PHYSIOLOGICAL CHARACTERISTICS OF HUMAN RED BLOOD CELL GHOSTS*

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ABSTRACT

The properties of ghosts prepared by hypotonic hemolysis at various ratios of cells (*C*) to hemolyzing solution (*H*) have been studied. At all ratios, hemoglobin (Hb) was found to be distributed equally between the ghost and supernatant compartments. Techniques employing Fe^{59}-labelled Hb showed that during hemolysis all of the Hb is exchangeable and that following hemolysis the ghost is impermeable to Hb.

Ghosts containing defined fractions of their original Hb were prepared by appropriately altering the ratio *C/H*. When washed and suspended in 0.17 M $NaCl-PO_4$—buffered media, the ghosts returned to their initial volume, recovered normal shape, and behaved as osmometers. The rate of rehemolysis of these reconstituted ghosts was observed to be proportional to the concentration of Hb in the ghosts. The rate of rehemolysis was accelerated by the addition of *n*-butyl alcohol (BA). For a given concentration of BA, temperature, and Hb content the rate of rehemolysis was minimal around the isoelectric point of Hb. Rehemolysis by BA was inhibited by the addition of sucrose to the medium. K influx and outflux were measured and found to be increased by the addition of BA and not influenced by the presence of sucrose. These results on the rehemolytic characteristics of ghosts are consistent with and support the colloid-osmotic theory of hemolysis.

INTRODUCTION

The properties displayed by ghost systems are generally considered to represent properties characteristic of the plasma membrane of intact cells. This assumption may not always be justified since certain properties of ghosts show some dependence upon the method used for their preparation (Ponder, 1952). Thus, necessarily, the equivalence of the behavior of ghost and intact cells should be established for each type of ghost studied. The purpose of this investigation is to examine the diffusion of hemoglobin during the initial

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hemolysis of intact cells, the rehemolysis of the resulting ghosts, the relation of these, and other properties to intact cells, and to evaluate the mechanism of lytic hemolysis. A preliminary report of this paper has been previously given (Hoffman, 1954).

Materials and Methods

The human blood used in this study was obtained from an arm vein of healthy young adult males; rabbit blood was collected by heart puncture. The blood was defibrinated or had sodium citrate added as an anticoagulant. The cells were washed three times with 20 volumes of 0.17 M NaCl–PO_4 (see Parpart *et al.*, 1947). Following the third wash the cells were packed and used as described below. For experiments in which unwashed cells were used, the collected blood was centrifuged and the buffy coat and plasma discarded. In most experiments hematocrit values were determined on the packed cells using the method of Parpart and Ballentine (1943). All preparations and experiments were carried out at room temperature.

Ghosts, used in the rehemolysis experiments, were prepared by the hemolysis of 1 volume of packed cells rapidly injected into various volumes (see below) of vigorously stirred distilled water. At the end of 45 minutes the ghosts were reversed either by the addition of sufficient 5 M NaCl to return the total solution to isotonicity or by diluting the hemolysis mixture with a large volume of isotonic saline buffered with phosphate. The ghosts were centrifuged at 3600 $\times$ *G* for 20 minutes, washed three times (again with 20 volumes) with an isotonic medium the composition of which depended upon the particular design of the experiment. The relatively slow rates of rehemolysis, as presented in Table IV, were measured by suspending usually 1 ml. of packed ghosts but sometimes 2 ml. (for ghosts containing small amounts of Hb) into flasks containing 100 ml. of suspension medium. These flasks were rolled to maintain continuous mixing. Aliquots were removed at intervals, centrifuged, and the optical density of the supernatant determined at 540 mμ (or at 413 mμ) on a Beckman spectrophotometer. Rapid rates of rehemolysis, as illustrated in Fig. 1, were followed using a densimeter modified slightly from the one described by Mawe (1956). No differences in the results are observed if rehemolysis is studied using ghosts prepared by the alternative method given immediately below.

Ghosts, used in the cation flux measurements, were prepared by altering the above procedure in the following fashion: 1 volume of packed cells from unwashed citrated blood (4 mg. citrate/1 cc. blood) was completely hemolyzed with approximately 10 volumes of distilled water. After 30 minutes the ghosts were reversed with 5 M NaCl. The ghosts were washed once with 0.17 M KCl–PO_4 (pH 7.5), then washed three times with 0.17 M NaCl–PO_4 (pH 7.5), and finally suspended (hematocrit value = 10 to 15 per cent) in a 0.17 M NaCl–PO_4 (pH 7.5) medium. A trace quantity of K^{42} was added after the suspension had been allowed to equilibrate for 30 minutes at 20°C. The ghosts, supernatant, and mixture were analyzed for radioactivity, using a well type scintillation counter; Na and K were determined using a Baird flame photometer. Ghost water was measured gravimetrically.

It is convenient to define the term *reconstituted ghosts* as referring to ghosts which have been processed by either of the above procedures; namely, ghosts which have been reversed and subsequently washed with isotonic media. It is clear that *reversal*

or *reversed*, operationally, refers to the addition of sufficient concentrated salt to return to isotonicity (relative to plasma) the hypotonic hemolysate. The response of the ghost to this alteration in the osmotic pressure of its environment will be described in the section on results.

The initial hemolysis in both of the above types of preparation was always checked by the method given in Hoffman *et al.* (1958) to insure that no intact red cells were present in the systems.

For centrifuging at 3600 × *G* an International clinical centrifuge was used; for all other separations a Servall centrifuge type SS-1 was employed. All values of pH were measured with a Cambridge research model pH meter.

Fe^{59}–labelled hemoglobin (Hb*) was prepared and obtained from rabbits by the method previously described by Hoffman (1958).

I^{131}–labelled albumin was obtained from Abbott Laboratories, Chicago, and dialyzed prior to use to remove any free I^{131}.

The K fluxes were calculated using the unsteady state equations developed by Sheppard and Martin (1950). The influx, i_{M_K}, in mM/liter ghost water/hour is calculated from the relation:

$$i_{M_K} = \frac{\frac{d(X)_g}{dt}(\mathrm{K})_g}{X_m - X_g}$$

in which (K) represents the potassium concentration in mM/liter; X, the specific activity (counts per minute ÷ (K)); subscripts g and m, refer to the ghost and medium compartments, respectively.

The outflux, o_{M_K}, in mM/liter ghost water/hour, is given by:

$$o_{M_K} = i_{M_K} - \frac{d(\mathrm{K})_g}{dt}$$

The inward, i_k, and outward, o_k, rate constants, in reciprocal hours, are obtained from:

$$i_k = \frac{i_{M_K}}{(\mathrm{K})_m}\,;\; o_k = \frac{o_{M_K}}{(\mathrm{K})_g}$$

RESULTS

1. Permeability and Intracellular State of Hemoglobin.—Table I, A shows the resulting distribution of Hb* (Fe^{59}–labelled Hb) between the ghost and medium compartments when 1 volume of packed normal rabbit red cells was hemolyzed in the presence of 10 volumes of a hypotonic solution containing a small amount of Hb*. Upon the completion of hemolysis the mixture was centrifuged at 15,000 × *G* and equal volumes of packed ghosts and their supernatants were appropriately diluted and counted. It is seen that Hb* is partitioned in both compartments to approximately equal concentrations. When the hypotonic solution is made isotonic prior to the addition of the cells

hemolysis is prevented and the measurement represents the amount of the suspension fluid resident with the cellular compartment (Table I, B).

The above experiment does not indicate whether the ghosts are continually permeable to Hb* after hemolysis or are only permeable transiently during the time of hemolysis alone. To decide between these alternatives the following experiment was performed. The red cells of two different rabbits were used: one in which the Hb had been previously labelled with Fe^{59}; the other contained normal unlabelled Hb only. Packed cells obtained from these two samples of blood were each hemolyzed with 10 volumes of distilled water. This procedure insured that the resulting hemolysates were of the same tonicity and hence, that no alteration of the hemolysis condition could occur. The ghosts and their hemolysates were then separated as in the above experiment. The ghosts derived from the unlabelled cells were resuspended in the hemolysate taken from the labelled cells. The converse exchange was also made in mixing

TABLE I

Hemolysis of Rabbit Red Cells in the Presence of Fe^{59}-Labelled Hemoglobin (Hb)*

Shown in the table are the counts per minute (C.P.M.) of equal volumes of packed ghosts and their supernatants after separation. See text for discussion.

Experiment sequence	Ghosts (G)	Supernatant (S)	$\frac{G}{S}$
	C.P.M.	*C.P.M.*	*C.P.M.*
A. Hb* + cells + time	5782	5694	1.015
B. Hb* + salt + cells + time	282	6928	0.041

ghosts containing Hb* and the hemolysate from the unlabelled cells. After standing at room temperature for 30 minutes the ghosts and supernatants of each were again separated and counted. Following correction for the amount of contaminating Hb* contained in each switch the results always showed, within an error of less than 5 per cent, that no Hb* escaped from the ghosts originally containing Hb* and that no Hb* entered the ghosts derived from the unlabelled cells. Thus, these results support the view that the ghost is permeable to Hb (in either direction) only at the time of hemolysis.

In Table II are presented experiments which consider the distribution of unlabelled Hb between the ghost and supernatant compartments after hemolysis has occurred. In Experiment C of Table II 1 volume of packed rabbit cells containing Hb* was hemolyzed with 10 volumes of distilled water. After the completion of hemolysis the ghosts and their supernatants were separated centrifugally as before and equal volumes of each were diluted, counted, and the Hb concentration determined spectrophotometrically. It can be seen that both the Hb concentration and the counts per minute are essentially the same in the ghosts and supernatants. This table also shows that the relative

specific activities of the two compartments are equal after hemolysis takes place.

Experiments of the type described in Table I, A and Table II, C have been performed on rabbit cells at other ratios of volume of packed cells (C) to volume of hemolyzing solution (H) varying from one-half to one-fifteenth. The results of these experiments likewise showed that for every ratio of C/H studied the distribution of Hb* in the former type and the bulk concentration of Hb in the latter type were equal in the two compartments upon the completion of hemolysis. This means, coupling these results, that all the intracellular Hb is exchangeable at the time of hemolysis, that is there is no bound Hb, since the relative specific activities of the two compartments are the same.

TABLE II

Bulk and Labelled Hemoglobin Diffusion to Equal Concentrations on Both Sides of Membrane at the Time of Hemolysis

One volume of rabbit red cells (containing Hb*) was hemolyzed with 10 volumes of distilled water. After completion of hemolysis the ghosts were separated from the hemolysate and equal volumes of each were diluted the same amount for the measurements taken below. Experiment C differs from D in that the ghosts of D were partially reversed by the addition of concentrated NaCl prior to their separation. O. D. is the optical density at 540 mμ; C.P.M. refers to the counts per minute; V_h means hemolytic volume; V_o means initial volume. See text for further explanation.

Experiment	Component	O.D.	C.P.M.	O.D. $\frac{G}{S}$	C.P.M. $\frac{G}{S}$
C (near V_h)	Ghosts (G)	0.203	771	1.030	1.043
	Supernatant (S)	0.197	739		
D (near V_o)	Ghosts (G)	0.254	937	1.337	1.333
	Supernatant (S)	0.190	703		

A number of the above types of experiments have been repeated with normal unlabelled human red cells. It was found that human I^{131}–labelled albumin would enter the cell only at the time of hemolysis, a result similar to that reported in Table I. This indicates that molecules normally incapable of permeating intact cells can enter into the ghost interior at the time of hemolysis. It is presumed that a limitation of this process is the size of the particular molecule chosen. Further, analogous studies on human cells at different ratios of C/H always showed that at equilibrium the bulk distribution of Hb in the ghost and supernatant compartments was equal, a result also found with rabbit cells.

When *reversal* is induced by the addition of sufficient salt to restore isotonicity (Bayliss, 1924) the ghost returns to its initial volume (Parpart, unpublished experiments) (see Ponder 1935, 1942, but compare Ponder 1951 *a*).

During this volume change the ghost remains impermeable to Hb. Thus, the amount of Hb remaining in the ghost following hemolysis becomes concentrated during reversal. (This transition is visible in the gross since the transparent mixture becomes opaque upon reversal and is accompanied by a dramatic change in color from dark to bright red.) These effects of reversal are illustrated by Experiment D in Table II. Here, the experiment was performed at the same time and in a manner identical with Experiment C but was partially reversed with a small amount of 5 M NaCl prior to the separation of the ghosts from their supernatant solutions. The results show the increase in Hb concentration in the ghost compartment, relative to the supernatant concentration, that occurred in response to reversal. This can also be seen in the comparison of the corresponding absolute values of Experiments C and D. Thus, as a consequence of reversal, the ghosts have undergone a stable volume change.

2. Rehemolysis of Ghosts.—Rehemolysis of ghosts is defined as a process analogous to the hemolysis of intact cells, when the membrane again becomes permeable to Hb. The characteristics of this rehemolytic event displayed by ghosts and its relationship to the hemolysis of intact cells will be described in this section.

The phenomenon of rehemolysis can be demonstrated quantitatively in the following manner. In each of two vessels packed unwashed normal human cells are hemolyzed with distilled water so that the ratio of C/H is 1/2.5. These systems are subsequently diluted so that the final ratio of cells to medium is 1/500: the first vessel is diluted with distilled water; the second, with isotonic saline buffered with phosphate to pH 7.55. To a third vessel the same volume of packed cells is added directly to 500 volumes of distilled water. Aliquots from each vessel are removed, centrifuged, and the concentration of Hb in the supernatant determined. The ghosts in vessels 2 and 3 undergo only a single hemolysis. The ghosts in vessel 1 should undergo two hemolyses: the first comparable to the hemolysis in vessel 2; the second, rehemolyzing during the next dilution should bring the system to a state equivalent to that of vessel 3. In Table III are shown typical results of this type of experiment. It is seen that the optical density of vessel $1 = 3 > 2$. It was independently established by the methods described by Hoffman *et al.* (1958) that 100 per cent hemolysis took place in vessel 2 and therefore in the first hemolysis of vessel 1. Thus, under these conditions rehemolysis appears to be all-or-none and during rehemolysis Hb appears to diffuse to equilibrium.

Again employing the procedures referred to in Table I, I^{131}–labelled albumin and Hb* could be incorporated into reconstituted (see Methods) human and rabbit ghosts, respectively, during the rehemolytic event. In addition, it was found that Hb in bulk would diffuse into human ghosts from a concentration greater outside than inside provided that rehemolysis was allowed to occur.

This latter point could be demonstrated using the following system: Ghosts

were made from the hemolysis of packed cells in a ratio $C/H = 1/37$, reversed, and washed twice with 0.17 M NaCl–PO_4 (pH 7.55). The more concentrated Hb solution was the supernatant derived from the hemolysis of packed cells in a ratio $C/H = 1/13$. One volume of the reconstituted ghosts was then mixed with 10 volumes of the Hb solution. Rehemolysis was insured because of the hypotonicity of the Hb solution relative to the ghosts. The mixture was then reversed by the addition of 5 M NaCl, centrifuged, and an aliquot of the ghosts pipetted into distilled water containing the solubilizing agent "antara" (see Hoffman *et al.*, 1958). The optical density of this supernatant was compared to that of the control; *i.e.*, a mixture handled identically but the 5 M NaCl was added to the Hb solution prior to the addition of the ghosts, thus preventing rehemolysis. The results of such an experiment always indicated that approximately 30 to 50 per cent of the expected increment was

TABLE III

Quantitation of Rehemolysis

Human red cells in vessels 2 and 3 were hemolyzed once at the indicated ratio of volume of cells (C) to volume of distilled water (H). The cells in vessel 1 were hemolyzed twice. See text for discussion. O.D. referes to the optical density of the supernatant at 540 mμ; V_h is the hemolytic volume; V_o is the initial volume of the cells.

Vessel	Sequence	C/H	O.D.
1	Cells + H_2O + time + H_2O + time + salt	1/2.5→1/500	0.503
2	Cells + H_2O + time + saline	1/2.5*	0.278
3	Cells + H_2O + time + salt	1/500	0.511

* $V_h = 1.72V_o$

found in the ghosts. It was felt that this was a significant amount since the optical density of the test system was generally a third to a half greater than that of the control system. Failure to achieve the theoretical distributions may be due to such factors as incomplete rehemolysis or the intractable nature of the system.

Given that Hb goes to diffusion equilibrium during the initial hemolysis, as discussed in section 1, it follows that ghosts can be prepared containing any desired fraction of their initial Hb by prior adjustment of the ratio C/H. The actual Hb content of the ghosts can be computed from this ratio if the hematocrit values of the packed cells and their hemolytic volume are known. Table IV shows that ghosts will rehemolyze at a rate proportional to the amount of Hb they contain. This rate of rehemolysis can be accelerated by the addition of *n*-butyl alcohol, the rate being dependent upon the concentration. For any given concentration of *n*-butyl alcohol the rate was greater the higher the temperature. Sucrose added to the extracellular phase considerably decreases the rate of rehemolysis.

These data are consistent with the proposal of Jacobs (1931), Netsky and Jacobs (1939), Jacobs and Stewart (1947), Davson and Ponder (1938), Wilbrandt (1941), that hemolysis induced by lytic agents proceeds by a Donnan mechanism. This has been called *colloid-osmotic hemolysis* by Wilbrandt (1941) in which the cation-impermeable barrier of the membrane is destroyed, resulting in a swelling of the cell and leading to eventual hemolysis. For a system of ghosts the rate of rehemolysis would depend upon the amount of Hb (colloid) they contain, under any constant set of experimental conditions. That this is so can be seen in the data presented in Fig. 1 and Table IV.

TABLE IV

Rehemolysis of Reconstituted Human Red Cell Ghosts in 0.17 M $NaCl$-PO_4 (pH 7.55) in the Presence of n-Butyl Alcohol. 24° C.

Sucrose substituted in osmotic equivalent for NaCl. See text for discussion. *C* is the volume of cells hemolyzed in *H* volumes of distilled water.

Experiment	Ratio C/H	Butyl alcohol	Sucrose	Time to 50 per cent rehemolysis
		moles/liter	*moles/liter*	
E	1/2	0	0	90* min.
	1/5			268* min.
F	1/2	0.45	0	66 min.
	1/5			108 min.
	1/10			174 min.
G	1/10	0.50	0	30 min.
	1/20			390 min.
H	1/4.5	0.37	0	10.5 hr.
			0.030	41‡ hr.

* Refers to time to 10 per cent rehemolysis at 37°C.

‡ Refers to time to 10 per cent rehemolysis.

Further, if the mechanism of hemolysis is colloid-osmotic then the rate of rehemolysis should be minimal at the isoelectric point for Hb (pH 6.8) and accelerated on both sides (see Adair and Adair, 1928). Table V gives the determinations of the rates of rehemolysis of ghosts, containing the same fraction of their initial Hb, suspended in NaCl–PO_4 media of different pH values. The rate of rehemolysis is more rapid on the acid side but is not accelerated on the alkaline side of the isoelectric point. A possible explanation for this is that the ghosts undergo an adjustment in volume due to exchange of OH^- for Cl^- (*cf.* Jacobs and Stewart, 1942). To test this interpretation the above experiment was repeated except that the ghosts were suspended in NaCl–PO_4 solutions of the same pH but of different tonicities. The rate of rehemolysis

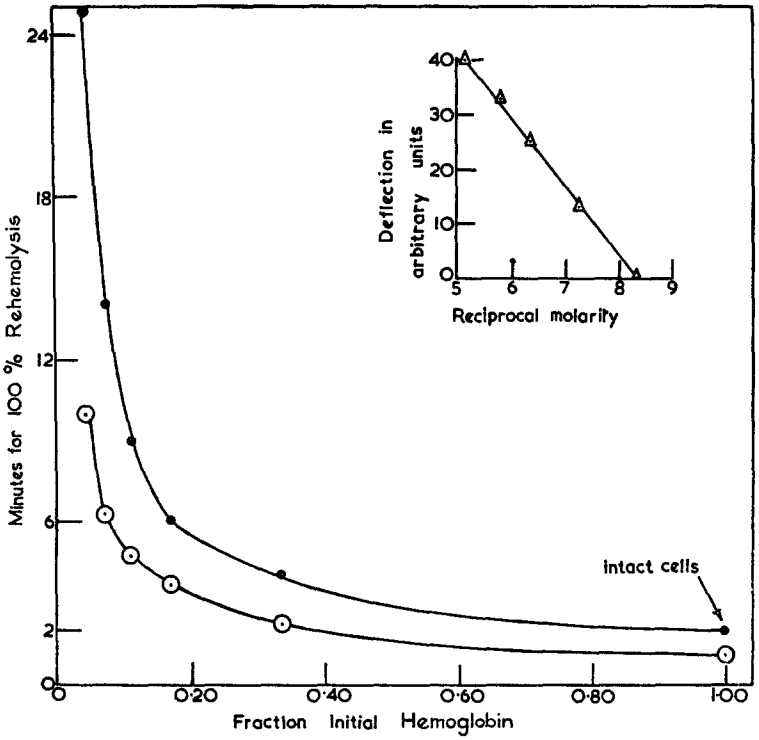

FIG. 1. This graph shows the time in minutes for 100 per cent rehemolysis (ordinate) in butyl alcohol of ghosts (and intact cells) containing various fractions of their initial hemoglobin (abscissa). The two curves represent different experiments. The fraction of initial Hb the ghosts contained was obtained from the relation $(C/[C + H])$. The inset shows the relative density (ordinate) of ghosts plotted against the reciprocal of the molarity of their suspension medium (abscissa).

TABLE V

Rehemolysis of Reconstituted Human Red Cell Ghosts Containing the Same Amount of Hemoglobin and Prepared at the Same pH But Suspended in NaCl-PO_4 Media of Different pH. 0.4 m Butyl Alcohol. 24° C.

See text.

C/H	pH	Time to 50 per cent rehemolysis
		min.
1/4	6.48	51
1/4	6.87	170
1/4	7.55	170

was found to be inversely related to the tonicity. Hence, the assumption of an opposing volume change on the basic side of the isoelectric point seems valid.

It appears, then, that fixing the internal pH at the time of hemolysis would circumvent the volume adjustment due to the pH differential between the ghost and the suspension medium used to induce rehemolysis. When ghosts are

TABLE VI

Rehemolysis of Reconstituted Human Red Cell Ghosts Containing the Same Amount of Hemoglobin But Prepared in NaCl-PO_4 Media of Different pH in Which They Were Also Suspended, As Indicated Below.

0.4 m Butyl Alcohol. 25° C.

See text.

C/H	pH	Time to 50 per cent rehemolysis
		min.
1/5	6.47	70
1/5	7.00	155
1/5	7.57	135

TABLE VII

This Table Shows the K and Na Concentrations (in mM/Liter Ghost Water) and the Per Cent Water of Reconstituted Human Red Cell Ghosts (C/H = 1/10)

Ghosts were suspended in a medium whose cation content is shown buffered to pH 7.55 with phosphate. These experiments are the same as those presented in Table VIII in which the K flux data are recorded. Sampling times shown refer to the time after the addition of K^{42} to the medium. Subscripts *g* and *m* refer to the ghost and medium compartments, respectively. All experiments performed at 20°C. See text for discussion.

Experiment	Time	$(K)_g$	$(Na)_g$	$(K)_m$	$(Na)_m$	H_2O
	min.					*per cent*
I	3	15.3	180	1.9	171	90.3
	37	11.4	189	3.9	168	90.2
J (+ 0.3 M butanol)	3	19.7	174	3.3	165	90.3
	37	8.2	192	3.8	165	91.0

prepared by hemolysis in dilute (0.02 M) phosphate buffer solutions of different pH and in which they are subsequently washed and suspended, any complicating volume change would be minimized. Table VI gives the result of such an experiment and shows that under these conditions the rate of rehemolysis is accelerated on both sides of the isoelectric point. It is an interesting fact that the results (Fig. 3, p. 675) of Teorell (1952) corroborate the interpretation given to the above data.

3. Potassium Permeability of Ghosts.—Tables VII and VIII present the

results of measurements of K flux in reconstituted human ghosts. The ghosts used in Experiments I and J were reversed with concentrated NaCl. Table VII shows the initial and final K and Na concentrations in the ghost and medium compartments and the water content of the ghosts. The fluxes were measured generally during the first 50 minutes after the addition of K^{42} to the medium with 3 aliquots being removed for analysis. During this experimental period the approach to isotopic equilibrium appeared to be described by a single exponential curve. The average fluxes for the experimental period are recorded in Table VIII. In addition, the ratio of the rate constants and the ratio of the specific activities of the terminal sample are also presented.

It was found that ghosts which were immediately separated from tagged medium usually contained 20 to 30 per cent of the medium radioactivity. Since the trapped medium measured with I^{131}–labelled albumin or Hb* was

TABLE VIII

This Table Shows the K Influx (i_{M_K}) and outflux (o_{M_K}) (in mMK/Liter Ghost Water/Hour) of Reconstituted Human Red Cell Ghosts

Analytical data for these experiments are given in Table VII. Also shown are the ratio of the inward (i_k) and outward (o_k) rate constants and the ratio of the specific activity of the ghost (X_g) and medium (X_m) compartments at the time of the terminal sample. See text for symbol reference and for further explanation.

Experiment	i_{M_K}	$^{o}{}_{M_K}$	i_k/o_k	X_g/X_m
I	2.8	9.7	1.3	0.09
J (+0.3 M butanol)	4.7	25	0.8	0.21

between 3 and 4 per cent, this was taken to mean that a certain fraction of the ghosts were freely permeable to K. It was thus necessary to correct the observed radioactivity in the ghost fraction for computation of the fluxes. This was done by plotting the radioactivity of the ghosts against time. The resulting straight line was extrapolated to zero time and the value so obtained was subtracted from the radioactivity of the ghost compartment at the various sampling times. This procedure assumes that the size of the freely permeable portion of the ghosts does not change over the experimental period. The K contents obtained by flame analysis were not corrected for this inhomogeneity (see below). This means that the actual fluxes must be lower than those calculated above since the $(K)_g$ of Table VII gives only the average K in the ghosts. I and J represent the results of a single paired experiment.

It is seen that both the influx and outflux of K are increased by *n*-butyl alcohol (J) over those of the control (I). The fluxes obtained for each of these cases are the same when 30 mM/liter sucrose is added to the medium.

When cells are washed with isotonic buffered saline prior to hemolysis in

their preparation as ghosts the above cation properties are lost. Rather, the results appear to be similar to the observations made by Davson and Ponder (1938) on rabbit red cells. It has not been possible to understand the cause of this difference although the loss of Ca^{++} may be a related phenomenon (*cf.* Hoffman *et. al.*, 1958).

4. Inhomogeneity.—The inhomogeneity with respect to K permeability can be established using a separating technique based on density differences (*cf.* Chalfin, 1956; Hoffman, 1958). In Table IX will be found the results of an experiment in which one-half of the ghosts after hemolysis were reversed with K^{42}-enriched KCl (series P) while the remaining half were reversed with unenriched KCl (series O). But for this alteration in procedure the ghosts in both series P and O were treated in identical fashion for the rest of the normal preparation to yield reconstituted ghosts. To the final ghost suspension

TABLE IX

Inhomogeneity of Reconstituted Human Red Cell Ghosts with Respect to K Permeability

See text for explanation.

Level	Per cent total K^{42} activity	
	Series P	Series O
1 (top)	5.7	35.7
2	6.7	35.7
3	11.2	21.9
4	26.9	13.5
5 (bottom)	49.5	5.7

of series O a small amount of K^{42} was added, the suspension stirred, and allowed to stand for 5 minutes. The ghosts of both series P and O were then centrifuged at 20,000 $\times$ *G* for 30 minutes in an air turbine. The packed ghosts in each of the centrifuge tubes were marked into five equal portions. Each level was pipetted off, diluted the same amount, and counted. The per cent of the total counts found in each level is given in Table IX. The specific activity of level 1 ghosts in series O was the same as that of the suspension medium. It can be seen that the ghosts in the bottom two-fifths of each series are the most impermeable to K. Thus, to a first approximation, the K^{42} in the bottom ghosts of series P was not washed out during their preparation and the K^{42} did not enter this bottom fraction after their reconstitution. These density differences have been used by Hoffman and Tosteson (1956) to obtain a more homogeneous suspension of ghosts.

Another line of evidence that the type of inhomogeniety described above exists in the ghost suspensions is that the measured hematocrit value is always greater than the hematocrit value computed from the values of $(K)_g$, $(K)_m$,

and the concentration of K in the suspension mixture. An indication that the ghosts used by Teorell (1952) also possess this inhomogeneity is reflected in the $(K)_g$ values in his Figs. 9 and 10.

An explanation of the origin of the inhomogeneity in the reconstituted ghost suspension possibly stems from the following observations. During the preparation of the ghosts, in the washings following reversal, some rehemolysis was always seen to occur. Since no ghosts were lost, this implies that the final suspension would be composed of at least two types of ghosts, namely, once and twice hemolyzed cells. A separate experiment in which all the ghosts were hemolyzed twice showed that by the time the first sample was separated (3 minutes after the addition of K^{42} to the medium) the specific activities of the ghost and medium compartments were the same. The ghosts used in this particular experiment were isolated (centrifugally) after their reversal with concentrated KCl, and rehemolyzed again in the same C/H ratio used initially; thereafter their preparation was continued in the standard manner. Thus, this result is compatible with the expectation that the density of twice hemolyzed cells would be less than that of singly hemolyzed cells (since they would contain a proportionately smaller amount of Hb) thereby correlating with the K permeability and density measurements referred to before. The conclusions reached above, however, do not rule out the possibility that other forms of inhomogeneity may be present.

5. Osmometry, Non-Electrolyte Permeability, and Shape Changes.—The inset of Fig. 1 shows that reconstituted ghosts behave as osmometers within the physiological range. The relative deflection of the densimeter is directly proportional to the ghost volume as Parpart (1935) found for intact red cells. The experiment given in the inset is typical of the results obtained with each group of ghosts represented by the points on the other curves in Fig. 1. Although the straight line relationship is obtained in each case the absolute amount of deflection was found to be greater the less Hb the ghosts contained.

Non-electrolyte permeability was measured using a modified densimeter (Mawe, 1956). The reconstituted ghosts were introduced into 0.3 M glycerol in 0.17 M NaCl–PO_4 (pH 7.50) and the extent of the shrinkage and the time course of their subsequent swelling back to their initial volume were recorded. For intact cells and for ghosts whose C/H ratio was 1/8, 1/12, and 1/20 the corresponding average times for total deflection were 52, 58, 62, and 60 seconds respectively. Thus, the permeability of reconstituted ghosts to glycerol does not appear to be significantly different from that of intact cells.

Shape changes of constant volume of reconstituted ghosts were observed either directly using a phase contrast microscope or by the method described by Hoffman, Collier, and Deitch (1956) in which light transmitted through a suspension of stirred ghosts decreases upon the addition of an agent which induces sphering. This latter method has the advantage of being free from the

possibility of skin fat influencing the results (see Teorell, 1952; Trotter, 1956). Reconstituted ghosts appear mainly as biconcave discs but some crenated discs are usually present. Upon the addition of non-lytic amounts of rose bengal or uranyl nitrate the ghosts immediately become spheres. When plasma or crystalline serum albumin is added to a suspension of ghosts sphered by either of the above agents the ghosts immediately revert to discs. The capacity to undergo these reversible transformations of shape does not seem to depend upon the Hb content of the reconstituted ghosts.

DISCUSSION

The behavior of reconstituted ghosts described above provides a basis for evaluating their relationship to intact cells. Comparisons using criteria such as shape, osmometry, and permeability indicate that these ghosts are remarkably similar to intact cells. In addition, the properties of the lysis induced by butyl alcohol do not appear to differ from those of intact cells (Netsky and Jacobs, 1939; Jacobs 1952; Parpart and Green, 1951). These resemblances favor the interpretation that the process of rehemolysis of ghosts cannot in principle be different from the process of hemolysis displayed by intact cells.

For the present viewpoint one advantage in studying ghost systems is that the colloid-osmotic mechanism of lytic hemolysis can be tested directly. As was discussed earlier, for any given series of ghosts the rate of rehemolysis depends upon the amount of Hb they contain. In particular, this relationship can be quantitatively demonstrated using a different plot of the data presented in Fig. 1. Thus, in Fig. 2 the time to reach 100 per cent rehemolysis is plotted against the reciprocal of the fraction of the initial Hb. It can be seen that the two separate experiments are fitted reasonably well by straight lines and that each line goes through the point obtained for intact cells. The corrections used, noted in the legend of Fig. 2, to convert the data to slightly different units on the abscissa do not alter these straight line relationships. The reason for the difference between the slopes of the two curves represented is referable to the different concentrations of butyl alcohol used. This method of plotting assumes that for all the ghosts in each experiment the average initial volume, V_o, hemolytic volume V_h, and degree of induced cation permeability are the same and independent of the amount of Hb the ghosts contain. This equates the time necessary to swell from V_o to V_h to the measurement of volume itself, thereby yielding an analogous Boyle-van't Hoff plot as given in the inset of Fig. 1. Included in Fig. 2 is a corresponding plot of the osmotic pressure of various concentrations of purified ox hemoglobin (unpublished observations of Dr. G. S. Adair).[1] The fact that straight lines are obtained for both types of measurements emphasizes that the osmotic swelling and subsequent rehemolysis of the ghosts proceed at a rate proportional to the

[1] The author wishes to thank Dr. G. S. Adair, Cambridge University, for allowing him the use of these observations.

amount of Hb they contain. Thus, these data lend direct support to the colloid-osmotic mechanism of hemolysis.

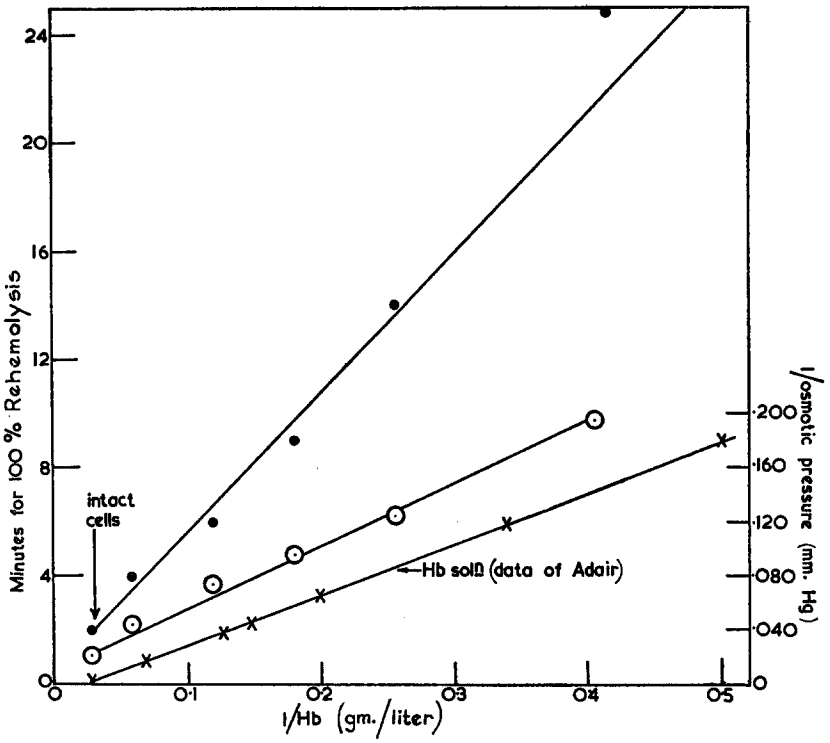

FIG. 2. This graph uses the same data as those presented in Fig. 1. For the two experiments (symbols: ●, ⊙) the time for 100 per cent rehemolysis (left ordinate) is plotted against the reciprocal of the Hb concentration (abscissa). The abscissa has been changed by correcting the values in Fig. 1 using an hematocrit value, Hct = 90 per cent, a hemolytic volume, V_h = 1.60, and 35 gm. Hb per 100 ml. of intact cells, by the relation:

$$\frac{C + H}{(35)(C)(\text{Hct})(V_h)}$$

The reciprocal of the osmotic pressure (right ordinate) of solutions of purified Ox Hb (symbol, X) is plotted against the same abscissa.

This colloid-osmotic mechanism quite generally describes many diverse forms of lytic hemolysis: for example,[2] by anionic detergents (Love, 1950), cationic detergents (Love, 1954), alcohols (Parpart and Green, 1951), ultraviolet radiation (Leu *et al.*, 1942), x-irradiation (Ting and Zirkle, 1940), photodynamic hemolysis (Davson and Ponder, 1940), heat (Wilbrandt, 1941),

[2] No attempt is made to cover this extensive literature.

hemolysins (Bernheimer, 1947), hydrostatic pressure (Hoffman and Chalfin, unpublished results). In each of these instances, as stressed by many of these authors, the agent appears to act by affecting the membrane in such a manner that the cation permeability is increased. This allows a Donnan swelling which continues until the hemolytic volume is reached. This swelling and likewise the subsequent hemolysis can be inhibited by adding to the cell's environment molecules such as sucrose, which in being relatively impermeable balance the internal osmotic force (Netsky and Jacobs, 1939; Wilbrandt, 1954). Objections to the colloid-osmotic mechanism have been raised by Ponder (1947, 1948 *a*, *b*, *c*, 1951 *b*, 1955) on the basis that although lytic agents in relatively low concentrations promote the exchange of Na for K no appreciable changes in cell volume are observed. This situation may be partially reconciled by the suggestion of Jacobs (1951) that the anion permeability of the cells under these conditions might be decreased thus offering protection by opposing the tendency to swell. On the other hand, as emphasized by Parpart and Green (1951), the important consideration may be the relative rate at which (relative, say, to their free diffusion constants) the cations move across the membrane. Relatively slight alterations in cation flux lead to slow changes in the cation composition but it does not necessarily follow that these changes lead to immediately detectable swelling or hemolysis (Parpart and Green, 1951). It appears that only in circumstances under which the resistance of the membrane to the diffusion of cations is very greatly decreased can swelling and hemolysis be expected to occur in any short period of time; *e.g.*, minutes to hours (Hoffman and Tosteson, unpublished experiments, see Tosteson, 1955). What is still necessary as Ponder (1951 *b*) has noted, is that measured rates of hemolysis should be predictable from known cation fluxes before the colloid-osmotic mechanism will be quantitatively descriptive of the process leading to hemolysis.

The above theory applies to the forces responsible for the swelling of the cell to its hemolytic volume. The process of hemolysis itself occurs, by definition, at the hemolytic volume, but is concerned with the changes in the membrane which allow Hb to escape. The cell appears to swell from a disc to a sphere without any increase in the surface area of its membrane (Jacobs, 1927). The volume at which the cell becomes permeable to Hb is indistinguishable from its spherical volume prior to hemolysis (Hoffman *et al.*, 1958; Castle and Daland, 1937; Guest and Wing, 1942). Thus, hemolysis is associated with a slight increase (stretch) in the surface area of the membrane (*cf.* Ponder, 1948 *c*). The gross changes in the molecular organization of the membrane must be transient since the ghost recovers almost immediately its impermeability to Hb as referred to before.

The experiments presented in Table IV indicate a method for estimating the tension of the ghost membrane which must be overcome if hemolysis is to

occur. It is seen in Table IV, G that ghosts with $C/H = 1/20$ require 6 hours to reach 50 per cent rehemolysis. In a similar separate experiment it was found that ghosts with $C/H = 1/30$ did not rehemolyze at all in 24 to 36 hours using this same concentration of butyl alcohol although swelling could be demonstrated indirectly using their osmotic resistance (Parpart *et al.*, 1947; Wilbrandt, 1941) as a relative index of their volume. It appears that the latter ghosts did not rehemolyze because they were unable to develop sufficient osmotic pressure to overcome the tension of the surface. If it is assumed that a $C/H = 1/30$ defines the limiting Hb concentration a ghost can contain without rehemolyzing then the tension can be calculated in the following manner. Ghosts hemolyzed at a ratio $C/H = 1/30$ contain a final Hb concentration approximately equivalent to 1.64 gm./100 cc. of ghosts. The osmotic pressure of such a Hb solution using the data of Gutfreund (1949) equals about 6.5 cm. H_2O or 6370 dynes/cm.^{2}. Using the formula (see Harvey, 1954):

$$\sigma = \frac{Pr}{2} = \frac{(6370)(3 \times 10^{-4})}{2} = 0.96 \text{ dynes/cm.}$$

in which σ = tension of the surface, P = internal pressure, and r = radius of spherical ghost in centimeters. This result, at best an approximation, is not unreasonably different from values observed for other types of cells (Harvey, 1954) but considerably higher than Norris (1939) measured for *Triturus* red cells.

As discussed before it is possible that the major contribution of the observed inhomogeneity arises as a result of the rehemolysis of a portion of the ghosts during their preparation. If this is so then for reasons set forth in the above paragraph these twice rehemolyzed ghosts are not involved in the measurements of rehemolysis as given in Tables IV, V, VI, or in Fig. 1.

The experiments presented in section 1 of the results show that Hb goes to diffusion equilibrium at the time of hemolysis. This conclusion was reached previously by Parpart (unpublished) on the basis of a different experimental procedure as well as by Hoffman *et al.* (1958) employing still another technique. Teorell (1952) obtains 6.0 per cent as the non-solvent volume for a system of ghosts hemolyzed at a $C/H = 1/5$; this value is what would be expected if Hb was partitioned to equal concentrations between the ghost and supernatant compartments at the time of hemolysis. Bateman *et al.* (1953) working with small angle x-ray scattering found that Hb in normal human cells existed in random orientation in agreement with results obtained from concentrated Hb solutions. On the other hand, Ponder (1942) and Moskowitz and Calvin (1952) present evidence, in marked contrast to the above conclusions, that Hb does not go to diffuison equilibrium at the time of hemolysis and that this residual Hb forms a structural component of the membrane (recently summarized by Ponder, 1955). Inspection of Table II, Experiment D, provides a

probable explanation for the interpretation Ponder (1942) placed on his results. Reversal, in the present context, as shown in Experiment D serves to concentrate Hb in the ghost since its volume is reduced without any loss of Hb. The concentration of Hb per unit volume of reversed ghosts is, therefore, higher than in an equivalent volume of hemolysate. Failure to achieve 100 per cent hemolysis would also yield a concentration of Hb higher in the ghost compartment than in the supernatant compartment; this could be caused by using a low hematocrit value for C or too small a volume of H or both. This criticism could likewise apply to the results of Szekely *et al.* (1952) and Gardos (1954). The results of Moskowitz and Calvin (1952) can be explained by the fact that high alkalinity favors the rehemolysis of ghosts. No single step hypotonic hemolysis procedure would be expected to provide Hb-free ghosts as pointed out by Hillier and Hoffman (1953). It must be concluded that the existence of a residual or structurally fixed Hb is incompatible with the present available evidence and should, therefore, be discarded.

It is important to emphasize the differences in the properties of the reconstituted ghosts used in this work from those of the ghosts studied by Teorell (1952) and likewise Stein (1956). The preparation used by Teorell (1952) was obtained by hemolyzing cells with distilled water ($C/H = 1/5$) and subsequent incubation overnight at 4°C. Teorell (1952) found that the resulting ghosts were osmometers over a considerable range of tonicities. Since this means that the ghosts were permeable only to water and since the tonicity of the original ghost suspension medium was equivalent to that of the hemolysate (*i.e.* 20.4 mM), it follows that the ghosts remained cation-impermeable when they were placed in a medium isotonic with plasma. The ghost cation analyses of Teorell (1952) support this conclusion. Teorell also found that the original volume the ghosts occupied was the same as that of the intact cells from which they were derived. In marked contrast to this situation, as discussed before, the ghosts used in the present study, upon reversal, underwent a considerable change in their ionic composition (Table VII; Table IX, series P) but also returned to their initial volume, as Parpart found. Apparently, this difference in the properties of the two ghost systems can be understood by considering that given enough time (incubation at 4°C. in the case of Teorell) the ghosts shrink back to their initial volume and in so doing become relatively impermeable to cations, the process being essentially a slow recovery. On the other hand, the return to initial volume and recovery of impermeability can be accelerated, in unincubated systems, by the addition of concentrated salt to the hemolysate. The above implies that the ghost becomes relatively impermeable to cations only after it has regained its initial volume, for both types of ghosts behave as osmometers thereafter.

Although the ghost suspended in its hemolysate will revert to its original volume upon reversal, this volume subsequently changes to a new stable

level when the ghosts are washed or resuspended in an isotonic solution of different ionic composition. The reconstituted ghosts used in this study shrink on being washed with NaCl–PO_4. This is reflected in the lower than expected water content of the ghosts given in Table VII. A separate study of their osmotic resistance also indicated that reconstituted ghosts were of smaller volume than the original intact cells. Teorell (1952) observed this shrinkage with his preparation of ghosts as well as a swelling when they were suspended in KCl. It is reasonable to assume that the process of a differential Na and K permeability described by him for this volume change is operative in reconstituted ghosts. Confirmation of this mechanism must await further study.

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