[Chem. Pharm. Bull. 25(8)2098—2104(1977)]

UDC 615.31.015.45.076.9 : 612.111-08

# Effect of Drugs on Erythrocytes. I.[1] Influence of pH, Osmotic Pressure and a Few Drugs on Erythrocytes' Destruction

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(Received February 25, 1977)

In order to examine the effect of parenteral solutions on erythrocytes, periodical changes in erythrocyte count were measured by HIAC automatic particle counter and, at the same time, erythrocytes were observed by a scanning electron microscope. It was found that HIAC particle counter is convenient for counting of blood cell number and that changes in the counts of cells in the range of 10—150 μm can be used as an index for the destruction of blood cells.

It was also found that removal of ascorbic acid from tetracycline preparations prevented destruction of erythrocytes and that chloramphenicol sodium succinate does not disrupt erythrocytes even in a high concentration.

These results indicated that erythrocyte destruction by parenteral solution was possibly due to the pH and osmotic pressure of the drug solution and to additives rather than or in addition to the direct action of drugs. Re- examination of the test methods for hemolysis and establishment of judgement standards are desirable.

**Keywords**——hemolysis; *in vitro* test; HIAC automatic particle counter; scanning electron microscope; pH; osmotic pressure; tetracycline hydrochloride; chloramphenicol sodium succinate; ascorbic acid

Harmful effect of parenteral drugs has become a social problem in recent years and, since Akaishi[3] reported that many parenteral solutions showed to have hemolytic activity when human blood was incubated with these solutions, several reports on the hemolytic action of parenteral solutions have been published in Japan.[3–8] There are some reports on hemolysis by parenteral solutions in foreign countries, for example on surface-active agents, anesthetics and commercial parenteral solutions.[9–14] Effect of parenteral solutions on erythrocytes is an important problem for human health, and there has also been a report on a possible relation between parenteral intramuscular solutions with high hemolytic activity and quadriceps muscle contraction, and between hemolytic effect of chloramphenicol preparations and aplastic anemia.[3]

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When *in vitro* test on hemolysis is carried out, hemolysis often differs according to other test methods used, and the occurrence of hemolysis also differs according to the concentration of drugs[9,10,13,14] or to the kind of inorganic salts added.[11] It is wrong, therefore, to judge hemolytic effect of drugs from the result of merely one experiment. It is especially necessary to take a great care in judging a relation between the results of hemolysis experiments and tissue or functional damage.

In the present study, influence of pH and osmotic pressure of several buffer solutions and a few drugs on erythrocytes were examined from changes in the erythrocyte count. At the same time, morphological changes of erythrocytes were examined using a scanning electron microscopy.

### Experimental Materials and Methods

**Blood**——Human blood was obtained from the medial cubital vein, 3 ml, using a disposable plastic syringe (6 ml) with a 22-gauge (×1 1/4″) needle. The blood was transferred to a vessel, 6 glass beads (4 mm diam.) were added, and the whole was swirled gently for 5 min. Fibrin clot thereby formed was removed and this defibrinized blood was used as a sample. Blood was drawn during the morning and used for the test within 12 hr.

**Drugs**——Tetracycline Injection (L Co., Lot No. 04771-525) containing 100 mg (potency) of tetracycline hydrochloride and 250 mg of ascorbic acid.

Chloramphenicol Injection (S Co., Lot No. 3345) containing 1 g (potency) of chloramphenicol sodium succinate.

Tetracycline Hydrochloride Powder (L Co., Lot No. 215).

Ascorbic acid and NaCl used were the special reagent grade.

**Preparation of Buffer Solutions**——0.14 M Veronal-acetate buffer, 0.067 M phosphate buffer, 0.26 M citric acid-sodium citrate buffer (hereafter abbreviated as citrate buffer), and NaCl solution were used. They were filtered through a membrane filter (Millipore, pore size 0.45±0.02 μm) and adjusted with Beckmann pH-meter Model SS2 to pH 2.1, 3.9, 4.8 and 6.3 for veronal, phosphate and citrate buffer, to pH 9.8 for phosphate buffer, and to pH 6.3 for NaCl solution.

**Preparation of Solutions with Various Osmotic Pressure**——Hypertonic solutions were prepared by the addition of NaCl and hypotonic solutions by dilution, and osmotic pressure ratio was adjusted to 0.25, 0.5, 1, 2 and 3. Osmotic pressure was measured by the freezing point depression, using a Knauer Halbmikro-osmometer Type M (Knauer Co., Berlin).

**Measurement of Erythrocytes**——The apparatus used were HIAC automatic particle counter, Model PC-305 SSTA (5 channels), automatic sampling stage Model BS-1331 (10—20 ml), and a sensor Model D-5-150 (5—150 μm), all of High Accuracy Product, Carifornia. This whole system will be abbreviated to HIAC hereafter. The channel size was set in the ranges of 5—6, 7, 8, 9, and 10—150 μm.

A wide-mouthed glass bottle was washed with ultraclean distilled water filtered through a Millipore filter of 0.45±0.02 μm pore size, 200 ml of the test solution and a polyethylene coated magnetic stirring bar (5×20 mm) were placed in it, and exactly 0.2 ml of the blood was added. Number of erythrocytes was counted at definite intervals. Revolution of the stirrer was set at 6.5 of HIAC stirring control-dial, revolution was started 30 sec before measurement and stopped with completion of the measurement.

**Observation on Erythrocytes**——Hitachi-Akashi scanning electron microscope, Model MSM-4 (Akashi Seisakusho, Tokyo) and Eiko ion-coater Model IB-3 (Eiko Engineering, Ibaragi) were used.

The blood sample was fixed in 10 volumes of 0.9% glutaraldehyde in 0.1M phosphate buffer solution (pH 7.4) for 2 hr, washed 3 times with distilled water (by centrifugation at 1500 rpm for 5 min), and 1 drop of it was placed on a small piece of glass (*ca.* 7×7 mm) and allowed to dry in air at room temperature. This sample was mounted on an aluminum stub (15 mm diam.) with silver conduction cement (Silvest P-255) and then shadowed with gold in a ion-coater to give surface conductivity. The blood cells were then observed under a scanning electron microscope, and some areas were selected at random and photographed.

## Result and Discussion

### Effect of Osmotic Pressure and pH on Erythrocytes

**Measurement of Erythrocyte Counts**——One of the methods for the measurement of erythrocyte count is that using a Coulter counter, but this is liable to be affected by electricity because the counter utilizes electric resistance, and it is difficult to measure one sample over a long period of time. For this reason, we used measurement with HIAC which utilizes

changes in light blockage, and this was found to be very convenient for the measurement of the size of blood cells and their distribution.

Particle size determination by HIAC is made from conversion of the area of a particle passing through the sensor into diameter of a circle. When human erythrocytes, with a diameter of 7—9 μm and thickness of 1—2.4 μm, forming a beconcave shape, are counted by HIAC, concentration of erythrocyte suspension is important, because the sensors provided with HIAC have the optimal particle concentration (number of particles/ml) and the particles above that number will not be counted individually. Ideally, cell suspension of $2\times10^6$ dilution is desirable but, at this concentration, the suspension will appear translucent, and changes in color and turbidity cannot be observed. The 1000-fold diluted blood suspension used in the present experiment was most suited for these observation, and erythrocytes were detected in the range of 10—150 μm by HIAC.

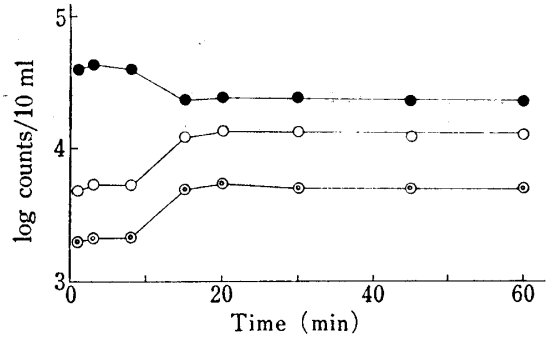


Fig. 1. Destruction Curve of Erythrocytes in Veronal-Sodium Acetate Buffer, pH 3.9, Osmotic Pressure Ratio 3

—●—, 10—150 μm; —○—, 5—6 μm; —◎—, 7, 8, 9 μm.

**Effect of Osmotic Pressure**——In hypertonic solutions, in which the erythrocytes of 10—150 μm decreased and smoll shrinked cells of 5—9 μm increased inversely. After a definite period, all the counts in any of the ranges became constant (Fig. 1). When such a pattern is observed, there will be no destruction of the erythrocyte membrane, the cells will have shrunk to a certain size, and this state is maintained for some time. Fig. 2 is a scanning electron micrograph of normal erythrocytes and those in a hypertonic solution with osmotic pressure ratio of 3.

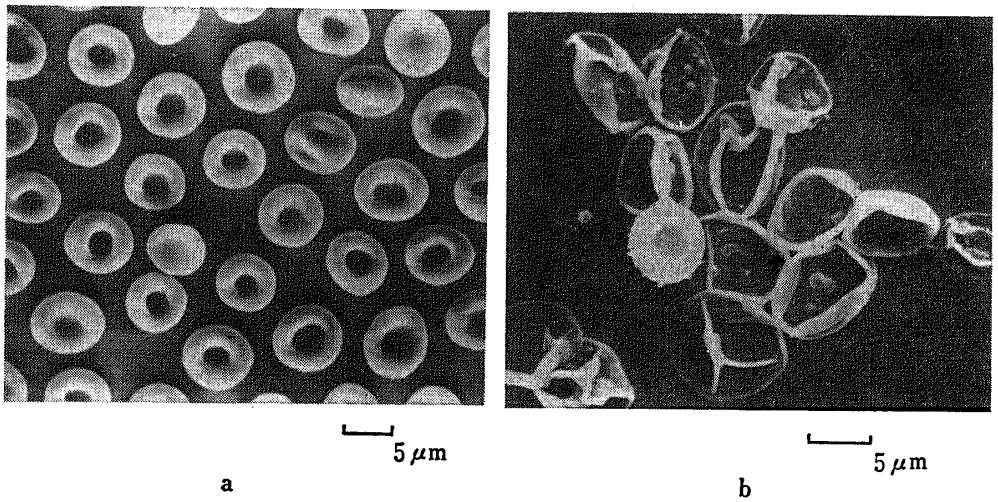


Fig. 2. Scanning Electron Micrograph of Erythrocytes

a, normal human erthrocytes in normal saline.
b, erythrocytes in hypertonic veronal-sodium acetate buffer, pH 3.9, osmotic pressure ratio 3.

In a hypotonic solution, differing from that in hypertonic solution, the erythrocyte membrane undergoes destruction and counts of cells in the range of 10—150 μm show a rapid decrease on a short time, and continues to decrease with time. At the same time, counts of particles in other ranges increase slightly (Fig. 3). This fact indicates that erythrocytes are disrupted and their membraneous pieces are counted as particles of below 9 μm by HIAC.

When this state is observed with the scanning electron micrographs, acanthocytes begin to appear after 1 min and destruction of blood cells progresses with decrease in the particle counts of 10—150 μm range (Fig. 4).

It was thereby found that destruction of erythrocytes with morphological changes in blood cells can be known from the counts of particles in the range of 10—150 μm as an index. Therefore, erythrocyte counts with changes on osmotic pressure of sodium chloride solutions were compared with particles only in the range of 10—150 μm (Fig. 5). As will be clear from this graph, destruction of blood cells increase suddenly when the osmotic pressure ratio falls below 0.40 and the destruction increases with lowering of osmotic pressure.

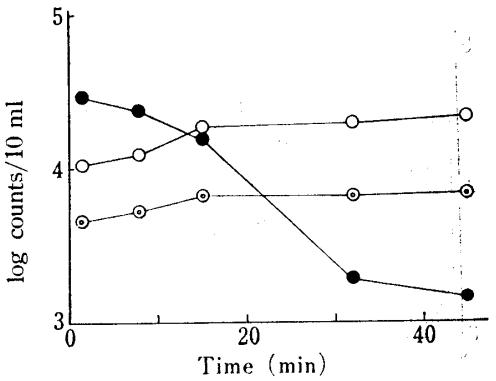


Fig. 3. Destruction Curve of Erythrocytes in Veronal-Sodium Acetate Buffer, pH 3.9, Osmotic Pressure Ratio 0.5

—●—, 10—150 μm; —○—, 5—6 μm; —◎—, 7, 8, 9 μm.

**Effect of pH**——Figure 6 shows the relation between changes in pH and destruction of erythrocytes. While the destruction of erythrocytes hardly occurs in the alkaline of pH, the destruction progresses when pH becomes below 3.3.

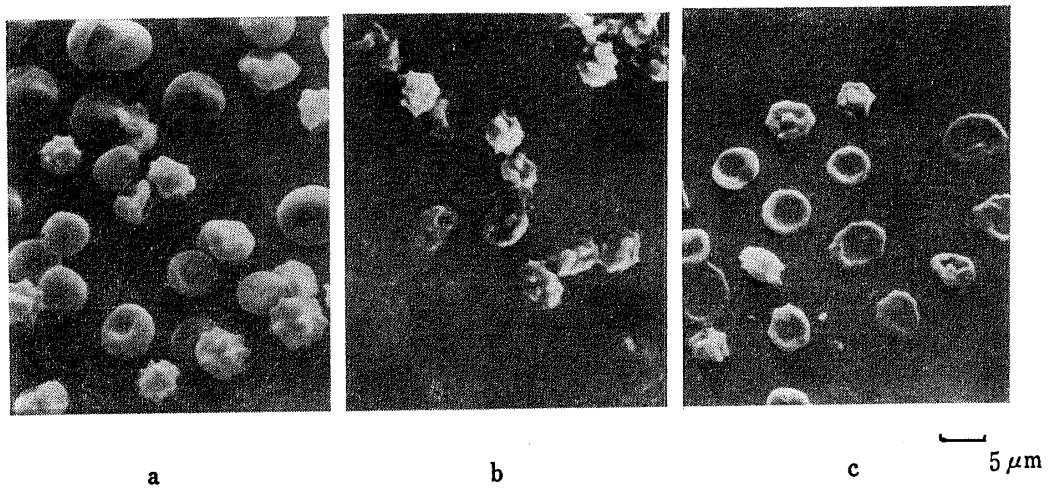


Fig. 4. Progressive Change on Shape of Cells in Veronal-Sodium Acetate Buffer, pH 3.9, Osmotic Pressure Ratio 3

a, after 1 min; b, after 15 min; c, after 45 min.

Safety range, in which destruction of erythrocytes would be difficult to occur, was examined by changing pH and osmotic pressure of the four kinds of buffer solution, and its result is illustrated in Fig. 7. However, there are some buffer solutions in which the destruction of erythrocytes occur easily, and the results were graded differently according to each buffer; the values were graded minus (—) if the destruction did not occur in the veronal buffer in which destruction usually occurrs most easily, and graded plus (+) if the destruction did occur in the citrate buffer, with strong destruction graded double plus (++) or triple plus (+++). From this grading, the area surrounded by a broken line in Fig. 7 may be termed a safety capacity of a human body into consideration, and this result should not, *per se*, be applied to injections in humans.

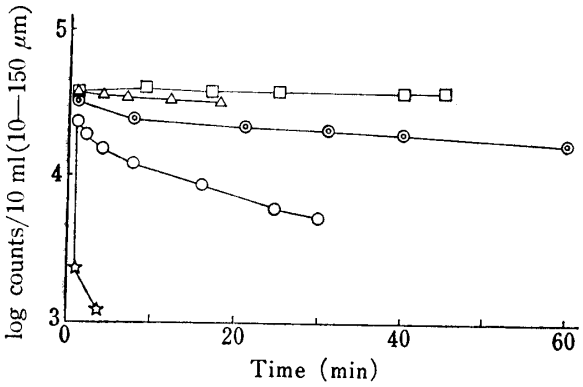


Fig. 5. Destruction Curve of Erythrocytes in NaCl Solution with Various Osmotic Pressure

Osmotic pressure ratio —□—, 2, 3; —△—, 0.50; —◎—, 0.45; —○—, 0.40; —☆—, 0.33.

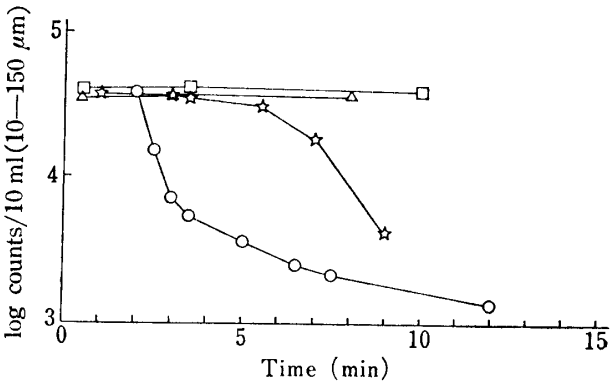


Fig. 6. Destruction Curve of Erythrocytes in Isotonic Veronal-Sodium Acetate Buffer with Various pH

—□—, pH 9.8, 7.3; —△—, pH 4.8; —☆—, pH 3.3; —○—, pH 2.1.

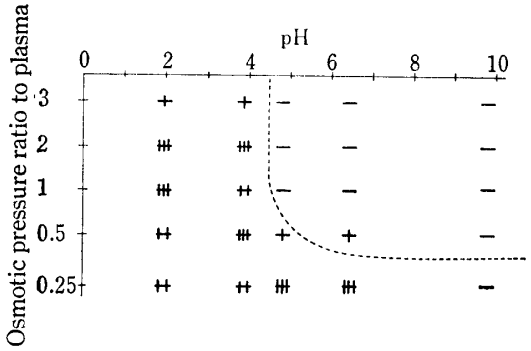


Fig. 7. Safety Range for Hemolysis according to pH and Osmotic Pressure

Buffer: veronal-acetate buffer, phosphate buffer, citrate buffer and NaCl solution.
+, some hemolysis observed in acetate buffer.
++ +++, hemolysis observed in all solutions.
−, no hemolysis observed in veronal buffer.

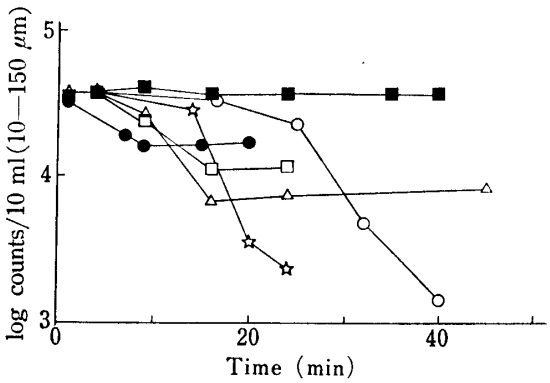


Fig. 8. Destruction Curve of Erythrocytes in 200 ml Saline containing 10 mg Tetracycline·HCl and Various Concentration of Ascorbic Acid

Ascorbic acid added —■—, 0 mg; —○—, 25 mg; —☆—, 50 mg; —△—, 100 mg; —□—, 150 mg; —●—, 200 mg.

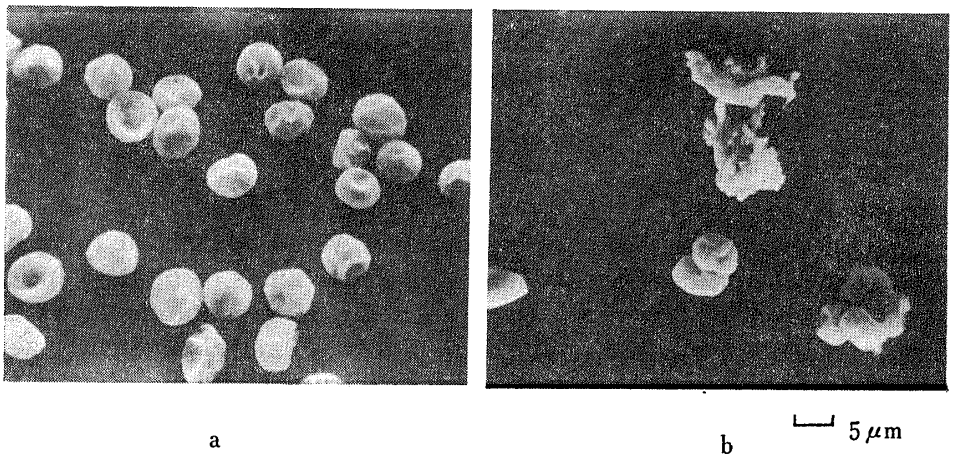


Fig. 9. Effect of Tetracycline·HCl (10 mg) containing Ascorbic Acid on Shape of Cells in Saline (200 ml) (Scanning Electron Micrograph)

a, containing 25 mg ascorbic acid, pH 3.9.
b, containing 200 mg ascorbic acid, pH 3.0.

## Effect of Several Drugs on Erythrocytes

**Effect of Tetracycline Preparations**——The tetracycline preparation of L Co. contains ascorbic acid as additive. Therefore, ascorbic acid was added to tetracycline hydrochloride in various ratios and effect of amount of ascorbic acid added on the counts and morphological changes in erythrocytes was examined (Fig. 8 and 9). While tetracycline hydrochloride alone had almost no effect in the concentrations used, addition of ascorbic acid increases the destruction of erythrocytes with increasing amount added. The counts only decreased within the time of measurement with up to 50 mg of ascorbic acid added but, above 100 mg of the acid added, the counts began to increase. This was considered to be due to the aggregation of blood cells or pieces of the cell membrane, which were counted as particles in the range of 10—150 μm. These facts suggest that hemolytic effect of tetracycline preparation is due chiefly to ascorbic acid added rather than to tetracycline hydrochloride itself.

These results indicate it is necessary to consider the physical properties and amount of an additive included in parenteral preparations.

**Effect of Chloramphenicol Preparations**——Figure 10 is a scanning electron micrograph of erythrocytes with the addition of chloramphenicol sodium succinate. Fig. 10 a, shows that there is hardly any change in erythrocytes at a concentration (75 μg/ml) of chloramphenicol corresponding to several fold of the maximum blood level obtained after administration of 1 g/day. Naturally, there was no change in the erythrocyte count. Fig. 10 b, shows the blood cells in a solution with 5000 μg/ml of chloramphenicol. It will be seen that blood cells have undergone swelling but there is no destruction of the cells.

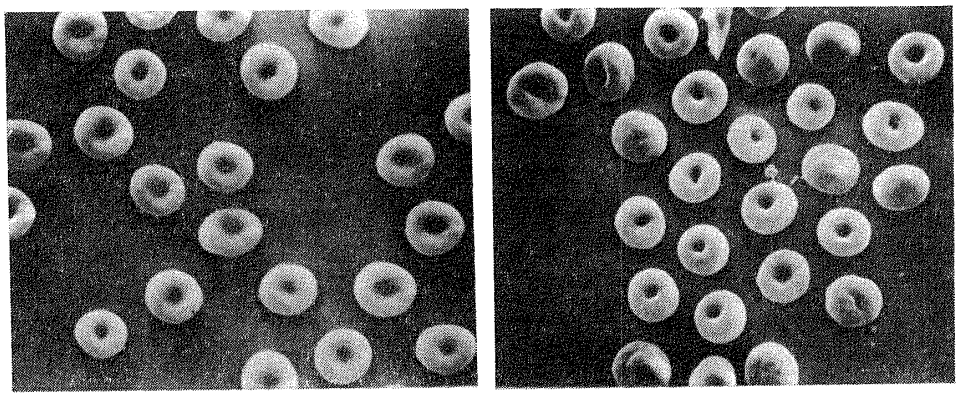


Fig. 10. Effect of Chloramphenicol Sodium Succinate on Erythrocytes in Saline (200 ml) (Scanning Electron Micrograph)

a, containing 15 mg chloramphenicol sodium succinate, pH 6.1.
b, containing 1 g chloramphenicol sodium succinate, pH 5.8.

These results seem to indicate that there is less possibility of chloramphenicol sodium succinate affecting the erythrocytes directly to produce blood damage. This preparation had judged to be hemolytic (卌) by other *in vitro* experiments but hemolytic effect could not be observed under our experimental condition and concentration tested.

## Conclusion

Commercial parenteral solutions may produce hemolytic damage due to pH and osmotic pressure of the solution or their additives, rather than to the direct action of drug itself on erythrocytes by a certain method, but by another method, such a damaging effect on erythrocytes is not occurred except that the drug itself *e.g.* ascorbic acid may produce hemolytic damage.

It is important to recognize that the hemolytic damage showed to occur in this study would not be likely to occur to the same degree *in vitro*. Re-examination should be made on numerous parenteral solution which have been branded as hazardous parenteral solution for hemolytic activity on these years, with judgement made from consorted results of various methods.