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The Kinetics of Resealing of Washed Erythrocyte Ghosts

Robert M. Johnson

Department of Biochemistry, Wayne State University, School of Medicine,
540 E. Canfield Avenue, Detroit, Michigan 48201

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Summary. The kinetics of resealing, defined as the recovery of impermeability to macromolecules, of well-washed human erythrocyte ghosts has been determined. The resealing process is first-order at temperatures above 20–25 °C in isotonic salt, with rate constants ranging from 0.01–0.15 min^{-1}. Below 20 °C, resealing occurs, but a long lag period is observed. Other erythrocyte membrane properties suggest a transition at about 20 °C, and it is possible that resealing rates are a measure of membrane fluidity. A temperature-induced reduction of resealed ghost volume was also observed. The effect of ionic strength on resealing parameters was determined. Low ionic strength buffers prevent resealing, which is also consistent with resealing as a lipid-related event. The effect of microtubule disrupting drugs and changes in the method of preparing ghosts are also described.

Most biochemical studies of erythrocyte membranes have used the washed ghost preparation pioneered by Dodge, Mitchell and Hanahan (1963). In contrast, studies of cation and nonelectrolyte transport or recovery of impermeability to solutes ("resealing") have for the most part, utilized an unwashed ghost preparation obtained by hypotonic hemolysis of erythrocytes, followed immediately by restoration of isotonicity with salt (for a review, *see* Bodemann & Passow, 1972). This divergence in membrane studies was in part due to the belief that extensively washed ghosts cannot be resealed (Steck, 1972*a*). More recently, however, it has become clear that washed erythrocyte membranes can reseal, and become impermeable to macromolecules (Kirkwood, Hudson & Brown, 1972; Steck, 1972*b*; Shin & Carraway, 1973; Taverna & Langdon, 1973; Boxer, Jenkins & Tanner, 1974), ATP (Bramley, Coleman & Finean, 1971; Hanahan, Ekholm & Hildebrandt, 1973), and to a certain extent, mannitol and related carbohydrates (Jung, Carlson & Balzer, 1973). This resealing involves reasonably intact ghosts, and can be distinguished from the formation of small impermeable vesicles from fragmented ghosts (Steck *et al.*, 1970). The resealing characteristics of the erythrocyte ghost, like many

other properties (Ponder, 1961; Bramley *et al.*, 1971; Bramley & Coleman, 1972; Hanahan *et al.*, 1973), depend on the exact preparation procedure followed. We wish to report here the kinetics of the recovery of membrane impermeability to macromolecules of well-washed ghosts prepared in low ionic strength buffers; in particular, the temperature dependence, ionic strength dependence and the effect of hemolyzing and washing solutions containing varied concentrations of NaCl and EDTA.

Materials and Methods

Bank blood in CPD solution was obtained from the Southwest Michigan Red Cross Blood Center, and used within one week of donation. Blood type does not seem to affect our results. Ghost preparation followed the procedure of Dodge *et al.* (1963), as modified by Kirk (1968). The erythrocytes were washed 3 times in 5 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1 mM Na_2 EDTA, removing the buffy coat each time, and the packed cells were hemolyzed by the addition of 2.85 volumes of 5 mM Tris-Cl, pH 7.4, 7 mM NaCl, 1 mM Na_2 EDTA. The ghosts were collected by centrifugation at $13{,}000 \times g$ for 20–40 min, washed twice in the hemolyzing buffer, and twice in 5 mM Tris-Cl, pH 7.4, 7 mM NaCl. At this point, the ghosts are white or slightly pink. During the washes, care was taken to remove the pellet found at the bottom of the centrifuge tube, which is reported to have protease activity (Fairbanks, Steck & Wallach, 1971). The ghosts are kept cold during the procedure. Seen in the phase microscope, the preparation contains intact ghosts, with approximately equal proportions of disc- and cup-shaped cells. Ghost preparations are used immediately, as they rapidly (within a few hours) lose their ability to reseal, as measured by our techniques. Overnight storage at 0 °C destroys all resealing properties. This may be due to slow resealing of ghosts occurring at 0 °C (*see below*) resulting in a preparation which can no longer take up macromolecules, or to some unknown alteration of the ghosts occurring during storage at 0 °C in low-ionic strength buffers. The preparations appear intact by phase microscopy even after a week's storage under these conditions; however, storage in buffers of lower osmolarity than that used here is known to lead to extensive fragmentation (Bramley *et al.*, 1971), and it is likely that changes such as loss of protein occur during storage at the osmolarity used in this preparation.

The above procedure describes our routine preparation. For studies of the effect of preparation buffers on ghost properties, 5 mM Tris-Cl, pH 7.4 with different concentrations of NaCl or Na_2 EDTA was employed. These variations will be noted as they occur.

Measurement of Resealing Rates

5 mM Tris-Cl, pH 7.4, was used throughout. A buffer solution with NaCl and specific concentrations of macromolecules or small molecules, to which the intact membrane is normally impermeable, was brought to the experimental temperature (usually 37 °C) in a shaking water bath. A small aliquot of freshly prepared packed ghosts, still in the final wash solution, was placed in the bath for exactly 2 min, which suffices to bring them to the experimental temperature. They are then mixed with the buffered macromolecule solution at time zero. Normally, 2.0 ml ghosts was mixed with 3.0 ml buffer. Aliquots (0.2–0.3 ml) of this incubation were taken at various times and quenched in 11 ml ice-cold 5 mM Tris-Cl,

pH 7.4, 140 mM NaCl. Resealing is stopped by the combination of low temperature and dilution, while isotonic saline preserves those ghosts that have resealed. Controls show that quenched aliquots can be stored on ice up to 1 hr without altering the results. The ghosts are collected by means of a 25-min spin in a clinical centrifuge. The supernatant was carefully aspirated. The pellet was suspended in 5 ml cold 5 mM Tris-Cl, 140 mM NaCl, pH 7.4 with a vortex mixer, centrifuged and the wash aspirated as before. The packed ghosts were lysed with 1.5 ml 0.1% NH_4OH, and the membranes pelleted in the clinical centrifuge. The supernatant was analyzed for the particular probe molecule used. Most of the experiments reported here used hemoglobin as the macromolecule trapped during resealing, because it is readily available and easily quantitated. In such cases, the absorbance at 415 or 542 nm was measured. The hemoglobin solution used was a hemolysate extensively dialyzed against the incubation buffer. In a typical run, the ghost concentration is 2×10^9 ml^{-1}, and hemoglobin is 30 mg ml^{-1}. In a few experiments, hemoglobin from Sigma was used, with no difference in the result. In other experiments, [^{125}I]-β-lactoglobulin, [^{14}C]-inulin (Amersham-Searle), or [^{14}C]-mannitol (New England Nuclear) were used, and the lysed ghost supernatant was analyzed by gamma counting, absorbance, or scintillation counting as appropriate. An aliquot of the incubation mixture was also assayed for the amount of probe molecule, and the fraction incorporated by the washed resealed ghosts calculated. From this number, and the number of ghosts in the incubation determined by Coulter counting, the average volume of the resealed ghosts is determined.

In resealing experiments performed to compare the effects of temperature or ionic strength, all incubations were begun within a 10-min period in order to minimize the effects of ghost instability.

Since hemoglobin was used in many of the resealing experiments, the question of its binding to the membrane must be considered (Dodge *et al.*, 1963). The evidence that the hemoglobin found in resealed ghost membranes is trapped within the ghost's internal volume, rather than nonspecifically bound to the ghost membrane, is as follows: a) no hemoglobin is found in the ghost pellet after incubation at 0 °C or in the absence of salt; b) at pH 7.4 (in contrast to pH 6.2) no hemoglobin binding to washed ghosts is observed; moreover, hemoglobin bound at pH 6.2 is released at pH 7.4 and above (Mitchell, Mitchell & Hanahan, 1965); c) the kinetics of resealing are very similar, whether hemoglobin, β-lactoglobulin, or inulin is used as a probe (Fig. 5); d) the hemoglobin found in ghosts prepared in high salt buffers (Dodge *et al.*, 1963) is very likely trapped within resealed membranes or derived from nonlysed erythrocytes, rather than absorbed to the membrane surface since lowering the ionic strength will release the hemoglobin (Bramley *et al.*, 1971); e) lysis of the resealed ghosts with saponin rather than with low ionic strength buffers does not change the kinetics of resealing.

Determination of Ghost Number and Size Distribution

The ghost preparations, before incubation, were diluted with 0.9% saline and counted in the Coulter Model B. In general, the recommendations of Miller, Wuest and Cowan (1972) were followed. Size distribution plots were determined with the Model J plotter attachment. The ghost preparations contain $6 \pm 0.5 \times 10^9$ particles ml^{-1}, except when NaCl is omitted from the ghost preparation buffers. The ghosts fragment under these conditions and particle counts reach 20×10^9 ml^{-1}. The percentage of the ghost volume accessible to hemoglobin in preparations before incubation was determined by mixing equal volumes of the ghost preparation and a dialyzed hemoglobin preparation in 5 mM Tris-Cl, pH 7.4, 7 mM NaCl at 0 °C. The ghosts were immediately centrifuged and the absorbance of the supernatant was compared to the absorbance expected on the basis of a one-half dilution:

$$\text{Percent of ghost volume accessible} = (A_o - A)/A \times 100$$

where A_0 is the absorbance of the original hemoglobin solution and A is the absorbance of the supernatant after mixing.

Hemoglobin concentrations in ghost preparations were determined by absorbance at 415 or 532 nm (Drabkin, 1946), or by the cyanmethemoglobin method (Aculute, Ortho Diagnostics).

Results

Kinetics of Resealing

The rate of resealing of washed erythrocyte ghosts in isotonic salt solutions can be determined from the amount of impermeant molecule trapped within the resealed ghosts as a function of time. From the concentration of impermeant molecule in the incubation medium, which can be assumed constant throughout the run, and the number of ghosts present, as determined by Coulter counting, the apparent resealed volume per ghost can be calculated. This is plotted as a function of time in Fig. 1, for an experiment using hemoglobin as impermeant molecule, in an isotonic salt solution at 37 °C. Resealing, measured in this way, is an apparent first-order process (*see* insert Fig. 1) with $k = 0.078\ \text{min}^{-1}$ and a final resealed volume of 14.0 fl. The final resealed volume remains unchanged

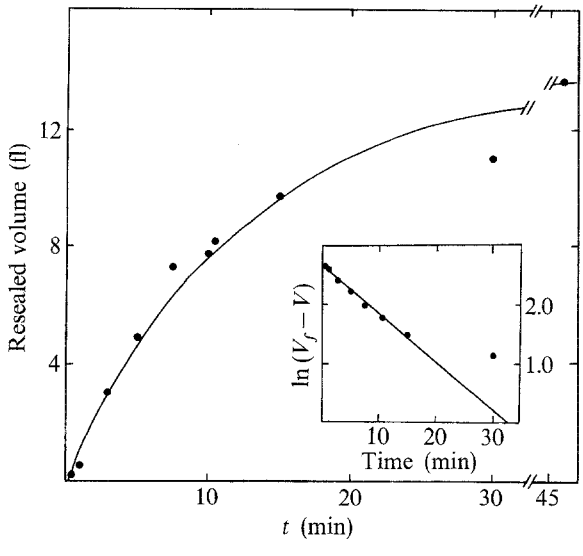

Fig. 1. The resealing of washed erythrocyte ghosts, as a function of time using hemoglobin incorporation as a measure of resealing. Resealed ghost volume is calculated from the amount of hemoglobin and the number of ghosts in the incubation. 5 mM Tris-Cl, pH 7.4, 134 mM NaCl, 37 °C. The line is the calculated graph for first-order kinetics with $k = 0.078\ \text{min}^{-1}$, and final resealed volume = 14.0 fl. *Inset:* semilogarithmic plot of the data

if the incubation is followed for 2 more hours. Variation of either ghost or macromolecule concentration over a 10-fold range does not affect the kinetics. The measured limiting volume per ghost varies considerably between experiments, ranging from 10 to 20 fl, at 37 °C, when hemoglobin is the impermeant molecule. The upper limit of this range is smaller than the average volume of the unsealed ghosts, which we estimate from Coulter Model J size distribution plots to be about 50–60 fl, in agreement with Palek, Curby and Lionetti (1972).

Temperature Dependence of Resealing Kinetics

Resealing plots for different temperatures are shown in Fig. 2. In these incubations, NaCl concentration was 140 mM. At the lower incubation temperatures, a long lag time is seen before resealing begins. This lag time becomes progressively shorter as the temperature is raised. Above about 20 °C resealing kinetics become first order. The length of the lag period at temperatures $\leqq 10$ °C is not known. While resealing at 0 °C does not occur to any measurable extent during the first 5–6 hr, ghosts stored at that temperature overnight (ca. 20 hr) will contain trapped hemoglobin. This lag period appears to reflect a temperature-dependent rearrangement of the membrane occuring prior to the observation of resealing at measurable rates.

Above 20 °C, the resealing time course is close to exponential. The first-order rate constant increases with temperature for a typical washed ghost preparation as shown in Table 1. V_0 is the limiting value for apparent

Table 1. Kinetic parameters for resealing, as a function of temperature

Temperature (°C)	k (min^{-1})	V_0 (fl)	Modal volume (fl)
20	0.013	30.5	60
22.5	0.018	31.5	61
25	0.036	29	28
30	0.077	19	28
35	0.114	17	28
40	0.134	14.5	30

The values are obtained for the experiment shown in Fig. 2. V_0 is the final apparent resealed volume, taken as the asymptote of the resealing curves. Modal volume is obtained using the Coulter Model J plotter. The experiments performed at 10°, 15°, and 17.5 °C are omitted from this table as the kinetics are not exponential. The modal volumes obtained for ghosts incubated at these temperatures for 5–6 hours are: 10°, 65 fl; 15°, 50 fl; 17.5°, 55 fl.

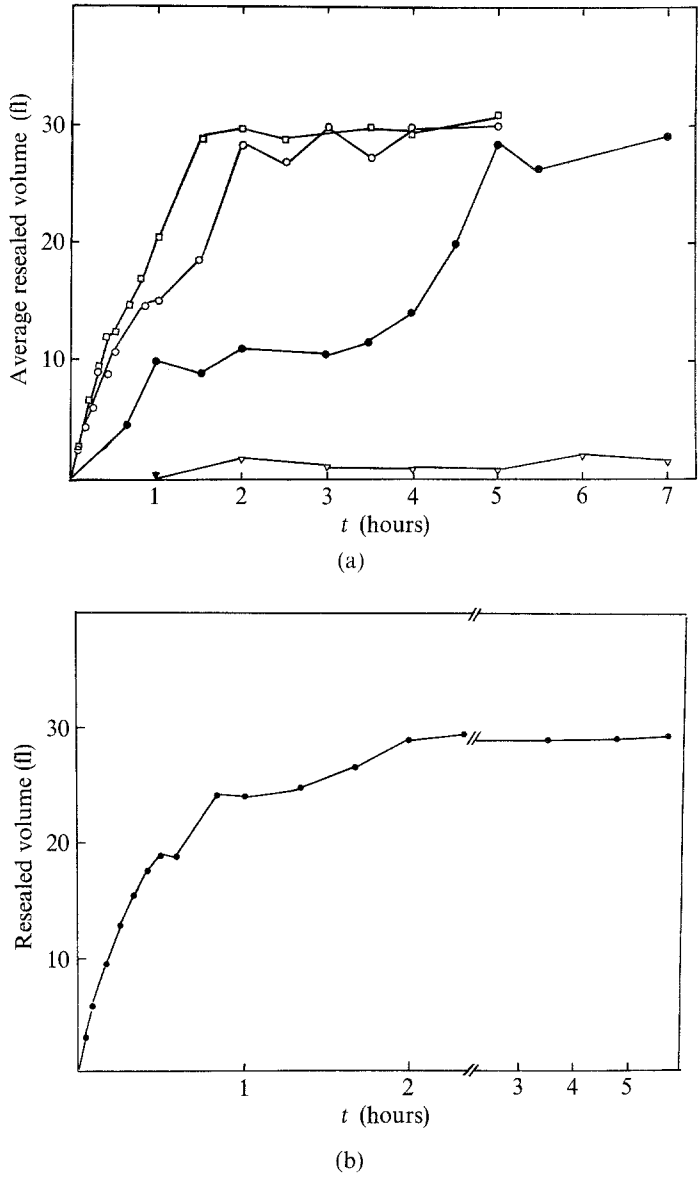

Fig. 2. Variation of resealing kinetics of washed ghosts with temperature. 1.6×10^9 ghosts ml^{-1} in 150 mM NaCl, 5 mM Tris-Cl, pH 7.4. Hemoglobin as impermeant marker. The points are the averages of two runs. (*a*) Resealing at 10° (▽), 15° (●), 20° (○), 22.5° (□). (*b*) Resealing at 25°. (*c*) Resealing at 30° (●), 35° (○), 40° (△)

resealed volume at long times. An Arrhenius plot of this data can be constructed (Fig. 3). This plot is not linear, but has a break at approximately 28 °C, suggesting a change in membrane properties at that tempera-

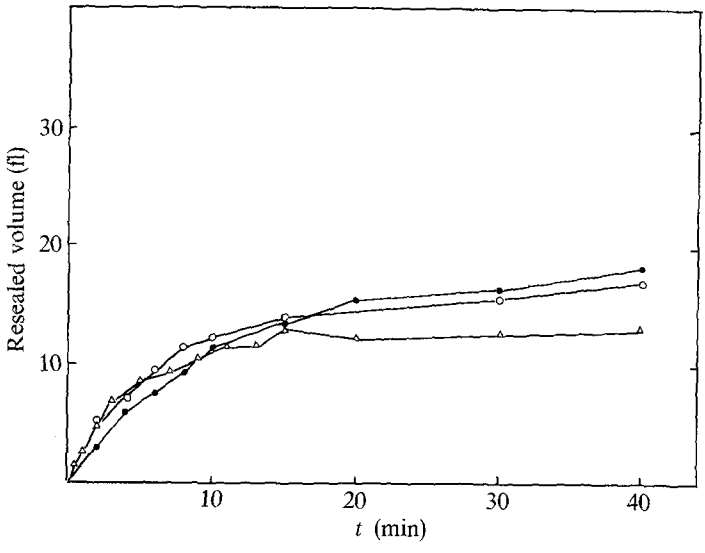

Fig. 2 (c)

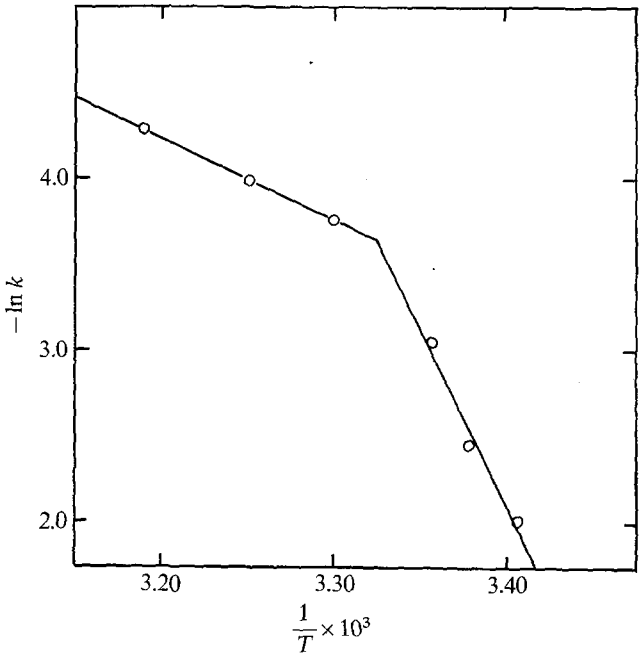

Fig. 3. Arrhenius plot of the data of Fig. 2

ture. The calculated activation energies for the low and high temperature regions of Fig. 3 are 41.0 kcal mol^{-1} and 9.1 kcal mol^{-1}, respectively. In contrast to the rate constant, the apparent resealed ghost volume de-

creases as temperature is raised. There are at least two possible explanations for this observation: a) increasing numbers of ghosts fail to reseal as temperature increases; b) ghosts enclose a smaller average volume as temperature is increased. It is difficult to unequivocally eliminate either possibility. Fragmentation of ghosts by endocytosis, which would drastically lower the ghost volume, has been observed at 37 °C if ionic strength is low (Steck *et al.*, 1970; Katsumata & Asai, 1972), or in the presence of MgATP in isotonic salt (Penniston, 1972). Our resealing experiments are done under different conditions, and we have not observed extensive fragmentation at incubation temperatures below 45 °C. Ghosts incubated at temperatures above 45 °C do fragment, as indicated by Coulter counting and size distribution plots.

Part of the variation in final resealed volume is correlated with a reduction in ghost volume. At the conclusion of the experiment shown in Fig. 2, the modal ghost volume was determined for each temperature. The resealed ghosts were diluted in 0.9% saline, and size distribution plots were obtained using the Coulter Model B with Model J plotter. The samples were thermostated at the respective incubation temperatures in a water-jacketed vessel. The model B counter corrects for the changes in conductivity brought about by temperature changes, so that values obtained at different temperature are comparable. The instrument was calibrated with fresh normal erythrocytes or Dade Hematology Control reference, and the erythrocyte modal volume was assumed to be 90 fl. The modal volumes of the resealed ghosts are also listed in Table 1. The modal size of freshly prepared unsealed ghosts is usually 55–60 fl. There is a fair correlation between the final resealed volume derived from the amount of hemoglobin in resealed ghosts at the conclusion of resealing, and the volumes directly determined with the Coulter counter. In most cases, the volume determined by resealing is about one-half the directly determined volume. This data suggests that (1) at temperatures of 25 °C or above, erythrocyte ghosts shrink in size from the 50–55 fl characteristic of unresealed ghosts to about 28–30 fl, and (2) that at a given temperature, about half of the ghosts are able to reseal and trap macromolecules.

Dependence of Resealing Kinetics on Ionic Strength and Tonicity

The ionic strength of the medium is important to ghost resealing. Fig. 4 shows resealing at 37 °C, with hemoglobin as impermeant molecule, in the presence of different NaCl concentrations. Little resealing occurs

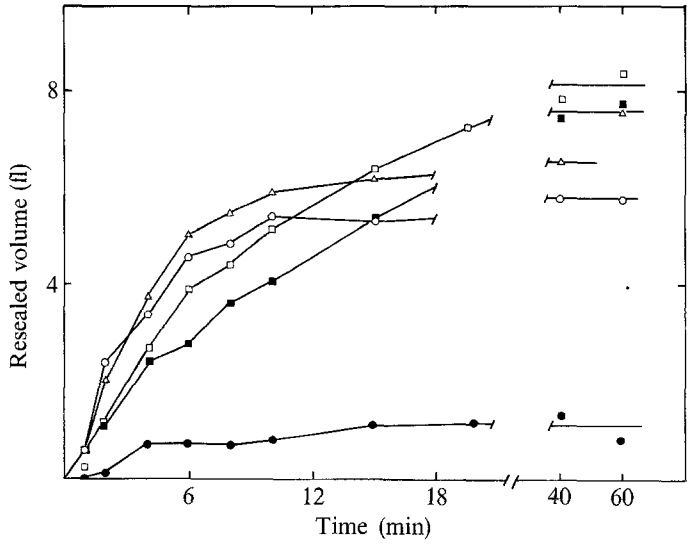

Fig. 4. Variation of resealing kinetics of washed ghosts with NaCl concentration. 2.4×10^9 ghosts ml^{-1} in 5 mM Tris-Cl, pH 7.4 with different NaCl concentrations, 25 °C. Hemoglobin as impermeant molecule. [NaCl] = 5 mM (●), 18 mM (○), 30 mM (△), 52 mM (■), 98 mM (□)

in the absence of salt. The final resealed volume of the ghosts increases with the NaCl concentration of the medium. This is seen in Fig. 4 and Table 2, which lists V_0 for two separate resealing experiments at 37 °C. The final resealed volume reaches a maximum when incubation NaCl is about 60 mM; further increases of NaCl do not increase V_0. The lowest NaCl concentration that will permit resealing varies between preparations, but at least 15 mM NaCl is always required for any appreciable resealing to occur. There are two qualitative effects of lowered medium NaCl on the rate of resealing. First, below 90 mM NaCl the kinetics are no longer first order. The low salt curves can be fitted by a two-exponential equation

Table 2. Relation between NaCl concentration of the incubation medium and V_0, the final average resealed volume of the ghosts. (Two separate experiments are given)

NaCl (mM)	V_0 (fl)	NaCl (mM)	V_0 (fl)
5.5	1.0	7	0.6
18	5.8	12	2.1
30	6.6	14.5	2.7
52	7.6	24	6.2
98	8.2	34	8.3
		54	13.6
		147	13.8

with an initial fast process, but the quality of the data do not justify elaborate fitting procedures. Secondly, at lower NaCl concentrations, resealing rates appear to be increased. Since the process is not first-order, rate constants are not obtainable. The half time for resealing can be estimated from Fig. 4, however, and is about 3 min in 18 and 30 mM NaCl, rising to about 6 min in 54 and 98 mM NaCl. This increase in half time with NaCl is a qualitatively consistent finding in resealing experiments. At NaCl concentrations above 60 mM, the resealing half times tend to be similar, as do final resealed volumes. If NaCl is replaced by sucrose no resealing is observed. Sucrose concentrations from 50 to 300 mM have been tested, and in no case is resealing observed; moreover, after 2 or 3 hr of incubation at 37 °C in sucrose media, the ghosts have undergone extensive fragmentation.

Use of Other Impermeant Probes

Fig. 5 shows the time course of resealing, using hemoglobin, [^{131}I]-β-lactoglobulin, [^{14}C]-inulin and [^{14}C]-mannitol as impermeant markers for resealing. This experiment was performed at 37 °C in 90 mM NaCl. In general, only macromolecules can be resealed within our ghost prepa-

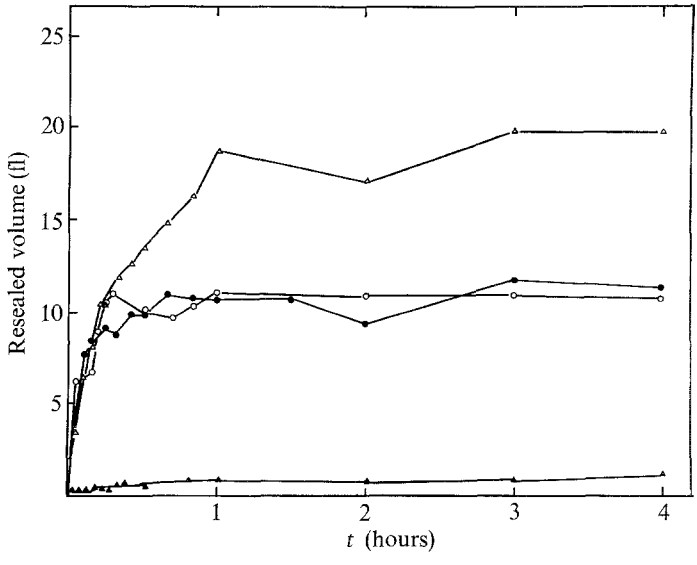

Fig. 5. Resealing of washed ghosts measured by the incorporation of different impermeant molecules in 5 mM Tris-Cl, pH 7.4, 90 mM NaCl, 37 °C. 1 mM [^{14}C]-mannitol (▲), 18 mg/ml hemoglobin (●), 0.30 mg/ml [^{131}I]-β-lactoglobulin (○), 1 mg/ml [^{14}C] inulin (△)

ration, which is prepared in 7 mM NaCl. The washed membrane does not recover impermeability to small molecules, as exemplified by mannitol. Using a different ghost preparation, it was also shown that the washed ghosts are unable to recover impermeability to ^{22}Na (data not shown). Because the NaCl concentration is 90 mM in this experiment, the kinetics of resealing are not exponential. These graphs clearly show, nevertheless, that the kinetics of resealing is essentially the same for hemoglobin and β-lactoglobulin (MW = 36,000). This provides further evidence that results obtained using hemoglobin as impermeant probe molecule are in fact due to resealing rather than hemoglobin adsorption. More inulin (MW = 5,000) is trapped during resealing than either hemoglobin or β-lactoglobulin. This is not a consistent finding. In a large number of experiments using both hemoglobin and inulin as impermeant probes, the apparent resealed volume of the ghosts as measured by inulin was usually slightly less than the volume as measured by hemoglobin, but occasional experiments show inulin volumes larger than hemoglobin volume (as in Fig. 5), or inulin volumes which are about one-half of hemoglobin volume. The reason for this inconstant ratio of measured resealed volumes is not known.

Effect of Microtubule Inhibitors on Resealing

The temperature dependence of resealing is consistent with an involvement of microtubules in the recovery of ghost membrane integrity, since microtubules depolymerize at 0 °C and reform at 37 °C (Omstead & Borisy, 1973); moreover, there is a report postulating the involvement of microtubules in the maintenance of normal erythrocyte shape (Jacob, Amsden & White, 1972). For these reasons, the effect of vinblastine and colchicine on the resealing rate was determined (Fig. 6). Neither of these microtubule inhibitors affect resealing, even at the concentrations used here, which are reported to sphere intact erythrocytes (Jacob *et al.*, 1972); the drug concentrations are about 10^3 higher than the binding constants for their interaction with tubulin, the subunit of microtubules. Direct measurement of [^{3}H]-colchicine binding to erythrocyte membranes suggests that tubulin is not present (Johnson, *submitted for publication*). When resealing experiments are followed for long time periods, an increased final resealed volume of the ghosts is sometimes seen in the presence of these drugs. It is possible that this is a reflection of some nonspecific effect of these drugs on the membrane, so as to increase cell volume (Seeman, Chau-Wong & Moyyen, 1973), but we have not studied this phenomenon further.

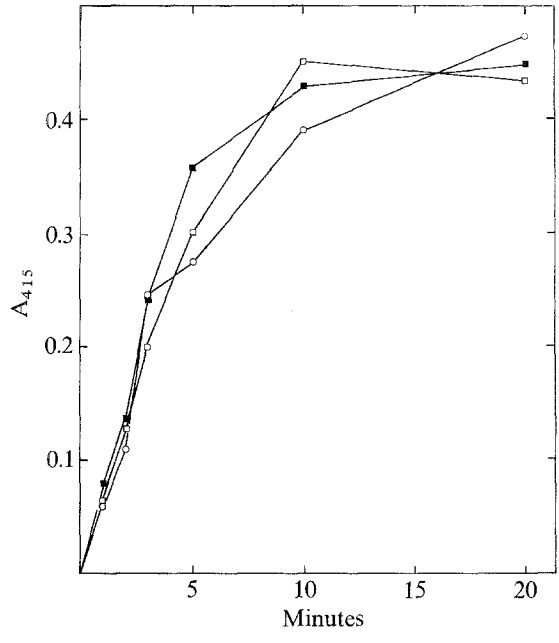

Fig. 6. Resealing of washed ghosts in the presence of tubulin binding drugs. 5 mM Tris-Cl, pH 7.4, 110 mM NaCl, 37 °C. The absorbance of hemoglobin incorporated into ghosts during incubation is plotted as a function of time. No additions (○), 3 mM colchicine (□), 100 μM vinblastine (■)

It is clear, however, that formation of a putative microtubule structure is not necessary for the ghost's recovery of macromolecular impermeability.

Effect of Changes in Ghost Preparation

Ghosts were prepared as described, but the salt concentration in the washing buffers was varied from the usual 7 mM NaCl up to 100 mM NaCl. Resealing experiments in 90 mM NaCl, 37 °C, using these different preparations are shown in Fig. 7. The ability of the ghosts to reseal and trap hemoglobin is diminished by higher salt concentrations in the preparation buffer. This result can be explained if the "leakiness" of the ghost is inversely related to the salt concentration of the preparation buffer. In this model, the ghost membrane prepared in higher salt (e.g. 40 mM NaCl) would be relatively impermeable to macromolecules, and will not trap hemoglobin during the resealing experiment because the macromolecule is not able to enter the ghost volume. Ghosts prepared in low salt

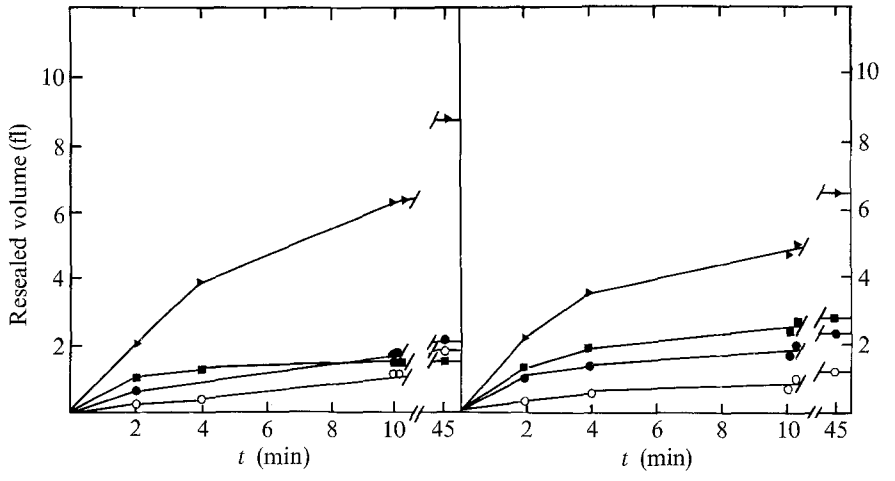

Fig. 7. Resealing of ghosts washed in buffers of varied ionic strength. Hemoglobin is the impermeant molecule. Ghosts were washed in 5 mM Tris-Cl, pH 7.4 with different amounts of NaCl. Resealing kinetics were determined as described in Materials and Methods in 90 mM NaCl, 37 °C. NaCl in washes: 7 mM (▲); 15 mM (■); 25 mM (●); 40 mM (○). *Right panel:* 1 mM EDTA included in lysis and first two washes. *Left panel:* EDTA omitted

buffers will be "leakier", and permeable to macromolecules, but will nevertheless recover impermeability during incubation at 37 °C in high salt concentrations. Evidence for this model can be obtained from direct measurements of the accessible internal volume of the ghost preparation before incubation. Such measurements are given in Table 3. Membranes prepared in 5 mM Tris without NaCl are listed, although such preparations are extensively fragmented, as indicated by the particle counts and microscopic observation. Increased salt concentrations in the preparation buffer are associated with greater retention of hemoglobin (Dodge *et al.*, 1963), with less effective resealing, as indicated by the diminished final resealed volume, and with a decrease in the internal volume of the ghost which is accessible to added hemoglobin, suggesting that ghosts prepared in high salt are less permeable to macromolecules. The addition of EDTA to the wash buffers decreases the amount of retained hemoglobin, without affecting resealing properties of the ghosts (Fig. 7). EDTA has the additional advantage of greatly increasing the reproducibility of resealing experiments; ghosts washed in NaCl alone show considerable variation between preparations, as Fig. 7 and Table 3 show. A resealing experiment was carried out in a similar way using washed ghosts prepared in phosphate buffers of varied osmolarities (7, 15, 25 and 40 mOsm), exactly as described by Dodge *et al.* (1963), with results closely resembling those shown in

Table 3

Salt conc. (mM)	1 mM EDTA	Particles/ml $\times 10^{-9}$ (a)	Percent accessible (b)	Apparent resealed volume (fl) (c)	Residual hemoglobin in preparation (d)
0	–	19.9	84	1.45	—
7	–	5.8	92	0	0.55
15	–	6.3	94	0.13	0.44
25	–	6.2	74	0	1.24
40	–	4.1	84	0	5.0
0	+	16.2	100	2.42	—
7	+	6.2	100	12.5	0.12
15	+	5.9	100	3.84	0.22
25	+	5.8	85	1.46	0.51
40	+	6.2	56	0.57	1.36

Ghosts were prepared from a single unit of blood. The lysis and wash buffers contained 5 mM Tris-Cl, pH 7.4 and the indicated amounts of NaCl. Ghosts were washed four times. When EDTA was used, it was present in the lysis step and the first two washes only.
(a) Particles per ml determined in the Coulter counter in 0.9% saline.
(b) Percent accessible is the fraction of the ghost internal volume which is permeable to hemoglobin, determined as described in Materials and Methods.
(c) Apparent resealed volume is determined from the amount of hemoglobin trapped by the ghosts during a 2-hr incubation at 37 °C in 140 mM NaCl.
(d) The residual hemoglobin in the washed cells is calculated from the amount of hemoglobin per ml and the cell count. It is expressed as percentage of the mean corpuscular hemoglobin (MCH), which is taken to be 29 pg (Blood and Other Body Fluids, FEBS Handbook, ed. Altman and Dittmer).

Fig. 7 except that the extent of resealing appears to be less in phosphate buffer.

Fig. 8 shows the relation between the resealable volume of ghosts prepared in different salt concentrations (with 1 mM EDTA in the first two washes), and the amount of hemoglobin contamination in the preparation. Not shown in the figure are two further concentrations of NaCl, 80 and 100 mM, in which the resealable volume was zero, and the contaminating Hb was 68 and 87 percent MCH, respectively. These results imply that ghosts prepared in low ionic strength buffers have increased permeability to macromolecules, resulting in both greater trapping of macromolecules during subsequent resealing experiments and less residual hemoglobin contamination in the preparation. We therefore agree with the suggestion of Bramley *et al.* (1971) that the hemoglobin contamination of washed ghost preparations is primarily attributable to hemoglobin trapped within impermeable (resealed) ghosts rather than to adsorption of hemoglobin to the membrane surface, at least at pH 7.4.

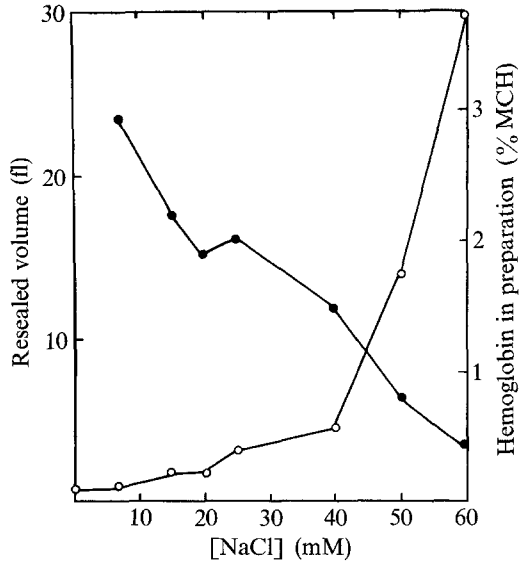

Fig. 8. Relation between the ability of a washed ghost preparation to reseal and the amount of hemoglobin in the preparation. Erythrocytes were lysed and washed in 5 mM Tris-Cl, pH 7.4 with the indicated amounts of NaCl and 1 mM EDTA. EDTA was omitted from the final two washes. Resealing to hemoglobin was measured after a 1-hr incubation at 37 °C in 100 mM NaCl. Hemoglobin contamination in the preparations is expressed as percent of MCH, assuming 29 pg of hemoglobin per erythrocyte. Resealed volume (●); hemoglobin in preparation (○)

SDS Gel Electrophoresis

Electrophoresis according to Fairbanks *et al.* (1971) was performed on the ghost preparations whose resealing properties are described in the previous section. Typical patterns are shown in Fig. 9. The Coomassie blue staining patterns are very similar for these ghosts prepared in Tris-Cl and ghosts prepared in phosphate buffers (Fairbanks *et al.*, 1971), and the numbering system of Fairbanks *et al.* can be used to describe the bands. In Tris buffer, as compared with phosphate, band *6* is relatively diminished. This band has been identified with glyceraldehyde-3-phosphate dehydrogenase (Tanner & Gray, 1971). The effect of EDTA (gels 5–8 as compared to gels 1–4) is to lower hemoglobin retention and band *4.2* in the ghosts. The effect of lowering salt concentration in the wash buffer is to diminish the relative amounts of bands *4.5* and *7*. There does not, however, seem to be any extensive alteration in the ghost protein composition brought about by washing the ghosts in different NaCl concentrations, although resealing properties are altered (Fig. 7).

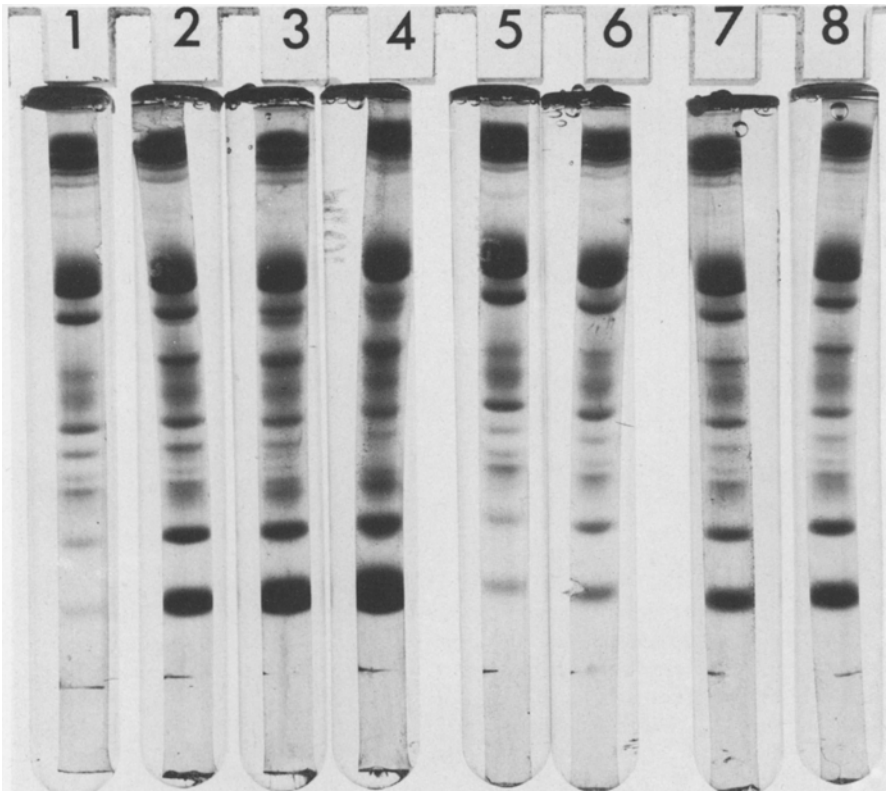

Fig. 9. SDS gel electrophoresis of washed ghost preparations. The washing buffers were 5 mM Tris-Cl, pH 7.4. Gels 5–8 show ghosts washed in 1 mM Na_2EDTA. The NaCl concentrations in the washes were: 1 and 5–7 mM; 2 and 6–15 mM; 3 and 7–25 mM; 4 and 8–40 mM. The dye front is marked with India ink; the fastest moving band is hemoglobin (H)

Discussion

It has long been known (Hoffman, Tosteson & Whittam, 1960) that unwashed ghosts reseal more effectively at 37 °C than at lower temperatures. Our extensive data for washed ghost resealing, defined as recovery of impermeability to macromolecules, show a similar temperature dependence, but the long lag period in the lower temperature kinetic plots is unexpected. It is possible that a phase transition of the membrane is involved. It may be suggested that the membranes at the preparation temperature (4 °C) exist predominantly in a more ordered structure from which resealing may occur less rapidly in relative terms. At temperatures

below 20 °C, the complex kinetics may reflect the low rate of the phase transition. Thus, the early time points in the low-temperature kinetic plots represent combined resealing of more and less ordered structures and perhaps a number of intermediate forms from which resealing may be proceeding at a number of intermediate rates. While part of the sigmoidal nature of the plots at 15 and 20 °C may reflect the increasing availability of fluid membranes which are both able to reseal more rapidly and enclose a greater average volume of solution than the more ordered membranes, it is also possible that cooperative effects, little understood at this juncture, explain the kinetics.

Generally, the clear-cut transition temperatures observed calorimetrically and by other techniques in model membranes containing one or a few closely related types of phospholipid are not seen when the membrane contains a mixture of lipids, as do natural membranes (Oldfield & Chapman, 1972). This is especially true when the membrane is rich in cholesterol. Artificial membranes containing cholesterol show lateral phase separations extending over a wide temperature range (Shimshick & McConnell, 1973). Erythrocyte membranes contain about 20% cholesterol by weight (Van Deenen & de Gier, 1964). There is some evidence, however, for a distinct phase transition or separation in erythrocyte membranes in the vicinity of 20 °C. For example, choline N-methyl protons are resolvable at 31 °C using 220 MHz NMR (Kamat & Chapman, 1968), but not at 18 °C (Glaser *et al.*, 1970). Oldfield and Chapman (1972) interpret this data as indicating a membrane lipid transition from a rigid to a mobile state between these temperatures. Elford and Solomon (1974) reported that the cation permeability of intact erythrocytes changes at approximately 20 °C, as does glucose transport (Hankin & Stein, 1972). Melchior and Morowitz (1973), on the basis of dilatometric measurements, suggest that ghosts undergo a phase transition around 22 °C; the macroscopic viscosity of erythrocyte ghosts indicates a transition at 18–19 °C (Zimmer & Schirmer, 1974).

Ghost membrane resealing may be related in simple molecular terms to the well-known ability of amphiphile lipids to form closed vesicles spontaneously in aqueous solution (Bangham, Hill & Miller, 1974). Phospholipids do not form closed vesicles below their transition temperature (Dervichian, 1964). We therefore suggest that ghost resealing to macromolecules occurs only if the membrane lipids are in their fluid state, and that resealing itself may be a useful indicator of lipid fluidity in erythrocyte membranes.

A reduction in ghost volume that occurs at higher temperatures is indicated by both resealing kinetics and Coulter size distribution plots.

This behavior of washed ghost membranes contrasts with the increase in ghost size observed when unwashed ghosts are resealed (Teorell, 1952; Palek *et al.*, 1971). Unwashed resealed erythrocyte ghosts expand from a post-hemolytic volume of approximately 60 fl to a final volume of about 140 fl after reversion and resealing. The washed ghosts in our experiments are also approximately 60 fl post-hemolysis, but this volume remains unchanged, if the incubation temperature is less than 22.5 °C (Table 1), and decreases at higher temperatures. The expansion of resealed unwashed ghosts has been explained as a consequence of recovered cation impermeability of the membrane (Seeman *et al.*, 1969). This is consistent with our observations, since our washed ghosts do not recover impermeability to small molecules and cations. Alternatively, some change in the putative contractile or structural system of the erythrocyte has been implicated in these volume changes (Palek *et al.*, 1971), and this system may be disrupted in extensively washed ghosts. This possibility is harder to assess. Alterations in membrane proteins (Hoogeveen *et al.*, 1970; Bramley *et al.*, 1971) and lipids (Dodge *et al.*, 1963) are known to occur during isolation of washed ghosts, but there is less agreement as to the extent to which native membrane structure is lost (Bretscher, 1973; Staros, Haley & Richards, 1974; Whiteley & Berg, 1974). In the absence of unequivocal evidence for an erythrocyte contractile system, cation permeability offers an adequate explanation for the absence of post-resealing swelling in washed ghosts. It is more difficult to explain the surprising reduction in ghost volume at higher temperatures. For example, at 35 °C, resealing experiments indicate a ghost volume of 17 fl, and the Coulter counter, 28 fl. It is known that intact erythrocytes will shrink at temperatures above 45 °C, resulting in microspherocytes (Ponder, 1955). The washed cell membrane may well be more labile to this heat-induced shrinkage, because of alterations during preparation. It is of interest to note that Humphries and McConnell (1974), using the exchange broadening of a spin label trapped within the human erythrocyte ghost (not washed and resealed at 37 °C), have estimated the internal volume to be about 22 fl.

The dependence of resealing on salt concentration is less complex. Any NaCl concentration above approximately 15 mM will permit resealing. The extent of resealing (final apparent resealed volume) and the half time change as medium NaCl is raised, up to about 50 mM NaCl, and the kinetics of resealing is biphasic when NaCl is low. The early rapid phase of resealing is progressively abolished by increasing salt, disappearing at about 100 mM NaCl where resealing is exponential. It is likely

that the ionic strength, rather than the tonicity, of the incubation medium is the more important factor in resealing. Indeed, ghosts incubated at 37 °C in a medium of low ionic strength will fragment, whether or not isotonicity is restored with sucrose. It is reasonable that media of low ionic strength inhibit resealing, if that process reflects the spontaneous reassociation of a separated lipid bilayer. The ghost membrane lipids are negatively charged due to the presence of phosphoserine (ca. 10% by weight) (Van Deenen & de Gier, 1964), and it is known that bilayer formation from charged lipids, e.g. liposome formation, is affected by the salt concentration of the medium (Bangham, de Gier & Greville, 1967). The earlier report of Bodemann and Passow (1972) that ionic strength is not a decisive factor in resealing probably can be attributed to the fact that their experiments were always conducted at 35 mOsm (equivalent to 5 mM Tris-Cl, pH 7.4 with 13 mM NaCl) or above, an ionic strength which permits resealing to occur. Rates were not directly measured by these workers.

The permeability barrier that results from resealing these washed ghost membranes is clearly different from an intact lipid bilayer. Liposomes are impermeable to most small molecules (Bangham *et al.*, 1974), whereas these resealed ghosts are not. The ghost lipid transition suggested by the temperature dependence is therefore a necessary precondition for resealing, but the nature of the actual permeability barrier is determined by other features of the ghost membrane. Our results, like those of others (Bodemann & Passow, 1972; Bramley & Coleman, 1972; Jung *et al.*, 1973), show that the ability of erythrocyte ghost membranes to recover impermeability is profoundly dependent on their mode of preparation.

For example, as the NaCl concentration of the hemolysis and washing buffers is increased, the ability to trap macromolecules is progressively lost, while the proportion of the ghost internal volume accessible to macromolecules declines (Fig. 7 and Table 3). This suggests that the size of the defects or "holes" in the membranes varies inversely and continuously with preparation buffer osmolarity or ionic strength. Another important determinant of the resealed ghost's permeability characteristics is the presence or absence of EDTA in the preparation buffers. EDTA appears to affect macromolecule and small molecule resealing differently. It is possible that recovery of impermeability toward small molecules is dependent on the presence of Ca in the membrane. EDTA is known to remove essentially all of the Ca of the erythrocyte if it is included in the washing buffers (Harrison & Long, 1968). Fig. 7 shows that EDTA in the hemolysis buffer does not notably affect macromolecule resealing, although its pres-

ence will abolish the membrane's ability to reseal and retain cations, even in unwashed ghosts (Hoffman, 1962; Bodemann & Passow, 1972). Washed ghosts prepared with EDTA-containing buffers show great permeability to macromolecules with unimpaired and reproducible resealing ability (Table 3). Phase microscopic examination of such preparations shows that the ghosts are intact. Similar conclusions about the effectiveness of EDTA in increasing permeability while maintaining the structural integrity of ghosts have been reached by Bramley and Coleman (1972), using bicarbonate rather than Tris buffers. Addition of Ca^{2+} to the resealing incubation medium profoundly affects the resealing kinetics *(manuscript in preparation)*, and it is likely that this is a reflection of the still obscure effects of Ca on phospholipid membranes (Triggle, 1972).

In a related recent study, Mueller and Morrison (1974) have demonstrated that ghosts washed five or six times in phosphate buffers will recover impermeability to macromolecules if 1 mM $CaCl_2$ (Segrest *et al.*, 1973) is added to the isotonic NaCl incubation solution. Resealed ghosts were separated from unresealed stroma by centrifugation on dextran gradients. The resealed ghosts were shown to trap lactoperoxidase and hemoglobin which could be subsequently released by hypotonic lysis, although no attempt was made to quantitate the process. The results of the present paper show that Ca is probably not an absolute requirement for macromolecule resealing. This finding may be of value if the presence of Ca is undesirable in a preparation of resealed washed membranes.

The phenomenon of time- and temperature-dependent recovery of impermeability to macromolecules described here may be relevant to biochemical studies of the ghost membrane. Membrane labeling by macromolecules, such as lactoperoxidase or membrane-specific antibodies, will be affected if the resealing processes described here are concurrent with the labeling procedure. It should be emphasized that this well-washed ghost preparation does not recover any appreciable impermeability to cations or small molecules.

In summary, these experiments show that the recovery of impermeability to macromolecular solutes in washed ghost membranes can be observed and characterized kinetically. The temperature and ionic strength dependence are consistent with the idea that resealing primarily involves reformation of an intact lipid bilayer, and there is little direct involvement of membrane proteins in this process. In particular, it is unlikely that microtubules are a factor in membrane resealing (Fig. 6). Ghosts with different resealing properties (Table 3) have similar protein compositions (Fig. 9). This does not, of course, eliminate the possibility that hemolysis in low

salt buffers causes a rearrangement of the membrane proteins which is reflected in the resealing properties.

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