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On How Macromolecules Reduce Hemoglobin Loss in Hypotonic Hemolysis

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ABSTRACT

When human red cells are hemolysed in hypotonic solutions containing macromolecules, the hemoglobin loss from the individual cells is reduced although the number of cells hemolysed is not affected. The evidence strongly suggests this is a colloid osmotic effect but an additional condition is also necessary if hemoglobin is to be retained. The cell must reseal,at least to hemoglobin and macromolecules. There is some evidence which points to the role of the macromolecule in this process. Further, at least in the case of dextran,a minimal size of about 2000 daltons is required for suppression of hemoglobin liberation and it is suggested that this limit may be set by the diffusion coefficient.

On the definition of hemolysis

Since quantitatively hemoglobin (Hb) is the major component of the red cells comprising some 98% of the total non-aqueous mass,it is not surprising that Hb loss has become the yardstick for hemolysis. This is satisfactory provided hemolysis is a quasi-All-or-None process in which the fraction of the total Hb of all the cells released is, allowing for some individual cellular variations, a fair estimate of the fraction of cells lysed. This important question has quite naturally been tackled by several workers (2,3,7,18,19,26,28).All came to the conclusion that in hemolysis induced either hypotonically,with saponin, or by freeze-thawing, Hb loss from the individual cell was essentially All-or-None. More recently the problem of the residual Hb has been taken up probably largely because of the need for obtaining Hb-free red cell membranes for electron microscopy and other investigations (20,25). These results suggest that Hb is not a structural element of the membrane and that Hb loss is not necessarily accompanied by significant losses of membrane component. This does not

imply,however , that the membrane is entirely unaltered,even though, for a short time at least, it can exhibit permselectivity (23).Further,Hb is not the only component lost during hemolysis; not unexpectedly the loss of other components seems to be inversely related to their molecular size (9). Other components could be used to estimate hemolysis,but this would offer no obvious advantage.

In the hemolytic systems used to check its All-or-None nature hemolysis is of this type.However, if human red cells are exposed to hypotonic solutions containing a macromolecule such as dextran,Ficoll^{R} or poly(ethylene glycol) (PEG) (1,13,14),albumin (10,11) or Hb itself (Marsden,unpublished data), the pattern of hemolysis is changed dramatically. The correspondence between the fraction of Hb released and the fraction of cells which have lost some Hb no longer holds even approximately and Hb loss is thus no longer an index of the resistance of the cells to a noxious stimulus. Under these circumstances the cells reseal spontaneously to Hb before this has equilibrated with the suspending medium.

Fig. 1 shows the relation between mean loss of cellular Hb and the osmolality of the suspending solution. Curve 0 is the curve typical of hypotonic hemolysis with the Hb loss complete,or nearly so, at lower osmolalities in the absence of macromolecules.When,however,the latter are present,the hemolysis curves reach maxima below complete loss depending on the macromolecular concentrations; the maximal Hb loss is less at higher macromolecule concentrations.

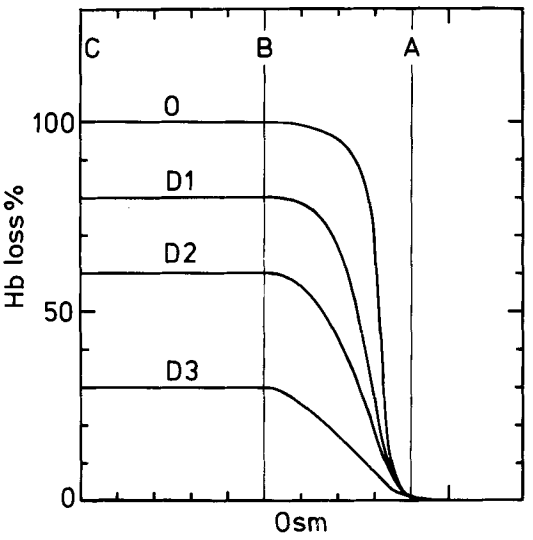

Fig.1.Diagram showing the pattern of Hb loss from red cells as a function of the osmolality (Osm) of the suspending solution in the absence (0) and presence of dextran.Curves D1,D2 and D3 refer to different concentrations of the latter,D1 is the lowest and D3 the highest.The curves are adapted from Reference 1.

From the shape of the curves it was concluded that they all represented cells with similar osmotic resistances,the only difference being that in the presence of the macromolecule the hemolyzed cell did not lose all its Hb.Thus, in the region BC in Fig.1 all the cells are hemolyzed,whereas in AB the number of cells hemolyzed varies between none and all. These conclusions were confirmed by phase-contrast microscopy (1),microinterferometry (14) and microspectrometry (1).

These findings illustrate the shortcomings of using a large intracellular molecule as a marker of cellular events. Thus,although the external macromolecule reduces the Hb loss from the individual cell, it does not prevent Hb loss from occurring and thus,the number of cells which liberate Hb is, at least to a first approximation, not affected.The macromolecules thus do not offer protection against hemolysis.It is possible that failure to appreciate this point has been responsible for attempts to use macromolecules as protectives in other hemolytic systems (21).

Characteristics of hypotonic hemolysis in the presence of macromolecules.

All investigations have been conducted in the region BC in Fig.1 that is with all cells lysed.

There are three interesting characteristics of macromolecular-induced reduction in Hb release. Firstly, although Hb loss could be greatly reduced,some Hb was always lost, the minimal loss being never less than about 20% (1,14,17). Secondly, the uptake of foreign substances during hemolysis also seems to be linked to this value.Thus,although dextran molecules up to a molecular weight of about 100.000 (Stokes-Einstein radius = 7.0 nm) can enter the cell during hemolysis (17) they do so only if the Hb loss exceeds about 20%. Now there is evidence that the first part,at least, of the Hb escaping from the cell leaves faster than can be accounted for by diffusion (8) and this would be the case if there was a convective fluid outflow in this initial period. Since a macromolecule can presumably only enter by diffusion,it would thus be unable to move inward until the outward fluid flow was slower than its diffusion rate.We might therefore predict that smaller dextran molecules with larger diffusion coefficients would enter earlier than large molecules, and the results of Östling (17) are in accord with such a tendency.

Since dextran uptake appeared to occur only if the Hb loss exceeded about 20% it is probable that any Hb loss in excess of this value is by diffusion and the time during which this loss occurs should be proportional to its magnitude. We may thus expect that more macromolecules will diffuse into cells which lose more Hb and this was confirmed by Östling (17). This inverse relation between

uptake and loss was also illustrated in a striking fashion when ferricyanide ions were present in the hypotonic macromolecular solution at room temperature (27). Hb and methemoglobin (MHb) were determined in the ghosts. When the MHb content was plotted against the degree of hemoglobin liberation a bell-shaped curve was obtained. This can be explained as follows. The more Hb that is lost, the greater the amount of ferricyanide entering the cell, and thus the MHb/Hb ratio rises.However, as the total cellular hemoglobin content (Hb+MHb) decreases at higher degrees of Hb liberation MHb is also reduced. This result is understandable only if the cells reseal before both the Hb and ferricyanide ion concentrations inside and outside the cell have equilibrated.

Low molecular solutes such as sucrose and raffinose added to the dextran--containing hemolysing solutions become locked in the ghosts (13,17).Further, recent studies at 0 ^{o}C have yielded indirect evidence that the ghosts are also presumably cation impermeable (see below).

The third characteristic of interest is the lower size limit to the macromolecule,below which it does not reduce Hb loss from the cell. Thus,whereas neither sucrose (mol.wt.342 daltons) nor raffinose (mol.wt.504 daltons) reduce Hb liberation, the effect is apparently fully developed already at a molecular weight (dextran) of about 2 000 daltons (1).

The mechanism of the macromolecular effect

1. Resealing

There is good evidence that the reduction in Hb liberation correlates well with the calculated osmotic pressure of the external dextran,provided the osmotic contribution of any dextran which has entered the cell is subtracted from it (16).

The different diffusion coefficients (D) of Hb (22) ($6.9 \cdot 10^{-7} cm^2 sec^{-1}$) and KCl (6) ($1.9 \cdot 10^{-5} cm^2 sec^{-1}$) mean, if the escape of the components is purely diffusive,that hypotonic hemolysis can be regarded as taking place essentially in two phases. If we assume that there is no discrimination of the porosity between Hb and KCl the ratios of their half-times of escape by diffusion are given by the reciprocals of the ratio of their diffusion coefficients (24).It can then be calculated that when nearly all the electrolyte has equilibrated, about 80% of the Hb will still be left in the cell. Thus during the time in which the major part of the Hb is escaping from the cell there should be no other osmotically significant components present. The real value for the osmotic pressure of the cellular Hb during this time will be less than the theoretical value,since the reflexion coefficient will be below unity,although greater than zero. If,however, the external macromolecule is to prevent some

of the Hb leaving the cell, this can only be achieved if the cell once more becomes impermeable to Hb. The problem of resealing thus becomes the crux of the matter.

Now there is some evidence which suggests that the macromolecule does affect the ability of the cell to reseal. It has been reported that red cells hemolyzed hypotonically at 0 °C and pH 6.0 remain permeable to both electrolytes (20) and Hb (4) and that they can be induced to reseal if warmed up to 37 °C (4,20). When red cells were hemolyzed hypotonically (20 mmol·l^{-1} NaCl, 4 mmol·l^{-1} $MgSO_4$, pH 6 in the presence of Ficoll^R (M_W = 400.000) at 0 °C they retained nearly as much Hb as at 23 °C (Zade-Oppen, unpublished data). The cells still at 0 °C were then restored to isotonicity (external medium) by adding hypertonic Ficoll^R-free NaCl solution buffered at pH 7.5. They were then incubated at 37 °C with the aim of resealing them to electrolyte. After washing, their osmotic resistance was redetermined and was found to be increased to such an extent as to suggest that the cells had a very low electrolyte content and therefore must have become "electrolyte tight" before the restoration of isotonicity. The macromolecules have thus also initiated resealing to ions.

The resealing of the cells in the presence of macromolecules suggests that they may interact with the membrane. Since the effectiveness of dextran in reducing Hb loss appears to be essentially independentof its molecular weight (1,16), a possible dextran-membrane interaction promoting sealing might also be independent of molecular weight.

Although it seems profitless to speculate on a possible macromolecule-membrane interaction, it is unlikely that this is related directly to the perturbing effect of the macromolecule on water structure. Micelle formation in a nonionic surfactant is fairly widely regarded as a useful system for studying water-structure perturbants (15). In this case, however, the macromolecules have dissimilar effects; whereas PEG depresses micelle formation, the other two polymers, dextran and Ficoll^R promote it (12).

2. Rate of penetration of external macromolecules into the red cell.

Even when the cell is permeable to Hb and still larger molecules (9,13, 17) the reflection coefficients are not zero and osmotic forces are therefore still operative. If the external macromolecule is to achieve colloid osmotic balance with the residual internal Hb, it must be able to penetrate slowly enough to maintain an adequate concentration difference between the inside and outside of the cell. This may be the factor which determines the smallest macromolecule which is effective in reducing Hb liberation. Fig. 2 shows the diffusion coefficients of sucrose, raffinose and some dextrans standardized to 20 °C in relation to their molecular weight.

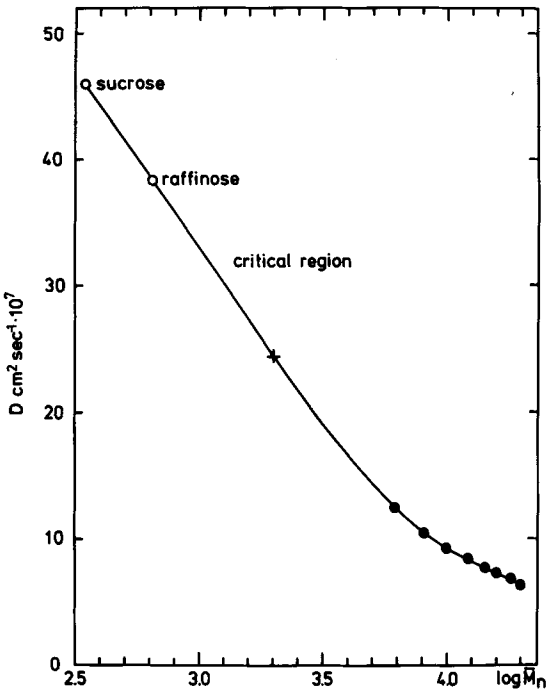

Fig.2 Diffusion coefficients standardized to 20 °C, of sucrose, raffinose and some dextran fractions (•) plotted against the logarithms of the molecular weights (M_n). The point (+) indicates the diffusion coefficient of the smallest dextran fraction tested (1). Hb has a similar Stokes-Einstein radius (3.2 nm) to a dextran with a molecular weight of about 20.000 (log = 4.3). The ability to reduce Hb loss develops in a critical region. The dextran data were obtained from Reference 3.

3. The relation between the macromolecular concentration and hemoglobin liberation.

There is a clear relation between the Hb liberated and the calculated net osmotic pressure of dextran (concentration outside minus concentration inside), assuming a reflexion coefficient of unity (16). Further the macromolecules promote resealing of the cell. How then does the osmotic relation, with some residual Hb in the cell, arise? Apparently resealing initiated by the macromolecule does not become final until colloid osmotic balance has been attained. As soon as this state is reached, the cell must reseal, very rapidly, at least to Hb and macromolecules, if Hb is to be retained and the then existing colloid osmotic balance is to be maintained.

REFERENCES

1. Davies,H.G.,Marsden,N.V.B.,Östling,S.G. & Zade-Oppen,A.M.M.: The effect of some neutral macromolecules on the pattern of hypotonic hemolysis. Acta Physiol Scand 74:577-593,1968.
2. Dienes,L.: Zeigen die Blutkörperchen einer Blutkörperchen-Aufschwemmung bei der Hämolyse messbare individuelle Verschiedenheiten? Biochem Z 33:268-274,1911.
3. Dodge,J.T.,Mitchell,C.& Hanahan,D.J.: The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch Biochem Biophys 100:119-130,1963.
4. Funder,J. & Wieth,J.O.:Chloride transport in human erythrocytes and ghosts: A quantitative comparison. J Physiol (Lond.) 262:679-698,1976.
5. Granath,K. cited by Grotte,G.:Passage of dextran molecules across the blood--lymph barrier. Acta Chir Scand Suppl. 211:1956.

6. Handbook of Chemistry and Physics.Rubber Publishing Company,Cleveland,Ohio 43rd Edition p. 2229,1961-1962.
7. Handovsky,H.:Untersuchungen über partielle Hämolyse. Arch Exper Pathol Pharmakol 69:412-430,1912.
8. Heedman,P.A.: Hemolysis of individual red blood cells.Exp Cell Res 14:9-21, 1958.
9. Hjelm,M.,Östling,S.G. & Persson,A.E.G.: The loss of certain cellular components from human erythrocytes during hypotonic hemolysis in the presence of dextran. Acta Physiol Scand 67:43-49,1966.
10. Lowenstein,L.M.: The effect of albumin on osmotic hemolysis.Exp Cell Res 20:59-65,1960.
11. Marsden,N.V.B.: Some observations on erythrocyte lysis. Acta Physiol Scand 31:Suppl. 114,41-42,1954.
12. Marsden,N.V.B.: The role of water in the interactions of some solutes in aqueous dextran gel systems. Acta Univ Upsal,Abstr Uppsala Dissertations in Med 123,1972.
13. Marsden,N.V.B. & Östling,S.G.: The accumulation of dextran in human red cells after haemolysis. Nature (Lond) 184:723-724,1959.
14. Marsden,N.V.B.,Zade-Oppen,A.M.M.& Johansson,L.P.: The effect of dextran on the dry mass distribution in osmotic hemolysis. Exp Cell Res 13:177-181,1957.
15. Mukerjee,P.: The Nature of the association equilibria and hydrophobic bonding in aqueous solutions of association colloids. Adv Colloid Interface Sci 1:241-275,1967.
16. Nylund,L.,Östling,S.G.,Marsden,N.V.B. & Zade-Oppen,A.M.M. In preparation.
17. Östling,S.G.: Permeability of human red cells during hypotonic fractional mass hemolysis in dextran. Acta Univ Upsal, Abstr Uppsala Dissertations in Med. 88:1970.
18. Parpart,A.K.: Is osmotic hemolysis an all-or-none phenomenon? Biol Bull 61: 500-517,1931.
19. Saslow,G.: On the supposed partial liberation of haemoglobin from the mammalian erythrocyte. Quart J Exp Physiol 19:329-335,1929.
20. Schwoch,G. & Passow,H.: Preparation and properties of human erythrocyte ghosts. Mol Cell Biochem 2:197-218,1973.
21. Strumia,M.M.& Strumia,P.V.: Recovery and survival of human red cells frozen with albumin,dextran and lactose-albumin. In Proc 9th Congr Int Soc Blood Transf,Mexico 1962 (Ed.L.Hollander,Basel,Karger,61-68,1964).
22. Tanford,C.: Physical Chemistry of Macromolecules,John Wiley, New York,p.358, 1961.
23. Teorell,T.: Permeability properties of erythrocyte ghosts. J Gen Physiol 35:669-701,1952.
24. Vink,H.: Model treatment of diffusion processes. Acta Chem Scand 18:409-414, 1964.
25. Weed,R.I.,Reed,C.F. & Berg,G.: Is hemoglobin an essential structural component of the red cell membrane? J Clin Invest 42:581-588,1963.
26. Wilbrandt,W.: Folgt die Hämolyse dem Alles-oder-Nichts-Gesetz? Experientia (Basel) 1:91,1945.
27. Zade-Oppen,A.M.M.: Limited $Fe(CN)_6^{-3}$ ion permeation into hemolyzing red cells. Proc.5th Int Biophys Congr Copenhagen: p. 103,1975.
28. Zade-Oppen,A.M.M. & Marsden,N.V.B.: The Phase Contrast Appearance of Hemolysing Red Cells and the Determination of Fragility. Acta Soc Med Upsal 73:91-112,1968.

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