BBA Report

BBA 71122

The effect of pH at hemolysis on the reconstitution of low cation permeability in human erythrocyte ghosts

S. LEPKE and H. PASSOW

Max-Planck-Institut für Biophysik, Frankfurt/Main, Kennedyallee 70, and Max-Planck-Institut für Molekulare Genetik, Berlin, Ihnestr. 63–73 (Germany)

(Received December 12th, 1971)

SUMMARY

Human red blood cells were hemolysed at 0°C in hypotonic media of varying pH (range 4.5–10.0). Subsequently the pH in the hemolysate was readjusted to 7.2 and the ghosts were incubated at 37°C for 45 min. The curve relating the yield of resealed ghosts to pH showed a maximum after hemolysis at pH 6 and a broad shoulder in the range between pH 7 and 9. The experiments suggest that during hemolysis buried ionizable groups become exposed to the ambient medium. The ability of these groups to return to their original locations within the hydrophobic interior of the membrane seems to depend on the charge which they assume during their exposure to the aqueous environment. However, alternative explanations cannot be excluded.

Ghost populations obtained by hypotonic hemolysis of red blood cells are "kinetically inhomogeneous"[1,2]. Some of the ghosts regain the same low cation permeability as the intact erythrocytes from which they were derived. The others do not. The reconstitution of the membrane after hemolysis seems to be predominantly of the "all-or-none" type: there are few ghosts which cannot be classified as being either "reconstituted" or "leaky". The ratio between reconstituted and leaky ghosts depends on the conditions at hemolysis[2] and on the treatment of the membrane thereafter[3]. The present communication deals with the effect of pH at hemolysis on the yield of reconstituted erythrocyte ghosts.

For an understanding of our experiments, it is necessary to recall in some detail the events which take place during the preparation of reconstituted erythrocyte ghosts

(Fig. 1). Shortly after hemolysis in a hypotonic medium nearly all of the ghosts become impermeable to hemoglobin. However, only a certain fraction recovers a low permeability to K^+ and Na^+. Upon restoration of isotonicity by the addition of alkali salts ("reversal") the ghosts which belong to this fraction shrink. The originally transparent suspension turns opaque. At the same time the added alkali salt equilibrates between the medium and the interior of those of the ghosts which are still leaky. Continued incubation, especially at elevated temperatures, leads to recovery of low cation permeability in many of the originally leaky ghosts[3]. If the incubation is performed at 37°C, this "resealing process" is completed after 45–60 min. A continuation of the incubation at 37°C beyond this length of time does not significantly increase the yield of reconstituted ghosts. A certain fraction of the ghosts still remains leaky.

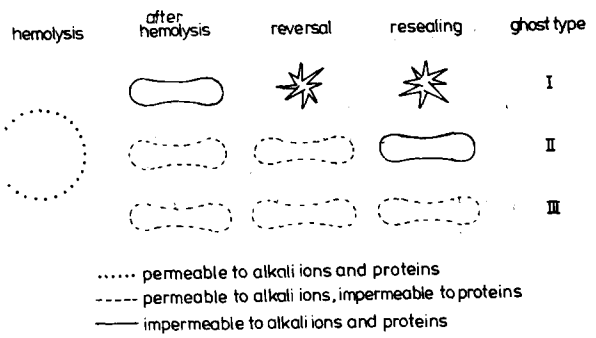

Fig. 1. Schematic representation of the events which take place during the preparation of red cell ghosts (Bodemann and Passow[2]). This schema applies if the erythrocytes are hemolysed at room temperature or above. If hemolysis is performed at 0°C, the ghost membrane is still slightly permeable to ^{131}I-labeled albumin after hemolysis. However, at the end of the resealing period complete impermeability is reestablished.

In order to facilitate the discussion of the described events, the various types of ghosts were designated Type I–III. Type I and Type II ghosts are both essentially impermeable to K^+ and Na^+. However, Type I ghosts reseal before, Type II ghosts after reversal by the addition of salt to the hemolysate. Type III ghosts are leaky for cations.

The ratio of the three types of ghosts is a function of temperature at hemolysis. The lower the temperature, the higher the proportion of Type II ghosts. At 0°C the reconstitution of cation impermeability is so slow that salt added for reversal equilibrates between ghosts and medium before resealing occurs. There are few if any ghosts of Type I. After incubation at 37°C, about 60–70% of the ghosts trap the incorporated alkali salts. Only 30–40% of the ghosts stay leaky. Hence, the final result of hemolysis at 0°C, reversal at that temperature, and subsequent resealing is a suspension which contains almost exclusively Type II and Type III ghosts. The indicated percentages of Type II and Type III ghosts are obtained when hemolysis and all other operations are performed close to pH 7.0.

In the present experiments on the effects of pH at hemolysis, the cells were

hemolysed at 0°C where the yield of Type II ghosts is maximal and where the percentage of Type I ghosts is negligible. For hemolysis, 2.0 ml of a 50% cell suspension (w/v) in isotonic saline were added to 20 ml of 4 mmoles/l $MgSO_4$ solutions containing, respectively, 0, 1, 2, 5, 10 mmoles/l NaOH, or 1, 3, 10 mmoles/l acetic acid. After hemolysis, the hemolysate was kept at 0°C for another 10 min. During this period, the pH was measured, isotonicity was restored, and the pH was readjusted to 7.2 ± 0.05. This was achieved by adding 2.0 ml of solutions containing 1.99 moles/l KCl and, respectively, 0, 10, 20, 50, 100 mmoles/l acetic acid, or 10, 30, 100 mmoles/l Tris hydroxide. If necessary, further quantities of acetic acid or Tris hydroxide were added until the desired pH value of 7.2 was established. The hemolysate was transferred to 37°C and kept at that temperature for 45 min. At the elevated temperature, the pH dropped to about 6.9. After incubation the ghosts were spun down at 27 000 X *g* for 5 min, the supernatant was removed, the sediment was resuspended in buffered NaCl solution (141 mmoles/l NaCl, 25 mmoles/l Tris chloride, pH 7.2), and incubated at 37°C for 20 min. During this incubation period most of the K^+ in the leaky ghosts (Type III) was released into the medium. After two further washes in the Tris-buffered NaCl solution, the ghosts were again resuspended in that solution and incubated at 37°C. The concentration of the ghost suspension was 5% (w/v). The time course of K^+ loss was followed (Fig. 2). The absence of an initial rapid phase of

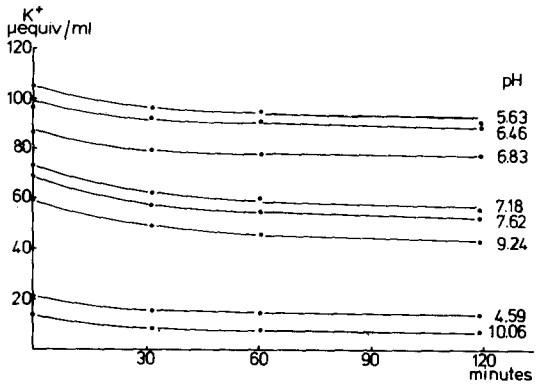

Fig. 2. K^+ leakage at pH 7.2 into Tris-buffered NaCl solution from red cell ghosts prepared by hemolysis at the various pH values indicated on the curves. Temperature at hemolysis, 0°C. Prior to the start of the experiment, the leaky ghosts (Type III) were depleted of K^+ as described in the text. Ordinate: K^+ content of the ghosts expressed in μequiv/ml of original red cells. Abscissa: time (min).

K^+ loss indicates that the Type III ghosts were successfully depleted of K^+ prior to the start of the measurement of net K^+ efflux. The figure further shows that, regardless of the total amount of K^+ incorporated into the ghosts at the various pH values at hemolysis, the resealed ghosts are fairly impermeable to K^+. Plotting the K^+ content of the ghosts as measured after 30 min of incubation at pH 7.2 against the pH as measured shortly after hemolysis, yields the result depicted in Fig. 3. Obviously the amount of incorporated K^+

reaches a maximum if hemolysis is performed under conditions where the pH in the final hemolysate assumes a value of about 6.0.

In terms of the scheme represented in Fig. 1 the variation of K^+ incorporation with pH at hemolysis should be due to variations of the ratio between Type II and Type III ghosts in the suspensions. At the maximum of the upper curve in Fig. 3 about 90% of the total ghost suspension should consist of Type II ghosts. The justification for this assumption can be derived from direct estimates of the Type II and Type III ghosts in the ghost suspensions whose K^+ contents are represented in Figs. 2 and 3. It is feasible to separate the two types of ghosts by centrifugation through a sucrose cushion[2]. The ghost suspension is layered on top of a 43% sucrose solution (w/v) containing 50 mmoles/l NaCl and 25 mmoles/l Tris, pH 7.2. After 90 min of centrifugation at 42 300 × *g* the leaky ghosts (Type III), whose density is entirely determined by the density of their membranes, are sedimented. The resealed ghosts (Type II), whose density is the mean of the densities of their membranes and of the trapped intracellular fluid, remain in the original suspension medium on top of the sucrose cushion. Since both types of ghosts recover their impermeability to proteins[2] the hemoglobin content of the bottom fraction, expressed in percent of the total hemoglobin content of the original ghost suspension, yields the percentage of Type III ghosts. Fig. 3 shows that this percentage varies inversely with the K^+ content of the total ghost suspension. This demonstrates that the variations of the K^+ content are, in fact, due to variations of the proportion of Type II ghosts as a function of pH.

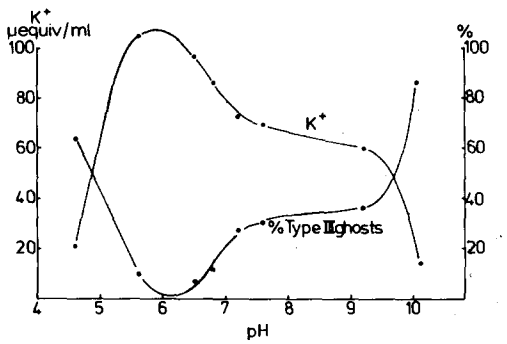

Fig. 3. K^+ content of ghosts as a function of pH at hemolysis. The K^+ content was measured after incubation in Tris–NaCl solution for 30 min. Data from Fig. 2. The lower curve represents the percentage of leaky ghosts (Type III). This percentage was determined by separating leaky from resealed ghosts by centrifugation through a sucrose cushion as described in the text. Ordinate: (a) K^+ contents expressed in µequiv/ml original red cells; (b) fraction of leaky ghosts (Type III), in percent. Abscissa: pH measured in the hemolysate at the temperature of hemolysis (0°C).

Fig. 4 shows that the K^+ content per ghost is roughly constant and independent of pH at hemolysis. Regardless of pH at hemolysis, the Type II ghosts assume the same volume and hence trap the same amount of K^+ when the pH is readjusted to 7.2 prior to the

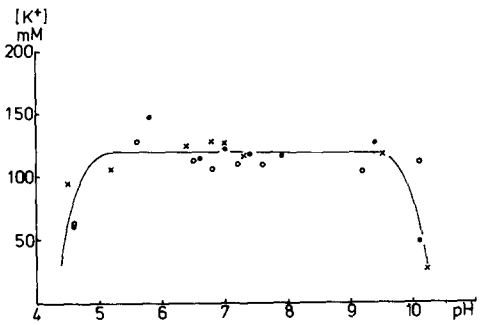

Fig. 4. K^+ concentration within resealed ghosts (Type III) as a function of pH at hemolysis. The various points refer to three similar experiments. The intracellular K^+ concentration was calculated as follows: The K^+ content of the ghosts of a measured volume of ghost suspension was divided by the fraction of Type II ghosts as determined by centrifugation on a sucrose cushion (*cf.* text). This yields a relative measure of the K^+ content per resealed ghost. Multiplication by the fraction of Type II ghosts of the volume of original red cells from which the ghosts were derived, provides a measure of the volume of Type II ghosts. Division of the calculated K^+ content by the calculated volume gives the K^+ concentrations indicated on the ordinate. Ordinate: K^+ concentration in mmoles/l original red cell volume. Abscissa: pH at hemolysis.

initiation of the resealing process by rewarming of the cold hemolysate to 37°C. The deviations at the extreme ends of the pH scale are presumably due to some hemolysis of the ghost. The calculated K^+ concentration inside the ghosts is lower than the K^+ concentration in the hemolysate after reversal. The K^+ concentration in the ghosts is expressed in mmoles/l of original erythrocyte volume and was calculated on the assumption that the volume of the ghost is identical with the volume of the intact red cell. However, since the ratio of the volume of erythrocytes to ghosts is unknown, the absolute values of the calculated concentrations represented in Fig. 4 are subject to some uncertainty. The difference between the calculated K^+ concentrations and the K^+ concentrations in the hemolysate could be accounted for if one assumes that the volume of the ghosts is smaller than the volume of the red cells from which they were derived.

The relationship between the fraction of Type II ghosts and pH as depicted in Fig. 3 is interesting for two reasons. First, for practical purposes it is important to know that hemolysis in an acid solution, which leads to a pH of about 6.0 in the final hemolysate, gives an optimum yield of 85–95% of intact ghosts. Secondly, the demonstrated relationship may provide some information about the factors on which the ability of the membrane to reseal depends.

The decisive influence of pH on the yield of Type II ghosts strongly suggests that ionizable groups play an important role in the reconstitution of the membrane after hypotonic hemolysis. The persistance of the effect of pH at hemolysis after readjustment of the pH in the final hemolysate to 7.2 and after resealing at that pH indicates that the ionizable groups are exposed to the ambient medium only at the instant of hemolysis. After the equilibration of the cell contents between ghosts and medium, when the forces which

lead to hemolysis have disappeared, the ionizable groups are buried again within the hydrophobic interior of the membrane. However, although necessary, the burial of these groups is not sufficient for resealing since the original low cation permeability of the intact erythrocyte membrane is only restored if burial of the ionizable groups is followed by an incubation period at 37°C.

Although the described pH dependence points to the participation of ionizable groups in the restoration of the membrane after hemolysis, it does not provide enough information for the formulation of a hypothesis of the mechanism by which these groups influence the recovery of the integrity of the membrane structure. Interactions between the buried groups themselves and with other membrane constituents may be involved. The total electrostatic free energy of interaction between the buried groups as well as the displacement of the pK values of specific groups which participate in intermolecular or intramolecular bond formation may contribute to a stabilisation of the membrane. Specific bonds may be established by ion pair formation, by ion–dipole interactions, or by charge fluctuations between basic groups (for a review, see ref. 5). In addition, the electrical charge of the buried groups may considerably affect molecular conformation and hence the hydrophobic interactions between membrane proteins and lipids.

If hemolysis would be accompanied by a shift of ion pairs from a hydrophobic environment of low dielectric constant to an aqueous medium of high dielectric constant, the strength of the ionic bond would be reduced and the degree of dissociation of the exposed groups would be changed. The pH in the hemolysing medium would determine the balance between the number of positively and negatively charged groups of the original ion pairs. Since the burial of the unpaired charged groups in a hydrophobic medium is associated with a considerable increase in free energy, those groups whose mates changed their state of ionisation after exposure are unlikely to return to their original locations in the membrane after hemolysis. This should reduce the probability for successful resealing during subsequent incubation at 37°C.

The characteristic shape of the relationship between pH at hemolysis and the number of resealable ghosts would suggest that – if ion pair formation plays a role at all – more than two types of charged groups are involved. An identification of these groups is difficult since the exposed groups may return to their original locations before the pH in the membrane assumes the value measured after complete equilibration between cell contents and hemolysing medium. Hence Fig. 3 does not necessarily correlate the yield of Type II ghosts to strictly pertinent pH values. Nevertheless, it would seem plausible to assume that carboxyl groups on the one hand and amino and imidazole groups on the other form ion pairs which interconnect protein molecules traversing the erythrocyte membrane. At pH 6.0, when all three groups are ionized, ion pair formation should be at a maximum. Below pH 6.0 the number of COO^- groups would be reduced and a net positive charge should exist. At pH values above 6.0 the number of positively charged imidazole groups, and above pH 9.0 the number of NH_3^+ groups would be diminished. The result should be a negative net charge. As described above, the appearance of negative or positive net charges

should reduce the probability for a successful resealing and hence could account for the pH dependence of the reconstitution of the ghost membrane represented in Fig. 3.

It should be reemphasized that totally different hypotheses may explain our findings equally well. For example, Morrison and Neurath[4] obtained evidence which suggests that there exist three different proteinases in the red cell membrane. In crude ghost suspensions prepared by osmotic hemolysis and prolonged incubation at 4°C, one of the proteinases shows a pH optimum at pH 3.0, the other two show optima at pH 8.0. One could postulate, therefore, that the maximal yield of resealable ghosts obtained after hemolysis at pH 6.0 is associated with the minimum of proteinase activity in this pH range. However, a necessary corollary of this hypothesis is the somewhat implausible assumption that (1) the proteinases perform their action on the permeability controlling sites at the instant of hemolysis at 0°C but not during the subsequent resealing period at 37°C, and (2) the activity of the proteinases is confined to a pH-dependent fraction of the cells, *i.e.* to those cells which will turn into Type III ghosts.

This work was performed during a stay of the authors at the Max-Planck-Institut für Molekulare Genetik, Berlin. Dr. Wittmann's hospitality is gratefully acknowledged. We thank Drs. V. Rudloff, J. Riordan, and D. Schubert for helpful discussions. Supported by the Deutsche Forschungsgemeinschaft, SFB 38.

REFERENCES

1 Hoffman, J.F. (1962) *J. Gen. Physiol.* **45**, 837.
2 Bodemann, H. and Passow, H. (1971) *J. Membrane Biol.*, in the press.
3 Hoffman, J.F., Tosteson, D.C. and Whittam, R. (1960) *Nature* **185**, 186.
4 Morrison, W.L. and Neurath, H. (1953) *J. Biol. Chem.* **200**, 39.
5 Timasheff, S.N. (1970) in *Biological Polyelectrolytes* (Veis, A., ed.), Vol. I, p. 1, M. Dekker, New York.