyte stock suspension to a solution of the haemolysin in 1000 μl phosphate buffered saline pH 7.4, giving a final haematocrit of 0.3 per cent. After 10 or 30 min incubation at room temperature the degree of haemolysis was determined by measuring the absorbance at 540 nm. Per cent haemolysis was calculated from the values obtained at different haemolysin concentrations and from a control which contained distilled water and was used as the value for 100 per cent haemolysis. For inhibition experiments isotonic solutions of HR in different concentrations were added to solutions of the haemolysins which had been adjusted to cause 50 per cent haemolysis (H_{50}). The erythrocyte stock suspension of 5 per cent haematocrit was added as described. After incubation for 30 min at room temperature, the absorbance at 540 nm was measured. Absorbance by HR at this wavelength was negligible up to a concentration of 10^{-2} M. For higher concentrations the data was corrected using a corresponding blank solution of HR. Since it was difficult to achieve the 50 per cent haemolysis in every case, values between 30 and 70 per cent were accepted and converted into relative haemolysis 1 [12].

For biphasic haemolysis curves a hypotonic buffer solution was chosen which caused 50 per cent haemolysis upon addition of the erythrocyte stock suspension. Increasing amounts of the haemolysin were given to the solution in the presence and in the absence of HR (2×10^{-2} M). After a 10 min incubation at room temperature the degree of haemolysis was determined and the data converted into relative haemolysis as described above.

RESULTS

In order to characterize the amphiphilic substances used according to their haemolytic potency, the concentrations which caused haemolysis were determined. The increasing extent of haemolysis for increasing concentrations can be seen in Fig. 2 and shows the wide range of haemolytic potency of the compounds used. The values for 50 per cent haemolysis (H_{50}) were taken from these curves and are listed in Table 1 in decreasing order.

When washed human erythrocytes and HR in increasing concentrations were added to an amount of haemolysin corresponding to the H_{50}, inhibition of haemolysis occurred for each of the compounds used (Fig. 3). The effect was strong in the case of saponins, the phenothiazine derivatives, and polidocanol and significantly weaker for sodium deoxycholate, sodium dodecyl sulfate and cetyltrimethylammonium bromide. The inhibition was dose-dependent, reaching 100 per cent in every case. The degree of inhibition was not influenced when the single components of the mixture were added in a different order. Even preincubation of erythrocytes with HR or preincubation of the haemolysin with HR did not alter the effect significantly.

For comparison the AH_{50} values were calculated,

Table 1. The haemolysins are listed according to their haemolytic activity which is expressed by the H_{50} values

Haemolysin	H_{50} [M]*	AH_{50} [M]†	Haemolysin : HR‡ Molecules	Shift factor§	CMC [M]‖
Aescin	2×10^{-6}	3×10^{-4}	1 : 150	5**	10^{-5} [24]
Digitonin	2×10^{-6}	7×10^{-4}	1 : 350		
CTAB	1.5×10^{-5}	2×10^{-2}	1 : 1333	2	2.8×10^{-4} [10]
Polidocanol¶	9×10^{-5}	9×10^{-4}	1 : 10	2	
SDS	1×10^{-4}	7×10^{-3}	1 : 70	2	3.4×10^{-3} [10]
Chlorpromazine	2.7×10^{-4}	7.5×10^{-4}	1 : 3	14	5.4×10^{-3} [25]
Sodium deoxycholate	6×10^{-4}	3.5×10^{-3}	1 : 6	2	2.4×10^{-3} [26]
Promethazine	1×10^{-3}	2.6×10^{-4}	4 : 1	17	
Thiazinamium	1×10^{-2}	6×10^{-4}	17 : 1	4	

* Concentration of haemolysin which is required to generate 50 per cent haemolysis in an erythrocyte suspension of 0.3 per cent haematocrit after 30 min incubation in phosphate buffered saline pH 7.4 at room temperature. The values were taken from Fig. 2.

† Concentration of HR which is needed for 50 per cent inhibition of haemolysis caused by the H_{50} of the haemolysin. The conditions for incubation were the same as described above. The values were taken from Fig. 3.

‡ Numerical relation of molecules haemolysin and molecules HR at the AH_{50}.

§ Shift factor: quotient of the haemolysin concentrations in presence and in absence of 2×10^{-2} M HR, where the curve reaches the value 1 of relative haemolysis [Figs. 5(a–c)]. The experimental details are given in Fig. 5.

‖ The critical micelle concentration (CMC) for the haemolysins were taken from the literature where available. The literature is cited in brackets.

¶ A mean mol. wt of 600 was taken since polidocanol comprises a mixture of polyoxyethylene laurylethers.

** The effect on the specific haemolysins aescin and digitonin was not studied in hypotonic solution. The given value was taken from Fig. 4 and represents the shift at 50 per cent haemolysis.

i.e. the concentration of HR which reduced 50 per cent haemolysis to 25 per cent (AH_{50} = 50 per cent antihaemolysis). Table 1 shows that inhibition by HR expressed by the AH_{50} values cannot be directly correlated to the haemolytic potency which is expressed by the H_{50} values. If the molecules of HR which caused 50 per cent antihaemolysis were calculated the difference between the amphiphilic substances used became apparent (Table 1). 1333 HR molecules per molecule of haemolysin were necessary to obtain the AH_{50} of the positively charged cetyltrimethylammonium bromide, whereas in the case of thiazinamium, which also carries a positive charge, only 1 HR molecule for 17 molecules haemolysin is needed.

If the haemolysins were arbitrarily divided into three groups it is seen that the agents with a strong haemolytic potency (aescin, digitonin, cetyltrimethylammonium bromide) needed more HR molecules for inhibition than substances with moderate (polidocanol, sodium dodecyl sulfate, chlorpromazine, sodium deoxycholate) or weak (promethazine, thiazinamium) haemolytic properties. The value for the critical micelle concentration for each compound

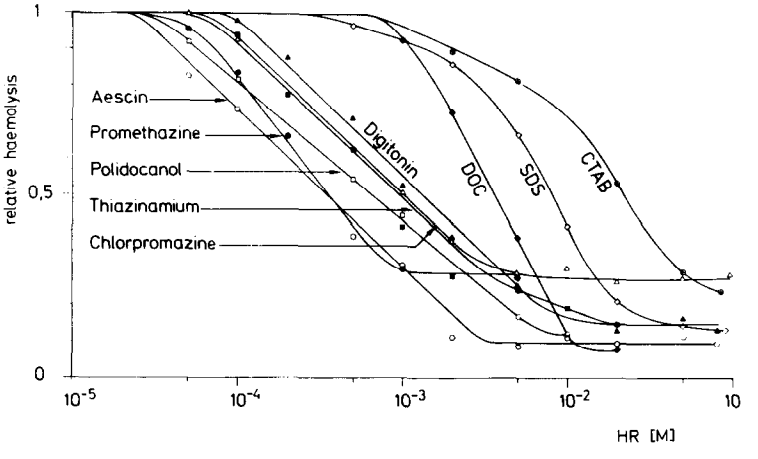

Fig. 3. Effect of HR on haemolysis caused by different haemolysins. HR in different concentrations was added to solutions of the haemolysins in phosphate buffered saline, in a concentration corresponding to the H_{50} (total volume 1 ml). Sixty-six microlitres of the erythrocyte stock solution (5 per cent haematocrit) were added, incubated for 30 min at room temperature, and the absorbance at 540 nm was determined. The initial value of haemolysis was taken as relative haemolysis 1 [11]. The values obtained after addition of HR were calculated accordingly.

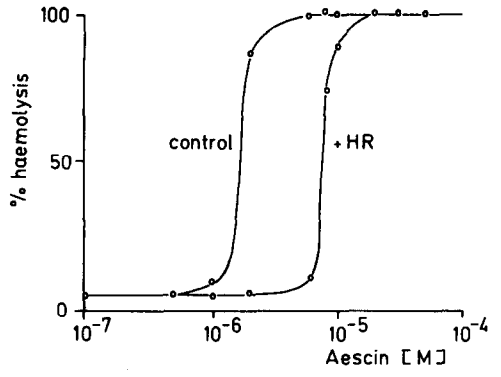

Fig. 4. Effect of HR on aescin haemolysis. Erythrocytes (66 μl of a stock solution with 5 per cent haematocrit) were added to 1 ml phosphate buffered saline containing increasing amounts of aescin in the presence and absence of 2×10^{-2} M HR. The mixture was incubated for 10 min at room temperature.

when available was taken from the literature and is listed in Table 1. No correlation between the critical micelle concentration and the AH_{50} seems possible.

In another experiment, inhibition of haemolysis by HR was analyzed at increasing concentrations of the amphiphilic substance. Figure 4 shows a dose–effect curve for aescin at a HR concentration of 2×10^{-2} M. The parallel shift of the curve suggested a competition between the two compounds. Similar results were obtained from the other haemolysins.

The influence of HR on the biphasic action of unspecific haemolysins was also examined. An erythrocyte suspension was added to hypotonic buffer solution which caused about 50 per cent lysis. The changes in absorbance at 540 nm, caused by haemolysins in increasing concentrations were observed in the absence and in the presence of HR (2×10^{-2} M). Three types of curve alterations were obtained [Figs. 5 (a–c)]. In the case of polidocanol, sodium dodecyl sulfate and thiazinamium, the entire

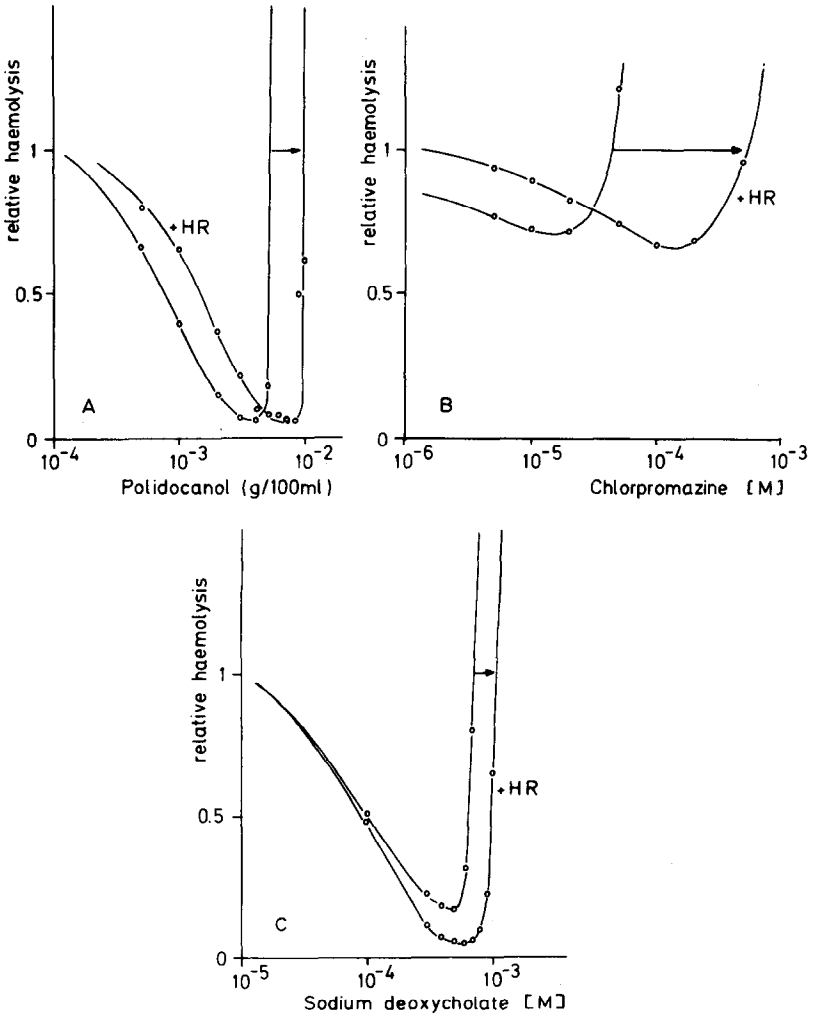

Fig. 5. Effect of HR on the biphasic haemolysis curve of unspecific haemolysins: (a) Polidocanol; (b) chlorpromazine; (c) sodium deoxycholate). Erythrocytes (66 μl of a stock solution with 5 per cent haematocrit) were added to 1 ml hypotonic buffer solution which had been adjusted to cause 50 per cent haemolysis. The buffer contained increasing amounts of the haemolysin and the procedure was carried out in the presence and absence of 2×10^{-2} M HR. The mixture was incubated for 10 min at room temperature. The initial value of hypotonic haemolysis was taken as relative haemolysis 1.

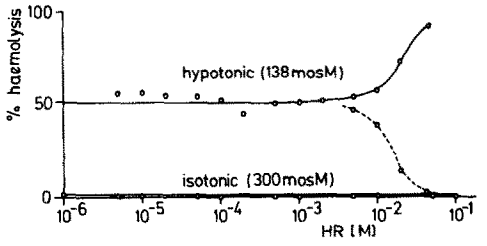

Fig. 6. Haemolytic properties of HR. Haemolysis in isotonic medium: 66 μl of the erythrocyte stock suspension (5 per cent haemotocrit) were added to 1 ml isotonic phosphate buffered saline containing increasing amounts of HR and incubated for 30 min at room temperature (curve at the bottom). For haemolysis in hypotonic solution, 66 μl of the erythrocyte stock solution were added to 1 ml hypotonic buffer solution containing HR in different concentrations and incubated for 10 min at room temperature. Solid line: the HR solution was adjusted to 138 mOsM for any used concentration. Dotted line: HR was dissolved in hypotonic (138 mOsM) buffer.

pattern was shifted to higher concentrations without changing the degree of protection or the slope of the haemolysis curve. Figure 5(a) shows the effect for polidocanol. A similar pattern was obtained for chlorpromazine [Fig. 5(b)] and promethazine (not shown) with the exception that the shift was far stronger. A different pattern could be seen when HR was present during the action of sodium deoxycholate on red blood cells in hypotonic solution [Fig. 5(c)]. The lytic effect of the detergent was moved to higher concentrations, as in the case of the other substances. The protecting effect, however, occurred at the same concentration in the presence or in the absence of HR, but was more pronounced in the presence of HR. This experiment was done also with different concentrations of HR. No shift of the stabilizing limb of the curve could be seen at any concentration used.

Since the shift of the lytic part of the haemolysis curve to higher concentration of amphiphilic compound was different for each haemolysin at a constant concentration of HR (2×10^{-2} M), a shift-factor was calculated.

If one compares the different shift-factors which are listed in Table 1, a strong inhibitory effect of HR on the chlorpromazine and promethazine caused haemolysis is noticed, which cannot be seen from the AH_{50} values.

Since HR is surface active (the critical micelle concentration was determined and found to be about 10^{-3}M) its haemolytic properties in isotonic and hypotonic solution were examined. No haemolysis could be detected under isotonic conditions (Fig. 6).

An apparent protection against hypotonic haemolysis, which was observed at higher concentrations (Fig. 6, upper curve, dotted line) could be due to the altered osmolarity upon addition of HR. If the added solution was kept at a osmolarity of 138 mOsM throughout the investigated concentration range, the rate of haemolysis was increased above 10^{-2} M HR (Fig. 6, upper curve). This indicates the osmotic nature of the observed effect.

DISCUSSION

The most interesting finding of this study is the variety of haemolysins which can be inhibited by HR ranging from negatively (sodium dodecyl sulfate, sodium deoxycholate) to positively charged molecules (cetyltrimethylammonium bromide, thiazinamium), molecules with condensed ring-systems (saponins, phenothiazines) and with long aliphatic chains (sodium dodecyl sulfate, cetyltrimethylammonium bromide, polidocanol). There was no feature in common among these compounds except their capability of forming micelles. The inhibitory potency of HR was quantitatively quite different, even for closely related lysins like the saponins or phenothiazines or for substances with the same charge.

HR exerted a stronger effect against saponin haemolysis *in vitro* than glycyrhizin, which was found as inhibitor and suggested as protective agent by Segal *et al.* [17]. Comparing the inhibitory potency of HR with their data for glycyrhizin, HR is about 10 times more potent. Whether HR protects erythrocytes *in vivo* against saponin haemolysis remains to be investigated.

There is no simple explanation for the mechanism of the observed inhibition of haemolysis by HR.

Two types of protective agents can be distinguished between by changing the order of addition of the single components to the reaction mixture [23]. Enhanced inhibition after preincubation of HR with erythrocytes would mean, according to Ponder [23], that the inhibitor protects the erythrocyte membrane, whereas enhancement after preincubation of HR with the lysin would suggest a reaction between these two components. Glycyrhizin for instance seems to protect the membrane and thus prevent the access of the haemolysin to its receptor [17]. On the other hand, Cho and Proulx [14] found an enhancement of the inhibitory effect after preincubation of membrane constituents with the haemolysins acyl carnitins, lysolecithins, and acyl cholins. This suggests a nonspecific interaction of the lysins with the inhibitors. In the case of HR, however, the order of addition apparently had no influence on the degree of inhibition. Both types of inhibition mechanisms seem possible: (1) reaction of HR with lysins; (2) protection of the membrane by HR.

(1) *Reaction of HR with the lysins*

Considering the big differences in the structure and charge of the haemolysins used, only an unspecific hydrophobic interaction between HR and the lysin should be possible. Formation of HR micelles could give the possibility of intercalation of the haemolysins. This mechanism would be in agreement with the competitive character of the inhibition, since the free lysin concentration which caused haemolysis can be reached again when the binding capacity of the HR complexes is saturated. The fast inactivation of the lysin without influence of preincubation favors a unspecific hydrophobic interaction. On the other hand, micelle formation has not been found to have an effect on the haemolytic action of amphiphilic substances [2]. Even mixed micelles obtained from

a mixture of two detergents did not inhibit their haemolytic activity [2]. Furthermore, in preliminary experiments (D.B. Wildenauer, B. Becker, unpublished results) we could not detect mixed micelles in a chlorpromazine (3×10^{-4} M)/HR (5×10^{-3} M) solution by chromatography on a Sephadex G 25 column which had been equilibrated with 5×10^{-3} M HR.

(2) *Protection of the membrane against the action of lysins*

Unspecific binding of the haemolysins to the membrane is thought to be the first step in haemolysis caused by amphiphilic substances [3, 4, 9]. Protection of erythrocytes by combination of HR with the surface and thus competition with the binding of the haemolysins could possibly be another mechanism for the inhibitory action of HR on haemolysis. The failure to enhance inhibition by preincubation should not necessarily exclude this possibility if the affinity to the binding site is in the same range as that of the haemolysins.

The differences in the AH_{50} values would then represent the different affinity of the haemolysins for the membrane binding sites. This would be an interesting mechanism and would mean that HR behaves in regard to the binding like a haemolysin without having a stabilizing and destabilizing effect on the erythrocyte membrane (Fig. 6).

Since the watersoluble rutin derivative HR represents a mixture of mono-, di-, tri-, and tetra-substituted rutosides, different actions of the single components were taken into consideration. To exclude this possibility, the supernatant of a solution of HR which was preincubated with erythrocytes was used for inhibition experiments. The same inhibitory activity was found as in a control solution. There seems to be no specific component of the HR mixture which was bound to the erythrocyte membrane being responsible for the observed effects.

There is no explanation for the observation that the stabilizing part of the sodium deoxycholate haemolysis curve is not shifted to higher concentrations [Fig. 5(c)].

Our results cannot be used to explain the protective effects of HR on oedemas [22] or on smooth muscles [20] which are thought to be based on an unspecific membrane protection [20, 22].

The only common feature seems to be an unspecific protection of cells against membrane damaging events.

Using a wide variety of amphiphilic substances, the inhibitory action of HR on haemolysis was studied. Reversible interaction of HR with components of the reaction mixture has been found, but further work is needed to distinguish between the possible mechanism, either reaction of HR with haemolysins or reaction with binding sites of the membrane.

Acknowledgements—The authors wish to thank A. von Nieciecki for the determination of the CMC of HR and aescin. We are grateful to Susan Metzger for reading the manuscript.

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