partly due to its limited biliary excretion and its slow rate of disappearance from the various tissues.

9. Part of the blood erythromycin is loosely bound to red blood cell component or adsorbed on the cells.

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Osmotic Concentration and Osmotic Pressure in Injectable Solutions*

By I. SETNIKAR and OLIMPIA TEMELCOU

A method for determining the osmotic pressure of injectable solutions by measuring the variations of the red-cell volume is described. By means of this method, substances of pharmaceutical interest can be classified into different groups according to their diffusibility through the erythrocyte membrane and their action upon it. It was demonstrated *in vitro* and *in vivo* that for many substances the iso-osmotic concentration is not equivalent to the isotonic concentration and that the confusion between iso-osmia and isotonia can have dangerous consequences.

IT IS COMMON KNOWLEDGE that 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.

Therefore, the osmotic concentration, measured by physical methods 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 membranes, otherwise a solution found to be iso-osmotic is hypotonic for the cells.

This distinction would be of little practical importance were it possible to accept the view of Szekely and Goyan (2) to the effect that, of the substances in pharmaceutical use, those freely diffusible through the cell membranes are exceptional. The researches performed with a hemolytic method by Husa, *et al.* (3–8), demonstrate that, on the contrary, many substances in common pharmaceutical use, at a concentration iso-osmotic with blood, cause hemolysis for the very reason that they are unable to counterbalance the intracellular osmotic pressure.

While the hemolytic method can demonstrate very clearly the difference between solutes which are diffusible through the membrane of red cells and those which are not, it is not so easy to determine the isotonic concentration because, for this purpose, it is necessary to start from the premise that for all solutes there is a single ratio between isotonic concentration and hemolytic concentration, whereas it has been shown that, on the contrary, this ratio may vary from 1.4 to 3.1 (9).

Efforts have therefore been directed to the search for a method of direct determination of isotonic concentration and it has been found that this could be done fairly simply by means of a suitable modification of the hematocrit method used by Eijkman (10). This paper describes the method employed and the results obtained therefrom.

METHOD

Human blood was drawn from a forearm vein; rabbit blood by cardiac puncture. The syringes used for drawing the blood were moistened with an 0.65% (isotonic) solution of NaF containing 5% heparin. The blood samples were centrifuged, the separated red cells were added to an equal volume of the solution under examination, and this suspension was centrifuged for thirty minutes at 3,000 r. p. m. in Wintrobe's hematocrit tubes. To determine the volume that the red cells would maintain in an isotonic solution, a similar test was performed mixing the red cells with the plasma of the same specimen of blood.

RESULTS

NaCl.—The ratio between the volume of rabbit

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Fig. 1. In contact with plasma, red cells maintained a volume equal to that which they would take up in contact with an 0.93% solution of NaCl. This concentration is, therefore, isotonic with the red cell specimen used.

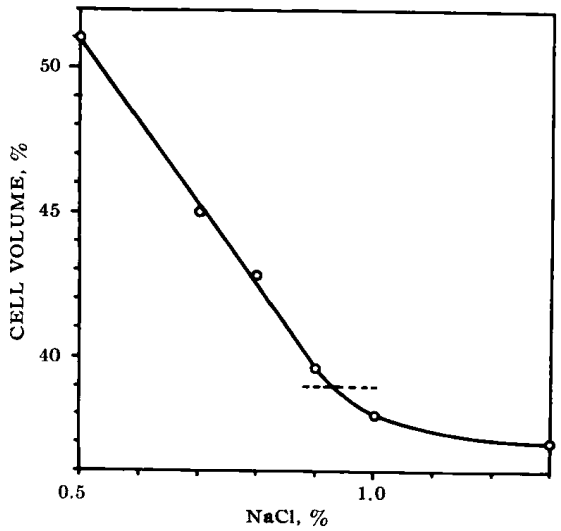

Fig. 1.—Effect of the NaCl concentration upon the volume of rabbit red cells. The dotted line represents the volume of the cells suspended in plasma (isotonic volume). For these red cells the isotonic concentration of NaCl was 0.93%.

Urea.—Solutions of urea from 1 to 2.6% (a 1.8% solution is iso-osmotic) caused complete hemolysis. The addition of NaCl at concentrations from 0.5 to 0.9% to an iso-osmotic solution of urea prevented laking, and the volume of the red cells was equal to that determined by solution of NaCl at the same concentration but without urea. Thus, urea does not of itself have a hemolytic effect, as in the opinion, for example, of Ebina (11); laking is brought about by the incapacity of this substance to counterbalance the intracellular osomotic pressure. In other words, as regards osmotic pressure, it is as if urea were not present in solution.

The incapacity of urea to exert an osmotic pressure can be demonstrated also *in vivo*. If 15 cc./Kg. of a 1.8% solution of urea is injected intravenously into rabbits, extensive hemolysis is observed, due to the destruction of about 3% of the red cells. A similar phenomenon is observed when one administers the same quantity of distilled water. Hemolysis can be entirely avoided by rendering the solution of urea isotonic with a 0.9% solution of NaCl.

Dextrose.—While dextrose exerts an osmotic pressure equal to its concentration on the erythrocytes of rabbits, on human erythrocytes the isotonic concentration is almost twice the iso-osmotic concentration (Fig. 2). The membranes of human erythrocytes would therefore seem to be partially permeable to dextrose. It is interesting to note that the resistance of human red cells increases in solutions of dextrose; the cell volume in hypotonic solutions can attain values practically twice those which usually precede hemolysis.

This increase in cell resistance perhaps accounts for the results obtained by Grosicki and

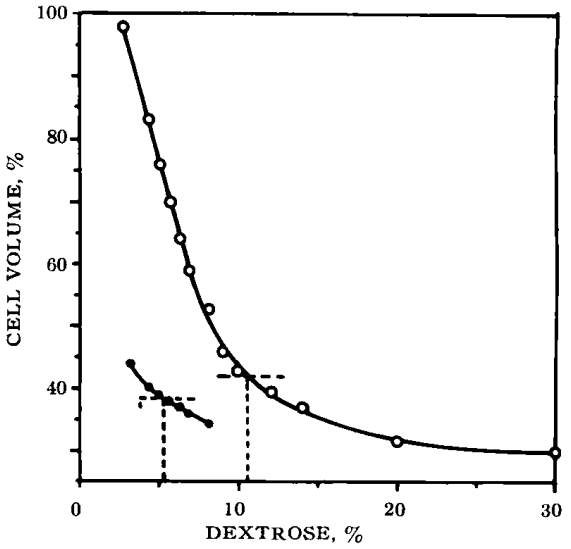

Fig. 2.—Effects of dextrose solutions at different concentrations upon the volume of the red cells of the rabbit and of man. The dotted line indicates the isotonic volume. O—O, human red cells; ●—●, rabbit red cells.

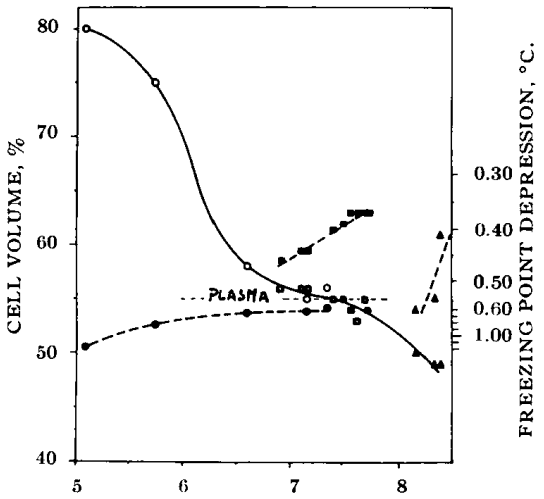

Fig. 3.—Effects of the pH upon the volume of red cells and thus also upon intracellular osmotic pressure. The cations concentration was maintained constant to 155 meq./L. of Na^+.

Left ordinate, cell volume; right ordinate, depression of the freezing point on a scale so adjusted that, for a solution of NaCl, the depressions of the freezing point would correspond to the cell volume; abscissa, pH of the buffer-red cell mixture.

The isotonic cell volume is shown by a dotted line. The straight line and the empty signs represents the cell volume, the dotted line and the full signs give the cryoscopic depression. O— Buffers of acetic acid-sodium acetate, □— buffers of $NaH_2PO_4 - Na_2HPO_4$, △-buffers of barbital-sodium barbital. Note the poor correlation between the osmotic concentration measured by the cryoscopic method and the osmotic pressure exerted by the solutions upon the red cells.

hemolytic concentration of dextrose for human red cells and those for rabbit red cells.

Procaine Hydrochloride.—At its iso-osmotic concentration (5.05%), procaine hydrochloride causes hemolysis of the red cells of the rabbit. In contra-

render the solution isotonic it is not sufficient to add 0.9% of NaCl but it is necessary to add this salt at a concentration of about 1.3%, as if the procaine increased the permeability of the membrane to sodium chloride.

It is interesting to note, however, that it is enough to add a 3.3% solution of dextrose (0.6 isoosmolar) to the same solution of procaine hydrochloride to have a solution which is isotonic for rabbit red cells, as if dextrose not only abolished the permeabilizing effect of procaine but rendered the cell membrane partially impermeable to procaine.

Saponin.—This substance has an intense hemolytic effect up to a concentration of 0.005–0.001% even if dissolved in an 0.9% solution of NaCl. The behavior of rabbit red cells placed in NaCl and dextrose solutions at different concentrations and in the presence of saponin at 0.05% was checked, and the results were very similar to those obtained with procaine hydrochloride at 5.05%.

$ZnSO_4$.—Zinc salts are of particular interest inasmuch as Hartman and Husa (6) observed by means of their hemolytic method that $ZnSO_4$ "protects" the red cells to such an extent that the isotonic solution of the salt would be 400 times more dilute than the iso-osmotic concentration. Cadwallader and Husa (12) have described similar results for zinc acetate. By our method, however, it can be shown that $ZnSO_4$ precipitates plasma proteins and causes hemolysis up to a concentration of 155 m*M*. The results described by Hartman and Husa can be confirmed only if blood and $ZnSO_4$ solution are mixed in the volumetric proportion of 1:50 (as these authors did), but it can also be demonstrated that the absence of laking is due to a precipitation and a denaturation of hemoglobin. One cannot, therefore, accept the conclusion that solutions of $ZnSO_4$ are isotonic at concentrations 400 times lower than the iso-osmotic concentration because not a protective action but a precipitating and denaturating action by the zinc ion is implicated.

Effects of the pH of Solutions on Intracellular Osmotic Pressure.—At physiological pH values negative charges prevail in red cell hemoglobin and about 50 meq./L. of cations are required for electrical neutralization. As hemoglobin is an ampholyte, its negative charges diminish when the environment becomes acid, releasing cations which, being unable to diffuse in the extracellular fluid through the cell membrane which is impermeable to them, attract anions from the extracellular fluid. A diminution of pH, therefore, involves a rise in intracellular osmotic pressure and, conversely, an augmentation of pH causes a fall in intracellular osmotic pressure. The isotonic concentration must, therefore, depend to some extent upon the pH of the solution.

Figure 3 gives experimental proof of this hypothesis. Although the cations concentration of the solution under examination was kept constant (155 meq./L. of Na^+), it may be observed that the cell volume increases in acid solutions and decreases in alkaline solutions, demonstrating that the osmotic pressure of red cells increases in contact with acid solutions and decreases in contact with alkaline solutions. Here again the osmotic concentration of the various solutions, measured by determining the depression of their freezing points, was not closely

DISCUSSION

Similar experiments carried out on substances of pharmaceutical interest showed that these could be classified into the following groups:

Group 1.—Substances whose iso-osmotic concentration is isotonic: NaCl (0.9%), KCl (1.19%), sodium thiosulfate N. F. (2.98%), sodium borate U. S. P. (2.6%), sodium propionate N. F. (1.47%), sodium benzoate U. S. P. (2.25%), sodium barbital (3.14%), sorbitol (5.48%), and dextrose U. S. P. (5.5.%) for rabbit red cells.

Group 2.—Substances which do not exert any osmotic pressure: urea, succinic dinitrile, antipyrine, aminophylline, ethanol, propylene glycol, sodium pentobarbital, Tween 80.

Group 3.—Substances whose isotonic concentration is higher than their iso-osmotic concentration: dextrose (as regards human red cells), glycine, sodium salicylate.

Group 4.—Substances which increase the permeability of the erythrocyte membrane to NaCl: procaine hydrochloride, adiphenine hydrochloride, ethanol, and propylene glycol at higher than 10–20% concentrations, Tween 60.

Group 5.—Substances with a pronounced hemolytic action: saponin, sulfuric esters of methylandrostenediol and of testosterone.

Group 6.—Substances which exert a protective action similar to that exerted by dextrose as regards the increase in permeability caused by procaine: dextrose, sorbitol.

Group 7.—Substances precipitating proteins: $ZnSO_4$ and all precipitants of proteins.

SUMMARY

When wishing to render an injectable solution isotonic, the main consideration should be the permeability of the cell membrane to the various solutes composing the solutions and the action of these solutes upon the cell membrane. In other words, *iso-osmia* (which can be determined by physical methods based on one of the colligative properties) is equal to *isotonia* only when all the solutes of the solution are unable to diffuse freely through the cell membrane. If this is not the case, isotonia can be determined only by measuring the osmotic effect of a given solution directly upon the concerned cells.

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