Biochemical Pharmacology, Vol. 33, No. 12, pp. 1851–1857, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
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THE EFFLUX OF SPIN LABEL ENTRAPPED IN HUMAN ERYTHROCYTE GHOSTS WHEN SUSPENDED IN HYPOSMOLAR SOLUTIONS. THE EFFECT OF CHLORPROMAZINE, TRIFLUOPERAZINE, NICARDIPINE AND SOME OTHER MEMBRANE ACTIVE SUBSTANCES

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(*Received* 4 *October* 1983; *accepted* 28 *November* 1983)

Abstract—Human erythrocyte ghosts were loaded with the spin label tempocholine. Once entrapped in the ghosts, this spin label, carrying a positive charge, is not able to penetrate through intact ghost membranes. The ghosts were loaded with spin label to a concentration high enough to introduce exchange broadening of the electron spin resonance (ESR) signal with a relatively small signal amplitude. The efflux of the spin label brought about by hyposmolar stress was studied. The appearance of the label in the relatively large external volume gave rise to an increase of the ESR signal amplitude since the concentration of the spin label outside the ghosts was in the range in which exchange broadening can be excluded. The duration of the efflux following hyposmolar stress was less than half a minute. After this time, the ghosts resealed spontaneously and without restoration of the normal osmolarity. A number of membrane active substances were studied for possible influence on the efflux of spin label induced by hyposmolar stress. The drug substances 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.

A great number of substances have been found to protect erythrocytes against osmotic lysis. According to Seeman [1] their action involves an increase of the surface of the red cell membrane. These substances, known as membrane expanders, include a variety of anaesthetics and tranquilizers of different structures.

A number of methods have been utilized for the study of these characteristics, including the measuring of osmotic haemolysis, membrane area and volume of erythrocytes. The technique of spin labelling has also been applied to the study of interaction between human erythrocyte ghost membranes and drug substances, including membrane expanders. In these experiments a spin label was covalently coupled through SH-groups of membrane proteins. Phenothiazines [2], a number of antitumour drugs [3] and propranolol [4] induced changes in the ESR-spectra indicating a redistribution between weakly and strongly immobilized spin label molecules. These results were interpreted as conformational changes of membrane proteins brought about by the drugs.

In this report, the spin labelling technique has been applied in a somewhat different form to the study of the influence of some membrane active substances on haemolysis. Human erythrocyte ghosts were loaded with the spin label tempocholine, carrying a positive charge. Once entrapped inside the resealed ghosts, tempocholine is not able to penetrate the intact ghosts membrane [5]. The ghosts were loaded with the spin label to a concentration high enough to introduce exchange broadening of the ESR signal. Any change of the membrane permeability leading to leakage of the spin label from the interior of the ghosts to the surrounding medium, of a volume which was much larger than the interior of the ghosts, will result in an enhancement of the ESR signal amplitude. This technique has previously been used by Humphries and McConnell [6] for the study of immunochemical lysis of erythrocyte ghosts.

In the present study the spin-label-loaded ghosts were suspended in a series of buffered solutions of decreasing osmolarity. The relative amplitude of the ESR signal for each sample increased as the result of an increasing efflux of spin label from the ghosts. The influence of membrane active substances was investigated by comparing aliquot samples with and without the actual substances.

The advantage of the spin label technique for efflux studies in the present case arises because the concentration of a reporter molecule such as tempocholine can be measured without previous separation of the ghosts from the external solution.

MATERIALS AND METHODS

Tempocholine. Tempocholine iodide, 4-[*N*-(2-hydroxyethyl) - *N,N* - dimethylammonio] - 2,2,6,6 - tetramethyl-1-piperidinyl-oxy iodide, was prepared by reacting 4-[*N*-(2-hydroxyethyl)-*N*-methylamino]-2,2,6,6-tetramethyl-1-piperidinyl-oxy [7] with

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methyl iodide in ether, orange crystals (methanol), m.p. 239–241°.

Preparation of erythrocyte ghosts loaded with spin label. Unless otherwise stated, all reactants were stored and most operations were carried out close to 0°. Centrifugations were carried out at +4°. Human erythrocytes (concentrate, blood-bank blood) were washed three times with buffer solution (A) containing 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 70%.

Haemolysis. An aliquot of buffer solution (A), 1.5 ml, and 4.5 ml of H_2O were added to 1.2 ml of washed erythrocytes. The suspension was thoroughly mixed and centrifuged at 10,000 *g* for 10 min.

Resealing with inclusion of spin label. An aliquot of the supernatant, 3 ml, was carefully removed and replaced by 3 ml of resealing solution containing 10 mM HEPES, 50 mM KCl, 1.0 mM $MgCl_2$, 0.3 mM $CaCl_2$, 200 mM tempocholine iodide and 1 mM ATP disodiumphosphate, and adjusted to pH 7.3–7.6. Thorough mixing gave a final tempocholine iodide concentration of about 83 mM. Resealing was accomplished by incubation at 37° for 1 hr and then chilling in an ice bath for 10 min. The suspension of spin-label-loaded ghosts was then centrifuged for 10 min at 1500 *g*, the supernatant was discarded and the ghosts were washed five times with 6 ml portions of buffer solution (A).

Electron spin resonance (ESR) measurements. The ESR spectra were recorded by use of a Varian E-9 spectrometer. The samples were contained in a small aqueous solution cell kept at a fixed position, as previously described [8]. The spectra were recorded at 9.1 GHz at a microwave power of 5 mW. The modulation amplitude was 0.1 G (0.01 mT).

Generally eight samples were prepared with decreasing osmolarity by use of buffer solutions (A) and (B). The latter had the same composition as buffer solution (A), but contained no NaCl. Each sample was prepared from 100 μl of spin-label-loaded ghosts (diluted 1:1 with buffer solution (A)), mixed with 300 μl of different combinations of buffers (A) and (B), so that the final concentrations corresponded to values between 0.155 and 0.040 M NaCl.

To 300 μl of the actual buffer solution, 4 μl of the membrane active substance to be tested dissolved in an appropriate solvent was added immediately before mixing with the spin-label-loaded ghosts.

After mixing, which was performed with the ghosts and buffer solutions kept close to 0°, the samples were transferred to the ESR cell. The spectra were recorded at +20° 2 min after mixing, and the recording was completed about 2 min later.

Presentation of the results. The results are given in diagrams where the signal amplitude of the spectral component $M_I = 0$, normalized on the values observed for physiological osmolarity (0.155 M NaCl), is plotted against the actual sample concentration of NaCl.* The results of some experiments were also presented as the percentage of the total spin label concentration present in the external solution and in the ghosts. The concentration values were obtained from calibration curves relating the signal amplitude to the spin label concentration. The percentage of the concentration in the ghosts was obtained by subtracting the values observed for the external solution (supernatant) from the total concentration of the spin label measured after destruction of the ghost membranes by the addition of deoxycholic acid so that the spin label became distributed throughout the sample.

Substances tested for membrane activity. Acetylsalicylic acid, amphotericin B, chloroquine diphosphate, chlorpromazine HCl, colchicine, concanavalin A, 1-decanol, deoxycholic acid (sodium salt), glutaraldehyde, lysophosphatidylcholine, procain HCl, salicylic acid, saponin, testosterone, trifluoperazine dihydrochloride, α-tocopherol, and vitamin-A–alcohol were obtained from Sigma Chemical Company or Fluka AG. Nicardipine HCl was obtained from Yamanouchi Pharmaceutical Company, Tokyo, Japan; Felodipine HCl and nifedipine HCl were obtained from AB Hässle, Mölndal, Sweden, and verapamil from Knoll AG, Ludwigshafen am Rhein, West Germany. The substances were used as supplied.

RESULTS

Spin-label-loaded ghosts suspended in hyposmolar solutions. No membrane active substances added. Figure 1 shows the signal amplitude normalized on the physiological osmolarity (0.155 M NaCl) from

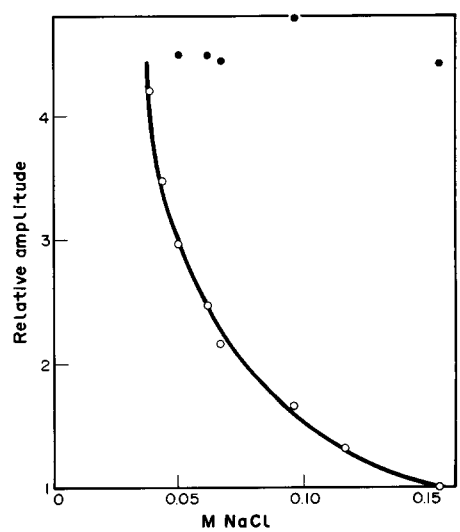

Fig. 1. The ESR signal amplitude, normalized on the physiological osmolarity (1.00 for 0.155 M NaCl) recorded from human erythrocyte ghosts loaded with the spin label tempocholine, in the range of osmolarity between 0.155 and 0.040 M NaCl. ○, no drug substance added; ●, with 1 mM deoxycholate.

* For practical reasons the electrolytic composition of the samples is given as the concentration of NaCl in Figs 1–5. Osmolarity and concentration of NaCl are used as approximately equivalent concepts in the text. The total osmolarity is somewhat larger than the concentration of NaCl, as the buffer salts must be included (10 mM).

spin-label-loaded ghosts in the osmolar range between 0.155 M and 0.040 M NaCl. The signal amplitude increases in a non-linear way when the osmolarity is decreased. This result indicated an efflux of spin label from the interior of the ghosts out into the surrounding external solution, brought about by the decreasing osmolarity. The concentration of the spin label inside the ghosts is high enough to give rise to exchange broadening with a relatively low signal amplitude. The concentration of the spin label which has leaked out into the surrounding solution, the volume of which is relatively large compared with the interior of the ghosts, is in a range where no exchange broadening exists. Consequently, the signal amplitude derived from the spin label in the external solution varies directly as the concentration, and the leakage gives rise to an increased signal amplitude. It should be emphasized that the actual signal amplitude is a superposition of the exchange broadened signal from the spin label still present inside the ghosts and the signal, generally of normal type, derived from the spin label in the surrounding external solution. Therefore, the signal amplitude cannot be used directly for separately evaluating inside and outside concentrations of label.

Figure 2 shows the partition of the spin label between the ghosts and the external surrounding solution given in percent spin label for the same experiment as shown in Fig. 1. The data of Fig. 2 were obtained as described above (Materials and Methods). The amount of spin label inside the ghosts decreases, and increases in the external solution, with decreasing osmolarity.

Duration of efflux of spin label from the ghosts. The amplitude values recorded at 2 min after mixing of the suspension of ghosts and the solutions of NaCl did not increase during an observation time of about one hr at 20°, a finding valid for all concentrations investigated, i.e. 0.155 to 0.040 M NaCl. When the spin-label-loaded ghosts, initially contained in a solution of physiological osmolarity, are suspended in solutions of a lower osmolarity, there is an immediate leakage through the ghost membranes. However, the leakage stops very soon, and before the sample had been transferred to the ESR cell.

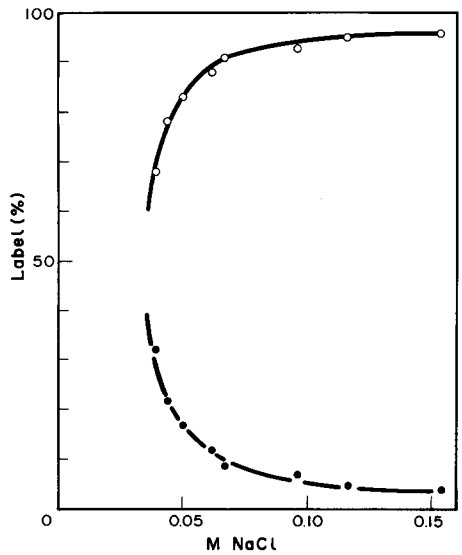

Fig. 2. Percentage partition of spin label between human erythrocyte ghosts loaded with tempocholine, and the external surrounding solution in the range of osmolarity between 0.155 and 0.040 M NaCl. No drug substance added. ○, inside the ghosts, ●, in the external solution.

However, when the experiments were performed with ghosts prepared and loaded with spin label in a buffer solution from which calcium and magnesium had been excluded, the signal amplitude was not constant but increased with time.

From Fig. 2 it can be seen that the concentration of the spin label in the ghosts is larger than in the external solution even after suspending the ghosts in the solution of the lowest NaCl concentration of the experiment, i.e. 0.040 M NaCl. Evidently, the efflux stops before an equalization between the inside and outside concentration is established.

An equalization of the inside–outside concentration was obtained only subsequent to treatment of the ghosts with membrane disrupting substances, such as the sodium salt of deoxycholic acid or saponins. The signal amplitude recorded in a sample of the complete system of ghosts and external solution after the addition of sodium deoxycholate to a concentration of 1 mM is included in Fig. 1. The increase was found to be about 6 times the normalized value observed with physiological osmolarity.

Leakage of spin label from ghosts at physiological osmolarity. Immediately after preparation and resealing of the ghosts in a solution of physiological osmolarity, i.e. 0.155 M NaCl, the external supernatant contained about 3–6% of the total amount of the spin label (Fig. 2). As the concentration of the external label was in the range where the signal amplitude varies directly as the concentration, and no exchange broadening was present, the contribution of the external spin label to the signal amplitude was relatively large. In an experiment in which the external part of the spin label was evaluated to be about 6%, the signal amplitude was decreased about 25% after the addition of ascorbate to a concentration of 2 mM, which selectively reduced the spin label in the external solution.

The ascorbate protected signal amplitude corresponding to the spin label inside the ghosts remained constant for an observation time of 30 min, provided the ascorbate had been added after the spin-label-loaded ghosts had been suspended in the solution of appropriate osmolarity. This observation is also valid for the lowest osmolarity investigated, i.e. 0.040 M NaCl. When the ascorbate was added prior to the suspension of the ghosts in the hyposmolar solutions, the signal amplitude was slightly reduced with time. Obviously, ascorbate cannot penetrate into the ghosts except during the short period following the transfer of the ghosts into hyposmolar solutions, i.e. simultaneously with the efflux of spin label.

In a semiquantitative experiment performed with spin-label-loaded ghosts suspended in a solution of physiological osmolarity, the leakage was much larger during an observation time of 24 hr when the ghosts were kept at 35°, compared with storing the ghosts at 0°.

The initial leakage at physiological osmolarity, which varied from batch to batch of ghost preparation, might be considered a nuisance. However, the measurements were based on recording the increase of efflux, and the results were generally normalized on the signal amplitude found for physiological osmolarity without added substances. Therefore, the presence of external spin label which gave rise to a background signal, seems not to have hampered the measurements in any essential manner.

Spin-label-loaded ghosts suspended in hyposmolar solutions. The influence of some membrane active substances. A number of substances known to have effects on membrane properties of erythrocytes, have been tested for a possible influence on the efflux of tempocholine from human erythrocyte ghosts when the ghosts are suspended in hyposmolar solutions. Most of the substances tested belonged to the group of membrane expanders or membrane stabilizers, which have been found to protect erythrocytes against osmotic lysis. The substances were selected rather arbitrarily from the very large number of substances found by Seeman [1] to protect erythrocytes against osmotic haemolysis.

Many of the active substances were found by Seeman to exhibit a biphasic influence, i.e. they protect the erythrocytes against osmotic haemolysis at low concentrations, but increase haemolysis when present at higher concentrations. In the search for substances which influence the osmotic leakage of tempocholine from the ghosts, the concentration of the substances was taken to 1 mM, a concentration high enough to increase the leakage in case of any activity. Some substances, such as saponin and the sodium salt of deoxycholic acid, were also included, which are known to bring about haemolysis by destruction of the erythrocyte membranes.

The results are collected in Table 1. Aliquots of the samples were tested with and without the actual substance for each osmolarity between 0.155 and 0.040 M NaCl. A number of substances, such as acetylsalicylic acid, chloroquine diphosphate, colchicine, dimethylsulphoxide, 1-decanol, and ethanol, had no influence on the osmotic efflux of spin label.

As expected, substances such as concanavalin A, deoxycholate and saponin brought about a complete leakage of spin label for all osmolarities investigated, evidently by destruction of ghost membranes.

Chlorpromazine, trifluoperazine and nicardipine. The results obtained with chlorpromazine are shown in Figs. 3 and 4. From Fig. 3 it can be seen that the curves representing the signal amplitude at different osmolarities are situated above the reference curve, indicating an increased efflux of tempocholine in the presence of chlorpromazine.* The increase of efflux relative to the reference varies with the concentration of chlorpromazine in the range tested, i.e. between 1 mM and 0.01 mM. The increase of efflux was largest at 1 mM, and decreased with decreasing chlorpromazine concentration. No increase relative to the reference could be observed at 0.01 mM.

Figure 4 shows the percentage of spin label concentration inside and outside of the ghosts in the presence of 1 mM chlorpromazine, and for the reference in the osmolar range between 0.155 and 0.040 M NaCl. The curves show the increased efflux of label from the ghosts in the presence of chlorprom-

* 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.

Table 1. Increase of the efflux of the stable nitroxide radical tempocholine from spin-label-loaded human erythrocyte ghosts when suspended in hyposmolar solutions. The effect of substances added to a final concentration of 1 mM relative to reference samples

Substance	Solvent for added substance	Increase of efflux of spin label relative to reference samples
Acetylsalicylic acid	Ethanol	–
Amphotericin B	DMSO	Large efflux: channel formation
Chloroquine diphosphate	H_2O	–
Chlorpromazine HCl	H_2O	+++ (see text)
Colchicine	H_2O	–
Concanavalin A	H_2O	Large efflux: membrane destruction
1-Decanol	Ethanol	–
Felodipine HCl	H_2O	–
Glutaraldehyde	H_2O	–
Lysophosphatidylcholine	DMSO	Large efflux: membrane destruction
Nicardipine HCl	H_2O	++ (see text)
Nifedipine HCl	H_2O	–
Procain HCl	H_2O	–
Salicylic acid	H_2O	–
Saponin	H_2O	Large efflux: membrane destruction
Testosterone	Ethanol	–
Trifluoperazine dihydrochloride	H_2O	++++ (see text)
α-Tocopherol	DMSO	–
Vitamin-A-alcohol	DMSO	–
Verapamil HCl	H_2O	–

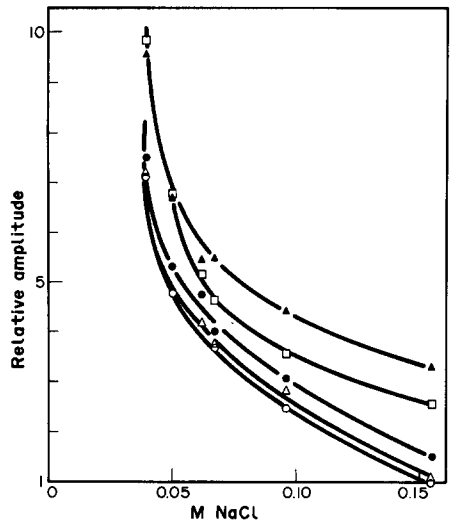

Fig. 3. The ESR-signal amplitude normalized on the physiological osmolarity (1.00 for 0.155 M NaCl) recorded from human erythrocyte ghosts loaded with the spin label tempocholine in the presence of chlorpromazine. The range of osmolarity was between 0.155 and 0.040 M NaCl. ▲, 1.25 mM; □, 0.5 mM; ●, 0.2 mM, △, 0.1 mM of chlorpromazine; ○, reference; i.e. no chlorpromazine added.

azine. The concentration of spin label in the ghosts is larger than in the external solution at the lowest osmolarity even in the presence of chlorpromazine. Evidently, the efflux of label stops before an equalization between inside and outside concentrations is established, even in the presence of chlorpromazine. However, it was noted that a slow leakage of label occurred in the presence of chlorpromazine following the initial efflux. The slow leakage amounted to about 15% within 20 min at physiological osmolarity. It is also to be noted that the largest increase of label efflux took place at physiological osmolarity in the presence of chlorpromazine (Figs. 3 and 4).

Trifluoperazine was found to increase the efflux of tempocholine in a way similar to that observed for chlorpromazine. The effect was somewhat larger than with chlorpromazine when the dose responses were compared in the range between 1–0.01 mM. As observed for chlorpromazine no effect could be detected with 0.01 mM trifluoperazine.

Nicardipine was also found to increase the efflux of spin label. The effect of nicardipine was somewhat smaller than that observed for chlorpromazine (Fig. 5).

The measurements performed on the supernatants after separation of the ghosts were made as a general check of the method (Figs. 2 and 4). Evidently, such measurements can be omitted for measuring the efflux brought about by added substances, an advantage of the spin label technique emphasized in the Introduction.

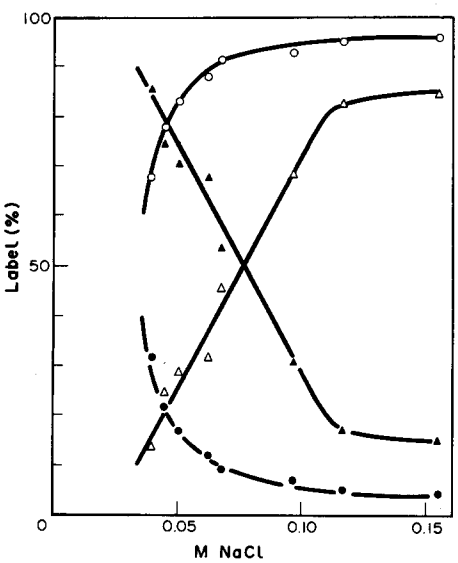

Fig. 4. Percentage partition of spin label between human erythrocyte ghosts loaded with tempocholine and the external surrounding solution, in the range of osmolarity between 0.155 and 0.040 M NaCl. With 1 mM chlorpromazine: △, inside the ghosts; ▲, outside the ghosts in the external solution. Reference, i.e. no drug substances: ○, inside the ghosts; ●, outside the ghost in the external solution.

DISCUSSION

When the spin-label-loaded human erythrocyte ghosts are subjected to a sudden osmotic stress or shock by suspension in hyposmolar solutions, there is an initial efflux of label through the membrane out into the external solution. Evidently, the initial phase involves an influx of water into the ghosts followed by an increase of volume and extension and stretching of the membrane so that pores are formed or enlarged, the width of which allows the passage of tempocholine molecules. After the efflux of a certain amount of spin label, the pores are closed or resealed rather rapidly. There is an inverse relation between the amount of efflux and the osmolarity. The lower the osmolarity, the larger is the efflux.

The label-loaded ghosts seem to react just like the ghosts of type I according to the classification by Hoffman [9] (see also [10]–[12]). By definition, type I ghosts are those which reseal immediately after haemolysis, and certainly before the 37° incubation

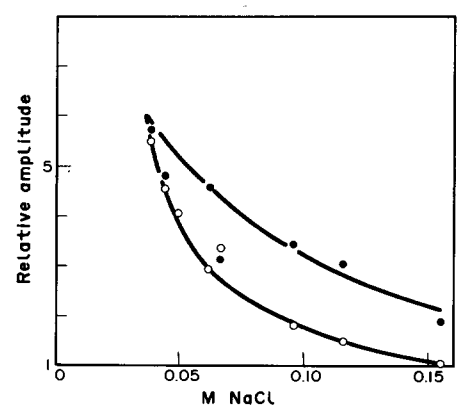

Fig. 5. The ESR signal amplitude normalized on the physiological osmolarity (1.00 for 0.155 M NaCl) recorded from human erythrocyte ghosts loaded with the spin label tempocholine in the range of osmolarity between 0.155 and 0.040 M NaCl. ●, with 1 mM nicardipine; ○, reference: no drug substances added.

designed to promote resealing of type II ghosts which only reseal after the physiological osmolarity is restored. It should be emphasized that the ghosts were loaded with the spin label under conditions valid for ghosts of type II, i.e. the spin label was added to the system simultaneously with restoration to physiological osmolarity (see Materials and Methods).

In addition to the efflux of spin label associated with hyposmolar shock, there is a slow leakage of label through the membranes. It is possible that the latter type of leakage is of an origin similar to that observed for a number of substances (cf. Richards and Eisner [12]). As the slow leakage increased when the ghosts were prepared and spin-label-loaded with the use of solutions from which calcium and magnesium ions had been excluded, the slow leakage might be connected with impairment of some mechanism necessary for maintaining normal membrane functions.

There is always a varying part of the total amount of spin label, 3–6%, present in the external solution at physiological osmolarity immediately after resealing and washing of the spin-label-loaded ghosts. The shape of curves of the type shown in Fig. 1 was also found to vary somewhat for different preparations of spin-label-loaded ghosts. The origin of these variations is not understood at present. It might be suggested that the age of the blood specimens is of importance, and that uncontrolled differences in the handling of the specimens, such as pipetting and centrifugation, could induce changes of the ghosts which lead to stretching and deformations of the membranes, subsequently leading to the formation of pores sufficiently large for the passage of tempocholine molecules.

The addition of the drug substances chlorpromazine, trifluoperazine and nicardipine induced dose-dependent increases of the efflux of spin label in the range of osmolarity investigated. In the presence of these drugs the ghosts also resealed very soon after the osmotic shock, i.e. they reacted as ghosts of type I. None of the concentrations of chlorpromazine (1 mM–0.01 mM) was found to diminish the efflux of spin label relative to the reference. In case of a decreased efflux, the curve should have been located below the reference curve, but such curves were not obtained. These findings seem to be inconsistent with the generally accepted theory, according to which substances such as chlorpromazine protect erythrocytes against osmotic haemolysis when present in low concentrations, i.e. for chlorpromazine in the range between 0.01–0.1 mM [1, 13], but increase haemolysis at higher concentrations.

However, it should be pointed out that efflux of the small positively charged tempochloline molecule with a mol. wt of 244 cannot be directly compared with the efflux of haemoglobin with a mol. wt of about 68,000. It is reasonable to assume that much larger pores are a prerequisite for the passage of haemoglobin molecules than those required for tempocholine molecules. It is suggested that the action of chlorpromazine involves an inhibition of the contractile protein skeleton of the membrane, which gives rise to increased extensibility of the membrane, and allows swelling of the erythrocytes or the ghosts to a larger volume when suspended in hyposmolar solutions. The increased extensibility leads to the formation of somewhat larger pores, or to a slower rate of resealing after the ghosts had been subjected to hyposmolar shock, with a subsequently increased efflux of the small tempocholine molecules. On the other hand, the increased extensibility will cause the erythrocytes or the ghosts to sustain a swelling to a larger volume before the membrane disrupts, with a concomitant efflux of haemoglobin. By this means the curve of haemolysis, including the value for 50% haemolysis, will be displaced to values of lower osmolarities, and the erythrocytes appear to be protected against osmotic haemolysis. At higher concentrations of chlorpromazine, the inhibition of the contractibility of the membrane proteins will increase so that the membrane no longer sustains even a moderate swelling with increased pressure, a situation which leads to a diminished osmotic resistance.

As nicardipine gave rise to results very similar to those observed with chlorpromazine and trifluoperazine, the interpretation given above is considered also to be valid for this drug.

Chlorpromazine, trifluoperazine and nicardipine are calcium antagonists (for a recent review see [14]), and are considered to exhibit their pharmacological action very probably by inhibition of calmodulin [15]. It has recently been emphasized that a number of membrane-stabilizing drugs also are calmodulin inhibitors and *vice versa* (Brewer [16] and Bereza *et al.* [17]). The results obtained here with chlorpromazine, trifluoperazine and nicardipine seem to be in conformity with these statements. The action of chlorpromazine, trifluoperazine and nicardipine on the permeability of spin-label-loaded ghosts is very probably regulated by calmodulin. Although nicardipine was found to increase the efflux of tempocholine, the two other calcium antagonists of the dihydropyridine class tested, i.e. felodipine and nifedipine (Table 1), had no influence on the efflux.

The negative results obtained with a number of substances (Table 1) generally classified as membrane stabilizers are considered to be connected with the fact that somewhat different properties of the erythrocyte membrane are studied. Membrane stabilization has mainly been studied by the efflux of haemoglobin and the measurement of erythrocyte area and volume, whereas the present study involves the efflux of the relatively small molecule of tempocholine. Consequently, complete conformity cannot be expected.

Acknowledgements—The authors are indebted to Dr Bengt Ljung and Dr. Johanna Deinum for valuable discussions, to AB Hässle, Mölndal for gifts of nifedipine and felodipine, to Dr. Toichi Takenaka, Yamanouchi Pharmaceutical Co., Tokyo for a gift of nicardipine, to Knoll AG, Ludwigshafen A.R. and AB Meda, Göteborg for a gift of verapamil, and to Dr. Lena Sandberg and Dr. Bo Samuelsson for supplying us with blood bank blood.

This work was supported by grants from The Swedish Natural Science Research Council.

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