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EXPERIMENTS IN PHYSICAL PHARMACY. VI. FACTORS INFLUENCING ERYTHROCYTE FRAGILITY AND ISOTONICITY DETERMINATION

Early interest in drug solutions having the same osmotic pressure as the body fluids was relegated to parenteral solutions, but during the past two decades, considerable attention has been given to the osmotic pressure of collyriums and of nasal preparations. Isotonic solutions are important because they cause no swelling or contraction of the tissues with which they come in contact and produce no discomfort when instilled into the eye, the nasal tract, the blood stream, or other body tissue.

Hypotonic solutions may be rendered isotonic by increasing the drug content or by the addition of some physiologically inert solute. The proportion of the solute to be added may be determined experimentally or calculated mathematically.

Students are familiar with the principle of the plasmolysis experiment from their biology courses. Drug solutions which come in contact with erythrocytes should have the same osmotic pressure as that of the cell content in order to maintain the cell volume or the normal tone of the cell. If red cells are placed in water or in sodium chloride solutions containing less than 0.9 per cent sodium chloride, water passes into the blood cells with a resultant increase in cell volume. If the pressure inside the cells is sufficiently great, the cells burst. Various factors influence the change in the cell volume, and students must be familiar with these.

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This paper presents experiments describing the factors influencing erythrocyte fragility, a quantitative study on osmotic fragility of erythrocytes, and a freezing-point-depression (FPD) method to determine isotonicity of given solutions.

Theory

Hemolysis. Only solutes which cannot pass through a barrier permeable to the solvent can exert an osmotic pressure (1); substances which can pass through the cell membranes cannot, therefore, counterbalance the osmotic pressure exerted by nondiffusible intracellular solutes. Thus, the osmotic concentration, measured by a physical method based on one of the colligative properties, is an expression of the osmotic pressure only when all the solutes present in solution are nondiffusible through the cell membrane.

The membrane of the erythrocyte is impermeable, or relatively so, to certain solutes. Hence, the volume of water in the cell is determined osmotically by the composition of the surrounding solution. The cell can swell or shrink in a reversible manner within limits, but at a critical volume, the tension on the membrane causes it to "rupture" (or become more permeable), and the cell's contents are released into the surrounding solution.

When red blood cells (or other cells) are surrounded by aqueous solutions of lower osmotic pressure than that inside the cell, there is an inward flow of water, and the cell volume is increased until the osmotic pressures inside and outside the cell are equal. If the membrane is too fragile to withstand the increased pressure, it will rupture before the osmotic pressures on both sides of the membrane are equal. This disruption of the red cell membrane accompanied by liberation of hemoglobin into the surrounding medium is termed *hemolysis*. The opposite process, i.e., the passage of water out of the red cell, results in a shrinkage or *crenation* of the cell.

By convention, a solution in which red blood cells (or other cells) maintain the same volume which they have in plasma or tissue fluid is said to be *isotonic*. If the cell volume increases, the external solution is *hypotonic*, and if it decreases the solution is *hypertonic*. Examples of solutions which are isotonic with many mammalian cells are 0.9 per cent NaCl and 5.51 per cent dextrose.

Certain chemicals and surface-active agents (2, 3) also cause hemolysis, but they do so by different mechanisms. Solutes such as urea, ammonium chloride, boric acid, and alcohol pass freely through the cell membrane and bring about hemolysis because they do not provide any osmotic pressure to balance that of cell contents. Other substances of pharmaceutical interest which do not exert any osmotic pressure are succinic dinitrite, antipyrine, aminophylline, propylene glycol, and sodium phenobarbital (2). Surface-active agents such as polysorbate 60 increase the permeability of the erythrocyte membrane to NaCl which normally does not pass through the membrane.

FPD. The normal freezing point or melting point of a pure solvent is the temperature at which the solid and liquid phases coexist in equilibrium under one atmospheric pressure. The vapor pressures of the liquid and the solid are equal at freezing point. If a solute is dissolved in the liquid the vapor pressure or the escaping tendency of the liquid solvent is lowered below that of the pure solvent. The temperature must drop in order to reestablish equilibrium between the liquid and the solid. In other words, the freezing point of the pure solvent is lowered by dissolving a solute in it. The magni-

tude of the FPD is proportional to the concentration of the solute (4).

The blood is an aqueous system containing dissolved solutes and it would have a freezing point lower than that of pure water. An aqueous solution of NaCl, 0.90 per cent, has the same freezing point as that of blood. Since water is the solvent in both the cases, the FPD of 0.90 per cent NaCl = FPD of blood. A solution in question is said to be isotonic if it has the same colligative property (FPD or osmotic pressure) as that of blood and if the erythrocyte membrane is impermeable to the dissolved solutes.

Apparatus and Materials

Needed for this experiment are a Beckmann cryoscopic apparatus, a cryoscopic thermometer (Heidenhain), a magnifying glass, a centrifuge, a colorimeter, a microscope, centrifuge tubes in rack, defibrinated or heparinized blood[1] (canine or bovine), pipets (10.0 ml. in 0.1 and 0.01), silver nitrate, sodium nitrate, sodium chloride solutions (0.20, 0.90, and 1.8 per cent), 3.6 per cent urea, 0.80 per cent ammonium chloride, and 1.9 per cent boric acid.

Experimental Procedures

The experiment can be performed over a two-week period with groups of two students. If desired, it can be performed in one laboratory period by omitting the part on "quantitative study of erythrocyte fragility." The latter procedure was followed in the physical pharmacy laboratory.

Effect of suspending erythrocytes in different concentrations of NaCl solutions. Erythrocytes are suspended in water and in hypotonic (distilled water or 0.20 per cent NaCl), in isotonic (0.90 per cent NaCl), and in hypertonic (1.8 per cent NaCl) solutions. The salt solutions are supplied in the laboratory. In each of the four labeled test tubes containing 5 ml. of each of the above NaCl solutions, 0.1 ml. of the given blood is pipetted. Each tube is sealed with parafilm[2] and inverted gently about three times to ensure complete mixing. The mixture is allowed to stand for 30 minutes. The tubes are held against a white background (filter paper), and the appearances of the contents are noted. Alternately, the tubes are centrifuged[3] (in which case centrifuge tubes should be used) at *ca.* 1500 rpm for 10 minutes and the appearance noted; the advantage of this procedure is that it hastens the rate of sedimentation. This alternate procedure was followed by the students in the medical school. Either procedure gave satisfactory results.

Effect of urea, ammonium chloride, and surface-active agent on the erythrocytes. This experiment illustrates that, although certain substances (urea, ammonium chloride, and surface-active agents) cause hemolysis, they do so by different mechanisms. Using the procedure described in the preceding section, the erythrocytes are suspended in 5 ml. each of the following solutions: 0.80 per cent ammonium chloride, 1.8 per cent urea, 2.5 ml. of 3.6 per cent urea + 2.5 ml. of 1.8 per cent sodium chloride, and 5 ml. of 0.90 per cent sodium chloride + one drop of 10 per cent polysorbate 60. It should be noted that the above concentrations of am-

[1]Coagulation of blood was prevented by the following two procedures. Bovine blood was obtained from a local slaughterhouse and was collected in a container with glass beads and agitated immediately. In some cases agitation with a brush was helpful in separating the fibrin. In the case of canine blood the container was premoistened with four to five drops of 5 per cent heparin solution. The osmotic effect due to the small amount of heparin would be negligible, and this procedure is preferred.

[2]Parafilm, available from supply houses

[3]The instructor or technician arranged for this.

monium chloride (0.80 per cent) and urea (1.8 per cent) in the final solutions are iso-osmotic with respect to red cells.

Quantitative study of red cell fragility. This part describes, in a quantitative manner, osmotic hemolysis of the red cells in sodium chloride solution of varying concentrations.

Ten milliliters of a series of the following concentrations of sodium chloride are prepared in eight test tubes by mixing appropriate amounts of 0.90 per cent sodium chloride solution and distilled water: 0.18, 0.36, 0.45, 0.54, 0.63, 0.72, and 0.90 per cent. To each of these tubes is added (by means of a pipet) 0.05 ml. of the blood which has been thoroughly mixed (the red cells sediment rapidly, and if the sample is not mixed different amounts of red cells may be added in each case). The tubes are now centrifuged at *ca.* 1500 rpm for 10 minutes. By this procedure whole red cells and fragments are sedimented. The clear supernatant solution is decanted into a cuvette and its optical density is measured on a spectrophotometer (Spectronic 20)[4]. The first tube containing 0 per cent NaCl (distilled water) is included as a reference tube, and the hemolysis of the red cells in this tube is taken as complete or 100 per cent for the purpose of calculations.

Treatment of data. From the optical density reading of the first tube showing complete hemolysis, the magnitude of hemolysis for each of the other tubes is given as a simple proportion—e.g., if the first tube gives an optical density value of 0.600, then a tube giving a value of 0.300 yields $0.600/0.300 \times 100 = 50$ per cent hemolysis.

[4]The instruction sheets covering the principle and operation of the instrument together with its labelled diagram were given to the students.

A plot is prepared showing per cent hemolysis in increasing order along the ordinate and sodium chloride solution in order of decreasing strength along the abscissa. The points are connected in a continuous curve. The shape of the curve would be expected to be sigmoid in character, i.e., convex toward the base at lower salt concentrations and convex upward as hemolysis nears completion.

Freezing point determination. The Beckmann cryoscopic apparatus[5] is portrayed in Fig. 1. In order to reduce the rate of cooling, the inner tube, containing the solution, the stirrer, and the thermometer[6], is partially insulated by being suspended in a larger test tube with an air space in between. The ice-salt bath temperature should be about –5°.

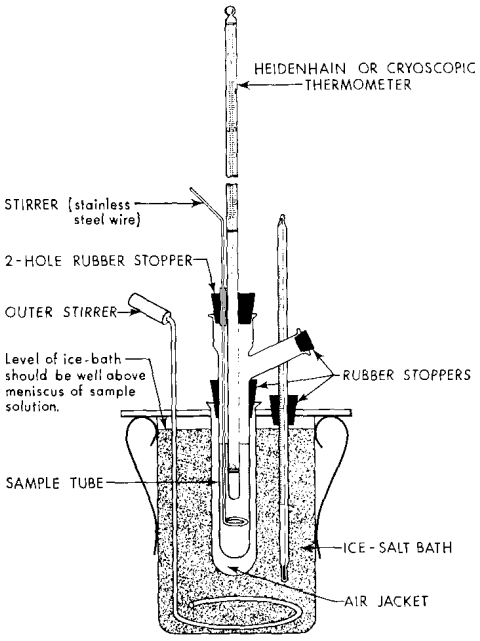

Fig. 1. The Beckmann cryoscopic apparatus

[5]The apparatus shown is of commercial design and is obtainable from major supply houses.

[6]The Heidenhain thermometer, with an operating temperature range of +1° to –5°, is very suitable. It has an advantage over the Beckmann thermometer in that it is comparatively easy to set.

About 20 ml. of the liquid under study is pipetted into the inner tube. The thermometer and the stirrer[7] are inserted with the thermometer carefully mounted in such a way that the bulb is about halfway between the bottom of the test tube and the upper surface of the liquid and concentric with the tube so that the stirrer can easily pass around it. If the thermometer is too close to the bottom a bridge of frozen solvent can easily form which will conduct heat away from the thermometer and result in low readings.

The motion of the stirrer should carry it from the bottom of the tube up to near the surface of the liquid; before starting the experiment, it is well to observe carefully how high the stirrer can be raised without too frequent splashing. The stirring should be continuous throughout the run and should be at the rate of about one stroke per second. The ice-salt bath should be stirred a few times per minute.

The test tube containing the liquid, stopper, thermometer, and stirrer is held in a beaker of ice-salt mixture, and the liquid is stirred until it visibly starts to freeze. The outside of the tube is wiped dry and warmed (with stirring) carefully with the hand to at least 1° above the freezing point. Then the tube is placed in the assembled apparatus, and temperature readings are taken every 20 (or 30) seconds with the aid of a magnifying glass. The thermometer is tapped *gently* before a reading is taken to prevent sticking of the mercury in the very fine capillary, and the temperature is estimated in tenths of the smallest scale division. Once freezing has occurred, the readings are taken for *ca.* 4-5 minutes; the temperature should remain fairly constant during that time. Supercooling will probably be experienced. A constant temperature shows that freezing is taking place.

Using the method described, the freezing points of the following liquids are determined: 1) pure distilled water, 2) 0.9 per cent NaCl, and 3) bovine blood or a prescription of 0.5 per cent silver nitrate ophthalmic solution rendered isotonic with $NaNO_3$.

Results and Discussion

The summary of the results outlined is based on the data reported by the pharmacy and medical students.

When the erythrocytes were suspended in 0.20 per cent NaCl, hemolysis occurred which was characterized by a clear pink solution, the cells having burst and released the hemoglobin. When they were suspended in isotonic (0.90 per cent) NaCl and hypertonic (1.8 per cent) NaCl, there was an absence of hemolysis marked by the sedimentation of the cells with a clear zone. Microscopic observations showed that the red cells retained their normal shape in isotonic solution while they were crenated in hypertonic solution.

The suspension of red cells into ammonium chloride and urea solutions, which were iso-osmotic with respect to the cells, produced hemolysis, indicated by a clear pink coloration of these solutions. These solutes pass through the membrane of the erythrocytes; therefore, they do not contribute to the osmotic pressure. The cells in the tube containing 0.90 per cent NaCl and 1.8 per cent urea failed to cause hemolysis, illustrating that urea played no part in hemolysis and that the urea solution behaved essentially like pure water. This mixture is then no different from 0.90 per cent NaCl as far as the erythrocytes are concerned. Hemolysis was,

[7] A coiled multiloop stirrer (designed by Walter S. Chen and easily prepared from a stainless steel wire by wrapping an end of the wire around glass tubing) is very efficient and stays in place around the thermometer during stirring.

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