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THE BLUE DEXTRAN EXCLUDED VOLUME OF THE HUMAN ERYTHROCYTE MEMBRANE

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SUMMARY

Permanent holes were produced in hemoglobin-free human erythrocyte ghosts by treatment with lysolecithin. A measured amount of the large probe molecule Blue Dextran 2000 was added to a known amount of the ghost suspension and the mixture made up to a known final volume. The blue dextran in the supernatant was determined and the ghost membrane volume excluded from blue dextran calculated. A plateau in the excluded volume versus amount lysolecithin per ghost curve was observed. This can be taken to indicate complete equilibration of blue dextran into the membrane interior.

The excluded volume of 8.7 ± 0.5 μm^3 is consistent with a membrane thickness of about 600 Å. This large value might be explained by the presence of a gel or low protein density structure at the membrane's inner face. Other evidence consistent with such a structure is summarized.

INTRODUCTION

One way of determining the volume of a membrane would be to determine the volume excluded from some probe molecule by the membrane. The excluded volume would depend on the size of the probe. We have used the very large probe Blue Dextran 2000 (average molecular weight $2 \cdot 10^6$) in measurements of the excluded volume of human erythrocyte membranes. Blue dextran is excluded from Sephadex gel filtration media. It might also be excluded from any gel or low density structure associated with the erythrocyte ghost.

Seeman [1] has shown that ghosts prepared by hypotonic lysis have only transient holes but that lysis by a saponin or lysolecithin produces permanent holes. These holes are large enough for passage of ferritin or colloidal gold. We have used lysolecithin to produce holes which are sufficiently large for equilibration of blue dextran into the ghost interior.

THEORY

A known number of ghosts are treated with lysolecithin, followed by the addition of a known amount of a concentrated blue dextran solution. The mixture

is then diluted to a pre-determined final volume. The absorbance of blue dextran in the supernatant is determined and compared with the absorbance of a known amount of the concentrated blue dextran solution diluted to a known final volume. This allows calculation of V_a, the volume of the membrane suspension which is accessible to blue dextran.

This can be expressed as:

$$V_a = \frac{W_t}{A_b}\left(\frac{A_s V_s}{W_s}\right) \tag{1}$$

where: W_t is the weight of concentrated blue dextran solution added to the membrane suspension, A_b is the absorbance of the blue dextran in the suspension supernatant, and A_s is the absorbance of weight W_s of the concentrated blue dextran solution diluted to volume V_s.

Since a small amount of residual hemoglobin is present in the ghost suspension, the total absorbance of the supernatant A_t must be corrected for the hemoglobin absorbance A_h:

$$A_b = A_t - A_h \tag{2}$$

A_h is the supernatant absorbance of a membrane suspension containing ghosts and lysolecithin but no blue dextran.

If V_t is the pre-determined final suspension volume and N_m is the number of ghosts in the suspension, the blue dextran excluded volume per ghost V_m is given by:

$$V_m = \frac{1}{N_m}\left[V_t - \frac{W_t A_s V_s}{W_s(A_t - A_h)}\right] \tag{3}$$

EXPERIMENTAL

Blue Dextran 2000 (Pharmacia Fine Chemicals) was dissolved in 20 mosM sodium phosphate buffer (pH 7.4), to give a 5 % solution. This concentrated blue dextran solution was centrifuged (4 times 40 min at 100 000 $\times g$) to remove very high molecular weight material. If this precaution is not taken, this material sediments along with the ghosts in excluded volume measurements. In order to test for such an effect after removal of this material, the concentrated blue dextran was diluted to a concentration similar to that used in the excluded volume measurements (0.1 %) and added to 12 ml scored polypropylene test tubes. The absorbance of a portion of the top layer of the solution was measured before and after centrifugation at 3000 $\times g$ for 20 min. Absorbance was measured to the nearest 0.001 unit on a Cary Model 16 recording spectrophotometer. No absorbance changes were observed.

Various dilutions of the concentrated blue dextran solution were made in order to verify that Beer's law was obeyed. The Beer's law curve was linear up to an absorbance value greater than 2.0, which is well above the value of about 1.1 used in the excluded volume measurements.

The lysolecithin was a Sigma product stated to be a mixture of lysolecithins having either palmitic or stearic acid substituted at the α position. Since its presence affects the absorbance of blue dextran, it was necessary to carry out measurements of

the blue dextran absorption spectrum at several different lysolecithin concentrations to see if an isosbestic point could be found. An isosbestic point was located at 657.5 nm. All excluded volume measurements were made at this wavelength.

The following procedure was used to determine the value of $A_s \cdot V_s/W_s$ (Eqns 1 and 3). Centrifuged concentrated blue dextran solution was carefully weighed into calibrated 25-ml volumetric flasks which were then diluted to the mark with water. Aliquots were added to scored tubes and centrifuged at $3000 \times g$ for 20 min. Samples from the upper layer of each tube were taken and the absorbance measured.

The scored tubes were 12-ml polypropylene test tubes having a machined calibration mark at a volume of approx. 9.0 ml. They were calibrated by weighing before and after the addition of water containing a trace of lysolecithin to the mark.

Red cell membranes were prepared by a procedure similar to that of Dodge et al. [2]. About 35 ml of human blood which had been stored at least 22 days in acid citrate–dextrose was placed in each of eight 50-ml polypropylene centrifuge tubes. The cells were spun down at $500 \times g$ in a refrigerated centrifuge and plasma and buffy coat removed. The cells were washed 3 times with 310 mosM sodium phosphate buffer (pH 7.4), after which the supernatant was removed. The washed cells were pooled and counted using a Coulter Counter, Model S. The concentrated cell suspension was stirred to achieve homogeneity while being added to eight scored tubes to the mark. The number of cells in each scored tube could therefore be calculated. The cells were quantitatively transferred from each scored tube to a 50-ml polypropylene tube containing 30 ml of cold 20 mosM sodium phosphate buffer (pH 7.4).

The ghosts were washed with cold hemolyzing 20 mosM buffer until little hemoglobin remained and the supernatant was only slightly pink. Five or six washings, in which the ghosts were centrifuged at $12\,000 \times g$ for 20–40 min, were required. Special care was taken so that very few ghosts were lost during the washing. After the last centrifugation a small dense mass of sediment was noted at the bottom of each tube. Probably this mass was mostly fragmented leucocytes. Its volume was only a few percent of that of the total ghost volume.

Varying amounts of a 20 % lysolecithin solution in 20 mosM sodium phosphate buffer (pH 7.4) were added to each tube with stirring to effect uniform membrane perforation. The suspension from each tube was transferred back to its corresponding scored tube with careful washing. The small dense mass of sediment stuck to the bottom of the tube and was discarded.

An aliquot of centrifuged concentrated blue dextran (approx. 0.25 g) was carefully weighed into each tube and 20 mosM sodium phosphate buffer (pH 7.4) were carefully added to the mark. The tubes were shaken and allowed to equilibrate at room temperature for about 1 h. Further standing gave no change in supernatant absorbance.

A series of eight ghost plus lysolecithin tubes was prepared in an identical way, except that blue dextran was not added. The supernatant absorbance from each of these tubes was used as the hemoglobin correction absorbance for the corresponding blue dextran containing tube.

The scored tubes containing blue dextran were centrifuged at $3000 \times g$ and 15–20 °C for 20 min. The hemoglobin correction tubes were centrifuged at $12\,000 \times g$ and 15–20 °C for 20 min. Upper portions of the supernatants of all tubes were removed and their absorbances at 657.5 nm were estimated to the nearest 0.001 unit. Ab-

sorbances of the blue dextran tubes, A_t, were approx. 1.1, while the hemoglobin correction absorbances, A_h, were generally in the range of 0.00–0.03.

Micro-Kjedahl analyses were performed on samples from the hemoglobin correction tubes. The nitrogen per ml of supernatant and the nitrogen per ml of total suspension were measured. This allows an estimate of the percentage of protein present which was solubilized.

Ghosts in the presence of various amounts of lysolecithin were observed with a phase-contrast microscope. Ghosts remained essentially intact, except at the very highest lysolecithin concentrations. At these concentrations only a few scattered membrane fragments could be observed.

RESULTS

The blue dextran excluded volume per red cell membrane as a function of the amount of lysolecithin present per red cell membrane is shown in Fig. 1. When no lysolecithin is present the volume per ghost is about 66 μm^3. This might be compared to the value of about 90 μm^3 for the normal red blood cell. The blue dextran must be excluded from a substantial number of the ghosts. Upon addition of lysolecithin the excluded volume drops and then becomes roughly constant up to a value of about $3 \cdot 10^{-10}$ mg of lysolecithin per ghost. Higher concentrations of the lysin result in fragmentation and solubilization of the membranes. The excluded volume then becomes negative due to adsorption of blue dextran by membrane fragments. The amount of nitrogen or the approximate amount of protein solubilized from the ghosts is also shown. This is essentially constant from 0 to about $3 \cdot 10^{-10}$ mg lysolecithin per ghost. Higher lysin concentrations give rise to extensive solubilization. Apparently there is a region of lysolecithin concentration where blue dextran can enter the membrane through the permanent holes, but no additional membrane protein is solubilized.

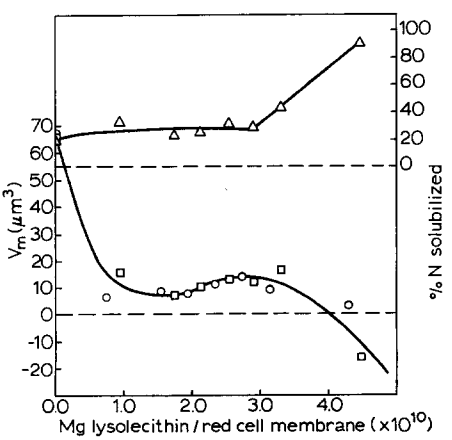

Fig. 1. Blue dextran excluded volume per red cell membrane vs lysolecithin present per red cell membrane. Conditions: hemoglobin-free ghosts, 20 mosM phosphate buffer (pH 7.4). The two symbols represent separate experiments on different blood samples. The upper curve represents the percent of total nitrogen of the ghost suspension which is in the supernatant vs the amount of lysolecithin present.

To test the reproducibility of our results we have made a series of measurements at a lysolecithin concentration of $2.0 \cdot 10^{-10}$ mg per ghost. The results are given in Table I. We assume that under these conditions a sufficient number of large permanent holes are present so that blue dextran can completely equilibrate across every ghost membrane. A series of eight measurements gave an excluded volume of 8.7 ± 0.5 μm^3. This quantity allows us to estimate the membrane thickness from which blue dextran is excluded. The surface area of the red cell membrane has been given as 145 ± 8.3 μm^2 [3]. Ghosts prepared using 20 mosM buffer would be expected to have a similar surface area since they have been shown to be approximately equal in diameter to normal red cells and to retain their biconcave shape [2]. If the excluded volume is divided by this surface area we obtain a thickness of 602 ± 73 Å.

TABLE I

EXCLUDED VOLUME PER GHOST AT ONE LYSOLECITHIN CONCENTRATION

mg lysolecithin per ghost ($\times 10^{10}$)	V_m (μm^3)	Average V_m and average deviation (μm^3)
1.92	8.64	
1.94	10.21	
1.98	9.48	
1.99	7.97	8.7 ± 0.5
2.00	8.64	
1.98	8.03	
1.99	8.31	
1.99	8.57	

These results were obtained on ghosts prepared under conditions (20 mosM buffer (pH 7.4)) such that they were essentially hemoglobin free. If ghosts are prepared under conditions of lower pH or higher osmolarity much more hemoglobin is retained along with some non-hemoglobin protein which includes a number of enzymes [2, 4–7]. Attempts were made to obtain excluded volumes at two other sets of conditions: 60 mosM phosphate buffer (pH 7.4); and 20 mosM phosphate buffer (pH 6.0). In both cases ghosts showed a bluish color, indicating blue dextran adsorption; and no plateau region, such as is seen in Fig. 1, was observed. Therefore, no reliable estimate of the excluded volume could be obtained. This adsorption of blue dextran to the membrane may be related to the fact that blue dextran is known to interact with at least two of the enzymes which are weakly held along with hemoglobin: pyruvate kinase [8, 9] and glutathione reductase [10].

DISCUSSION

Questions arise as to whether the results that have been presented represent true blue dextran excluded volumes. One possible criticism is that not all of the membranes have permanent holes sufficiently large for blue dextran equilibration or that a significant portion of the blue dextran is too large to pass through the holes. This objection is probably not valid since very high molecular weight material was removed

from the blue dextran prior to its use and because of the plateau in the excluded volume curve. At a level of $1 \cdot 10^{-10}$ mg of lysolecithin per red cell membrane the excluded volume is about 10 μm^3. A 2-fold increase in the amount of lysolecithin results in no further decrease in excluded volume, yet should certainly cause the production of more and larger permanent holes and thus ensure that all ghosts are sufficiently perforated for blue dextran equilibration.

Adsorption of blue dextran by the membrane could result in excluded volume values which are systematically too low. Adsorption, indicated by a blue ghost color, does occur if the ionic strength of the medium is too high. There is no indication of adsorption at an osmolarity of $20 \cdot 10^{-3}$, so charge repulsion between negatively charged membrane and blue dextran is evidently high enough to prevent it.

We have assumed that the number of ghosts in an experiment is equal to the number of red cells taken. However, some ghosts could be lost in washing and transfer between tubes or in the residue of high density material which was discarded after the addition of lysolecithin. We feel that such losses, which would give a systematically low excluded volume, are small.

Interaction of lysolecithin with the membrane results in permanent holes. Some of the lysolecithin must be held by the membrane while some, along with membrane lipid, is probably solubilized in micelles. The excluded volume of 8.7 μm^3 was determined at a concentration of $2.0 \cdot 10^{-13}$ g of lysolecithin per membrane. This should be compared to a value of about $13 \cdot 10^{-13}$ g for the cell membrane dry weight [2]. Our results appear to indicate some increase in excluded volume at higher lysolecithin levels. This could be related to additional lysolecithin uptake by the membrane.

A subtle error in the excluded volume measurements is associated with the fact that our results relate to the volume excluded from the blue chromophores and not, strictly speaking, to the volume excluded from the surface of the blue dextran molecule itself. If a substantial number of chromophores are in the interior of the average conformation of the blue dextran, then they cannot come in direct contact with the membrane surface even though the blue dextran molecule itself can. Thus, the blue chromophore excluded volume could be greater than the blue dextran excluded volume. We have implicitly assumed that these two volumes are approximately equal; but since the blue chromophore distribution in the average blue dextran conformation is unknown, we cannot prove this assumption.

A number of values have been reported for erythrocyte membrane thickness. Electron microscopy gives a value of 71 Å for the dense trilamellar structure [1]. X-ray diffraction studies on dried membranes indicate a value of 80–85 Å [11]. An interesting energy transfer experiment gives a value of approx. 65 Å for dried and 100 Å for rehydrated membranes [12]. There appears to be little doubt that the dense region of the membrane has a thickness of about 75 Å.

However, other measurements have yielded higher values for membrane thickness. We can mention work with the analytical leptoscope which gives values of 150–250 Å for membranes of different species dried on microscope slides at pH 6 [13, 14], and Mitchison's quantitative birefringence measurements of human red cell ghosts in glycerol which lead to a value of 5000 Å [15]. Additional discussion is given by Mitchison and by Ponder [16].

If our results for the value of the blue dextran excluded volume are accepted

as at least approximately correct, then a hydrated membrane thickness of about 600 Å must be explained. One possibility is that blue dextran is excluded from the region of the membrane due to charge repulsion between it and the negative charge of the membrane at one or both of the membrane faces. We consider this unlikely since an increase in the ionic strength of the medium results in actual blue dextran adsorption to the membrane. Thus, any repulsion is apparently not very strong. Another possibility is that the blue dextran is excluded from the membrane due to "ice like" water structure which it cannot penetrate. Finally, the presence of glycoprotein carbohydrate at the membrane's outer face might give rise to some blue dextran exclusion.

Our favored explanation is that there could be some gel or low protein density structure associated with the membrane's inner face [15]. This is consistent with a number of other observations.

Conventional electron microscopy of hemoglobin-free ghosts shows filamentous material associated with the membrane's inner face [17]. This material has an appearance consistent with that of a dried gel-like structure. Freeze-etching studies of whole blood cells show closer packing of protein molecules in the juxtamembrane area [18]. This might be related to the ordering of certain proteins at the membrane's inner face. Work in which the red cell membrane's inner face was exposed by ion bombardment and then studied by scanning electron microscopy reveals interesting structure, possibly due to adsorption of proteins at the inner face [19]. The work of Weed et al. on the deformability of the red cell membrane under various conditions should be mentioned [20]. These workers have suggested that a reversible sol–gel transformation can occur at the membrane's inner face.

The blue dextran excluded volume reported here refers to the hemoglobin-free red cell membrane. It is possible that the corresponding volume associated with pink or red ghosts, which have not only hemoglobin but also a number of other proteins adsorbed to the inner face [2, 4–7] might be even higher. It is unfortunate that, due to its adsorption, blue dextran cannot be used to measure this volume.

The use of lysolecithin or other lysins to produce large permanent holes in the red cell membrane should have other applications. The procedure exposes the inner face of the membrane to the external environment without membrane fragmentation. Thus, the inner face can be studied in a variety of ways without complications due to transport across the membrane. Hopefully, the lysins will concentrate in lipid bilayer regions of the membrane and not give rise to serious disruption of membrane structure and certain of its functions.

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REFERENCES

1 Seeman, P. (1967) J. Cell Biol. 32, 55–70
2 Dodge, J. R., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130
3 Westerman, M. P., Pierce, L. E. and Jensen, W. N. (1961) J. Lab. Clin. Med. 57, 819–824
4 Mitchell, C. D., Mitchell, W. B. and Hanahan, D. J. (1965) Biochim. Biophys. Acta 104, 348–358

5 Green, D. E., Mauer, E., Hultin, H. O., Richardson, S. H., Salmon, B., Brierley, G. P. and Baum, H. (1965) Arch. Biochem. Biophys. 112, 635–647
6 Bramley, T. A., Coleman, R. and Finean, J. B. (1971) Biochim. Biophys. Acta 241, 752–769
7 Duchon, G. and Collier, H. B. (1971) J. Membrane Biol. 6, 138–157
8 Blume, K. G., Hoffbauer, R. W., Busch, D., Arnold, H. and Lohr, G. W. (1971) Biochim. Biophys. Acta 227, 364–372
9 Staal, G. E. J., Koster, J. F., Kamp, H., Van Milligen-Boersma, L. and Veeger, C. (1971) Biochim. Biophys. Acta 227, 86–96
10 Staal, G. E. J., Visser, J. and Veeger, C. (1969) Biochim. Biophys. Acta 185, 39–48
11 Coleman, R., Finean, J. B., Knutton, S. and Limbrick, A. R. (1970) Biochim. Biophys. Acta 219, 81–92
12 Peters, R. (1973) Biochim. Biophys. Acta 318, 469–473
13 Waugh, D. F. and Schmitt, F. O. (1940) Cold Spring Harbor Symp. Quant. Biol. 8, 233–241
14 Waugh, D. F. (1950) Ann. N. Y. Acad. Sci. 50, 835–853
15 Mitchison, J. M. (1953) J. Exp. Biol. 30, 397–432
16 Ponder, E. (1955) Protoplasmatologia X (2), 1–123
17 Marchesi, V. T. and Palade, G. E. (1967) J. Cell Biol. 35, 385–404
18 Lessin, L. S., Wallace, N. J. and Ponder, E. (1969) J. Exp. Med. 130, 443–466
19 Levis, S. M., Osborn, J. S. and Stuart, P. R. (1968) Nature 220, 614–616
20 Weed, R. I., La Celle, P. L. and Merrill, E. W. (1969) J. Clin. Invest. 48, 795–809