Br. J. Pharmac. (1982), **77**, 141–145

EVIDENCE FOR CARRIAGE OF SILVER BY SULPHADIMIDINE: HAEMOLYSIS OF HUMAN ERYTHROCYTES

P.M. BALLINGER, B.S. BROWN, M.M. GRIFFIN & F.S. STEVEN

Department of Medical Biochemistry, University of Manchester, Medical School, Stopford Building, Oxford Road, Manchester M13 9PT

1 Human erythrocytes suspended in isotonic saline haemolyse in the presence of *both* Ag^+ ions and sulphadimidine.

2 Neither Ag^+ ions nor sulphadimidine on their own will haemolyse erythrocytes suspended in isotonic saline.

3 At constant Ag^+ ion concentration the degree of haemolysis of saline-suspended erythrocytes depends upon the concentration of sulphadimidine.

4 Human erythrocytes suspended in isotonic sucrose (chloride-free) haemolyse in the presence of Ag^+ ions.

5 Sulphadimidine in chloride-free sucrose competes with erythrocytes for Ag^+ ions resulting in stoichiometric protection of the erythrocytes from the haemolytic action of Ag^+ ions.

6 Haemolysis occurs when each erythrocyte receives approximately 1.2×10^9 Ag^+ ions whether suspended in saline or sucrose.

7 Sulphadimidine acts as a carrier for Ag^+ ions and so prevents their precipitation as AgCl when erythrocytes are suspended in saline.

Introduction

It has long been known that low concentration of Ag^+ ions will haemolyse erythrocytes (Hausmann & Kerl, 1920; Meneghetti, 1921; 1922; Ball, 1933). However, Ag^+ ions precipitate in the presence of Cl^- ions when the solubility product, $[Ag^+][Cl^-]$, is exceeded. The solubility product for AgCl at 25°C is 1.7×10^{-10} so that the maximum concentration of Ag^+ ion that can occur in isotonic saline is in the region of 1.1 nM. Thus the concentration of Ag^+ ion attainable in chloride-containing body fluids is very low, and this fact hampers the study of the pharmacological effects of the Ag^+ ion.

If Ag^+ ions could be carried under physiological conditions in the presence of Cl^- ions then their pharmacological effects could be studied. One Ag^+ ion carrier that has been reported is the sulphonamide sulphadiazine (Fox, 1968). Silver sulphadiazine is used in burn therapy (McDougall, 1972) as an antibiotic because it releases bacteriostatic amounts of Ag^+ ions whilst preventing the precipitation of Ag^+ ions as insoluble AgCl (Fox, 1968).

In the present study a related sulphonamide, sulphadimidine, has been shown to carry Ag^+ ions in the presence of Cl^- ions; this can only be achieved by complex formation. Its efficiency as a carrier has been demonstrated in a model system using human erythrocytes. This system offers a simple means of screening for other carriers of Ag^+ ions which is independent of an assay involving enzymes. In a further study (Ballinger, Griffin, Itzhaki & Steven, 1982) we have demonstrated that the carriage of Ag^+ ions by sulphadimidine may lead to alteration of proteolytic enzyme activity.

Methods

Blood

Blood was donated by three normal male subjects aged 19, 39 and 49 years; the data presented here are from the 49 year-old donor. Plastic syringes and containers were used throughout. Freshly drawn human blood (10 ml) was suspended in 100 ml isotonic saline and centrifuged for 5 min. The erythrocytes were washed three times in an excess of either isotonic (154 mM, 0.9% w/v) saline or isotonic (291 mM, 9.95% w/v) sucrose in the absence of anticoagulant, centrifuged, then resuspended in either saline or sucrose. The concentration of erythrocytes was such that 3.0 ml of cell suspension, after complete haemolysis with added saponin, gave an A_{530} of 0.20. The isosbestic point for oxyhaemoglobin and methaemoglobin has been reported by Joiner & Lauf

0007-1188/82/090141-05 $01.00

(1978) to be 527 nm. We chose to employ 530 nm as being close to the isosbestic point to ensure that any conversion of oxyhaemoglobin to methaemoglobin in these experiments did not invalidate our experimental data.

Haemolysis of erythrocytes suspended in isotonic saline

(a) In the presence of a constant concentration of sulphadimidine and increasing concentrations of Ag^+ ions Sodium sulphadimidine (100 μl) and 0–100 μl silver nitrate were added to each of a series of 10 ml centrifuge tubes. The contents were mixed and allowed to react for 5 min at room temperature to form silver sulphadimidine. Isotonic saline (1.0 ml) was added followed by 2.0 ml of erythrocytes suspended in saline. After mixing, the tubes were left for 10 min at room temperature for haemolysis to occur and centrifuged for 5 min to sediment any unhaemolysed cells. Degree of haemolysis was determined from the absorbance of the supernatants at 530 nm. Control tubes contained all the reagents except either sulphadimidine or silver nitrate.

(b) In the presence of a constant concentration of Ag^+ ions and increasing concentrations of sulphadimidine A series of 10 ml centrifuge tubes, each containing 100 μl silver nitrate and 0–150 μl sodium sulphadimidine, was treated as described in section (a).

Haemolysis of erythrocytes suspended in isotonic sucrose

(a) In the presence of increasing concentrations of Ag^+ ions A suspension of erythrocytes in sucrose, followed by 0–60 μl silver nitrate, were added to each of a series of 10 ml centrifuge tubes. The contents were mixed, allowed to stand for 10 min at room temperature, then centrifuged for 5 min. The degree of haemolysis was determined from the absorbance of the supernatants at 530 nm.

(b) In the presence of a constant concentration of Ag^+ ions and increasing concentrations of sulphadimidine Silver nitrate (40 μl) and 0–50 μl of sodium sulphadimidine (stock solution diluted 100 times) were added to each of a series of 10 ml centrifuge tubes. The contents were mixed, and allowed to react for 5 min at room temperature to form silver sulphadimidine. Then 3.0 ml of erythrocytes suspended in sucrose was added to each tube. After 10 min the tubes were centrifuged for 5 min. The degree of haemolysis was determined from the absorbance of the supernatants at 530 nm.

Total haemolysis

In each series of experiments, solid saponin (approximately 100 μg) was added to the control tubes after their absorbance had been determined. Saponin produced complete haemolysis in 10 min at room temperature, after which time the absorbance at 530 nm was again determined. This absorbance represented 100% haemolysis, and enabled the absorbance of other supernatants to be converted to per cent haemolysis.

Erythrocyte numbers

There was a linear relationship between erythrocyte number (haematocrit) and absorbance at 530 nm of fully haemolysed cells. An absorbance of 0.200 was equivalent to 34.3×10^6 erythrocytes in 3.0 ml of reaction mixture.

Absorbance

Absorbance at 530 nm was determined in an LKB Ultrospec 4050 spectrophotometer against a water blank.

Chemicals

All were of 'Analar' grade. Sulphadimidine sodium, B.P., (sulphamethazine) was obtained from ICI Pharmaceutical Division, Macclesfield, Cheshire as a 1.1 M solution in water. This was used as stock solution, 100 μl giving a final concentration in the reaction mixtures of 33.3 mM. Silver nitrate stock solution in water had a concentration of 3.0 mM, 100 μl giving a final concentration in the reaction mixtures of 100 μM. Saponin was obtained from Sigma, London.

Results and Discussion

Figure 1 shows that Ag^+ ions in the presence of sulphadimidine caused haemolysis of erythrocytes. Similar results were obtained for several batches of human erythrocytes. Minimal haemolysis (about 1%) took place in control tubes from which either sulphadimidine or Ag^+ ions had been omitted. Thus it can be concluded that both Ag^+ ions and sulphadimidine are necessary for haemolysis.

Each curve showed a threshold concentration of Ag^+ ions below which haemolysis did not occur. These Ag^+ concentrations were 8 μM, 30 μM and 56 μM for curves (a), (b) and (c) respectively. These threshold concentrations corresponded to 249×10^6 Ag^+ ions per erythrocyte for curve (a), 380×10^6 for curve (b) and 301×10^6 for curve (c). We do not

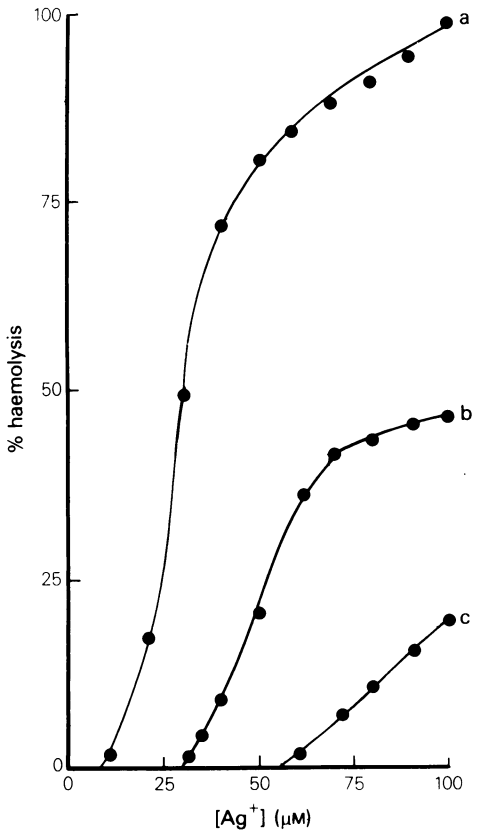

Figure 1 Haemolysis of erythrocytes suspended in isotonic saline and 33 μM sodium sulphadimidine by incremental addition of Ag^+ ions. Each tube contained 3.0 ml of reagents and haemolysis took place in 10 min at room temperature. Three concentrations of erythrocytes were used: (a) 58×10^6, (b) 141×10^6, and (c) 334×10^6 erythrocytes per 3.0 ml reaction mixture.

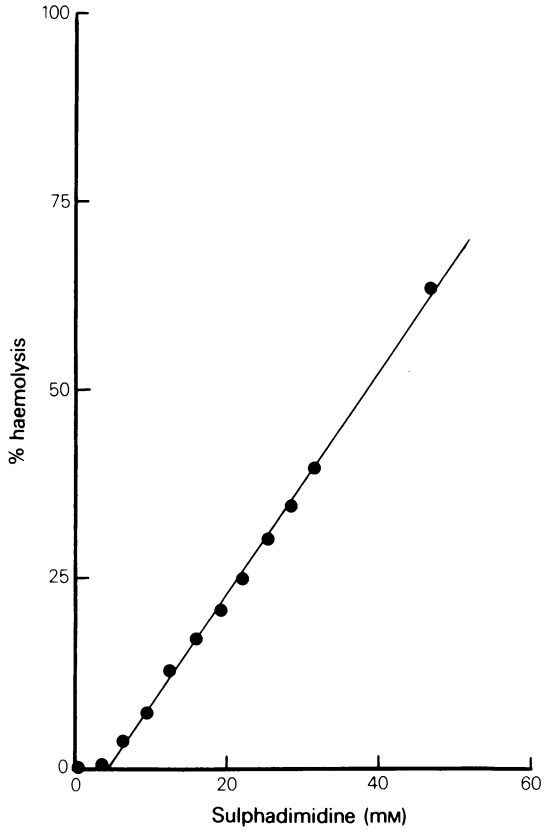

Figure 2 Haemolysis of erythrocytes suspended in isotonic saline in the presence of 100 μM Ag^+ ions and 0–50 mM sodium sulphadimidine. Each tube contained 47×10^6 erythrocytes in a final volume of 3.0 ml. Haemolysis took place in 10 min at room temperature.

know the reason for the observed threshold concentrations of Ag^+ before haemolysis occurred (Figure 1). We believe this threshold may represent the binding of Ag^+ to the erythrocytes at sites which have no significance for the stability of the cell membrane. A similar threshold was noted for the binding of low concentrations of ouabain to cells prior to the inhibition of the Na^+/K^+ pump (Baker & Willis, 1972). At constant erythrocyte number, degree of haemolysis was directly proportional to Ag^+ ion concentration.

Figure 2 shows that when the concentration of Ag^+ ions was held constant, the degree of haemolysis depended upon the concentration of sulphadimidine. The concentration of Ag^+ ions used, 100 μM, had been previously found sufficient to cause 100% haemolysis (Figure 1). Nevertheless, in the absence of sulphadimidine no haemolysis occurred because the Ag^+ ions were removed from solution by precipitation as silver chloride. In the presence of millimolar concentrations of sulphadimidine, Ag^+ ions were prevented from precipitating and thus became available to bring about haemolysis.

Figure 3 shows that when the concentration of Ag^+ ions was held constant, the degree of haemolysis was inversely related to erythrocyte number. From the slope of each curve in Figure 1 it can be calculated that, if all the Ag^+ ions are transferred to erythrocytes, haemolysis occurs when the average erythrocyte receives 1.2×10^9 Ag^+ ions.

The above observations suggest that both Ag^+ ions and sulphadimidine are necessary for the haemolysis of erythrocytes suspended in isotonic saline. The observation that no haemolysis occurred in the presence of sulphadimidine alone suggested that Ag^+ was the haemolysing agent while sulphadimidine was the carrier.

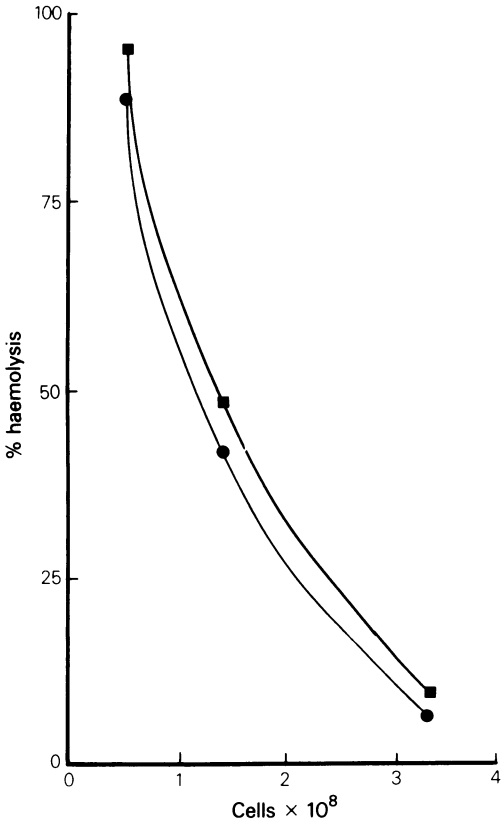

Figure 3 Haemolysis of erythrocytes suspended in isotonic saline and 33 mM sodium sulphadimidine. Variation of degree of haemolysis at constant Ag^+ ion concentration with number of erythrocytes in 3.03ml final volume. Conditions as described in Figure 1. Concentration of Ag^+ ions were (●) 70 μM and (■) 80 μM.

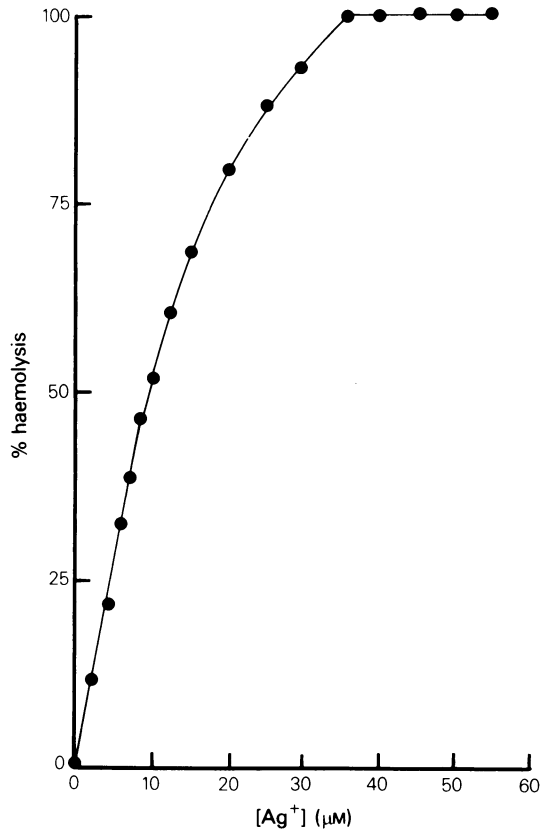

Figure 4 Haemolysis of erythrocytes suspended in isotonic sucrose by incremental addition of Ag^+ ions. Each tube contained 27×10^6 erythrocytes in a final volume of 3.0 ml, and haemolysis took place in 10 min at room temperature.

Confirmation that Ag^+ was the haemolysing agent was obtained from the experiment shown in Figure 4. When erythrocytes were suspended in isotonic sucrose, haemolysis occurred upon the addition of Ag^+ ions. No sulphadimidine was necessary because the system was free of chloride ions. The degree of haemolysis was directly proportional to Ag^+ ion concentration, total haemolysis being obtained when the Ag^+ ion concentration was 35 μM. From the slope of the curve in Figure 4 it can be calculated that haemolysis occurs when the average erythrocyte receives 1.25×10^9 Ag^+ ions. Similar figures were obtained for erythrocytes taken from two different subjects and suspended at several different concentrations. These figures are also similar to those obtained for erythrocytes suspended in isotonic saline.

Confirmation that sulphadimidine was acting as a carrier for Ag^+ ions was obtained from the experiment shown in Figure 5. If sulphadimidine were acting as a carrier one might expect it to protect erythrocytes from haemolysis by Ag^+ ions. Erythrocytes were exposed to 40 μM Ag^+ ions to which different amounts of sulphadimidine had been added previously. The concentration of Ag^+ ions used had been previously found sufficient to cause 100% haemolysis (Figure 4). In the absence of sulphadimidine complete haemolysis occurred. Addition of incremental amounts of sulphadimidine to the Ag^+ ions, before exposure to the erythrocytes, resulted in protection of the erythrocytes from the haemolytic effect of Ag^+ ions.

The degree of protection was directly proportional to the concentration of sulphadimidine, and protection was complete with 40 μM sulphadimidine. At this concentration every Ag^+ ion was bound to a molecule of sulphadimidine.

In this experiment sulphadimidine and the

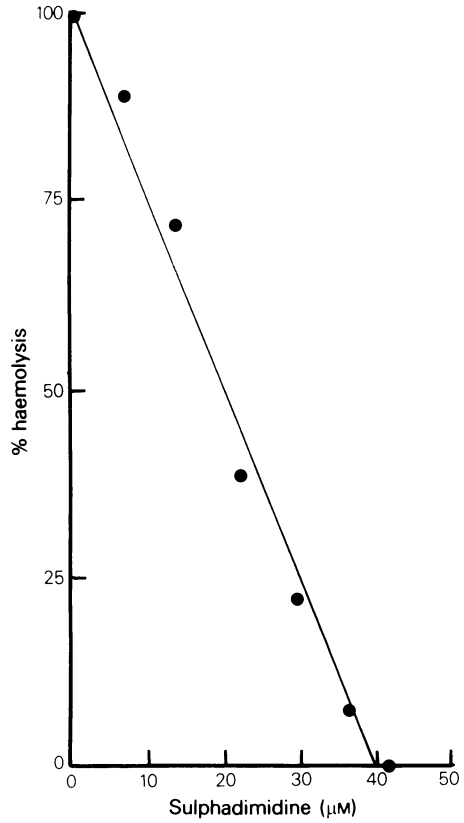

Figure 5 Haemolysis of erythrocytes suspended in isotonic sucrose in the presence of 40 μM Ag^+ ions and 0–40 μM sodium sulphadimidine. Each tube contained 27×10^6 erythrocytes in a final volume of 3.0 ml, and haemolysis took place in 10 min at room temperature.

erythrocyte membrane were in competition for a fixed quantity of Ag^+ ions. This experiment can only be carried out in the absence of Cl^- ions because in their presence AgCl precipitates.

A comparison of the data presented in Figures 1 and 5 indicates that with NaCl as medium, silver sulphadimidine is a more effective lytic agent than with sucrose as suspension medium for the erythrocytes. It is well known that erythrocytes shrink in the presence of isotonic sucrose due to the loss of cytoplasmic K^+ and consequent loss of water. It might be expected that the shrunken erythrocytes in sucrose experienced a smaller cytosolic pressure on their cell membranes, than the spherical erythrocytes suspended in NaCl. This might afford a possible explanation for differences in lytic effect observed in Figures 1 and 5.

Conclusion

Silver ions may be carried under physiological conditions as a complex with sulphadimidine. Silver ions can thus act on biological systems in the presence of chloride ions at concentrations that would result in the precipitation of AgCl were the sulphadimidine not present. Haemolysis of erythrocytes offers a convenient technique for the screening of compounds for the carriage of silver ions. Such studies have led to the use of silver sulphadimidine in the control of the proteolytic enzymes trypsin and chymotrypsin (Ballinger *et al.*, 1982). It is probable that other enzymes may also be modified by silver sulphadimidine.

We gratefully acknowledge many helpful discussions with Dr H. Bobinski of the Department of Physiology. Correspondence to F.S.S., please.

References

BALL, E.C. (1933). Hemolytic action of silver occurring as an impurity in chemically pure sodium chloride. *Biol. Bull.*, **64,** 277–288.

BALLINGER, P.M., GRIFFIN, M.M., ITZHAKI, S. & STEVEN, F.S. (1982). Evidence for carriage of silver by sulphadimidine: Inhibition of proteolytic enzymes. *Br. J. Pharmac.*, **77,** 147–151.

BAKER, P.F. & WILLIS, J.S. (1972). Binding of the cardiac glycoside ouabain to intact cells. *J. Physiol.*, **224,** 441–462.

FOX, C.L., JR. (1968). Silver sulphadiazine – a new topical therapy for *Pseudomonas* in burns. *Arch. Surg.*, **96,** 184–188.

HAUSMANN, W. & KERL, W. (1920). Oligodynamic hemolysis. *Biochem. Z.*, **112,** 122–123.

JOINER, C.H. & LAUF, P.K. (1978). The correlation between ouabain binding and potassium pump inhibition in human and sheep erythrocytes. *J. Physiol.*, **283,** 155–175.

McDOUGALL, I.A. (1972). Use of silver sulphadiazine in the treatment of burns at the Royal Perth Hospital. *Med. J. Aust.*, **1,** 979–981.

MENEGHETTI, E. (1921). Haemolysis and fixation with ethyl alcohol;; comparative haemolytic and fixative action of metals. *Arch. Sci. Biol.*, **2,** 279–317.

MENEGHETTI, E. (1922). Haemolytic and coagulating effect of metal ions. *Biochem. Z.*, **131,** 38–77.

(Received March 8, 1982. Revised April 30, 1982.)