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Factors Controlling the Resealing of the Membrane of Human Erythrocyte Ghosts after Hypotonic Hemolysis

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Summary. In accordance with former observations of Hoffman (1962*a*), ghost populations obtained by hypotonic hemolysis and subsequent restoration of isotonicity by the addition of alkali salts, were found to be composed of 3 types of ghosts. For our purposes it was useful to distinguish between: (1) ghosts which reseal immediately after hemolysis (type I); these ghosts are incapable of incorporating alkali ions which are added after hemolysis; (2) ghosts which reseal after the addition of alkali ions (type II); salt added to the hemolysate becomes trapped inside these ghosts in the course of the resealing process at temperatures above 0 °C; and (3) ghosts which remain leaky regardless of the experimental condition (type III). The discrimination between the various types of ghosts was partly achieved by a kinetic method first devised by Hoffman (1962*a*), and partly by sucrose density gradient centrifugation.

The relative sizes of the 3 fractions depend on the temperature at which hemolysis took place and on the time interval which elapsed between hemolysis and the addition of salt. At 37 °C the resealing process is fast. Many of the ghosts reseal before salt can be added to the hemolysate. Hence, the fraction of type I ghosts is high after hemolysis at that temperature. At 0 °C resealing is extremely slow. Hence, salt which has been added to the hemolysate at that temperature will enter the ghosts and become trapped during subsequent incubation at 37 °C. There are no ghosts of type I and many ghosts of type II (about 60%). Regardless of the temperature at hemolysis, there are always ghosts which do not reseal even after prolonged incubation at 37 °C. A method has been designed which permits the preparation of homogeneous populations of type II ghosts.

Complexing agents (ATP, EDTA, 2,3-DPG) may prevent the resealing of the ghost membrane. However, they exert this effect only at elevated temperatures and when present in the medium at the instant of hemolysis. At 0 °C, the presence of complexing agents in the medium at the instant of hemolysis has no effect on the subsequent resealing at 37 °C. The recovery of the ghost membrane takes place in spite of the continued presence of the agents and eventually leads to trapping of these agents inside the resealed ghosts.

The experiments support the contention that the complexing agents interact with a membrane constituent which is neither accessible from the inner nor from the outer surface of the cell membrane but becomes exposed during the hemolytic event when the complexing agents penetrate across the membrane. Apparently, at low temperatures membrane ligands are more successful in competing with the added complexing agents for this constituent than at higher temperatures.

Extending former observations of Hoffman, we found that not only Mg^{++} but also Ca^{++} facilitates the resealing process. Perhaps one or the other of the two alkaline earth ions is the membrane constituent which normally participates in the maintenance of the integrity of the red blood cell membrane.

In 1952, Teorell showed that erythrocyte ghosts reseal after the hemolytic event and then behave like osmometers. In 1953, Straub demonstrated that ATP could be incorporated into ghosts where it energized the active transport of Na and K. Since these discoveries were made, erythrocyte ghosts have become an indispensable tool for the investigation of the mechanism of active transport and of the factors which control passive permeability. In particular, experiments with red blood cell ghosts were essential in attempts to explore the effects of internal substrates, activators, and inhibitors on active transport (Post, Merrit, Kinsolving & Albright, 1960; Dunham & Glynn, 1961; Glynn, 1962; Hoffman, 1962*a*) and the effects of ATP and EDTA on passive ion permeability (Hoffman, 1962*b*; Lepke & Passow, 1968; Romero & Whittam, 1971).

A ghost suspension does not represent a homogeneous population (Hoffman, 1958, 1962*a*). A certain fraction of the ghost population is capable of resealing, i.e., of regaining a high degree of impermeability to cations. The rest of the ghosts remains leaky. The resealing process proceeds at rates which differ among the various ghosts of the population. If an alkali salt is added to a population of hemolyzed red blood cells, then those ghosts which were already resealed shrink. Those ghosts which are still leaky, equilibrate with the salt in the medium. As time goes on, their membranes reseal and the alkali salt becomes trapped inside the ghosts. This process can be accelerated by raising the temperature to 37 °C (Hoffman, Tosteson & Whittam, 1960). For our purposes, it proved to be convenient to discriminate between ghosts which, at the time when the salt is added, are already resealed and ghosts which are capable of resealing thereafter on incubation at 37 °C. We call those ghosts which resealed before the addition of salt, type I ghosts and those which reseal after addition of salt, type II ghosts. Even after prolonged incubation at 37 °C, there always remains a certain fraction of ghosts which does not reseal. Ghosts belonging to that fraction are designated type III ghosts. The present paper is devoted to a more detailed investigation of the factors which determine the yield of ghosts of the various types. In particular, the effects of variations of temperature at hemolysis were studied.

If hemolysis is performed at 0 °C more salt can be incorporated into the ghosts than after hemolysis at 25 °C or at 37 °C (Passow, 1969). In the course of the present work, this effect was found to be related to an increase of type II ghosts with decreasing temperature at hemolysis. Furthermore, former observations of Hoffman (1962*b*) on the role of alkaline earth ions and complexing agents in the hemolyzing fluid were confirmed and extended by studying the effects of these agents on the restoration of the ghost

membrane when present at various temperatures in the hemolyzing medium. The results were useful for the determination of optimal conditions for the preparation of resealed red blood cell ghosts. In addition, they provided some information on the factors which control the passive permeability of the red blood cell membrane to alkali ions.

Materials and Methods

Blood was withdrawn from apparently healthy donors and stored under sterile conditions in ACD buffer at 4 °C for no more than 2 or 3 days. After centrifugation at $1{,}600 \times g$, the buffy coat was carefully removed and ghosts were perpared as summarized in Fig. 1. Mixing of cells and medium during hemolysis was achieved by pipetting the erythrocyte Tris-Cl suspension into the well stirred hemolysis medium. 'Reversal' of hemolysis was performed by the addition to the hemolysate of a small volume of a concentrated solution of an alkali salt. The final alkali salt concentration was either 155 mmoles/liter (isotonic) or, more frequently, 100 mmoles/liter. Unless stated otherwise, salt was added for reversal at the temperature of hemolysis and 5 min after hemolysis. Transfer to 37 °C followed after another 5 min at the temperature of hemolysis. At the end of the resealing period, the ghosts were centrifuged down in a Sorvall RC 2-B refrigerated centrifuge at $34{,}800 \times g$ for 10 min and were further used as described under Results.

K and Na were determined by flame photometry after diluting the cell suspension in ice cold isotonic choline chloride solution and subsequent centrifugation at $34{,}800 \times g$. Corrections for trapped extracellular Na or K were made as described by Passow (1969).

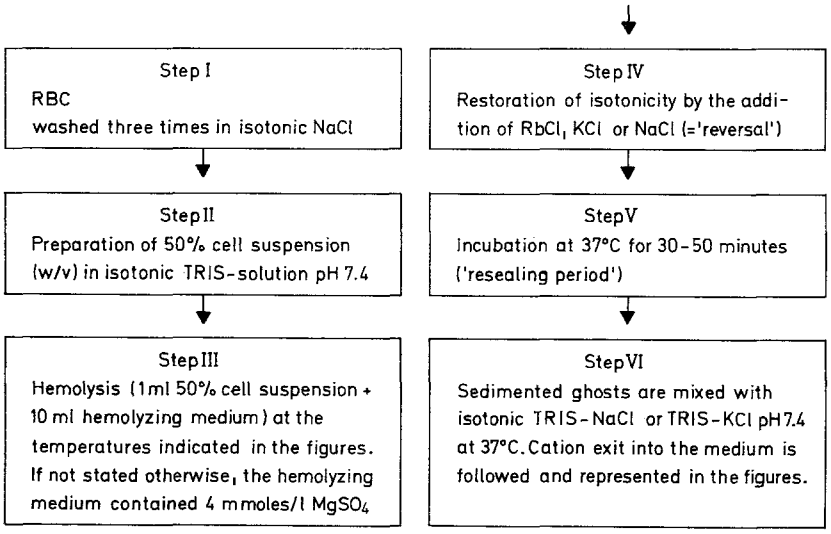

Fig. 1. Schematic representation of the experimental procedure employed in kinetic studies. The pH values indicated in the figure refer to room temperature. The pH of the Tris-Cl solution as well as of the cell contents varies with temperature. Hence the actual pH values as measured at the temperatures at which the various steps were performed varied somewhat. In the temperature range 0 to 37 °C, the following pH values were observed: Step III: 6.9 to 7.1, Step IV: 6.9 to 7.1, Step V: 6.95 to 7.15 (37 °C only), Step VI: 7.1 (37 °C only)

The incorporation of ^{22}Na, ^{24}Na, ^{86}Rb, and ^{131}I-albumin into the ghosts was studied by adding the radioisotopes either to the hemolyzing medium (i.e., during Step III), to the solution which was used to restore isotonicity after hemolysis (i.e., during Step IV), or 5 min before the end of the resealing period at 37 °C.

The efflux of radioactive alkali ions was measured at 37 °C after resuspending the resealed ghosts in isotonic solutions containing 10 mmoles/liter Tris-Cl, pH 7.4 (Step VI). Ghosts and medium were separated at suitable time intervals by centrifugation. The radioactivity in the ghosts was determined by counting aliquots of the supernatant and of the total cell suspension and taking the difference between the two results. The amount of extracellular alkali ions transferred from the original hemolysate into the Tris-NaCl medium was estimated by means of ^{131}I-albumin. The labeled protein was added to the original hemolysate 5 min before the end of the resealing period and the ratio of the radioactivity of ^{131}I and the alkali ion species under investigation was determined. After mixing the sedimented ghosts with the back exchange medium, the amount of ^{131}I-albumin in the resulting suspension was estimated and the corresponding amount of transferred extracellular alkali ions was calculated.

The simultaneous determination of the radioactivity of the alkali ions and of ^{131}I-albumin was performed as described by Oberhausen and Muth (1969).

For density gradient centrifugation, radioactively labeled ghosts were washed twice at 4 °C and resuspended to form a 30% (w/v) suspension in isotonic Tris-NaCl. A portion of 0.5 ml of this suspension was layered on top of a continuous sucrose gradient (43% sucrose at the top and 62% at the bottom of a tube of 36-ml capacity). The sucrose contained, uniformely from top to bottom, 25 mmoles/liter NaCl and 25 mmoles/liter Tris-HCl, pH 7.4. Centrifugation was carried out in a Spinco preparative ultracentrifuge model 50 B at 26,000 rpm for 60 min. A type SW 27 rotor was used. The resulting bands were photographed and collected by means of a cannula and syringe for the determination of the radioactivity.

The non-hemoglobin protein in the ghost ('membrane protein') was estimated as follows: The ghosts were solubilized by mixing one part of ghost suspension with 4 parts of glacial acetic acid. The light extinction of resulting clear solution was determined by means of a Zeiss spectrophotometer at 398 and 280 nm. The extinction at the former wavelength is a measure for the amount of hemoglobin. The extinction at 280 nm represents the total stromal protein, including hemoglobin. Since the amount of hemoglobin is known from determinations at 398 nm, it was possible to calculate the content of stromal protein. The method is not very accurate since it involves the formation of the difference between quantities of equal orders of magnitude. Nevertheless, the results were reasonably reproducible (*cf.* Table 4).

ATP was determined by an enzymatic test using glyceraldehyde phosphate dehydrogenase and glycerate kinase (Boehringer).

Results

Influence of Temperature at Hemolysis on the Composition of the Ghost Population – Estimation of the Various Fractions of Ghosts by Kinetic Measurements

Ghosts were prepared by the procedure described in Fig. 1. Rb was incorporated into them by adding to the hemolysate a concentrated RbCl solution which restored isotonicity (Step IV of Fig. 1). Fig. 2 represents the time course of Rb exit from the resealed ghosts into isotonic Tris-NaCl

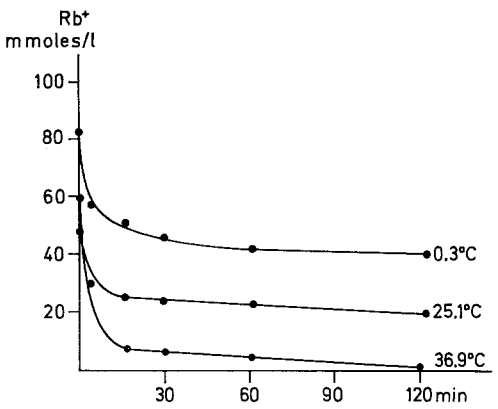

Fig. 2. Time course of Rb exit into isotonic Tris-NaCl medium. The ghosts were obtained by hemolysis at the temperatures indicated on the curves. Reversal 10 min after hemolysis by the addition of RbCl. Resealing at 37 °C for 40 min. *Ordinate:* Rb^+ content in mmoles/liter ghosts. *Abscissa:* time in minutes

solution (pH 7.4). The curves show that increasing the temperature at hemolysis decreases the amount of Rb which can be incorporated into the cells at 'reversal' of hemolysis. The Rb loss into the medium seems to be biphasic. Initially, Rb leakage is fairly rapid. After about 15 min and before reaching diffusion equilibrium, Rb leakage slows down and continues at a much lower rate. This peculiar time course of leakage is also found when K instead of Rb is incorporated into the ghosts.

The observed kinetics represent the average behavior of a population. One possible explanation would consist in the assumption that the rapid phase of cation exit is due to leakage from ghosts which did not reseal (i.e., from type III ghosts). At the end of the rapid phase, the resealed ghosts (type II) still contain a large amount of Rb which continues to escape slowly into the medium. An extrapolation of the later parts of the curves, relating cation loss to time, back to the origin should yield the total amount of Rb or K incorporated into the type II ghosts at the end of the resealing period. The variation of this amount with the temperature at hemolysis would represent the variation of the proportion of type II ghosts which resealed after reversal. The experiments shown in Fig. 3*a–c* lend support to such an interpretation and provide, in addition, some information on those ghosts which resealed after hemolysis before Rb was added for reversal.

Following a procedure first employed by Hoffman (1962*a*), ^{86}Rb was added at 3 different stages of the preparation of the ghosts: (1) at hemolysis, (2) at reversal, and (3) shortly before the end of the resealing period. Curve *1*

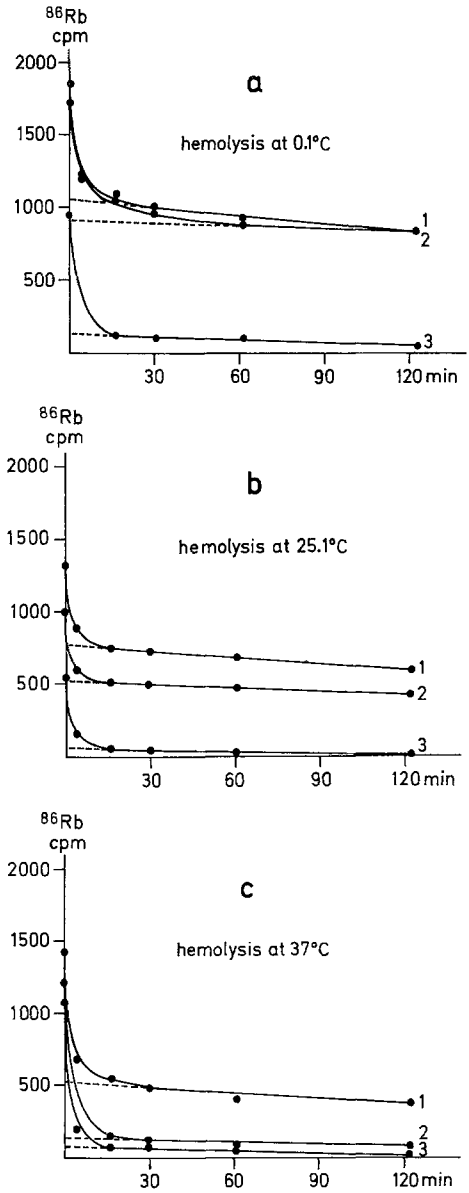

Fig. 3. Time course of ^{86}Rb exit into isotonic Tris-NaCl medium from ghosts which were loaded with $^{86}RbCl$: (1) during hemolysis, (2) during reversal with RbCl at the temperature of hemolysis, and (3) at the end of the resealing period at 37 °C. *Ordinates:* ^{86}Rb (counts/min) in the ghosts of 1-ml suspension. *Abscissas:* time in minutes

of Fig. 3*b* represents the ^{86}Rb efflux from ghosts obtained from red blood cells which were hemolyzed at 25 °C in the presence of a trace amount of ^{86}Rb in the hemolyzing medium. The radioactivity remained present

in the supernatant of the ghost suspension from the time of hemolysis until the end of the resealing period (Steps III–V). Under these conditions, radioactivity is incorporated into ghosts of type I and II, i.e., into those ghosts which reseal immediately after the hemolytic episode and those which reseal later after incubation at 37 °C (Step V). If the radioactivity is added after hemolysis (i.e., after Step III), during reversal (i.e., during Step IV), less radioactivity is trapped inside the ghosts (curve *2*). This is due to the fact that the type I ghosts largely recovered their impermeability before the addition of ^{86}Rb, while the type II ghosts are still leaky and capable of incorporating ^{86}Rb which is added at the time of reversal. Little radioactivity is taken up if ^{86}Rb is added 5 min before the end of the resealing period (Step V, curve *3*). Moreover, the radioactivity which is incorporated at Step V is rapidly lost after resuspension of the ghosts in Tris-NaCl solution, i.e., at Step VI. The rapid equilibration of the radioactivity indicates the presence of type III ghosts, i.e., ghosts which did not reseal.

A comparison of the various experiments depicted in Fig. 3 shows that the proportion of the three types of ghosts in the total population strongly depends on the temperature at which hemolysis was performed. In order to obtain a semiquantitative estimate of the distribution of the ghosts among the 3 types, the later portions of the curves, relating ^{86}Rb concentration to time, were extrapolated to zero time as described above. The differences between the initial ^{86}Rb contents and the intersections of the extrapolated curves with the ordinate give a measure for the number of leaky (type III) ghosts existing under the respective experimental conditions. The percentages of ghosts of type II and of type I + II are inferred from the intercepts of the extrapolated curves *1* and *2*, respectively, on the ordinate. Table 1 summarizes the results derived by this method from a number of experiments.

Table 1 shows that the yield of ghosts of type II increases if one lowers the temperature at which hemolysis is performed from 37 °C to 0 °C. After hemolysis at 0 °C, about 60% of the ghosts retain the capacity to reseal upon rewarming to 37 °C. A high yield of these ghosts is desirable for most permeability studies since they can be loaded at low temperature with salt and substrates and subsequently be resealed by incubation at 37 °C. At 0 °C the number of ghosts of type I which reseals spontaneously and, hence, cannot be loaded remains almost negligible. However, this number increases to 15 or 20% if hemolysis is performed at 25 °C or 37 °C. Similar numbers of ghosts (about 38%) remain leaky after a resealing period of 30 to 60 min (type III) at 0 °C and 25 °C. This number increases sharply to 75% if the temperature is raised to 37 °C.

Table 1. Percentage of ghosts of type I (resealed before reversal), type II (resealed after reversal), and type III (leaky) as a function of temperature at hemolysis [a]

Temperature at hemolysis	% of ghosts			No. of exp.
	Type I	Type II	Type III	
0 °C	3.86 ± 2.03	57.24 ± 9.00	38.90 ± 7.84	7
25 °C	17.17 ± 8.59	45.17 ± 11.04	37.66 ± 14.14	12
37 °C	15.43 ± 7.56	10.03 ± 2.84	74.54 ± 8.82	7

[a] The numerals represent mean values and the corresponding standard deviations.

If, in experiments of the type described above, K or Na is used in place of Rb, results are obtained which are completely analoguous to those described above. This is illustrated by an experiment (Fig. 4) in which, at 37 °C, the same batch of ghosts was first loaded with ^{22}Na at hemolysis and then with ^{24}Na during the subsequent reversal. Much more Na was incorporated at hemolysis than at reversal. This shows that the prevalence of ghosts of type I which was demonstrated to exist under the specified

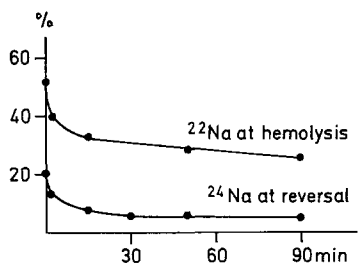

Fig. 4

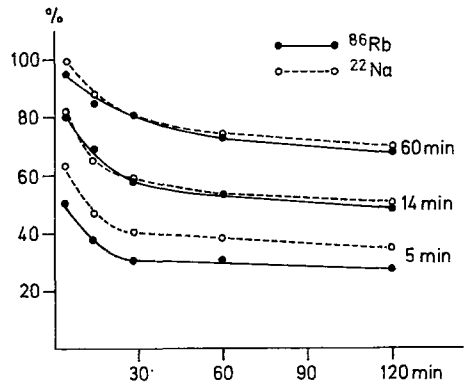

Fig. 5

Fig. 4. Exit of radioactivity from ghosts into which ^{22}Na and ^{24}Na were incorporated at hemolysis or at reversal. The radioactivity in the ghosts (ordinate) is expressed in percent of the radioactivity of ^{22}Na or ^{24}Na in a corresponding volume of the original hemolysate. Temperature at hemolysis and reversal: 37 °C. *Abscissa:* time in minutes

Fig. 5. Retention of ^{22}Na and ^{86}Rb by ghosts which, after hemolysis at 0 °C, were incubated at 37 °C for the time periods indicated on the curves. After hemolysis, the final (ideal) osmolarity was 35 mosm/liter (including 4 mmoles/liter $MgSO_4$ in the hemolyzing fluid). There was no reversal after hemolysis. The ordinate represents the radioactivity in the ghosts in percent of the radioactivity in a corresponding volume of the hemolysate. The abscissa indicates the time after resuspension of the sedimented ghosts in 35 mosm/liter Tris-NaCl, pH 7.4

conditions by measuring permeability to Rb can also be shown if the permeability to Na is estimated instead. A comparison of the behavior of incorporated ^{22}Na and ^{86}Rb (Fig. 5) is described in another context on page 8.

Influence of Temperature at Hemolysis on the Composition of the Ghost Population – Separation of the Various Fractions of Ghosts by Density Gradient Centrifugation

The findings presented above were explained by the assumption of the existence in the same population of three types of ghosts which differ with respect to their capacity of resealing for alkali ions. Attempts to separate the three ghost fractions by density gradient centrifugation will be described below. They were not completely satisfactory. Nevertheless, support for the inferences drawn from the kinetic studies was obtained.

Fig. 6 shows the band pattern observed after sucrose density gradient centrifugation of ghost populations obtained after hemolysis at 0, 25 and 37 °C, reversal at the temperatures of hemolysis, and subsequent resealing at 37 °C for 45 min. The centrifugation was carried out after washing the resealed ghosts two times in isotonic Tris-NaCl solution, pH 7.4. When hemolysis was performed at 0 °C, there were two bands. On top of the lower band there was a less dense, somewhat diffuse layer. If hemolysis was performed at 37 °C, one usually observed three bands. The position of the

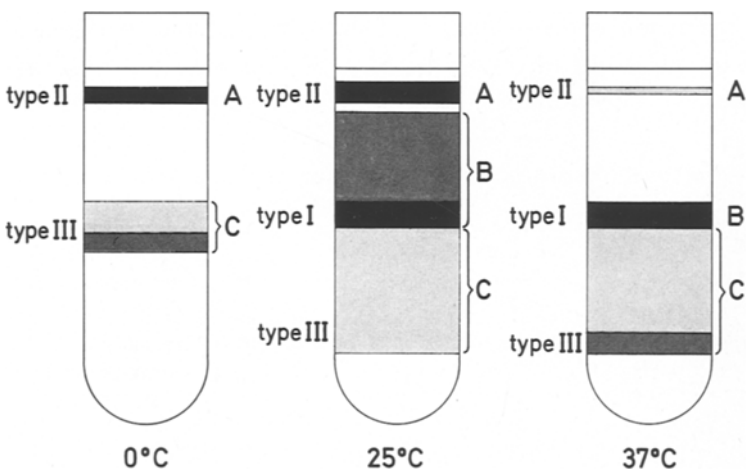

Fig. 6. Density gradient centrifugation of ghosts which were prepared by hemolysis at 0, 25 and 37 °C. The letters A, B and C designate the fractions which were collected separately and studied as described in the text. The roman numerals refer to the corresponding kinetically defined ghost types

uppermost band corresponded to that of the upper band obtained with ghosts which were prepared by hemolysis at 0 °C. However, this band was usually faint and sometimes barely detectable. The space between the two lower bands was, as a rule, slightly colored and uniformely turbid, indicating the presence of ghosts with a fairly wide range of densities. Ghost populations prepared by hemolysis at 25 °C exhibited a more complex band pattern. The uppermost band again corresponded to the upper band observed in ghosts prepared by hemolysis at 0 °C. The middle band had the same position as the middle band of a ghost population obtained by hemolysis at 37 °C. However, above this band a diffuse layer of ghosts reached almost up to the uppermost band, and below another diffuse layer extended down to the curvature of the centrifuge tube.

For the purpose of the present study, the contents of the centrifuge tubes were subdivided into three fractions only (A, B, C). The cells in the diffuse layers on top of the well defined bands were collected together with the cells in these bands. The diffuse layer below the lower of the two sharp bands observed in ghost suspensions which were obtained by hemolysis at 25 °C was collected separately. The definition of the three fractions is illustrated in Fig. 6.

The relative sizes of the various fractions of ghosts obtained after density gradient centrifugation could be estimated semiquantitatively after incorporation of ^{131}I-albumin into the ghosts at hemolysis. Table 2 shows that incorporation of the protein is feasible provided it is added at hemolysis. Regardless of the temperature at hemolysis and hence regardless of the proportion between ghosts of the various types in the suspension, the rate of release of incorporated ^{131}I-albumin into the medium is very slow (Table 3). Therefore, the amount of radioactivity found in the various

Table 2. ^{131}I-albumin content in the ghosts after resealing and two washings in ^{131}I-free medium [a]

Temperature at hemolysis	% of ^{131}I-albumin		
	Addition of radioactivity		
	(at hemolysis)	(at reversion)	(after the resealing period)
0 °C	100.0	43.5	2.2
25 °C	100.0	2.7	2.6
37 °C	100.0	4.7	3.7

[a] The amount incorporated at hemolysis was arbitrarily set equal to 100%. The ghosts were loaded either at hemolysis, at reversal, or at the end of the resealing period. Hemolysis and reversal were performed at 0, 25 or 37 °C, respectively.

Table 3. Efflux of ^{131}I-albumin from ghosts loaded at hemolysis at 0 or 37 °C, respectively [a]

Time (min)	Content of ^{131}I-albumin (% of initial value)	
	0 °C	37 °C
0	100.0	100.0
4	99.3	98.8
16	99.5	97.0
30	99.2	96.0
58	98.5	95.0
120	98.1	93.5

[a] After resealing, the ghosts were washed twice in isotonic Tris-NaCl solution. Subsequently, the ^{131}I-albumin efflux into that solution was measured at 37 °C.

Table 4. Relationship between ^{131}I-albumin incorporation and amount of membrane protein in the various ghost fractions obtained after density gradient centrifugation [a]

Temperature at hemolysis	Ghost fraction	$\left[\frac{^{131}\text{I-albumin, cpm}}{\varepsilon 280}\right]$
0 °C	"Total ghost suspension"	492
	A(=type II)	459
	C(=type III)	622
25 °C	"Total ghost suspension"	167
	A(=type II)	154
	B(=type I)	167
	C(=type III)	126
37 °C	"Total ghost suspension"	161
	B(=type I)	144
	C(=type III)	158

[a] For description of method *see* text. The results represent averages of three independent experiments. "Total ghost suspension" refers to the original ghost suspension before separation into the various fractions.

bands in the density gradient should be a measure of the number of ghosts of which these bands are composed. The retention of ^{131}I-albumin by the resealed ghosts of the various fractions obtained by density gradient centrifugation is demonstrated in Table 4 in which the ratio between the incorporated radioactivity and the amount of membrane protein in the respective band is presented. For a given temperature at hemolysis, this ratio

is, within the limits of the considerable experimental error the same for the three ghost fractions. This shows that the incorporated radioactivity is indeed a measure for the number of ghosts[1]. The described results emphasize that the term 'resealing' cannot indiscriminantly be used for alkali ions and proteins since ghosts of type III which remain leaky for alkali ions regain their impermeability to albumin molecules.

As in the experiments described in the previous section on the kinetics of alkali ion leakage, ^{86}Rb was incorporated into the ghosts (a) at the instant of hemolysis (i.e., during Step III of Fig. 1), or (b) during restoration of isotonicity by the addition of RbCl after hemolysis (i.e., during Step IV of Fig. 1). At the end of the subsequent resealing period at 37 °C, the ghosts were subjected to density gradient centrifugation: The resulting fractions A, B, and C were collected separately and the activity of the incorporated ^{86}Rb was estimated. In order to obtain some indication of the number of ghosts in each fraction, ^{131}I-albumin was incorporated at the instant of hemolysis into the ghosts. The amount of ^{131}I determined in each of the fractions was used as a measure of their relative sizes. Table 5 shows a result obtained by this method.

If hemolysis and reversal are performed at 0 °C, ^{86}Rb is found only in the uppermost band (fraction A). This result is obtained regardless of whether the tracer is added at hemolysis or during reversal. This finding suggests that fraction A is primarily composed of type II ghosts, i.e., of those ghosts which reseal only after incubation at 37 °C (Step V) and that fraction C contains primarily type III ghosts, i.e., ghosts whose impermeability to Rb is neither spontaneously restored nor restored by incubation at 37° C.

If hemolysis is performed at 37 °C, a correct determination of the ratio ^{86}Rb/^{131}I-albumin in the faint upper band, fraction A, is difficult to accomplish. Nevertheless, the data show that the incorporation of ^{86}Rb follows the same pattern as in a ghost population which was obtained by hemolysis at 0 °C. This suggests that fraction A represents ghosts of type II.

Fraction B contains much radioactivity if ^{86}Rb is present at hemolysis, but only very little, if the isotope is added during reversal. Hence, this fraction behaves like ghosts of type I which reseal in the time interval between hemolysis and reversal.

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1 It is interesting to note that the ratio ^{131}I-albumin/membrane protein is higher after incorporation of the protein at 0 °C than at 25 °C or at 37 °C. Perhaps, the final volume which the ghosts attain after hemolysis is larger if hemolysis is performed at the lower temperature. However, other factors may also contribute to this finding.

Table 5. Distribution of ^{86}Rb activity in the different fractions obtained after density gradient centrifugation [a]

Temperature at hemolysis	Ghost fraction	Content of ^{131}I-albumin (%)	Content of $^{86}RbCl$			
			added at hemolysis		added at reversion	
			(%)	($^{86}Rb/^{131}I$-albumin)	(%)	($^{86}Rb/^{131}I$-albumin)
0 °C	"Total ghost suspension"	100.0	100.0	1.00	100.0	1.00
	A(=type II)	65.2	97.3	1.49	97.8	1.50
	C(=type III)	23.7	0	0	0	0
25 °C	"Total ghost suspension"	100.0	100.0	1.00	66.2	0.66
	A(=type II)	37.6	51.6	1.37	51.6	1.37
	B(=type I)	43.6	34.8	0.80	9.7	0.22
	C(=type III)	2.2	0	0	0	0
37 °C	"Total ghost suspension"	100.0	100.0	1.00	36.0	0.36
	A(=type II)	0.1	12.4		16.5	
	B(=type I)	78.0	80.6	1.03	16.9	0.22
	C(=type III)	4.2	0.4	0.10	0.6	0.14

[a] ^{86}Rb was added either at hemolysis or at reversion. The amount of ^{131}I-albumin incorporated into the ghosts is representative for the number of ghosts. The proportion of $^{86}Rb/^{131}I$-albumin in fraction A was not estimated after 37 °C because of the very low number of ghosts.

Fraction C contains little if any radioactivity, regardless of whether ^{86}Rb was added at hemolysis or during reversal. The behavior of these ghosts suggests that they are identical with those of type III, i.e., that they are leaky for Rb.

A comparison of Fig. 6*a* and 6*c* shows that the fraction of leaky ghosts obtained after hemolysis at 37 °C exhibits a higher density than the corresponding fraction obtained after hemolysis at 0 °C. Possibly, ghosts prepared by hemolysis at the lower temperature release more membrane protein and less lipid into the medium than those prepared at the higher temperature. Alternatively, at the two higher temperatures, the ghosts may lose more lipid and less protein than at 0 °C (*cf.* footnote 1).

The distribution pattern observed with ghosts prepared by hemolysis at 0 and 37 °C was reasonably reproducible. In some experiments, the relative amounts of ghosts in fractions A, B and C corresponded roughly to the relative amounts of ghosts of types I, II and III as determined in simultaneous kinetic experiments by the methods described in the previous section.

In other experiments correspondence was rather poor, especially after hemolysis at 37 °C. The results of density gradient centrifugations of ghosts prepared by hemolysis at 25 °C were less reproducible than the previously described ones and more difficult to interpret.

The ghosts obtained after hemolysis at 25 °C consistently show two bands whose properties allow a comparison with the kinetically defined types of ghosts. The uppermost band, fraction A, shows the behavior anticipated for ghosts of type II. The lower diffuse layer of cells (fraction C) behaves like ghosts of type III (Table 5). However, a quantitative correlation with kinetic data proved to be impossible. Correspondingly, the behavior of fraction B was somewhat erratic. In some experiments (as in the experiment represented in Table 5), fraction B clearly showed the behavior of type I ghosts: radioactivity was primarily incorporated at hemolysis but not at reversal. However, frequently fraction B could not be adequately defined by the criteria employed in the hitherto described experiments.

The results of the density gradient centrifugation remain basically unaltered if the time of centrifugation is increased beyond 1 hr, the time allotted for sedimentation in the experiments described above. This indicates that the separation of the various ghost fractions occurs primarily on account of differences of density. Ghosts of type II (fraction A) reseal after the uptake of electrolytes. As a consequence, their water content is high, their density is low. They are located in the upper part of the density gradient. Ghosts of type I reseal before electrolytes become incorporated. They are shrunken, and hence penetrate deeper into the density gradient. Under the microscope the smaller size and higher concentration of the intracellular hemoglobin inside these ghosts as compared to type II ghosts is immediately apparent. The ghosts of type III seem to be leaky for sucrose. Therefore, their position at the lower end of the density gradient is determined by the high density of their dry matter (membrane constituents plus residual hemoglobin).

The difficulties encountered in our attempts to achieve a quantitative separation of the three types of ghosts by means of centrifugation into a sucrose density gradient are presumably caused by two factors: (1) The ghosts tend to form aggregates. Therefore, ghosts of the prevailing types are likely to carry with them ghosts of the other types. This would tend to reduce the resolution of the method. (2) In concentrated sucrose solutions ghosts become leaky for Rb, even if some other electrolyte (e.g., NaCl) is present. The ghosts may lose up to 50% of the incorporated ^{86}Rb within 1 hr, the time required for the density gradient centrifugation. This effect tends to increase the size of fractions B and C.

Preparation of Kinetically Homogeneous Ghost Populations

Although the separation by sucrose density gradient centrifugation posed a number of problems which we were unable to resolve, the results obtained with this method were presented at some length since they served as a basis for the development of a simple method for the preparation of suspensions which contain primarily ghosts of type II. The preparation of such suspensions may be useful in studies where kinetically homogeneous populations are required.

To obtain such populations, hemolysis is performed at 0 °C and the ghosts are loaded with the cations and substrates of one's choice at that temperature. Hemolysis at 0 °C eliminates the formation of ghosts of type I whose internal composition cannot be manipulated at will, since they reseal spontaneously before anything can be incorporated into them. After resealing at 37 °C the ghosts are washed twice in isotonic salt solution and are then resuspended to form a 30% (v/v) suspension. This suspension is layered on top of a sucrose cushion (43% sucrose in a solution containing 25 mmoles/liter NaCl and 25 mmoles/liter Tris, pH 7.4). Centrifugation at $34{,}800 \times g$ for 1 hr leads to the sedimentation of nearly all of the leaky ghosts. For this procedure, the SS 34 angle rotor of a Sorvall RC 2-B centrifuge may be used. The resealed ghosts form a skin on top of the sucrose solution. They can be aspirated into a pipette and be used for flux measurements after resuspension in a suitable medium. During removal of the ghosts from the surface of the sucrose cushion, mixing of the ghosts with the underlying sucrose solution must be avoided. Mixing may result in an irreversible damage to the membrane.

To demonstrate that centrifugation across a sucrose cushion leads indeed to a separation of leaky and resealed ghosts the following experiment was performed (Fig. 7): Ghosts were hemolyzed at 0 °C, loaded with ^{86}Rb, resealed at 37 °C for 50 min, and subsequently layered on top of the sucrose cushion as described above. Since the ghosts were neither separated from the hemolysate nor washed there was no loss of radioactivity from the leaky ghosts prior to the centrifugation across the sucrose cushion. ^{86}Rb distribution between resealed or leaky ghosts and the hemolysate was at equilibrium throughout the whole centrifugation procedure. After centrifugation, the resealed ghosts were collected and resuspended in isotonic Tris-NaCl solution. ^{86}Rb exit was followed. The initial point (zero time in Fig. 7) was obtained after correction for trapped hemolysate by means of an estimate of ^{131}I-albumin dilution as described under Materials and Methods. A comparison with the original batch of untreated ghosts shows that after the centrifugation procedure the initial rapid phase of ^{86}Rb exit disappeared.

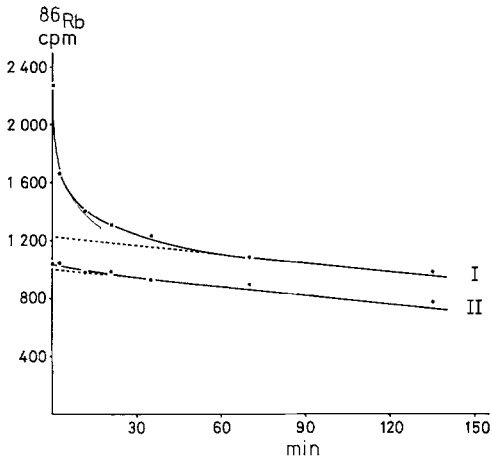

Fig. 7. ^{86}Rb efflux from type II ghosts before (curve *I*) and after (curve *II*) removal of leaky ghosts (type III). Hemolysis and reversion were performed at 0 °C in the presence of ^{86}Rb. Resealing was achieved by incubation at 37 °C for 50 min. The separation of intact ghosts of type II from leaky ghosts of type III was performed by centrifugation of the ghost suspension through a sucrose cushion as described in the text. *Ordinate:* ^{86}Rb content of the ghosts of 1.0-ml suspension in counts/min. *Abscissa:* time in minutes

Apparently, the separation procedure yields a fairly uniform suspension of type II ghosts.

The efficiency of the described separation technique can be inferred from the following experiment. After incorporation of ^{131}I-albumin during hemolysis at 0 °C and subsequent addition of ^{86}Rb at that temperature, the ratio ^{86}Rb/^{131}I-albumin in the hemolysate amounted to 0.477 (arbitrary units, average of 11 separate experiments). After washing the resealed ghosts twice, this ratio dropped to 0.340. This indicates the removal of ^{86}Rb from the ghosts which, although leaky for Rb, remain impermeable to ^{131}I-albumin. After separation of the intact ghosts from the leaky ones by centrifugation through the cushion the ratio ^{86}Rb/^{131}I-albumin was back to 0.477. The yield of intact ghosts (type II) was 58.5% and that of leaky ghosts (type III) was 32.5%. Nine percent of the ghosts could not be recovered.

For many purposes, a separation of leaky and intact ghosts is unnecessary. It is sufficient to avoid the formation of type I ghosts by hemolyzing and loading at 0 °C and to wash out the radioactivity which is trapped in the leaky ghosts. Any further loss of radioactivity from the resulting ghost population represents leakage from resealed ghosts of type II. If one wants to avoid washing the ghosts before the start of the flux measurements, it is even possible to resuspend the resealed ghosts after sedimentation

in the desired back exchange medium and to ignore the initial rapid phase of leakage. The permeability of the resealed membrane is represented by the later, slow phase of transfer.

Separation of ghosts by means of a sucrose cushion proved to be indispensable in experiments on the iodide permeability of red blood cell ghosts. Iodide penetrates so fast that the rates of leakage from broken and resealed cells overlap. The rate constant of iodide exchange across the membranes of isolated reconstituted ghosts were, within the limits of experimental error of the flux determinations, identical to those of intact red blood cells (Wood & Passow, 1971).

Time Course of Resealing

In the previously described experiments, the ghosts were kept at the temperature at which hemolysis took place for a fixed length of time, 5 min. Subsequently, salt was added to restore isotonicity ('reversal'). Those ghosts which did not reseal before reversal, incorporate the added salt. This salt is later trapped inside the cells when the resealing process continues. This process is accelerated by raising the temperature to 37 °C.

Fig. 8 shows by means of the kinetic method (*cf.* pp. 5–9) that increasing the time interval between hemolysis and reversal at 25 °C leads to an increase of the percentage of ghosts of type I at the expense of ghosts of type II. Table 6 illustrates the same phenomenon by means of data which were obtained by separating the ghost fractions on a density gradient.

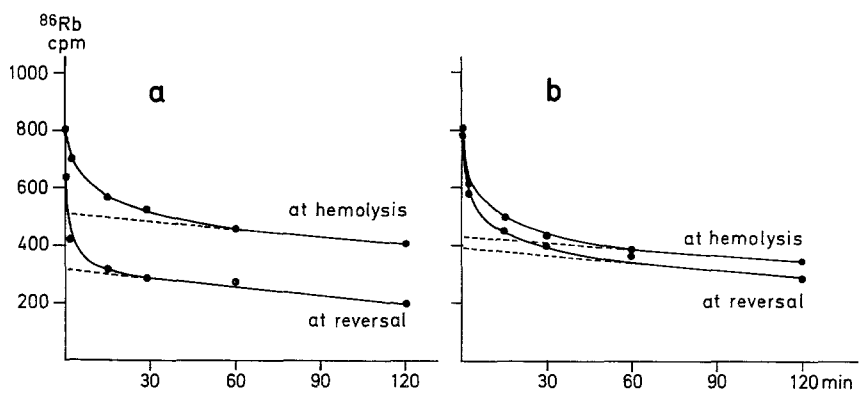

Fig. 8. Effect of varying the time interval between hemolysis and reversion on ^{86}Rb incorporation at hemolysis and reversal. Hemolysis and reversal were performed at 25 °C. The dashed lines were used to estimate the relative size of the three ghost fractions: (a) reversion 30 min after hemolysis; (b) reversion 5 min after hemolysis. *Ordinates:* ^{86}Rb content of the ghosts of 1.0-ml suspension. *Abscissas:* time in minutes

Table 6. Percentage distribution of the ghost fractions B(=type I, before reversal), A(=type II, resealed after reversal), and C(=type III, not resealed) as a function of the time interval between hemolysis and reversal[a]

Time between hemolysis and reversal	Type I (%)	Type II (%)	Type III (%)
1 min	35.58 ± 7.02	47.05 ± 3.20	17.37 ± 5.82
5 min	47.08 ± 6.45	38.75 ± 3.17	14.17 ± 4.44
30 min	59.90 ± 9.94	25.60 ± 5.37	14.50 ± 5.89

[a] The size of the fraction was determined by density gradient centrifugation. The numerals represent mean values and the corresponding standard deviations. 25 °C.

If hemolysis is performed at 37 °C, a continuation of incubation at that temperature to more than 5 min does not increase the yield of ghosts of type I. All ghosts which are potentially capable of resealing actually reseal within this time period; there are no ghosts of type II. If hemolysis and subsequent incubation take place at 0 °C the rate of resealing is so low that no ghosts of type I are formed within the time of experimental observation.

Resealing without Reversal

The previously described experiments suggest that ghosts can be resealed without restoration of isotonicity (i.e., without going through Step IV) by incubation of the hypotonic hemolysate at 37 °C. Fig. 5 shows that this is in fact observed. After hemolysis at 0 °C in the presence of trace amounts of ^{86}Rb and ^{22}Na, the hemolysate was transferred to 37 °C. The time course of resealing was followed by taking samples of the hemolysate after suitable time intervals. After centrifugation the sedimented ghosts were resuspended in Tris-NaCl media of the same tonicity which existed in the original hemolysate. The efflux of the radioactivity was followed. Fig. 5 shows that the amount of trapped radioactivity increases with increasing length of incubation prior to the flux measurements. This finding indicates that the addition of salt is not a necessary prerequisite for the reconstitution of the integrity of the ghost membrane after hemolysis. Fig. 5 also shows that the time course of ^{86}Rb leakage from the resealed ghosts is rather similar to that of ^{22}Na and, during the time period covered in the experiment, independent of the length of the resealing period which preceeded the flux measurements.

Effects of Alkaline Earth Ions and Complexing Agents in the Hemolyzing Medium

It has been shown by Hoffman (1962*b*) that alkaline earth ions and complexing agents considerably affect the yield of resealed ghosts if present in the hemolyzing medium. Hoffman hemolyzed the cells at room temperature only. In view of the marked influence of temperature at hemolysis on the properties of the resulting ghost population, it seemed worthwhile to extend Hoffman's work on the role of these agents by studying their effects at various temperatures.

If present in the hemolyzing medium at 0 °C, Mg^{++} has no effect on K retention of the ghosts. However, if present at 25 °C, there is a considerable increase of the amount of K retained by the cells after reversal and resealing at 37 °C (Fig. 9). The effect increases with increasing Mg^{++} concentrations. It remains to be decided whether this finding entirely reflects a specific action of Mg^{++} or if the augmentation of the tonicity of the hemolyzing medium also contributed to the result.

At concentrations below 0.01 mmoles/liter, Ca^{++} has effects similar to those of Mg^{++}. However, above this concentration the effect passes through a maximum. At 1.0 mmole/liter, Ca^{++} completely prevents the recovery of the impermeability of the ghost membrane to K (Fig. 9). At first glance, this result seems to conflict with previous findings of Hoffman (1962*b*). However, a closer comparison of Fig. 2 in Hoffman's paper with

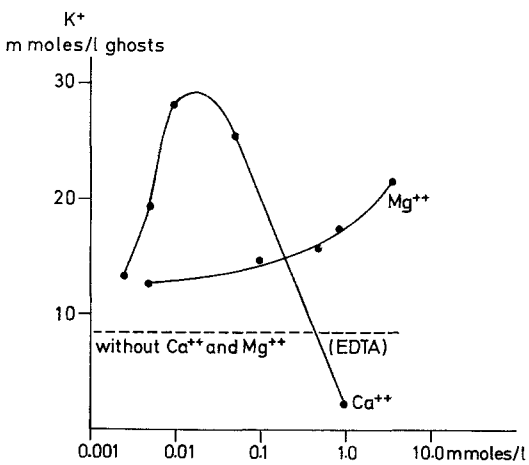

Fig. 9. Effects of Ca^{++} or Mg^{++} in the hemolyzing medium on the resealing of ghosts. Temperature at hemolysis, 26 °C. Reversal by KCl, final concentration in the hemolysate 150 mmoles/liter. Resealing at 37 °C for 2 hr. *Ordinate:* K^+ content of the ghosts of type II after 60 min of incubation in the absence of alkaline earth ions in Tris-NaCl, pH 7.2 at 37 °C. *Abscissa:* concentration of alkaline earth ions in the hemolyzing medium in mmoles/liter

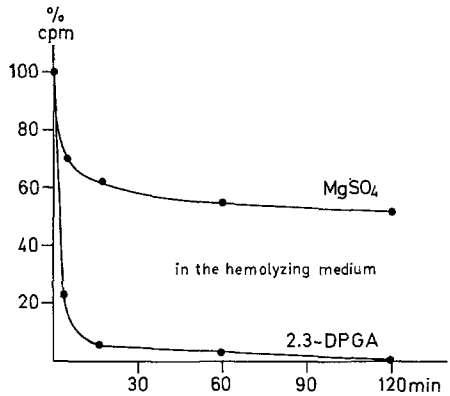

Fig. 10. Effect of 2,3-diphosphoglycerate (2,3-DPG) (1 mmole/liter) in the hemolyzing medium on ^{86}Rb retention of ghosts. Hemolysis at 23.5 °C. Reversal with KCl 5 min after hemolysis. Resealing at 37 °C for 1 hr. For comparison, the effect of the presence of 4 mmoles/liter $MgSO_4$ in the hemolyzing medium is also illustrated. The measurements were initiated by resuspension of the ^{86}Rb-loaded ghosts in Tris-NaCl. *Ordinate:* ^{86}Rb in percent of initial value. *Abscissa:* time in minutes

our data shows that we cover with many data points a concentration range which in Hoffman's paper is represented by two points only. Hence, he may have missed the maximum. We do agree, nevertheless, that at Ca^{++} concentrations above 0.1 mmoles/liter, K retention is lower than in the absence of Ca^{++}.

Complexing agents are capable of preventing the recovery of the ghost membrane from the hemolytic shock. However, they exert this effect only if present in the medium at the instant of hemolysis. If added after hemolysis, they do not interfere with the resealing process.

The efficiency of the complexing agents to alter the membrane during the hemolytic event is greatly affected by the temperature at which hemolysis takes place. After hemolysis at 25 °C or 37 °C, there is little or no resealing of the ghosts. However, if hemolysis is performed at 0 °C, the complexing agent does not prevent the subsequent resealing of the membrane for alkali ions during incubation at 37 °C. If the resealing process is allowed to take place in the presence of the complexing agents, then these agents themselves become trapped inside the ghosts.

Below, a number of experiments will be described which illustrate the effects of complexing agents.

Fig. 10 demonstrates leakage from ghosts which were obtained by hemolysis at 23.5 °C in the presence of 2,3-diphosphoglycerate (2,3-DPG) in the hemolyzing medium. This complexing intermediate of red cell metabolism completely prevents the recovery of the normal, low permeability to K. Density gradient centrifugation at the end of the resealing period shows that

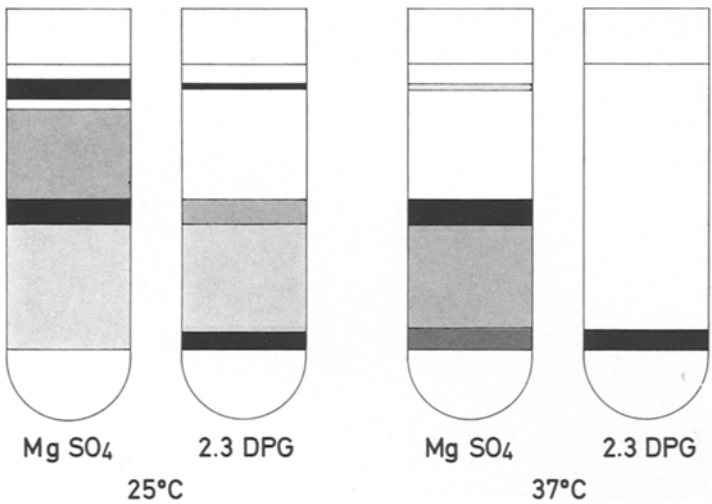

Fig. 11. Density gradient centrifugation of ghosts which were prepared by hemolysis in the presence of either $MgSO_4$ (4 mmoles/liter) or 2,3-DPG (4 mmoles/liter) at 25 °C and 37 °C, respectively

the ghost fractions A and B are greatly reduced while fraction C (ghosts of type III) is considerably increased (Fig. 11). This effect is even more pronounced if hemolysis is performed at 37 °C. If 2,3-DPG is present in the hemolyzing medium at that temperature, only one band can be found near the lower end of the density gradient. This band represents ghosts of type III, i.e., leaky ghosts.

Fig. 12 shows the incorporation of potassium and either ATP or ^{14}C-EDTA into ghosts which were subjected to a resealing period at 37 °C after hemolysis at 0, 20 or 37 °C in the presence of one or the other of the two agents. At the end of the resealing period the ghosts were washed 2 times in isotonic NaCl solution. This removed K and complexing agents from leaky cells (type III). Hence, the curves represent the intracellular concentrations in the ghosts of type I and II as a function of time. It is interesting to note that it is possible to incorporate into the ghosts complexing agents whose complex forming capacities were not neutralized by the addition of divalent metal ions. Incorporation was increased if the temperature at hemolysis was lowered. The subsequent elevation of the temperature to 37 °C during the resealing period did not enable the complexing agents to prevent the recovery of the membrane. This finding suggests that the complexing agents remove some metal ions from the red cell membrane which are only accessible to them when they penetrate across the membrane during the hemolytic event. There is no simple explanation for the finding that, on their way through the membrane, the complexing agents remove

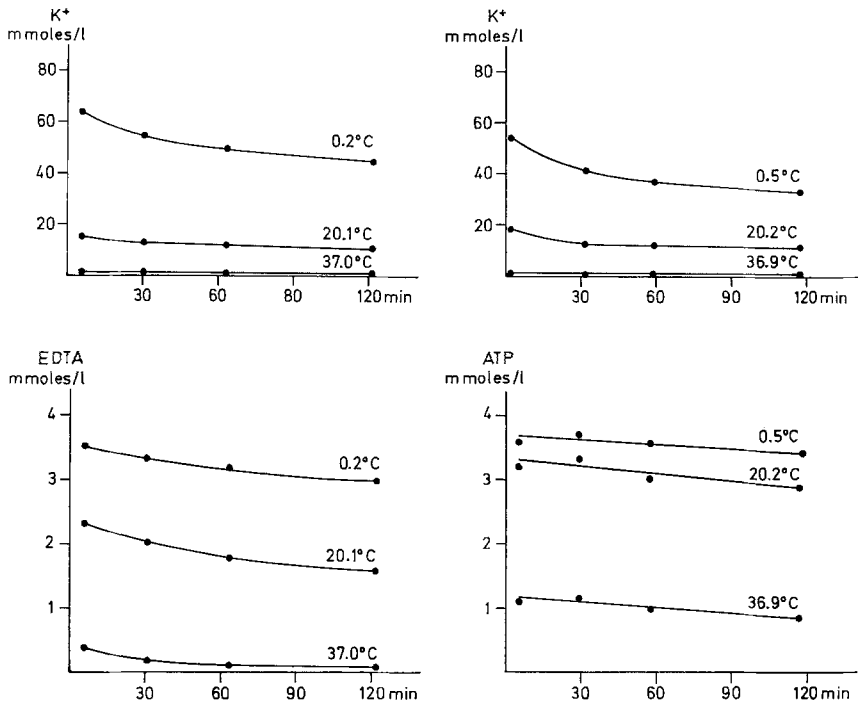

Fig. 12. Retention of K^+, ATP and EDTA by erythrocyte ghosts. Hemolysis at the temperatures indicated on the curves. The concentration of the complexing agents in the hemolyzing medium was 4 mmoles/liter. Reversal by addition of KCl, 5 min after hemolysis. Resealing at 37 °C for 60 min. *Ordinates:* K^+, EDTA and ATP concentrations within the ghosts in mmoles/liter ghosts. *Abscissas:* time in minutes

the metal ions only at elevated temperatures but not at 0 °C. Perhaps, at the lower temperature, the buried metal ions are not fully exposed to the penetrating chelators. Alternatively, the affinity of the membrane ligands to the fully exposed metal may exceed that of the penetrating ligands at low but not at elevated temperatures.

The described results are of practical significance since they show how complexing agents can be incorporated into the red cell ghosts. The incorporation of EDTA or ATP into ghosts during hemolysis at low temperature was used by Lepke and Passow (1968) in their work on the action of internal and external complexing agents on the K permeability of cells which were exposed to $CaCl_2$ and NaF.

Discussion

The starting point for the present investigation was the comprehensive work of Hoffman (1962*a*) on the heterogeneity of suspensions of red blood cell ghosts. Hoffman discriminated between three types of ghosts: (1)

Group I ghosts which appeared to recover their impermeability to Na but not to K without 'reversal', i.e., without restoration of isotonicity of the hemolysate by the addition of salt ('spontaneous recovery'). (2) Group II ghosts which required reversal for the restitution for the relative cation impermeability. After hemolysis, group II ghosts remained permeable to K and Na. Relative impermeability could be induced by increasing the tonicity of the medium. This 'induced recovery' could be enhanced by incubation at 37 °C. In addition to group I and group II ghosts, Hoffman observed that a certain fraction of the ghosts always remained leaky. These ghosts were designated group III ghosts.

For the interpretation of our data on the effects of temperature at hemolysis on the subsequent reconstitution of the ghost membrane, a differentiation between three types of ghosts proved to be essential. However, the description of our findings is facilitated if the group I and group II ghosts are redefined along somewhat different lines than in Hoffman's paper.

For the interpretation of our results, it is not necessary to discriminate between 'spontaneous' and 'induced' reconstitution. The experiments presented in Fig. 5 show that under our conditions, ghosts reseal without increasing the tonicity above the level reached in the hemolysate at the end of the hemolytic process (about 35 'ideal' milliosmoles). We did not compare the rates of resealing in the presence and absence of added salt at 37 °C. However, our experiments showed that the number of resealed ghosts reaches its maximal value within about 1 hr. This rate is similar to that observed by Hoffman *et al.* (1960) with 'reconstituted' ghosts, i.e., with ghosts suspended in a hemolysate whose osmolarity was restored to isotonicity by the addition of KCl after hemolysis. Although there may exist some small differences between the rates of resealing in the presence and absence of salt, under our conditions, the decisive variable for the reconstitution of leaky ghosts is, apparently, not the ionic strength but just the time for which the ghosts were kept at a particular temperature. At 0 °C, resealing is so slow that we could not measure it. At 37 °C, resealing is so fast that, if hemolysis is performed at that temperature, nearly all ghosts which potentially possess the capacity to reseal at that temperature will do so within less than a few minutes. Therefore, our definition of type I and type II ghosts only discriminates between ghosts which were already resealed at the time of reversal and those which are bound to reseal in the course of further incubation.

At the instant of reversal, salt enters the leaky ghosts. Subsequently, increasing numbers of leaky ghosts (type III) reseal (i.e., turn into type II

ghosts) and trap the salt which entered after reversal. If one waits until all ghosts which are potentially capable of resealing actually regained their relative impermeability, one obtains a maximal number of type II ghosts. This number decreases as the time interval between hemolysis and reversal increases. Correspondingly, the number of type I ghosts increases at the expense of type II ghosts (*cf.* Table 6). In summary, our definition of type I and type II ghosts is purely operational and does not imply the existence of any fundamental difference between type I and type II ghosts of a population. However, it presumes that the rates of resealing of the individual cells differ.

It is interesting to note that the slopes of the curves relating ^{86}Rb content of the resealed cells to time is fairly independent of the length of the resealing period preceding the flux measurements (Fig. 5). This finding suggests that, just like hemolysis, the resealing process is all or none.

This statement does not imply that resealing is necessarily a cooperative phenomenon. A gradual decrease of the size of a channel could eventually lead to a critical size below which the penetration for a given particle may fairly abruptly become impossible. The assumption that gradual changes in the membrane eventually lead to sudden changes of permeability is supported by the finding that, at 0 °C, the membrane of type II ghosts is much less permeable to ^{131}I-albumin than to ^{86}Rb. After transfer to 37 °C, the membrane reseals very quickly to albumin but much more slowly to Rb. In unpublished experiments, we were able to show the presence of ghosts which were impermeable for sucrose but not for Rb. Similarly, the slight discrimination between Na^+ and K^+ in ghosts of group I described by Hoffman (1962*a*) may fall into the same category and demonstrate that at the time when he performed his measurements, the channel size had decreased to a point where only K could penetrate.

The effects of alkaline earth ions and complexing agents on the ghost membrane suggest that some divalent cations, possibly Ca^{++} or Mg^{++}, participate in the maintenance of cation impermeability. However, the evidence presented so far does not rule out that, in the intact red cell membrane, Mn, Zn, or some other metal ion plays the role which, in our ghost preparations is played by Ca^{++} or Mg^{++}.

In contrast to Mg^{++}, Ca^{++} exerts a dual action on the ghost membrane. If present at concentrations below 0.01 mmoles/liter, it produces the described facilitation of the resealing process. At higher concentrations, it evokes K loss. This latter effect is also observed in ghosts which were resealed before exposure to the alkaline earth ions. (Romero & Whittam, 1971; Riordan & Passow, 1971).

The complexing agents exert their action on the ghosts during the hemolytic event while they are penetrating across the membrane (*cf.* page 20). On their way through the membrane, they can remove the 'membrane glue' (Hoffman, 1962*b*) only at elevated temperatures but not at 0 °C. This behavior enables one to incorporate into the ghosts complexing agents whose ligands are not neutralized by the presence of alkaline earth ions. After hemolysis at 0 °C in the presence of a complexing agent, elevation of the temperature to 37 °C leads to a recovery of the membrane is spite of the presence of the agent. This surprising finding possibly resolves some contradictions existing in the literature.

In his famous paper, Straub (1953) described the incorporation of ATP into red blood cell ghosts by means of hypotonic hemolysis in ice cold media. After transfer of the ghosts to 37 °C, active K and Na movements could be measured. These movements were energized by ATP. Straub's experiments were later criticized by Hoffman (1962*a*). On the basis of his observations about the effects of ATP and EDTA in the hemolyzing medium on the resealing of ghosts, Hoffman concluded that it was impossible to incorporate, in the absence of alkaline earth ions, complexing agents into the ghosts. No alkaline earth ions were present under Straub's conditions. Hence, Hoffman suggested that in Straub's experiments only intact red blood cells which escaped hemolysis accomplished the active transport. Hoffman performed hemolysis at 23 °C. At this temperature, there is, in fact, no resealing after hemolysis in the presence of ATP. However, as shown in Fig. 12 of this paper, under Straub's conditions, an incorporation of ATP into ghosts is certainly feasible. Straub hemolyzed one part of cells with two parts of hemolyzing medium. Under these conditions, some cells may escape hemolysis. At low pH values, the surviving cells are capable of taking up some ATP. Hence, it cannot be excluded that in Straub's experiments some of the active transport was accomplished by ATP-loaded intact red blood cells (J. F. Hoffman, *personal communication*). Nevertheless, ATP-filled ghosts must have contributed significantly to the observed effects.

The normal red blood cell contains many complexing agents as metabolic intermediates, notably ATP and 2,3-DPG. As can be inferred from the data presented in Pennel's paper (1964) on the composition of the red blood cells, the concentrations of these agents exceed those of the intracellular alkaline earth ions considerably. One should expect, therefore, that the penetration of the complexing agents across the membrane during hemolysis should contribute to the formation of type III ghosts. This does not seem to be the case at 0 °C since addition of Mg^{++} to the hemolyzing

medium does not affect the yield of type III ghosts. At 37 °C where the yield of type III ghosts reaches values up to 75%, the addition of Mg^{++} to the hemolyzing medium also does not reduce the number of type III ghosts. Only around 25 °C are the alkaline earth ions markedly effective in decreasing the yield of leaky ghosts (*unpublished results*). Hence, it remains unclear why a fairly reproducible fraction of the ghosts does not reseal even after prolonged incubation at 37 °C.

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