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THE INFLUENCE OF CHLORPROMAZINE ON HUMAN ERYTHROCYTES

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Abstract—The following effects of chlorpromazine on human erythrocytes could be observed: Protection against osmotic hemolysis; A change in the cell shape in isotonic medium; An increase of the mean cellular volume in isotonic medium; A small increase of trapped medium after centrifugation of the red blood cells; A decrease of the sedimentation rate of the erythrocytes. All these effects are maximal at the same drug concentration, suggesting a common mechanism. No influence of chlorpromazine on the metabolism of intact cells could be found in the drug concentration range used in these experiments. From the osmotic behaviour of the erythrocytes an influence of the drug on the rigidity of the cell membrane may be viewed.

THE DISCOVERY that erythrocytes can be protected against osmotic hemolysis by chlorpromazine[1–3] has prompted a number of investigations. In the recent literature there is evidence that this protective effect is found not only with chlorpromazine, but also with other compounds, including other phenothiazine derivatives, detergents and alcohols.[4,5] The mechanism of this protective effect has been studied.[5–7] In the case of chlorpromazine the decrease of osmotic fragility is associated with an increase of the critical hemolytic volume of the red blood cells.[5] This increase of the critical volume can explain the decrease of the osmotic fragility caused by the drug. Further studies concerning the influence of chlorpromazine on human erythrocytes were performed, in an attempt to approach the reason for the increase in critical volume.

METHODS

Human blood was collected from haematologically normal adult donors, utilizing heparin as anticoagulant. Whole blood was used, without further pretreatment, as washing of the red cells prior to the experiments appeared to have no influence on the protection against osmotic haemolysis. All NaCl solutions utilized were prepared by dilution of the buffered stock solution described by Parpart *et al.*[8] In the chlorpromazine experiments the drug was added to the NaCl solutions in advance. Incubations were performed at 25°, unless otherwise stated. The per cent haemolysis was determined by measuring the per cent haemoglobin, liberated in the supernatant. Haemoglobin was determined by the method of Crosby, Munn and Furth[9] or, when very small amounts of haemoglobin had to be measured, by the benzidine method of Bing and Baker,[10] as modified by Dacie.[11]

Measurements of the mean cell volume of the red blood cells under various experimental conditions and determination of the critical haemolytic volume were performed as described previously,[5] utilizing Hamburger type haematocrit tubes. In

other experiments the mean cell volumes of normal and drug-treated cells were compared by analysis of volume distribution curves with a model B Coulter electronic particle counter.

Trapped medium between cells, centrifuged in Hamburger type tubes was determined by adding 2% ^{14}C-labelled dextran (M.G. 60,000–90,000) to the medium; 0·05 ml of packed cells were resuspended in 2 ml isotonic salt solution. The radioactivity in the supernatant of this suspension was measured in a liquid scintillation counter, with the liquid scintillator described by Bray.[12] Na^+ and K^+ determinations were carried out with a flame photometer. Intracellular Na^+ concentrations were determined after haemolysing 0·05 ml packed cells in 3 ml distilled water. The Na^+ concentration measured in the haemolysate was corrected for NaCl in the trapped medium. The osmolarity of cell contents was measured with a freezing point osmometer (Advanced Instruments Inc.) after freezing (—40°) and thawing of packed cells. In preliminary experiments it appeared that this treatment caused complete haemolysis. The water fraction of red blood cells was measured via drying of packed cells at 90°, to minimum weight; corrections were applied for trapped medium.

Glucose-6-phosphate dehydrogenase was isolated from erythrocytes according to Marks *et al.*[13] The activity of this enzyme preparation was measured with the method described by Kornberg and Horecker.[14] Reduced glutathione was measured according to Grunert and Phillips[15] with the modification of Beutler.[16] ATP was measured by the firefly tail, luciferin–luciferase system as described by McElroy and Seliger,[17] according to the method of Addanki *et al.*[18] The assay was carried out in a liquid scintillation counter, recording counts between 15 and 27 sec after mixing. ATP was extracted from the cells as described by Feldheim *et al.*[19]

The sedimentation rate of red blood cells was measured in standard 150 mm sedimentation tubes, with mixtures of 0·8 ml blood and 0·2 ml 3·8% sodium citrate, containing varying concentrations of chlorpromazine.

RESULTS

In order to determine the chlorpromazine concentration, giving optimal protection against osmotic haemolysis, a NaCl solution was selected, giving about 80% osmotic haemolysis in the control. To a series of tubes, containing 10 ml of this NaCl solution and increasing chlorpromazine concentrations, 0·3 ml blood was added. The results are given in Fig. 1. In further experiments the same procedure was followed, utilizing varying red blood cell concentrations. In Fig. 2 the lowest chlorpromazine concentration giving optimal protection against haemolysis is plotted against the erythrocyte concentration. The maximum protection that can be obtained with chlorpromazine corresponds to about 10 m-osm: to achieve the same per cent haemolysis in the presence of the drug the NaCl concentration should be reduced with 10 m-osm, as compared with the control.

Comparison of haemolysis (as calculated from released haemoglobin) with K^+ loss at decreasing NaCl concentrations reveals, that some K^+ loss occurs already in isotonic medium, without concomitant haemolysis. This isotonic K^+ loss amounted to 5·6 per cent of the total cellular K^+ content in the control, and to 7·2 per cent in the presence of chlorpromazine, after 30 min incubation at 25°. If the K^+ loss at lower, haemolysing NaCl concentrations is corrected for this initial, isotonic K^+ loss, it appears that the K^+ release slightly exceeds haemoglobin liberation, over the whole

range from 0 to 100 per cent osmotic haemolysis. This relationship between potassium and haemoglobin release is not influenced by chlorpromazine (Fig. 3).

With human erythrocytes the decrease of osmotic fragility, caused by chlorpromazine, was associated with an increase of the critical haemolytic volume of 12 per cent: in

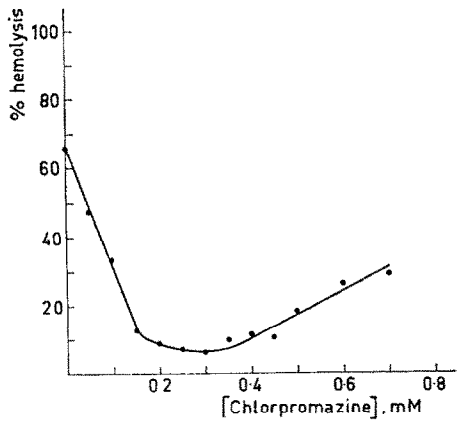

FIG. 1. The protection against osmotic hemolysis by chlorpromazine. Hemolysis was measured after 45 min incubation at 25°. Final NaCl concentration: 0·41 per cent.

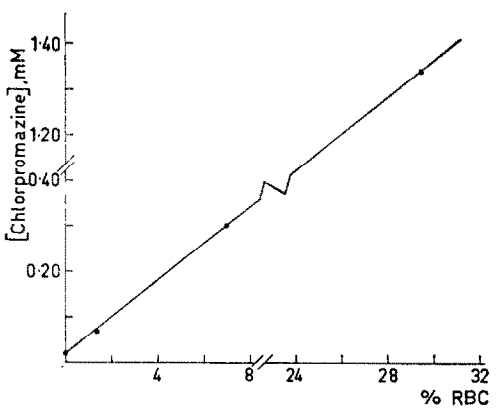

FIG. 2. The relationship between red blood cell concentration and the chlorpromazine concentration giving maximal protection against osmotic hemolysis.

the control the critical volume was $1{\cdot}77V_0$, increasing to $1{\cdot}98V_0$ in the presence of the drug (V_0 = mean cellular volume in 1 % NaCl, in the absence of chlorpromazine). As will be discussed below, this increase is considerably greater, than would be expected from the shift of 10 m-osm of the osmotic fragility curve. To explore this problem further, some morphological studies were undertaken. Photomicrographs of fresh human erythrocytes in isotonic NaCl in the absence (Fig. 4) and in the presence (Fig. 5) of chlorpromazine indicate an obvious change of cell shape, induced by the drug. The normal biconcave disks are transformed into pronounced cup-shaped cells, more or less looking like dented spheres. The cells with clear centres and very dark edges suggest this shape distinctly.

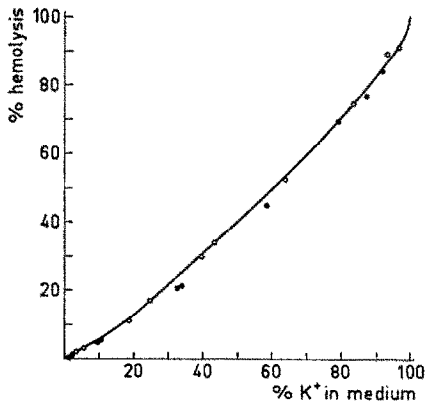

FIG. 3. The relationship between hemoglobin and potassium release during osmotic hemolysis Both Hb and K^+ are expressed in per cent of the total cellular content. ●—●: control; ○—○: chlorpromazine present.

Measurement of the mean cellular volume with Hamburger type haematocrit tubes reveals that chlorpromazine causes an increase of the haematocrit value in isotonic medium (Fig. 6). In 20 experiments the increase averaged 10·3 ± 2·3 (S.D.) per cent at the optimal drug concentration. This augmented haematocrit value could

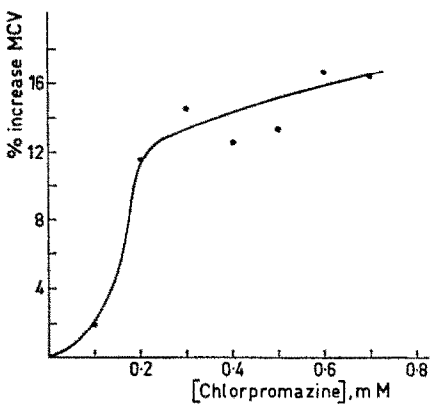

FIG. 6. The influence of chlorpromazine on the mean cellular volume (MCV) in 1% NaCl, as calculated from the hematocrit value.

be caused either by increased medium trapping or by a real swelling of the cells. To distinguish between these two possibilities trapped medium was determined with ^{14}C-labelled dextran. The experimental results showed a medium trapping of 5·8 (±0·5) per cent in the absence, and 6·9 (±0·3) per cent in the presence of chlorpromazine. This small increase of medium trapping will cause an increase of 1·1 per cent of the haematocrit value. The remaining 9·2 per cent apparently reflects a real increase of the mean cellular volume. Experiments with a Coulter electronic particle counter revealed a similar increase of the mean cellular volume; the shape of the volume distribution curve was not changed, indicating a volume increase of all individual cells.

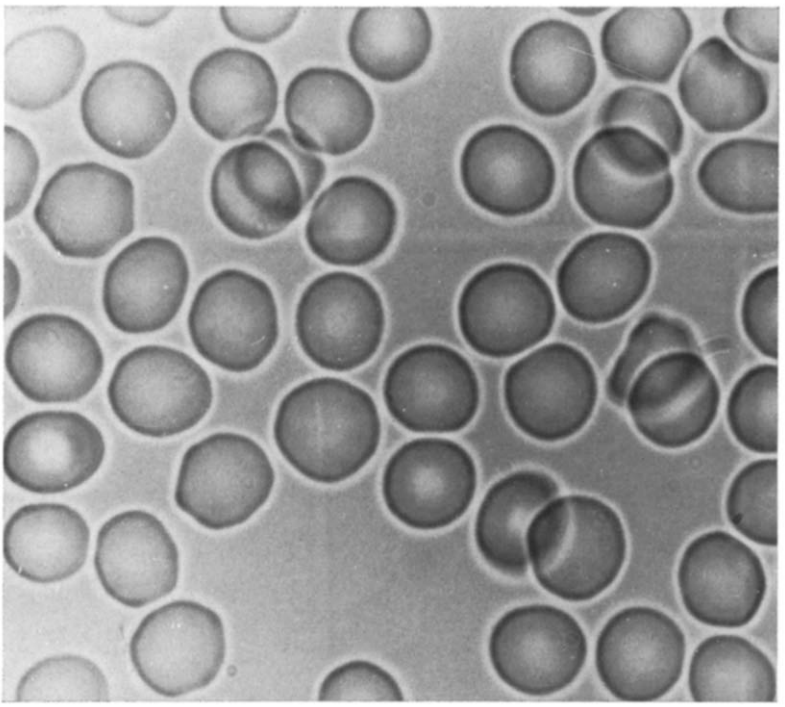

FIG. 4. Photomicrograph of fresh human erythrocytes in 1% NaCl.

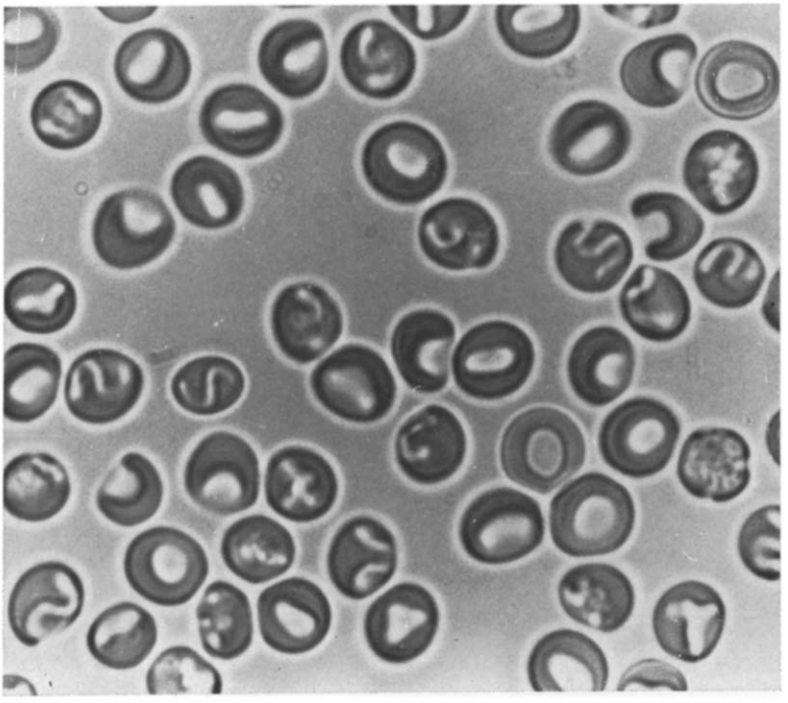

FIG. 5. Photomicrograph of fresh human erythrocytes in 1% NaCl, containing 10^{-4} M chlorpromazine.

Determination of the osmolarity of erythrocytes, disrupted by freezing and thawing showed that this drug-induced increase of the mean cellular volume is associated with a very small decrease of the intracellular tonicity. At a tonicity of the medium of 277 m-osm, measurements of intracellular tonicity revealed a mean value of 270 ± 8 (S.D.) m-osm in the absence, and of 264 ± 8 (S.D.) m-osm in the presence of chlorpromazine. Calculation revealed that the mean difference of the paired observations was significant ($P < 0{\cdot}01$).

Measurement of the intracellular Na^+ concentration indicates a significant net uptake of Na^+ ions in the presence of chlorpromazine. This uptake occurs apparently immediately after addition of the drug, and is not progressive in the course of time. The net sodium uptake induced by the drug amounted to 19 mEquiv/l. red blood cells in isotonic NaCl solution, as calculated from paired observations with and without addition of chlorpromazine. This sodium uptake was not counterbalanced by an equal potassium loss: only 1·5 mEquiv. K^+/l red blood cells is released to the medium under these experimental conditions. Apparently the net result is a considerable increase of the total cellular electrolyte content, caused by chlorpromazine. As paired observations were used, the figures were automatically corrected for the well known, slow K^+/Na^+ exchange that occurs in normal human erythrocytes when incubated in isotonic NaCl solution.[20]

The relationship between mean cellular volume and the reciprocal of the tonicity of the medium is depicted in Fig. 7, both in the absence and in the presence of chlorpromazine.

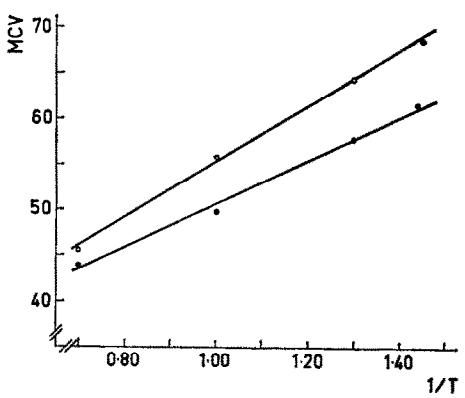

FIG. 7. The relationship between mean cellular volume (MCV) and the reciprocal of the tonicity of the medium ($1/T$). The mean cellular volume was measured in Hamburger type hematocrit tubes and corrected for trapped medium. ●—●: control; ○—○: 10^{-4} M chlorpromazine. Red blood cell concentration: 1½ per cent.

Chlorpromazine appeared to have a pronounced influence on the sedimentation rate of human erythrocytes, as shown in Table 1.

Carver demonstrated inhibition of isolated glucose-6-phosphate dehydrogenase by some phenothiazine derivatives.[21] This inhibition was confirmed in the present investigations: with a final chlorpromazine concentration of 4×10^{-4} M an enzyme inhibition of 30 per cent was found, under the experimental conditions described by

TABLE 1. THE INFLUENCE OF VARYING CHLORPROMAZINE CONCENTRATIONS ON THE SEDIMENTATION RATE OF HUMAN RED BLOOD CELLS AT ROOM TEMPERATURE

Sedimentation after:	Final chlorpromazine concn $\times 10^4$ M				
	0·0	2·5	5·0	12·0	20·0
1 hr	12·3	5·2	3·0	2·7	3·0
2 hr	25·1	15·8	6·8	5·1	6·4
3 hr	33·8	24·4	11·2	9·4	9·4
5 hr	41·2	34·5	18·8	15·3	16·1

Carver. Chlorpromazine did not influence the cellular reduced glutathione concentration in intact erythrocytes however. In a 8% red blood cell suspension the reduced glutathion concentration remained constant at a level of 680 μg/ml cells, during at least 4 hr, both in the control and after addition of $1{\cdot}1 \times 10^{-4}$ M chlorpromazine. In the GSH-stability test as described by Beutler[16] this chlorpromazine concentration did not have any influence. In similar experiments the intracellular ATP concentration appeared to be insensitive to chlorpromazine. An ATP concentration of 2·2 μmole/ml cells was measured after incubation at 37° during 1 hr, both in the presence and in the absence of $1{\cdot}1 \times 10^{-4}$ M chlorpromazine.

DISCUSSION

The relationship between red blood cell concentration and optimal chlorpromazine concentration with respect to the protection against osmotic haemolysis is demonstrated in Fig. 2. Assuming an equal drug uptake per cell at the point of maximal protection, irrespective the erythrocyte concentration, the figure suggests an equilibrium distribution of the drug between cells and medium. The extrapolated curve will intersect the ordinate at the equilibrium concentration in this case. At a given erythrocyte concentration all other chlorpromazine effects described in this paper are, within the experimental error, maximal at the same drug concentration, giving maximal protection against osmotic haemolysis. This suggests that the decreased osmotic fragility, the change of cell shape, the increase of the mean cellular volume and the decrease of the sedimentation rate may have the same physical chemical background.

The cup-shape, induced by chlorpromazine (Fig. 5), is much more pronounced than the cup forms that can be observed during the disk–sphere transformation in hypotonic NaCl solutions, as described e.g. by Ponder (see ref. 20, p. 10). A more or less similar change in cell shape was observed by Seeman with electron microscopy, after fixation of prochlorperazine ethane disulfonate treated cells.[7] This shape change is associated with an 8–13 per cent increase of the haematocrit value. Only about 10 per cent of this increase is accounted for by a rise of trapped medium. It seems reasonable to assume that the small increase of trapped medium is caused by the shape change. The remaining 90 per cent of the increased haematocrit value reflects an increase of the mean cellular volume. This swelling of the red blood cells appears to be caused mainly by Na^+ uptake, not counterbalanced by an equal K^+ loss. The potassium release in isotonic medium only slightly exceeds the potassium loss in control experiments without drug. In a particular experiment the increase of mean cellular volume (corrected for trapped medium) was 11 per cent. The amount of Na^+ taken up per ml red cells minus the amount of K^+ lost in excess of the control corresponded

to 0·11 ml isotonic medium. This would exactly account for the observed volume increase. A small correction must be made, however, for the decrease of the osmotic coefficient of intracellular haemoglobin, that should be expected on dilution of cellular contents.[22–24] The volume increase is indeed associated with a very small, but significant decrease of the intracellular tonicity. It should be emphasized that the data of intracellular tonicity have a comparative value only. Prior to freezing and thawing, the control and drug-treated, packed cells were adjusted to the same haematocrit (about 95 per cent) with isotonic medium. Therefore, some dilution of cell contents with isotonic medium cannot be avoided. This will result in too low tonicity readings, caused by the decreased osmotic coefficient of the haemoglobin. It is impossible to correct exactly for this effect; so the only conclusion that can be made is that the cell content of drug treated cells is slightly hypotonic with reference to control cells at the same tonicity of the medium.

In a previous paper[5] the decreased osmotic fragility, caused by chlorpromazine, has been ascribed to the increased critical haemolytic volume, that could be measured experimentally. The increase of the critical haemolytic volume seemed, however, much higher than was needed for the shift of about 10 m-osm of the fragility curve. This discrepancy is explained by the volume increase in isotonic medium. The curve, demonstrating the relationship between V and $1/T$ (Fig. 7) is shifted upwards in the presence of chlorpromazine. The critical haemolytic volume in the presence of chlorpromazine measured experimentally, corresponds reasonably well to the volume, calculated from this figure, extrapolating the upper curve to the NaCl concentration giving 50 per cent osmotic haemolysis, viz. $1{\cdot}98V_0$ and $2{\cdot}08V_0$ respectively (V_0 = mean cellular volume in 1 % NaCl, in the absence of chlorpromazine). The difference between these two figures may be explained by the not exactly linear relationship between V and $1/T$ at low NaCl concentrations (see ref. 20, p. 69).

The relationship between V and $1/T$ is given by the equation:

$$\frac{V}{V_0} = WR\left(\frac{1}{T} - 1\right) + 1,$$

where V = mean cellular volume, V_0 = mean cellular volume in isotonic solution, W = the water fraction of the cells at $T = 1$, T = tonicity (osmolarity of medium in per cent of the osmolarity of an isotonic solution) and R = an empirical constant.[25] Theoretically R should be equal to 1, if the cells would behave like perfect osmometers. To account for deviations found experimentally, Ponder introduced the empirical constant, having a value smaller than one. The significance of this correction factor R is still disputable. The possible explanations for its meaning comprise:[26]

(1) Leakage of ions from the cells to the medium;
(2) Binding of water of solvation to intracellular proteins;
(3) The concentration dependence of the osmotic coefficient of haemoglobin;
(4) A rigidity of the cell membrane, resisting changes in volume.

The value of R is not constant over a large range of NaCl concentrations; therefore, the relationship between V and $1/T$ will deviate slightly from the straight line. The mean value of R over a certain range of medium tonicities can be calculated from the

experimental data. To calculate R in the presence of chlorpromazine, the above equation should be written as:

$$\frac{V}{V_0'} = W'R'\left(\frac{1}{T} - 1\right) + 1,$$

where V_0' = mean cellular volume in isotonic, chloropromazine containing solution and W' = the water fraction of the cells at $T = 1$, in the presence of chlorpromazine.

Evaluation of the mean R value over the range $T = 1{\cdot}4$ to $T = 0{\cdot}7$ reveals an R value of 0·76 in the control and an R' value of 0·85 in the presence of chlorpromazine. The value of R found in the absence of the drug (0·76) is in good agreement with R values estimated by other authors over this tonicity range.[20,27] Considering the possible factors contributing to the correction term R, it appears that an influence of chlorpromazine (in the present concentrations) on the binding of water of solvation to intracellular protein, is a priori very unlikely. Nevertheless, assuming that binding of water of solvation would be the explanation for the physical meaning of R, the drug-induced uptake of isotonic medium would have some influence on the R value. This can be best illustrated by the following example. In a particular experiment the following data were measured: $W = 0{\cdot}70$; $R = 0{\cdot}76$; after addition of chlorpromazine V'_0 appeared to be $1{\cdot}103 V_0$ and $R' = 0{\cdot}85$. Under the above-mentioned assumption the osmotically active water fraction in the control would be $R.W = 0{\cdot}532$. In the presence of chlorpromazine 0·103 ml isotonic medium has been taken up per ml cells; therefore

$$W' = \frac{0{\cdot}70 + 0{\cdot}103}{1{\cdot}103} = 0{\cdot}728.$$

It is reasonable to assume that chlorpromazine will not influence the total amount of bound water per cell. Thus the osmotically active water fraction of drug treated cells would be:

$$\frac{0{\cdot}532 + 0{\cdot}103}{1{\cdot}103} = 0{\cdot}576 \quad \text{and} \quad R' = \frac{0{\cdot}576}{0{\cdot}728} = 0{\cdot}79.$$

So even under this extreme assumption only a fraction of the observed R change from 0·76 to 0·85 could be explained along these lines.

Concerning the concentration dependence of the osmotic coefficient of haemoglobin, a similar calculation reveals that the R-shift caused by chlorpromazine cannot be explained on this basis. Moreover, even in the control this concentration dependence of the osmotic coefficient cannot explain R values lower than about 0·95.[23]

A significant leakage of ions from the cells to the medium in excess of the control could be excluded experimentally. The data represented in Fig. 3 support this conclusion. Therefore the R-change induced by chlorpromazine presumably reflects an influence of the drug on the rigidity of the cell membrane.

Possibly there is a causal relationship between this influence of chlorpromazine on the rigidity of the membrane on the one hand, and the shape change and the momentary, limited uptake of Na^+ and water after the addition of the drug on the other hand. This eventual relationship can as yet not be surveyed, however.

With reference to the inhibition of isolated glucose-6-phosphate dehydrogenase by phenothiazine derivatives as described by Carver[21] and the possible metabolic dependence of the critical haemolytic volume,[28] it was investigated if the effects of chlorpromazine on intact human erythrocytes were associated with an influence on cellular metabolism. The unchanged intracellular ATP and glutathione concentrations in the presence of chlorpromazine make it very unlikely that the drug effects as described in this communication could be explained via an influence on cellular metabolism.

At the present time the most obvious interpretation of the experimental results appears to be an interaction of chlorpromazine with certain cellular structural entities as a common basis of the diverse drug effects. The physical chemical nature of this interaction remains to be further investigated.

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