XC. THE DETERMINATION OF THE QUANTITY OF FREE WATER IN ERYTHROCYTES.

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(*Received May 6th, 1930.*)

The erythrocyte can be considered as a two-phase system, free water and dissolved salts occupying the continuous phase, while water bound with haemoglobin and lipoids constitutes the dispersed phase. The total water content of the cell has been shown by drying methods to be 70 % of the entire volume of the cell, but it is of some importance, in studying the partition relations existing between corpuscles and plasma, to determine accurately the relative proportions of free and bound water to be found in the cell. "Free water" may be defined as that proportion of the cell water that is osmotically transferable and "bound water" as that proportion that is not osmotically transferable under the same experimental conditions. It will be seen that free water is then approximately equivalent to the "dispersion medium" of previous investigators, and bound water is a part of the "dispersed phase" (consisting of water bound with haemoglobin and lipoids). Two opposing conclusions have been reached by the method of determining changes in the volume of corpuscles in saline solutions of varying osmotic pressures after prolonged centrifuging. Gough [1924] finds that the free water occupies 30–35 % of the cell volume, while Ege [1927] finds a value of 60 % for the free water. These results have been examined in the present investigation.

Gough suspends washed sheep cells in saline solutions varying in strength from 0·6 % to 4 %, spins them in a water centrifuge (*ca.* 2500 r.p.m.) for 3 hours, and then measures the height of the deposit of sedimented cells in the haematocrite tubes. Plotting the strength of solution against the cell volume reached after centrifuging, Gough finds: (*a*) that the curve for increasing volume of free water abstracted with increased concentration of NaCl assumes an asymptotic form, indicating that the maximum amount of free water can be abstracted by solutions of 3 % NaCl, and (*b*) that one must exclude a volume of two-thirds of the bulk of the corpuscle as not taking any part in the osmotic equilibrium. He thus obtains a value for the free water of 30–35 %, with the concentrations of NaCl he employs.

Ege, using the same method, concludes that the free water occupies 60 % of the cell volume, and believes that the source of error in Gough's determination is incomplete sedimentation of the corpuscles when suspended in hyper-

tonic solutions. If the column of cells has not settled completely, owing to the presence of fluid in the interstices of the cells, the value for the diminished cell volume in hypertonic solutions does not represent the "true" volume, but a slightly higher value. This introduces a considerable error, according to Ege, in the amount of free water obtained from the cell[1]. Ege points out that centrifuging must be continued until the column of corpuscles is perfectly sedimented, *i.e.* until there is no interstitial fluid. The corpuscles have then attained their "true" volume as manifested by the column of blood taking on the translucent "appearance of laked blood" throughout its entire extent (Koeppe's criterion [1905]). If complete sedimentation takes place, as evidenced by the appearance of Koeppe's criterion, the value for the free water is found to be 60 % of the cell volume.

In the following experiments the question of complete sedimentation was considered and attention fixed on determining the maximum quantity of water that can be expressed from the cells (*i.e.* whether Gough's curve actually reached an asymptote in the concentration of NaCl used by him).

Exp. 1. Previous experimenters have used sheep cells freed from serum by washing three times in 0·85 % NaCl. These are then mixed thoroughly with equal volumes of NaCl solutions of varying concentrations and transferred to haematocrite tubes 10 cm. long. Similar preparations were made in this experiment, using NaCl solutions of from 3 to 20 %, and these were compared with preparations of whole unwashed blood (retaining the serum) mixed with equal quantities of the same NaCl solutions. The haematocrite tubes were spun in a high speed centrifuge (14,000 r.p.m.) for 10 minutes–1 hour. Constant volume was reached after about 12 minutes of spinning, but Koeppe's criterion was never observed even when the spinning was continued for 2 hours. The control in all cases was either washed cells (or whole blood) mixed with an equal volume of plasma. The determination is made by measuring the height of the column of sedimented cells in plasma and expressing this as percentage volume of the entire length of the column of liquid in the haematocrite tube. This is compared with the height of the sedimented column in the various saline solutions used, and the difference between readings in plasma and hypertonic saline, expressed as a percentage of the reading in plasma, gives the volume of free water transferred from the cell.

The average value for five experiments with washed cells gave 45 % free water, while the whole blood gave a value of 32 % for the free water. Similar discrepancies were noted for ox and rabbit cells, the cells washed in "isotonic" saline always giving higher values for the volume of free water. This would seem to be due to a factor hitherto neglected by previous investigators—that 0·85 % NaCl is not "isotonic" for most mammalian cells, and that many so-called "isotonic" solutions change the volume of the corpuscles by permitting

[1] Gough's estimation of the height of column of sedimented cells is much less sensitive to errors than Ege believes, and the difference between the results of the two investigators cannot be explained in this way.

an entrance of water[1]. In the following experiments, it was decided, as a consequence, to use only unwashed corpuscles, retaining the serum.

Exp. 2. The effect of time of spinning on the volume of cells was studied in an endeavour to determine the significance of the appearance of Koeppe's criterion.

Percentage volume of cells after spinning in haematocrite.

Time of spinning	5 mins.	10 mins.	12 mins.	14 mins.	16 mins.	20 mins.	30 mins.	1 hr.	2 hrs.
				Cells in 3 % NaCl.					
Ox	9·6	9·2	9·2	9·2	9·2	9·2	9·2	9·1	9·1
Sheep	15·4	15·1	15·0	15·0	15·0	15·0	14·9	14·9	14·8
Rabbit	12·3	11·8	11·8	11·8	11·8	11·8	11·8	11·6	11·6
Human... ...	14·3	13·8	13·8	13·8	13·8	13·8	13·8	13·2	13·2
				Cells in 8 % NaCl.					
Ox	8·5	8·2	8·2	8·2	8·2	8·2	—	—	—
Sheep	14·3	13·9	13·9	13·9	13·9	—	—	—	—
Rabbit	12·3	11·7	11·7	11·7	11·6	11·6	—	—	—
Human... ...	15·5	14·2	14·2	—	—	—	—	—	—

Constant volume was reached in some 10–12 minutes and prolonged centrifuging gave no appreciable decrease in cell volume. In no case did Koeppe's criterion appear[2], even with prolonged centrifuging with these hypertonic solutions. It would seem reasonable to expect its appearance after prolonged centrifuging at such high speed, according to Ege's results, but it was never observed, except under circumstances which will be explained later. It is important to observe the blank spaces in the table. These denote that cells in 8 % NaCl haemolysed after prolonged spinning, thus obscuring the readings, since the disintegrated cells were spread throughout the haematocrite tube and left no clear column of sedimented cells to be used as a basis for estimating cell volume.

Exp. 3. To determine the maximum quantity of water that can be expressed osmotically from the corpuscles, the cells were placed in concentrated saline solutions and spun until constant volume was reached. In many cases haemolysis took place and the cell volume could not be accurately measured on that account.

Tonicity	3 % NaCl	6 %	8 %	12 %	20 %
	Volume of free water osmotically transferred.				
Sheep	32·1	32·8	33·0	33·0	33·0
Rabbit	30·4	31·2	31·3	31·3	31·3
Human	31·3	33·1	33·2	33·2	33·2

According to theory, the amount of water leaving the cell varies with the concentration of the surrounding hypertonic fluid. Since we get no significant increase in the amount of water leaving the cell (*i.e.* since we have apparently reached the asymptote on Gough's curve relating cell volume to strength of

[1] Unpublished data of Ponder and Saslow demonstrate these points quantitatively.

[2] It has been shown by experiments of Ponder and Saslow (now in press) that Koeppe's criterion is not coincident with the volume as determined by more accurate means such as the colorimetric method.

NaCl solution used) even with such strong solutions, we may conclude that the maximum quantity of free water has been transferred from the cell. In addition, it can easily be calculated that if the interior of the cell is assumed to consist of 100 unit volumes of a 1 % solution and if the surrounding fluid is a 4 % solution, sixty unit volumes of water would have to pass from the cell to the surrounding medium for osmotic equilibrium to be reached. If, then, we obtain less than this quantity even with more strongly hypertonic solutions, the reason is not that we cannot obtain any more water from the cell, but rather that there is no more water free to leave the cell. Solutions of KCl, sucrose and glucose were also used as suspension media but were not as successful in extracting the free water from the cells as were solutions of NaCl in similar concentrations. The experiments on sheep, ox, rabbit and human corpuscles accordingly confirmed Gough's conclusion that the free water occupies 30–35 % of the cell volume, provided haemolysis did not occur.

The appearance of haemolysis in suspensions in contact with strongly hypertonic solutions or subjected to prolonged spinning in the haematocrite complicated the experiments, but suggested the possible source of Ege's high value for the free water. When haemolysis occurs, the column of sedimented cells is decreased in height by the haemolysed corpuscles streaming up along the sides of the haematocrite tube. If this continues for any length of time, the entire tube becomes filled with the contents of the haemolysed cells and at the bottom of the tube is seen a slightly denser sediment of cells which appears somewhat translucent. The translucency, however, is not due to the close packing indicating Koeppe's criterion (which Ege describes as the "appearance of haemolysed cells"), but is due to an actual haemolysis taking place. Furthermore, the height of sedimented cells when haemolysed cannot represent the true cell volume as the cell contents have been liberated throughout the entire length of the haematocrite tube, and the cell volume is therefore very much less than the true volume. Such haemolysis with apparently smaller cell volumes and, consequently, larger amounts of free water, would seem to be the source of Ege's high value for the free water to be extracted from the cell[1].

Summary.

Determination of the maximum change of volume of blood corpuscles in NaCl solutions of varying strengths gives a value of 30–35 % of the cell volume occupied by the free or osmotically transferable water contained in the corpuscle and 30–35 % for the value of the bound water, confirming Gough's figures.

[1] Ege cites experiments by the method of determining depression of the freezing point by equal volumes of water and blood corpuscle press-juice in support of his high value for the free water. This method is not a reliable means of determining the relative proportions of free and bound water as it assumes two questionable points: (i) that the bound water is inactive in producing freezing-point lowering, and (ii) that the dissolved sucrose cannot be added to the bound water as well as to the free water.

Washing cells in "isotonic" solutions, prolonged centrifuging, the occurrence of haemolysis and the use of Koeppe's criterion as indication of true cell volume are to be noted as possible sources of error in Ege's determination of 60 % free water in the cell.

REFERENCES.

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