Biochemical Pharmacology, Vol. 34, No. 1, pp. 31–38, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

ENTRAPPING OF THE SPIN LABEL TEMPOCHOLINE INTO HUMAN ERYTHROCYTES BY RESEALING AFTER HYPOSMOLAR STRESS. COMPARISON WITH HAEMOLYSIS. THE EFFECTS OF SOME MEMBRANE-ACTIVE SUBSTANCES

CARL LAGERCRANTZ*, THOMAS LARSSON and LARS TOLLSTEN
Department of Medical Physics, University of Göteborg, Box 33031, S-400 33 Göteborg, Sweden
and
KAREN PRUS
AB Ferrosan, Box 839, S-201 80 Malmö, Sweden

(*Received* 8 *May* 1984; *accepted* 9 *July* 1984)

Abstract—Human erythrocytes were subjected to a sudden hyposmolar stress by suspension in solutions of varying salt concentrations in the presence of the spin label tempocholine. The enlarged pores in the erythrocyte membranes produced by the influx of water, followed by stretching, allowed the passage of the spin label, so that a certain amount of tempocholine was entrapped when the erythrocytes spontaneously resealed with closing of the pores. The excess of spin label in the external solution was then reduced to a diamagnetic species by the addition of ascorbic acid. The positively charged tempocholine and the ascorbic acid did not penetrate properly resealed erythrocytes, so that the electron spin resonance (ESR) signal from the entrapped spin label constituted a measure of the effective resealing of the pores and rifts in the membrane subsequent to hyposmolar stress.

Some drug substances were found to influence the entrapping curves obtained when the amount of entrapped spin label was plotted against the osmolarity. Chlorpromazine, trifluoperazine, nicardipine, amperozide and haloperidol gave rise to a dose-dependent decrease of the entrapping of tempocholine, especially at low osmolarities. The exclusion of Ca^{2+} and Mg^{2+} ions from the solutions increased the action of chlorpromazine.

The protective action against haemolysis brought about by a number of membrane-active substances at low concentrations [2] had its counterpart in the entrapping curve observed with chlorpromazine at 0.1 mM.

It is suggested that the substances in this series exerted their action on the resealing process by interaction with the calmodulin system.

When human erythrocytes are subjected to a sudden hyposmolar stress by suspension in hyposmolar solutions, there is influx of water followed by an increase of the cell volume, which gives rise to stretching of the membrane, so that existing pores are enlarged or new pores are formed. Below a certain level of osmolarity of the external solution, the swelling of the red cells brings about pores or rifts in the membrane large enough for the passage of haemoglobin. After these initial events the erythrocyte ghosts, now more or less depleted of haemoglobin, reseal, i.e. the enlarged pores or rifts are spontaneously closed. However, ghosts have been found to reseal differently. According to Hoffman [1], ghosts have been classified into the following three classes: Type I: ghosts which reseal immediately after haemolysis; Type II: ghosts which only reseal after restoration of the normal tonicity; and Type III: ghosts which never retain their low permeability to cations.

Many substances have been found to influence the haemolysis of human erythrocytes. Some of these substances, such as deoxycholate, exert their action by disrupting the lipid bilayer, so that haemoglobin is able to leak through the defects formed in the membranes. Other substances have been found to have a biphasic influence on erythrocytes. Above a certain level of concentration, these substances cause lysis of the red cells, whereas they have a protective action against osmotic haemolysis at lower concentrations. According to Seeman [2], their action involves an increase in the surface of the red cell membranes. These substances, known as membrane expanders and membrane stabilizers, include a variety of anaesthetics and tranquilizers of different structures.

The action mechanism of the membrane expanders seems not to have been fully established, especially not their interaction with the resealing system. We have recently studied this problem in human erythrocyte ghosts subjected to hyposmolar stress in the presence of a number of membrane-active substances (Lagercrantz *et al.* [3]). In these experiments, the efflux of the spin label tempocholine entrapped in the ghosts, was measured. It was found that the duration of the efflux following hyposmolar stress was less than half a minute. After this time, the ghosts resealed spontaneously and without restoration to normal osmolarity. The drug substances

* To whom correspondence should be addressed.

chlorpromazine, trifluoperazine and nicardipine were found to increase the hyposmolar efflux of spin label. It was suggested that these substances, classified as calcium-antagonists and inhibitors of the calmodulin system, exert their action on the efflux of spin label by interaction with membrane proteins which maintain shape and tension of the erythrocytes.

This paper describes some experiments in which the resealing of human erythrocytes has been studied in more detail by measuring the entrapping of the spin label tempocholine. The erythrocytes were subjected to a sudden hyposmolar stress by suspension in solutions of varying salt concentrations in the presence of tempocholine. The enlarged pores or rifts formed in the erythrocyte membranes allowed passage of the spin label to the interior of the red cells, so that a certain amount of the spin label was entrapped when the erythrocyte membranes resealed. The excess of spin label in the external solution was then reduced to a diamagnetic species by the addition of ascorbic acid, which did not penetrate through properly resealed membranes. The ascorbic-acid-protected signal from the spin label entrapped in the erythrocytes constituted a measure not only of the effective pore width and duration of the open state brought about by hyposmolar stress, but also of the efficiency and extent of the resealing. Once entrapped inside the resealed erythrocytes, positively charged tempocholine was not able to penetrate the intact cell membranes [4, 5]. The membrane-active susbtances to be tested were added to the buffer solutions of varying osmolarities. Entrapping curves were obtained by plotting the ascorbic-acid–protected signal against the actual osmolarity.

The relation between the entrapping of tempocholine and the osmotic leakage of haemoglobin from the red cells was also studied. Haemolysis curves were constructed where the amount of haemoglobin in the external solution was plotted against the osmolarity. Chlorpromazine, trifluoperazine, nicardipine, amperozide and haloperidol were found to introduce dose-dependent changes in both the entrapping and haemolysis curves.

The advantage of the spin label technique in the form utilized here for studies of efflux and influx through pores in the erythrocyte membranes arises because the amount or concentration of a reporter molecule such as tempocholine can be evaluated without previous separation of the erythrocytes from the external solution. Thus the distinction of inside-outside localization of the spin label was brought about by using a molecular-spectroscopic property, i.e. exchange broadening, in the previously described efflux method [3], but is realized in the present method, i.e. the influx or entrapping method, by a chemical reaction in which the spin label outside the erythrocytes is reduced to a diamagnetic species.

MATERIALS AND METHODS

Materials. Tempocholine iodide was prepared as described earlier [3]. Ascorbic acid and sodium ascorbate were obtained from Fluka AG. Several of the membrane-active substances investigated in the present experiments had been tested with the efflux method [3]. Additional substances were amperozide (FG 5606), i.e. *N*-ethyl-4-[4,4-bis(fluorophenyl) butyl]-1-piperazinecarboxamide hydrochloride (Hogpax®), obtained from AB Ferrosan, Malmö, Sweden, and haloperidol, obtained from AB Leo, Helsingborg, Sweden.

Entrapping of tempocholine into human erythrocytes. Human erythrocytes (concentrate, blood-bank blood) were washed four times with buffer solution (A) with the following concentrations: 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 1 mM $MgCl_2$, 0.3 mM $CaCl_2$ and 154 mM NaCl, and adjusted to pH 7.4. After the last centrifugation the supernatant was removed so that the erythrocyte concentration was about 95%, as evaluated by haematocrite centrifugation. The erythrocytes were kept close to 0° during the preparation, and the preparative centrifugations were performed at 4° and 10,000 *g*.

Resealing with entrapping of spin label after suspension of the erythrocytes in hyposmolar solutions was performed as follows. To 50 μl of washed erythrocytes, 50 μl of a buffer solution was added, with the following concentrations: 10 mM HEPES, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 54 mM NaCl and 100 mM tempocholine iodide, followed by the addition of 400 μl of buffer solution, pH 7.4, with the concentrations: 10 mM HEPES, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$ and varying amounts of sodium chloride, so that the final concentrations corresponded to values between 0.145 and 0.020 M NaCl. The tempocholine iodide concentration was thus 10 mM in the samples. The erythrocyte suspensions were then incubated for 1 hr at 37°. After the incubation the suspensions were kept at 0° for 10 min.

Reduction of excess spin label to a diamagnetic species. To the chilled erythrocyte suspension (500 μl), 10 μl of 1 M ascorbic acid dissolved in H_2O (freshly prepared) was slowly added with gentle mixing. The pH of the mixture was now about 5.

Electron spin resonance (ESR) measurements. The ESR spectra were recorded as described earlier [3]. The erythrocyte suspension was transferred to the ESR sample cell about 3 min after the addition of the reducing agent, a time sufficient for the reduction of the excess of spin label in the external solution. The ESR spectra were recorded at 20°. The amplitude of the spectral component $M_I = +1$ was used as the actual measure of the amount of tempocholine entrapped in the erythrocytes.

Substances tested for influence on the entrapping of tempocholine. Membrane-active substances to be tested, such as chlorpromazine, were added to the appropriate concentration to the buffer solutions (400 μl) before the addition to the erythrocyte suspension.

Representation of the results. Figures 1(a), 1(b), 2, 3(a), 4, 5(a) and 6–8 show the relative amount of entrapped tempocholine as a function of the osmolarity of the external solution in which the erythrocytes are suspended. The osmolarity given as osM was taken as: 2 × (molarity of NaCl + molarity of tempocholine iodide). For practical reasons the contribution from the buffer salts was omitted.

The reference curves constitute the results obtained without any added membrane-active sub-

stances. A separate reference curve was made for every preparation of erythrocytes. Aliquots of the preparations were used for the reference curve and for the experiments in which drug substances were added. All measurements were made under experimental conditions as identical as possible, i.e. with the same microwave power, modulation amplitude and orientation of the ESR sample cell in the cavity. The ESR signal amplitude was recorded in arbitrary units and given as the relative signal amplitude in the figures.

Haemolysis experiments. Erythrocyte suspensions were prepared in buffer solutions corresponding to an osmolarity range between 0.310 and 0.060 osM. To 50 μl of washed erythrocytes (see above), 50 μl of buffer solution (A) was added, followed by the addition of 400 μl of a buffer solution, pH 7.4, with the concentrations: 10 mM HEPES, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, and varying amounts of sodium chloride, so that the final concentration corresponded to values between 0.155 and 0.030 M NaCl. The erythrocyte suspensions were then incubated for 1 hr at 37°. After the incubation the suspensions were kept at 0° for 10 min.

After separation of the cells by centrifugation (2500 *g*), 300 μl of the supernatant was removed for estimation of haemoglobin by the cyanomethaemoglobin method [6]. To 100 μl of the supernatant 10 ml of a solution containing 1 g $NaHCO_3$, 0.050 g KCN, and 0.200 g $K_3Fe(CN)_6$ diluted to 1 with H_2O was added. The reaction mixture was left standing for at least 20 min. The absorption was then recorded at 540 nm. The results were presented by plotting the absorption against the actual osmolarities.

RESULTS

Entrapping of tempocholine in the absence of added drug substances

A stable ESR signal that represents exclusively the spin label entrapped in the erythrocytes subsequently resealed to hyposmolar stress is a prerequisite for the present method. First of all, the label should not be able to penetrate through properly resealed erythrocyte membranes, neither from the inside nor from the outside. Positively charged tempocholine has been found to fulfil this condition [3–5].

The amount of tempocholine entrapped in the erythrocytes is proportional to the concentration of added tempocholine. Therefore, it is essential to use an added tempocholine concentration as high as possible for obtaining a measurable ESR signal amplitude in the ranges of osmolarity where the entrapping is low. However, the concentration of added tempocholine has to be kept below the concentration range of exchange broadening if a linear relation is to be obtained between the signal amplitude and concentration. As a compromise, a tempocholine concentration of 10 mM was used in the experiments of this series. This value was selected from an experimental curve where the signal amplitude was plotted against the tempocholine concentration. The signal amplitude is an increasing function up to about 10 mM, a value which also constitutes a maximum. At higher concentrations the signal amplitude decreases due to exchange broadening.

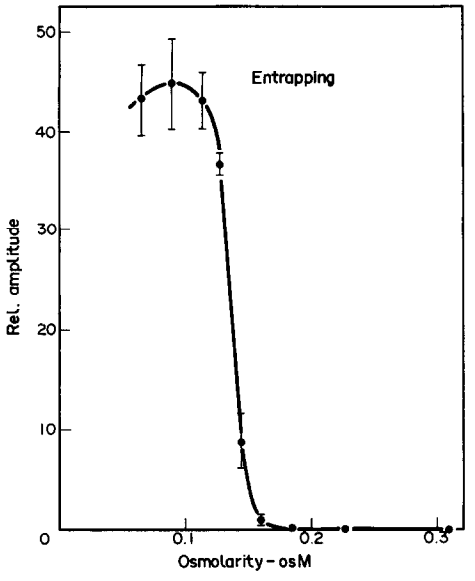
It is essential for the method that the excess of spin label remaining in the external solution subsequently to resealing could be converted to diamagnetic species. Selective reduction of the spin labels with sodium ascorbate was introduced by Kornberg and McConnell [7] for estimating the localization of labels in different compartments in their studies of inside-outside transitions of phospholipids in vesicle membranes. Sodium ascorbate, added in a 10-fold excess, was found to rapidly remove the paramagnetism of the external solution but had no direct influence on the spin label located inside the vesicles or in the membranes.

In conformity with this statements it was found that a large excess was required for the removal of tempocholine outside the erythrocytes when sodium ascorbate was used as the reducing agent. However, a 10-fold excess of sodium ascorbate gave rise to an unstable ESR signal, probably by damaging the erythrocyte membrane with leakage and reduction of the entrapped tempocholine. This interpretation seems to be in conformity with the report that high concentrations of ascorbate cause lysis of erythrocytes [8].

After some experimentation it was found that an appropriate reduction of the spin label outside the erythrocytes was achieved after the addition of a 2-fold excess of ascorbic acid. The pH of the erythrocyte suspension was about 5 after the addition of ascorbic acid, and about 7.4 when sodium ascorbate was used as the reducing agent. No trace of ascorbic radicals could be detected when the reduction was carried out with ascorbic acid, whereas reduction with sodium ascorbate gave rise to a superposition from relatively stable ascorbate radicals on spectral component $M_I = 0$ in the ESR spectrum of the spin label. It is very probable that the instability of the ascorbic radicals at the lower pH value (cf. [9]) displaced the equilibrium between ascorbate and its radical towards the latter form, thereby increasing the rate of the reduction of the spin label, and at the same time decreasing the required excess of the reducing agent.

The ESR signal from the entrapped tempocholine did not decrease more than 1–2% within half an hour after the addition of the 2-fold excess of ascorbic acid (20 mM). Experiments showed that a maximum of entrapped tempocholine was obtained after only a few minutes of incubation (37°). An extension of the incubation time to more than one hour did not decrease the ESR signal from the entrapped tempocholine. The stability of this ESR signal indicated that the signal was ascorbic-acid protected, i.e. the reducing agent did not penetrate through properly resealed erythrocyte membranes at the concentration used in this case. The stability of the ESR signal also indicated that the reduction of tempocholine located inside erythrocytes by the inherent redox system observed by Ross and McConnell [5] was of minor importance in the present study.

The rather low pH value, i.e. about 5, produced by the addition of ascorbic acid, was considered to be without influence on the amount of entrapped



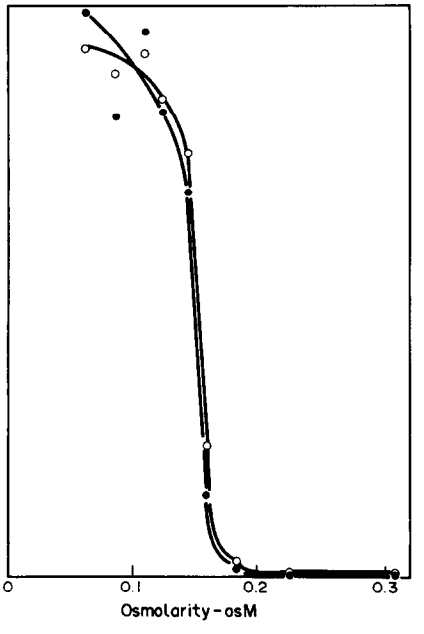


Fig. 1. (a) The ESR signal amplitude (relative units) from the spin label tempocholine entrapped in human erythrocytes subjected to hyposmolar stress by suspension in buffer solutions containing the spin label, and in the range of osmolarities between 0.310 and 0.060 osM. After incubation at 37° for 1 hr, the excess of spin label in the external solution was reduced to a diamagnetic species by the addition of ascorbic acid. The curve and the error bars (standard deviations) were constructed from measurements performed on eight consecutive days with erythrocytes of the same blood-bank blood. (b) Haemolysis and entrapping curves obtained when human erythrocytes of the same washed batch were suspended in buffer solutions in the osmolar range between 0.310 and 0.060 osM. ○ Haemolysis; ● entrapping of tempocholine.

tempocholine as the entrapping and resealing had been completed before the addition.

Preliminary observations indicated that the resealing was less rapid with aged erythrocytes. Consequently, the incubation time was extended and taken

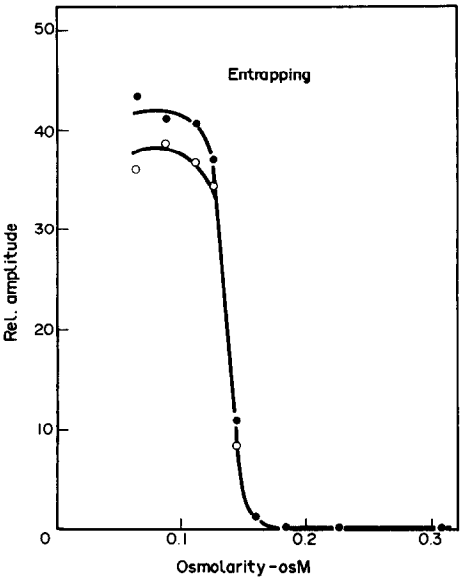


Fig. 2. Entrapping of tempocholine in human erythrocytes in the presence of the disodium salt of ethylenediamine tetraacetic acid (EDTA). ● Reference: no EDTA; ○ 2 mM of EDTA.

to be one hour in most of the experiments reported.

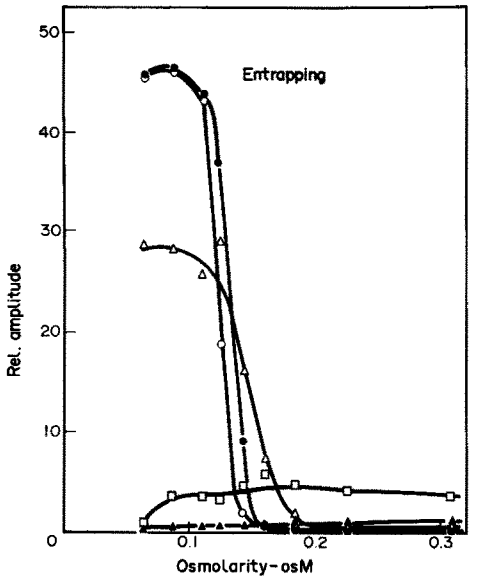
Figure 1(a) shows the entrapping of tempocholine into human erythrocytes when suspended in buffer solutions in the osmolar range between 0.310 and 0.060 osM. The amount of spin label entrapped was rather small or almost negligible at concentrations close to normosmolarity but increased steeply below 0.160 osM. Below 0.080 osM there was a maximum of entrapping, and at still lower osmolarities the entrapping decreased. It was not possible to extend the measurements below about 0.060 osM with the amount of erythrocytes in our samples, and the compositions of buffer solutions used in this work.

The entrapping curve in Fig. 1(a) was constructed from the mean values obtained from preparations and measurements performed on eight consecutive days using the same sample of blood-bank blood. The error bars indicate the standard deviations of the measurements. Evidently the deviations were rather small.

The results obtained when the buffer solutions contained 2 mM EDTA, leading to sequestering of Ca^{2+} and Mg^{2+} ions, are shown in Fig. 2. The entrapping of tempocholine was somewhat reduced at lower osmolarities. The stability of the ESR signal derived from entrapped tempocholine was also somewhat lower than observed for the reference, indicating a certain leakage of ascorbic acid into the erythrocytes in the presence of EDTA.

Haemolysis curves in the absence of drug substances

The haemolysis curve obtained when human erythrocytes were suspended in buffer solutions in the osmolar range between 0.310 and 0.060 osM is shown in Fig. 1(b). For comparison the corresponding entrapping curve was included. The scales of the curves were chosen so that the maximum of entrapping and haemolysis (100%) coincided. Obviously the two curves were situated very close to each other.



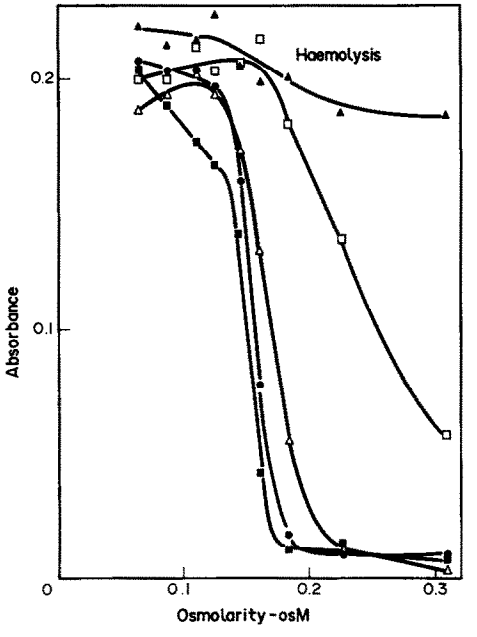


Fig. 3 Entrapping of tempocholine in human erythrocytes, (a), and haemolysis, (b) in the presence of chlorpromazine added to concentrations between 0.05 and 1 mM. ● Reference: no chlorpromazine; ■ 0.05 mM of chlorpromazine (haemolysis only); ○ 0.1 mM of chlorpromazine (entrapping only); △ 0.2 mM of chlorpromazine; □ 0.5 mM of chlorpromazine; ▲ 1 mM of chlorpromazine.

Entrapping of tempocholine in the presence of membrane-active substances

The results obtained with the efflux method had shown that chlorpromazine, trifluoperazine, or nicardipine brought about a dose-dependent increase of the efflux of tempocholine from spin-label-loaded human erythrocyte ghosts [3]. These substances were now tested for their influence on the entrapping of tempocholine into human erythrocytes.

*Chlorpromazine**. The amount of tempocholine entrapped in the presence of chlorpromazine added to a concentration between 0.1 and 1 mM is shown in Fig. 3(a). The erythrocytes were suspended in different buffer solutions in an osmolar range between 0.310 and 0.060 osM.

The amount of entrapped tempocholine was very small or almost negligible in the whole range of osmolarities investigated when chlorpromazine was added at a concentration of 1 mM. Chlorpromazine in a concentration of 0.5 mM gave rise to a small increase of the amount of entrapped spin label above about 0.160 osM. Below this osmolarity the amount of entrapped tempocholine was much smaller than that of the reference. Chlorpromazine added to a concentration of 0.2 mM gave rise to a curve situated somewhat over the reference in the range between 0.180 and 0.150 osM but below the reference at osmolarities lower than 0.150 osM.

It is clearly seen that the curve obtained with 0.1 mM of chlorpromazine is displaced to the left of the reference curve, and that the maximum of entrapping obtained with this concentration of chlorpromazine approximately coincides with that of the reference curve. Similar displacements were obtained with 0.05 and with 0.025 mM of chlorpromazine.

Trifluoperazine. This substance was found to have the most pronounced influence on the entrapping curve of the substances so far tested (Fig. 4). Even

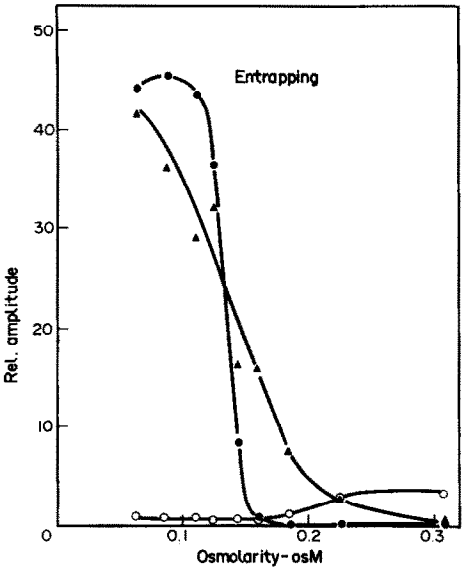


Fig. 4. Entrapping of tempocholine in human erythrocytes in the presence of trifluoperazine. ● Reference: no trifluoperazine; ▲ 0.1 mM of trifluoperazine; ○ 0.3 mM of trifluoroperazine.

* Chlorpromazine seems not to react with tempocholine or ascorbic acid. As stated earlier [3] no change of the signal amplitude could be detected when chlorpromazine was added to a concentration of 1 mM to a sample of tempocholine dissolved in HEPES buffer at pH 7.4 indicating that no redox reaction took place between these substances. Furthermore, no radicals could be detected when ascorbic acid was added to a solution of chlorpromazine (pH 5).

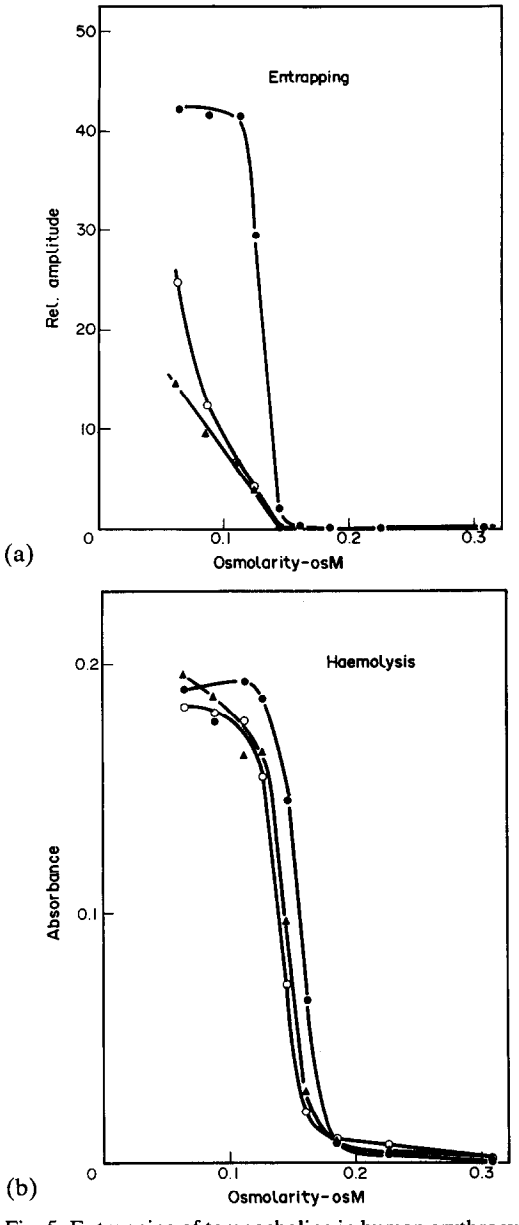


Fig. 5. Entrapping of tempocholine in human erythrocytes, (a), and haemolysis, (b) in the presence of nicardipine. The drug was added dissolved in 10 μl of DMSO/400 μl of buffer solution. ● Reference: no nicardipine but with 10 μl of DMSO; ○ 0.5 mM of nicardipine; ▲ 1 mM of nicardipine.

at a concentration of 0.1 mM, trifluoperazine gave rise to a decreased entrapping in the lower range of osmolarities. However, 0.1 mM trifluoperazine gave rise also to an increased entrapping in the osmolar range close to normosmolarity and in the middle range. Trifluoperazine added to a concentration of 0.3 mM gave rise to a small increase of the entrapping of tempocholine above 0.180 osM. Below this value the amount of entrapped spin label was almost negligible.

Nicardipine. This drug substance had a somewhat different influence on the entrapping curves, which were displaced towards lower entrapping in the range of lower osmolarities in comparison with the reference curve (Fig. 5a).

Exclusion of Ca^{2+} *and* Mg^{2+} *ions.* The effects of chlorpromazine were enhanced when the entrapping was performed with buffer solutions from which Ca^{2+} ions had been excluded, or sequestered together with Mg^{2+} ions by the addition of EDTA. The entrapping curves observed with 0.2 mM chlorpromazine with and without the addition of 2 mM EDTA, are shown in Fig. 6. The entrapping of the spin label is clearly reduced in the presence of EDTA, compared with the curve obtained with buffer solutions containing unsequestered Ca^{2+} (0.3 mM) and Mg^{2+} (1 mM) ions. An increase of the concentration of Ca^{2+} ions from 0.3 to 0.6 mM had no influence on any of the entrapping curves obtained with chlorpromazine. Similar results were obtained with trifluoperazine and nicardipine when Ca^{2+} and Mg^{2+} ions were excluded or sequestered from the buffer solutions.

Stability of the ESR signal. No decrease in the stability of the ESR signal from entrapped tempocholine was observed with chlorpromazine added in concentrations below 0.5 mM. Above this concentration the stability was lower, indicating increased leakage of ascorbic acid into the erythrocytes. The leakage of tempocholine through the erythrocyte membrane was considered to be of minor importance in view of the long incubation time (1 hr) before the addition of ascorbic acid, and the fact that the instability manifested itself by a decrease of the ESR signal from an initially relatively high level. Similar results were observed with trifluoperazine and nicardipine.

Amperozide and haloperidol. These psychotropic compounds, which were not included in our earlier study [3], affected the entrapping of tempocholine, and gave rise to curves with shapes somewhat different from those observed for chlorpromazine. Thus the curve obtained with 0.4 mM amperozide indicated increased entrapping at osmolarities close to normosmolarity and at osmolarities in the middle

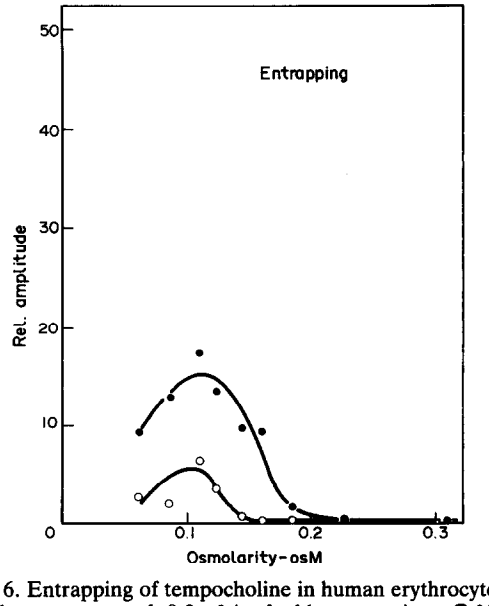


Fig. 6. Entrapping of tempocholine in human erythrocytes in the presence of 0.2 mM of chlorpromazine. ● No EDTA; ○ with 2 mM of EDTA.

range (Fig. 7). However, at a higher concentration of added amperozide (1 mM), entrapping was very low or negligible in the whole range of osmolarities investigated. The addition of haloperidol to a concentration of 1 mM had a relatively small influence on the entrapping, with a small increase in the middle range of osmolarity and a small decrease at lower values (Fig. 8). The exclusion of Ca^{2+} and Mg^{2+} ions from the buffer solutions had no influence on the entrapping curves observed with amperozide and haloperidol.

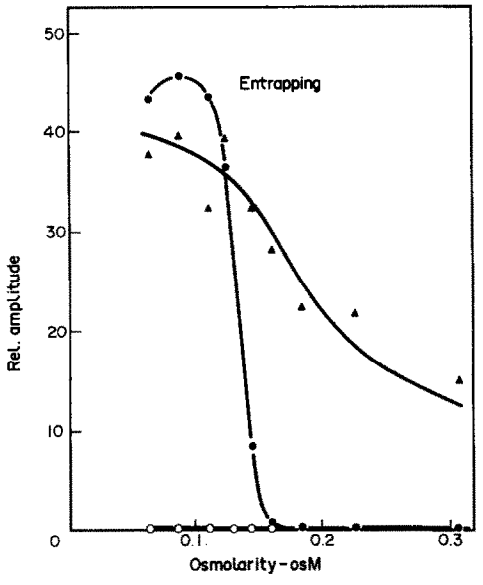


Fig. 7. Entrapping of tempocholine in human erythrocytes in the presence of amperozide. ● Reference: no amperozide; ▲ 0.4 mM of amperozide; ○ 1 mM of amperozide.

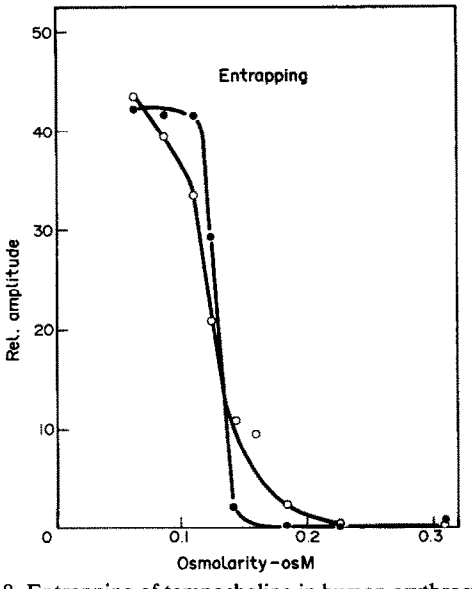


Fig. 8. Entrapping of tempocholine in human erythrocytes in the presence of haloperidol. The drug was added dissolved in 10 μl of DMSO/400 μl of buffer solution. ● Reference: no haloperidol but 10 μl of DMSO; ○ 1 mM of haloperidol.

Haemolysis curves obtained in the presence of membrane-active substances

It is well known that substances such as chlorpromazine and trifluoperazine increase osmotic haemolysis of human erythrocytes when these substances are present in concentrations above a certain level [2]. For comparison with entrapping of tempocholine, haemolysis curves were recorded with the drug substances in the same concentrations as those used in the entrapping experiments. As expected, the haemolysis increased with decreasing osmolarity. The extent of haemolysis increased with increasing concentration of added substances. The curves obtained with chlorpromazine are shown in Fig. 3(b). The extent of haemolysis is increased in a dose-dependent way in the range of concentration between 0.2 and 1 mM of chlorpromazine. The curve obtained with a concentration of 0.05 mM was displaced to the left of the reference curves (no added chlorpromazine) indicating a protective action against osmotic haemolysis, which is in conformity with the results reported by Seeman [2].

All of the curves in Fig. 3(b) exhibited a maximum of haemolysis, i.e. 100% of haemolysis, below an osmolarity of about 0.120 osM. Similar curves were obtained with the other substances in this series. However, nicardipine was found to exhibit a displacement of the haemolysis curves to the left of the reference at very high concentrations, namely at both 0.5 and 1 mM (Fig. 5b).

DISCUSSION

The curves obtained in the absence of membrane-active substances indicate a close coincidence between entrapping of tempocholine and haemolysis (Fig. 1b). Once pores or rifts have been formed in the erythrocyte membranes, they allow the simultaneous passage of both tempocholine and haemoglobin. The opening of the actual pores seems to take place in a very small range of osmolarity, so that no discrimination can be detected between pores large enough for the passage of tempocholine (mol.wt of 244) into the erythrocytes but too small for the leakage of haemoglobin (mol. wt about 68,000), and pores which allow the passage of haemoglobin.

The entrapping of tempocholine exhibits a maximum about 0.080 osM. Below this osmolarity the entrapping and resealing decreased, probably as the result of irreversible rifts and the disruption of some erythrocyte membranes (Fig. 1a).

The membrane-active substances in this series which could influence the entrapping and haemolysis gave rise to entrapping curves of different shapes, reflecting their individual structures and interactions with the erythrocytes.

Generally, there is an inverse relation between entrapping and haemolysis at the higher concentrations of added substances. Evidently, osmotic resistance is decreased, leading to the leakage of haemoglobin, and the ability of the erythrocyte ghosts to reseal is also decreased. When the concentration of added substances is decreased, the haemolysis decreases and the entrapping is increased. In the limit of no added substances, hae-

molysis and entrapping curves almost coincide, as pointed out above.

The protective action against haemolysis brought about by a number of membrane-active substances at low concentrations [2], also clearly seen in the haemolysis curve obtained with chlorpromazine (0.05 mM, Fig. 3b), has its counterpart in the entrapping curves. Thus, the curve obtained with 0.1 mM of chlorpromazine, Fig. 3(a), is situated to the left of the reference, indicating a displacement of the entrapping to a somewhat lower osmolarity, while the maximum of entrapping of the reference is maintained.

Evidently, haemolysis curves only constitute a measure of the total leakage of haemoglobin, but give no information about the resealing process. From haemolysis curves, it is not possible to distinguish between haemoglobin leaked from a certain fraction of the cell population which might give rise to ghosts not able to reseal at all, and haemoglobin leaked from cells which reseal after the loss of a minor part of their haemoglobin content.

The entrapping curves, on the other hand, reflect the ability of the erythrocytes to reseal after sudden hyposmolar stress. The entrapping curves clearly indicate that the resealing process is active even in the range of 100% haemolysis. This is illustrated in Fig. 1(b), where no membrane-active substances had been added. In the presence of membrane-active substances in this series, the resealing is still active in the range of 100% haemolysis, but is reduced in proportion to the concentration of the added substances, as seen with chlorpromazine, Figs. 3(a) and 3(b). The resealing is very small at a concentration of 1 and 0.5 mM, but only reduced to about half of the level of the reference at 0.2 mM of chlorpromazine.

The calmodulin system has been found to be inhibited by phenothiazine derivatives such as trifluoperazine [10]. It has been pointed out that many calcium antagonists also are inhibitors of the calmodulin system [11]. Calmodulin antagonists such as chlorpromazine have been found to alter the shape of human erythrocytes and to induce a transformation of discoid cells into cup forms. The control of erythrocyte shape was believed to be under regulation by calmodulin and to be mediated through its direct and indirect effects on the spectrin-actin cytoskeleton, and by control of intracellular ATP and Ca^{2+} levels [12].

In view of these statements and the results obtained with the drug substances in this series, together with the reduction of the entrapping after exclusion of Ca^{2+} ions, it seems very probable that the calmodulin system is also involved in the resealing process. It seems also reasonable to assume that the increased osmotic haemolysis brought about by substances such as trifluoperazine, is due to the inhibition of the calmodulin system, which impairs the ability of the skeletal proteins of the membranes to withstand the stretching of the membranes brought about by the entrance of water.

The results obtained with chlorpromazine indicate that the resealing process is maintained and highly effective in the range of concentration where this drug has a protective action against osmotic haemolysis (<0.1 mM of chlorpromazine, Figs. 3a and 3b). If we accept the statement that the decreased entrapping and resealing, observed at higher concentration of the substances here used (Figs. 3–8), are due to an inhibition of the calmodulin system, it seems difficult to assume that the protective action at lower concentrations is also connected with inhibition.

Therefore, it seems more probable that there is stimulation of the part of the calmodulin system assumed to be involved in the resealing process at the low concentrations of the substances used. The biphasic shape of the curves in which the relative haemolysis is plotted against the concentration of the membrane-active substances [2], seems also to be in conformity with this view of different modes of interaction at high and low concentrations as regards haemoloysis and resealing, when the latter is evaluated by the entrapping of tempocholine.

Finally, it should be pointed out that the individual shape of the entrapping curves obtained with the drug substances in this series probably reflects differences as regards their relative effects on lysis and resealing. Some substances might contribute more to the weakening of the cell membrane than to the inhibition of the resealing while others exhibit a reversed contribution. However, a closer analysis of these matters has not been performed so far.

Acknowledgements—The authors are indebted to Dr. Lena Sandberg, Sahlgrenska Sjukhuset, Göteborg, Sweden, for supplying us with blood-bank blood. This work was supported by grants from the Swedish Natural Science Research Council.

REFERENCES

1. J. F. Hoffman, *J. gen. Physiol.* **45**, 837 (1962).
2. P. Seeman, *Pharmac. Rev.* **24**, 583 (1972).
3. C. Lagercrantz, Th. Larsson, L. Sörskog and P. Lincoln, *Biochem. Pharmac.* **33**, 1851 (1984).
4. G. K. Humphries and H. M. McConnell, *Proc. natn. Acad. Sci. USA* **71**, 1691 (1974).
5. A. H. Ross and H. M. McConnell, *Biochemistry* **14**, 2793 (1975).
6. W. G. Zijlstra and E. J. van Kampen, *Clin. Chim. Acta* **5**, 719 (1960).
7. R. D. Kornberg and H. M. McConnell, *Biochemistry* **10**, 1111 (1971).
8. J. T. Rotruck, A. L. Pope, H. E. Ganther and W. G. Hockstra, *J. Nutr.* **102**, 689 (1972).
9. C. Lagercrantz, *Acta Chem. Scand.* **18**, 562 (1964).
10. B. Weiss, W. C. Prozialeck and T. L. Wallace, *Biochem. Pharmac.* **31**, 2217 (1982).
11. G. J. Brewer, U. L. Bereza, I. Mizukami, J. C. Aster and L. F. Brewer, *The Red Cell: Fifth Ann Arbor Conference* (Ed. G. J. Brewer), p. 187 Alan R. Liss, New York (1981).
12. G. A. Nelson, M. L. Andrews and M. J. Karnovsky, *J. Cell Biol.* **96**, 730 (1983).