PERMEABILITY PROPERTIES OF ERYTHROCYTE GHOSTS*

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INTRODUCTION

In research on the nature of the mechanisms of biological transport for electrolytes, non-electrolytes, and water, erythrocytes have been a much used model for single cell behavior. A large literature exists, which is growing rapidly owing to the introduction of radioactive tracers (*cf.* the monographs by Brooks and Brooks (1941) and by Ponder (1948 *a*). More recent works are reviewed by Teorell (1949) and by Steinbach (1951)). In particular, attention has been focussed on the "selective" distribution of potassium and sodium, often characterized by a higher concentration of K and lower concentration of Na inside the red cells than in the plasma. The current opinion is that this K-Na distribution is caused by "active transport" processes (*cf.* the recent works by Maizels and collaborators, for instance Flynn and Maizels (1949); Harris and Maizels (1951); also Sheppard, Martin, and Beyl (1951); Raker, Taylor, Weller, and Hastings (1950); Dunker and Passow (1950)).

In view of the seemingly very complicated permeability processes in intact erythrocytes, it was thought to be advantageous to attempt some investigations on hemolyzed erythrocytes,—the so called "ghosts." It was believed that these cells, probably devoid of many of the "living" constituents, could serve as somewhat simplified models, capable of shedding some light on the more elementary, physicochemical properties of the cell membranes.

It has long been known that the cell residues after hypotonic hemolysis are by no means disrupted or disintegrated cells; on the contrary, the "ghosts" seem to retain many of the properties of intact blood corpuscles (the older literature is given by Barón (1928)). A few previous papers deal with the cation permeability of ghosts (Barón (1928); Winokuroff (1929); Ponder (1935–36); Davson and Ponder (1938)). The evidence brought forward by these articles seems, however, contradictory and incomplete, particularly with regard to the volume conditions at different tonicities of the medium in which the ghosts were suspended. As regards cation permeability Ponder (1935–36

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page 632) claims that "reversion" restores the cation impermeability, while Winokuroff (1929 page 100) claims that the ghost cells completely lose their semipermeable properties.

In the present work the permeability properties of human erythrocyte ghosts have been reinvestigated along somewhat different lines. Special attention has been devoted to the "osmometric" properties of the ghost. It has been shown that *untreated* ghost cells behave as almost perfect osmometers, being semipermeable in the sense that the K and Na ions exhibit a slow diffusion, while water is rapidly transferred across the ghost membrane in such a direction as to secure continuous isotonicity with the external medium. Treatment of the ghosts with minute amounts of a lysin (Na oleate) induces a rapid cation ion exchange across the membrane, which seems to follow simple diffusion laws. Various miscellaneous observations were also made on the permeability to some other substances (glucose, sucrose, etc.) and on the shape transformations of the ghosts.

Methods

Preparation of Ghosts.—Venous blood from the cubital vein of healthy persons was drawn into a centrifuge tube with addition of 1 volume per cent of 0.5 per cent heparin (in 0.9 per cent NaCl) as an anticoagulant. The corpuscles were centrifuged off at 2500 R.P.M. and washed and recentrifuged twice with equal volumes of Ringer's solution. The final suspension was added to distilled water in the proportion: 1 volume cells + 5 volumes of H_2O. After gentle mixing and standing for half an hour the hemolysate was filtered through cotton wool and kept stoppered in an Erlenmeyer flask (with excess of air) till the following day (in the refrigerator at + 4°C.).

If the hemolysate proportion approaches 1:10, the ghosts are irreversibly destroyed as regards the permeability properties to be investigated. A proportion less than 1:6 on the other hand, does not give sufficient loss of hemoglobin.

Salt Media and Mixing Procedure.—The (1 + 5) hemolysate was added to NaCl, KCl, or non-electrolyte (sucrose, glucose) solutions of various concentrations, hypotonic as well as hypertonic with regard to the original tonicity in plasma (*ca.* 155 millinormality). A geometrical dilution scale was employed with the ratio 1:4; i.e., 1000, 250, 62.5, etc. mN. For the usual electrophotometric measurements of the volume changes of the ghosts the following mixtures were prepared: 3.6 ml. NaCl, etc. solution + 0.4 ml. buffer solution of pH 7.10 (+ 0.1 ml. of Na oleate N/1000) and finally 0.2 ml. of the hemolysate. Gentle but rapid mixing is important. The time for the hemolysate addition is recorded as zero-time.

All chemicals were *pro analysi.* The *buffers* were veronal + Na acetate mixtures according to Michaelis (1930): 50 ml. buffer stock solution + 60 ml. N/10 HCl + 240 ml. H_2O. The *oleate soap* stock solution was made by adding a small excess of oleic acid to N/100 NaOH, shaking, and filtering. The strongly opalescent solution was always heated before the dilution to the final N/1000 solution.

The calculated *"tonicity", i.e.* the total osmotic concentration of the mixed salt

media relative to the *hemolysate,* included all components and the electrolytes were assumed to be fully dissociated. The mean total alkali concentration of the hemolysate (1 + 5) was analytically determined to be 20.4 mN (13.4 K + 7.0 Na).

Table I summarizes the set up of the serial experiments and the corresponding total concentration and tonicity values.

Determination of ghost cell volumes was usually performed indirectly by an electrophotometric method similar to the one described by Wilbrandt (1938) (*cf.* Ponder, 1948 *a*, p. 76). This rests on the principle that the light scattering of a red blood cell

TABLE I

Design of the Serial Experiments

(*a*) Experimental set up

	ml.	Serial experiments				
Stock solution (KCl, NaCl, etc.*)	3.6	M/1	M/4	M/16	M/64	M/256
Buffer, pH 7.1	0.4	← M/25 →				
Na oleate	0.1	← M/1000 →				
Hmt (1 + 5)	0.2	← *ca.* M/49 →				

(*b*) Resulting concentrations

(C_e), total concentration, *mN*	843	215	57.9	18.6	8.8
Tonicity (Hmt = unity‡)	41.3	10.5	2.8	0.91	0.43
NaCl experiments:					
(Na_e), external [Na]	841	213	56.4	17.1	7.3
(K_e), " [K]	0.7	0.7	0.7	0.7	0.7
KCl experiments:					
(Na_e), external [Na]	4.1	4.1	4.1	4.1	4.1
(K_e), " [K]	838	210	53	13.7	3.9

* For non-electrolytes such as sucrose, etc. the stock concentrations were doubled.

‡ Hemolysate composition: 13.4 mN K + 7.0 mN Na = 20.4 mN total concentration.

suspension increases when the cells shrink. We have found this to be true in the case of the ghost cells. As the theoretical relationship between cell volumes and light scattering, and hence light absorption, seems to be highly involved (due to influence of shape, refraction indices, etc.) we had to resort to an empirical calibration of light absorption *versus* volume:

(*a*) *The light absorption* was determined with a selenium barrier photocell using parallel red light (Corning filter BG 4 transmitting wave lengths of $\geqq$ 720 mμ). The cell suspensions were kept in a cuvette of 1 cm. length, and in order to increase the sensitivity the cuvette was placed 2 cm. away from the photocell. The absorptivity coefficient (ϵ) was calculated according to the usual formula $\epsilon = \log \frac{I_o}{I}$, in which I_o is

the galvanometer reading (100 per cent) with water in the cuvette, I the corresponding figure obtained with the ghost cell suspension. Stirring was not found necessary over periods of at least 5 minutes. Between readings of the successive samples in a series, the suspensions were poured back into the Pyrex test tubes in which they initially were mixed.

(*b*) *Hematocrit volumes* giving the absolute volume were determined in the high speed hematocrit of Enghoff (1937) (3 minutes at 9600 R.P.M., with a mean radius of 10 cm.). The construction of the centrifuge tubes is illustrated in Fig. 1. The length of the cell column in the bottom capillary bore (comprising about 1.5 to 2 per cent of the total tube volume) was measured by means of a measuring microscope. All tubes were appropriately calibrated gravimetrically with mercury. Difficulties

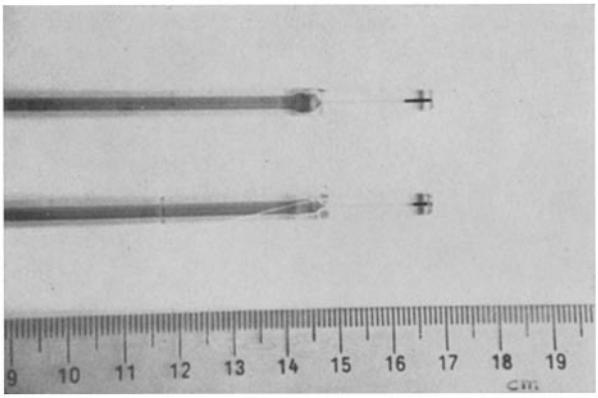

FIG. 1. Centrifuge tubes employed in the Enghoff hematocrit. Note the dark ghost cell sediment in the bottom capillary part (the upper tube an experiment with N/16 mN KCl, the lower tube with N/16 mN NaCl, both with oleate, after 30 minutes).

were sometimes encountered in media of the lowest tonicity (< 20 mN) because of diffuse boundaries between cell column and supernatant. In the higher tonicities, however, perfectly sharp boundaries were obtained.

(*c*) *The relation between light absorption and ghost cell volume* was determined from several separate experiments with simultaneous observations of hematocrit values and light absorptivity (ϵ) employing different media of various tonicities and varying time intervals after preparation of the cell suspension. If ϵ is plotted against hematocrit-per cent, an upwardly curved graph is obtained. This could, however, be transformed to a satisfactorily straight line by replotting the logarithm of ϵ (log ϵ) against hematocrit-per cent. In Fig. 2 are assembled all single determinations as well as the corresponding straight line fit (calculated according to appropriate statistical methods).

The *volumes* (V) to be presented in the following are all calculated from the "average straight line" of Fig. 2.

The scattering of the points may seem large, and is in the main probably

due to the fact that the cell counts in the hemolysates have varied on different occasions. A comparison between the results obtained by the average calibration line and a corresponding single calibration line shows, however, that the more convenient use of the average line does not introduce error of a magnitude that will invalidate the conclusions to be drawn.

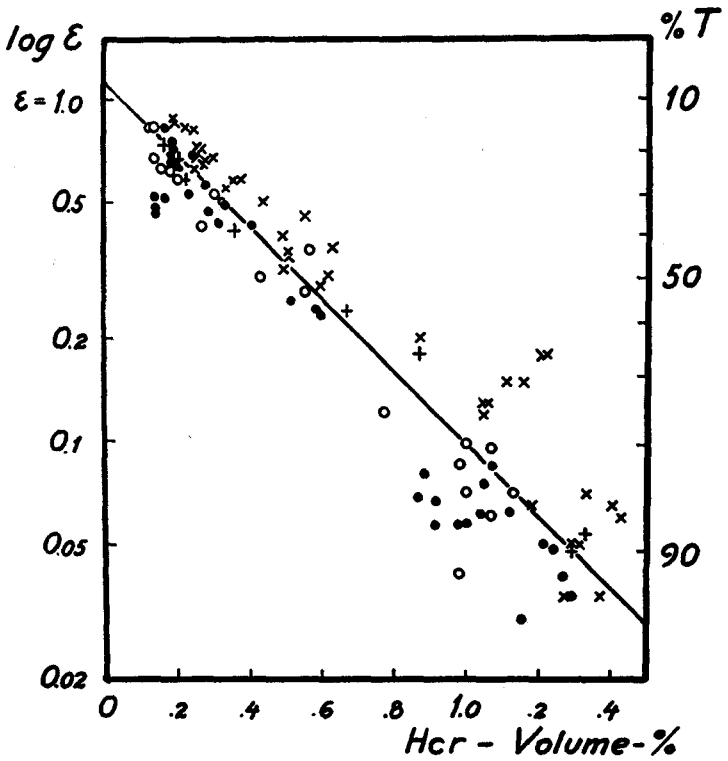

FIG. 2. Calibration curve of the relation between ghost cell volume and light transmission. The absorptivity coefficient ϵ plotted on a logarithmic scale gives a straight line *versus* the hematocrit volume (Hcr = volume per cent). T is per cent light transmission. The points mark four different series comprising KCl and NaCl experiments, with and without oleate addition.

Potassium and sodium determinations were performed by means of a flame photometer (Perkin & Elmer Corp., with internal Li standard) in suitable dilutions with an error estimated to be about ±5 per cent. No correction was attempted for mutual "interference" effects, which in cases of extreme proportions of K:Na might increase the error. The alkali determinations of the supernatants were straight forward, those of the ghost cell centrifugates (in the low speed centrifuge) necessitated a special correction procedure, because

it was impossible to achieve dense packing of the cells without admixture of the surrounding solution. The correction procedure was based on the use of a low concentration of inulin as a dilution indicator. This substance does not penetrate the ghosts, nor does it influence them osmotically, or apparently in any other respect.

Experiments aiming at *simultaneous measurements of cell volume changes and K and Na determinations* were run as follows:—

20 ml. hemolysate (1 + 5) was added to a mixture of 360 ml. NaCl or KCl (62.5 or 31.3 mN) + 40 ml. buffer pH 7.10 + 6.7 ml. of 10 per cent dialyzed inulin. This suspension was divided in three equal parts. The first was immediately subjected to Na and K analyses; the second was analyzed after 40 to 70 minutes; and the third portion, of which an aliquot was continuously followed photometrically for volume determinations, was left for about 60 minutes when 3.3 ml. of N/2000 Na oleate was added. The alkali analyses were performed 50 to 75 minutes after the oleate addition. In this way three different sets of data were obtained, one in an early phase after making the suspension, one just before the addition of the oleate and, finally, one showing the effect of oleate for approximately 1 hour (*cf.* Table IV and Figs. 9 to 11).

Detailed Procedure: (*a*) The ghosts were separated by first centrifuging for 15 minutes at 2200 R.P.M. in 150 ml. tubes, the supernatant being sucked off as completely as possible, after which the ghosts were recentrifuged in a small tube in an angle centrifuge in order to achieve still sharper separation.

(*b*) Of both supernatant and ghost deposit, 0.1 ml. was taken for *inulin analysis* (method of Corcoran and Page (1939) with minor modifications).

(*c*) The K and Na analyses were performed on 0.1 or 0.2 ml. samples diluted in 9.9 (9.8) ml. of N/100 LiCl (the latter being used as an internal standard in the flame photometer).

(*d*) The calculations of the alkali content referred to "pure" ghosts were performed according to the formula

$$\text{mN alkali content in "pure" ghosts, } a = \frac{gn - s^1}{n - 1}$$

Here s and g are the alkali figures obtained for the supernatant and ghost centrifugate respectively, n being the ratio (inulin concentration in supernatant): (inulin concentration in ghost centrifugate).

The inulin procedure yields ghost cell volume contents of the centrifugate

[1] $1/n$ is the fraction of supernatant, $(1 - 1/n)$ that of the ghosts. This leads to the obvious equation $a(1 - 1/n) + s \cdot 1/n = g$, which yields a. As an extra volume control to check the inulin procedure, direct cell counting was performed in a Bürker counting chamber on suspension and centrifugate (1:100). A simultaneous hematocrit determination was also made. These data allowed calculation of *the percentage of "pure" ghosts in the centrifugate* = 100 (*number in diluted centrifugate* × *hematocrit value*): (*cell number in suspension*).

which agree reasonably well with those obtained by direct cell counts. The discrepancies, although large in some cases, do not appreciably influence the final alkali concentration figures.

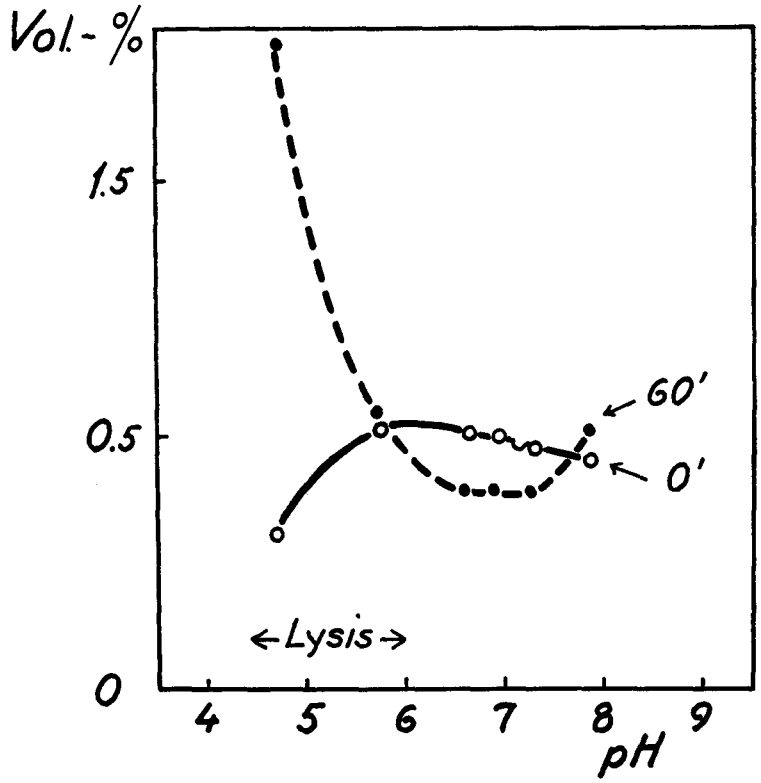

FIG. 3. The pH effect on the ghost cell volume (N/16 NaCl with oleate). The values after 60 minutes at pH < 6 are not reliable owing to agglutination (*cf.* Fig. 4 *a*) and lysis. pH 7.0 chosen as standard condition.

Photomicrography was performed according to conventional methods, either in dark field, or by phase contrast microscopy. The cell suspension was placed in a shallow quartz chamber (0.2 mm. height) (*cf.* the discussions on disc sphere transformation on page 689).

General Experimental Conditions

During the course of this investigation it was found that volume changes of the ghost cells were dependent not only on the main ion concentrations (of K and Na), the factors to be studied, but also on various other factors: the pH of the medium, the oleate concentration, and the temperature. Therefore it was

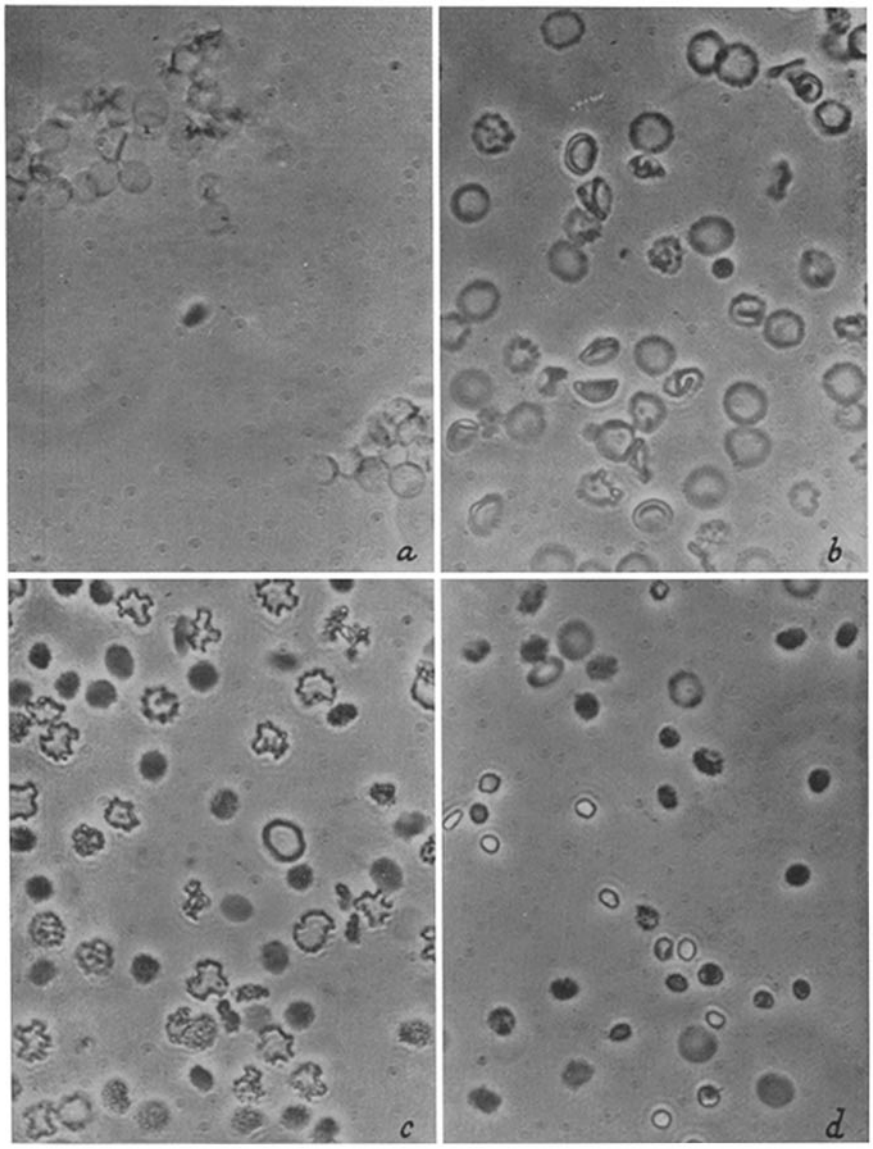

FIG. 4. Photomicrographs of the ghost cells at different pH. Phase contrast pictures corresponding to some of the 60 minute points of the experiment of Fig. 3. (*a*) pH = 4.72. (*b*) pH = 5.73. (*c*) pH = 6.90. (*d*) pH = 7.84.

necessary to find the optimal values of these and then maintain experimental constancy with regard to them.

The pH effect was rather marked as is seen in Fig. 3. Small portions of the universal buffer described above having pH values between 3.6 to 9.85 were added to a series of 62 mN NaCl solutions with M/1000 oleate, hemolysate added, etc., the photometric values followed as usual, and the reading after 0 and 60 minutes plotted against final pH of the suspension. It is to be noted that the maximal volume change takes place within the physiological pH range. A pH value of 7.0 was chosen as a standard condition.

TABLE II

The Effect of Different Na Oleate Concentrations

0.1 ml. oleate added in 4.3 ml. medium.

Salt (C_s = 56 mN)	Concentration of oleate added*	Volume		Lysis
		0 min.	30 min.	
		per cent	*per cent*	
NaCl	N/100	0.405	(1.08)	+++
"	N/200	0.415	(1.32)	+++
"	N/400	0.400	(1.17)	+++
"	N/800	0.420	0.328	—
"	N/1600	0.420	0.210	—
"	N/3200	0.420	0.215	—
"	0	0.395	0.425	—
KCl	N/1000	0.420	0.480	—

* Final concentration in suspension is 43 times less.

On *examination with the phase contrast microscope* some interesting observations were made as to the pH dependence of the shape of the cells (*cf.* Fig. 4). At the optimal pH of about 7 a polydisperse picture was obtained of mainly flat and strongly crenated cells. Toward a lower pH the cells had a smoother form and in the most acid media, about pH 5, at which lysis occurs, small pale agglutinated cells of spherical shapes were seen. In weakly alkaline media (pH about 8) the ghosts change to quite small, compact appearing bodies. The average diameters of the ghosts ranged in these experiments between 2.5 and 4 μ. Further consideration of the shape transformations will be deferred to the Discussion.

The *oleate concentration* is rather critical, in so far as too high concentrations cause lysis of the ghosts and too small amounts are without effect on the ion exchange and thereby on the volume changes. Table II shows that the maximal effect is produced between M/800–M/3200. M/1000 was chosen as the standard condition.

Some experiments were also made with *other fatty acid salts:* laurate and ricinoleate had about the same effect as oleate; palmitate was less effective, and stearate without effect. The behavior of the fatty acid salts in sucrose medium was different from that

in alkali chlorides; in the intermediate soap dilutions there was quick shrinking, which was followed by a phase of lysis.

Temperature did not exert any marked influence on the rate of volume change. The rate, measured by the slope of the curves at zero time, increased roughly by a factor of 1.3 for every 10° of increase in temperature; 25° was chosen as the standard condition.

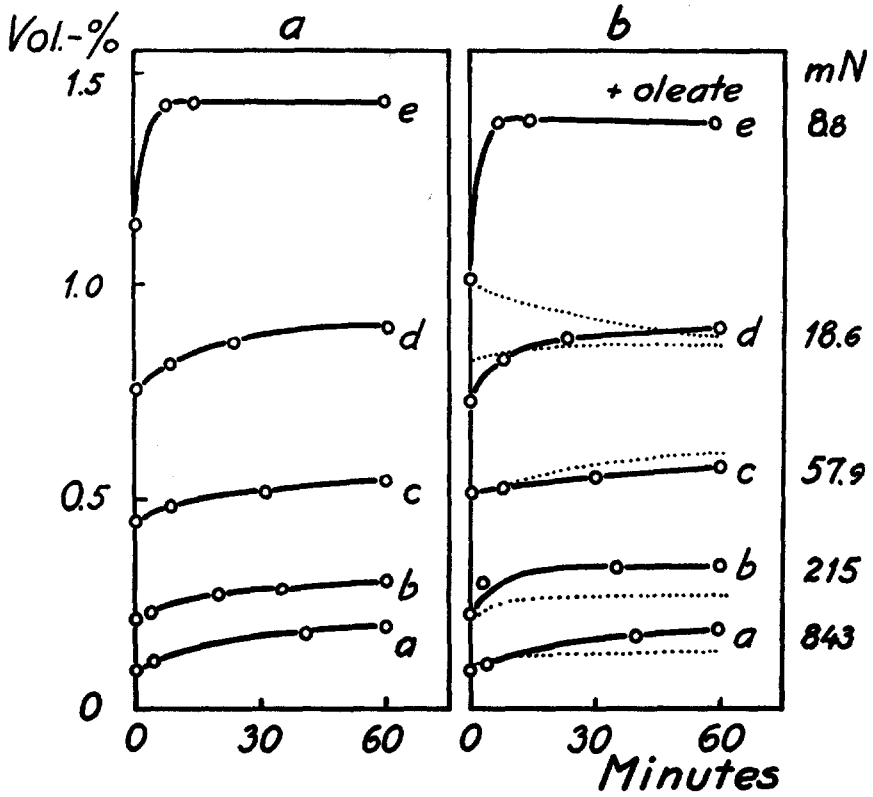

FIG. 5. Ghost cell volumes in KCl media of different concentrations. (Total concentration, C_e, in millinormality to the right. Curves *d* are close to isotonicity with the hemolysate.) (*a*) Without oleate. (*b*) With oleate. Dotted lines refer to volumes *calculated* according to the theory in the Appendix.

RESULTS

1. General Behavior of the Ghosts.—When the clear hemolysate containing the ghosts was added to hypertonic NaCl, KCl (or non-electrolytes) these solutions instantaneously acquired a marked reddish-grey turbidity as if a precipitate had been formed. The light absorption as recorded in the electro-

photometer jumped to a high value, the higher the more concentrated the medium. This *initial phase* value was attained within a few seconds and then the volume changed slowly for a period of about 30 minutes, after which an approximately constant value was approached at 60 to 90 minutes. The direction of the light absorption change during this *second* or *slow phase*

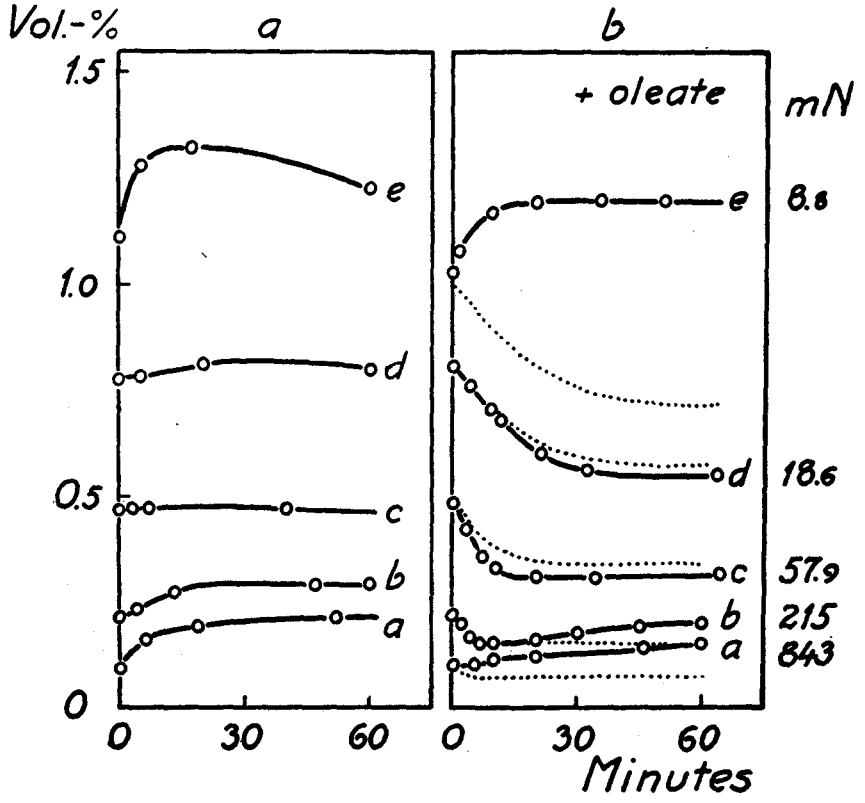

FIG. 6. Ghost cell volumes in NaCl media of different concentrations. (Total concentration, C_e, in millinormality to the right. Curves *d* are close to isotonicity with the hemolysate.) (*a*) Without oleate. (*b*) With oleate. Dotted lines refer to volumes *calculated* according to the theory in the Appendix.

depended on the conditions. With NaCl + oleate as a medium the absorption increased, signifying a volume shrinkage of the ghosts; the corresponding experiments with KCl showed a slow clearing up of the solution, indicating a swelling of the ghosts.

A detailed description and analysis of the observations follow, below. An over-all conclusion, however, is that *the ghost cells behave as comparatively perfect osmometers, permeable to water under all conditions and allowing a slow*

permeation of Na and K ions, particularly after conditioning with small amounts of fatty acid soaps.

2. The Ghosts as Osmometers during the Initial Phase.—A typical set of *volume* versus *time* curves for KCl and NaCl media of different concentrations is plotted in Figs. 5 and 6 respectively. It can be seen that the zero time value (= the initial phase) decreases with the tonicity of the medium used, but is

TABLE III

Ghost Volumes in the Initial Phase (Zero Time)

KCl and NaCl media of different concentrations, without and with addition of Na oleate.

Salt	Oleate	Medium concentration (C_e) (Tonicity in relation to hemolysate)					Figure No.
		843 (41.3)	215 (10.5)	57.9 (2.84)	18.6 (0.91)	8.8 (0.43)	
		Volume (V_o)					
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	
KCl	0	0.095	0.213	0.446	0.750	1.14	5 *a*
"	+	0.101	0.218	0.430	0.827	0.99	—
"	+	0.095	0.228	0.516	0.728	1.01	5 *b*
NaCl	0	0.088	0.210	0.468	0.775	1.11	6 *a*
"	0	0.119	0.228	0.496	0.870	1.11	—
"	+	0.101	0.220	0.480	0.810	1.03	6 *b*
"	+	0.095	0.197	0.490	0.810	1.14	—
"	+	0.135	0.220	0.516	0.870	1.11	—
Mean value, observed...		0.104	0.217	0.480	0.805	1.08	7
Calculated value*.....		0.104	0.216	0.488	0.821	1.00	7

* According to equation (1) ... $(C_e + 32)(V - 0.060) = 38.5$.

approximately the same with and without oleate addition, regardless of whether K or Na is employed as medium. These initial phase volumes are summarized in Table III, which also comprises four more series and the averages of these experiments. These average volumes in turn are plotted against total concentrations of the media in Fig. 7.

The experimental points conform rather well to a hyperbola of the equation[2]

$$(C_e + 32)(V - 0.060) = 38.5 \qquad (1)$$

The volume values *calculated* according to this expression are also found in Table III and agree satisfactorily with the observed figures.

Equation (1) is of the form of van't-Hoff–Mariotte's law for ideal osmotic

[2] Calculated by method of displacements of coordinates (*cf.* Running, page 53).

swelling-shrinking, *i.e. the volume of the ghost cells is inversely proportional to the concentration*, if certain allowances are made: First, that the least possible ghost volume, with practically all solvent water expelled, is taken to be 0.060 volume per cent of the suspension (= non-solvent volume); secondly that there

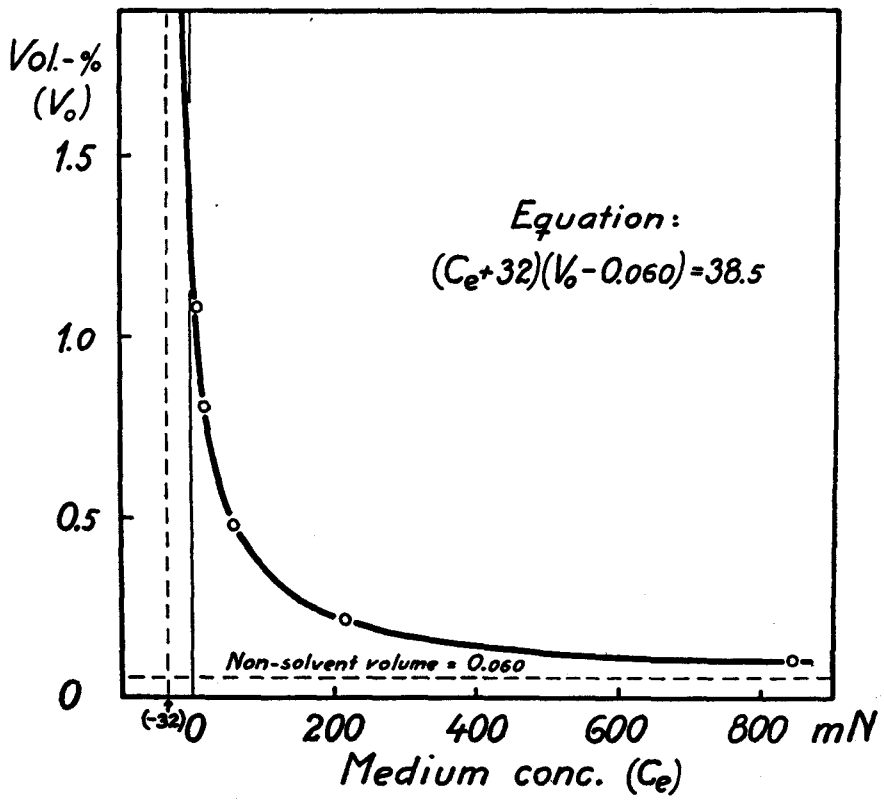

FIG. 7. The relation between the initial ghost cell volumes, V_o, and medium total concentration, C_e, of the medium. The open circles are mean values for the KCl and NaCl media of Table III. The solid line is a hyperbola calculated according to the modified van't Hoff–Mariotte equation (1). As to the significance of the asymptotes (the dashed lines) see the text.

exists, inside the cell, an internal pressure equivalent to a 32 mN NaCl solution. Further comments as to the physical justification of these values (which incidentally are the asymptote values of the hyperbola in Fig. 7) will be postponed to the Discussion (page 691). With these provisions, the experiments show that *the ghosts in hypertonic and not too strongly hypotonic media behave as reasonably perfect osmometers*. These conclusions are confirmed also by the fact that increase or decrease of the medium concentration by addition

of small volumes of, for instance concentrated NaCl, or H_2O, brings about an instantaneous change (within a few seconds) of the cell volumes in the expected direction (*cf.* the adjustment experiments in Table V). Apparently *the water permeation takes place with great speed,* in contrast to that of the alkali ions, which, as will be shown in the next section, pass across the ghost cell membrane comparatively slowly.

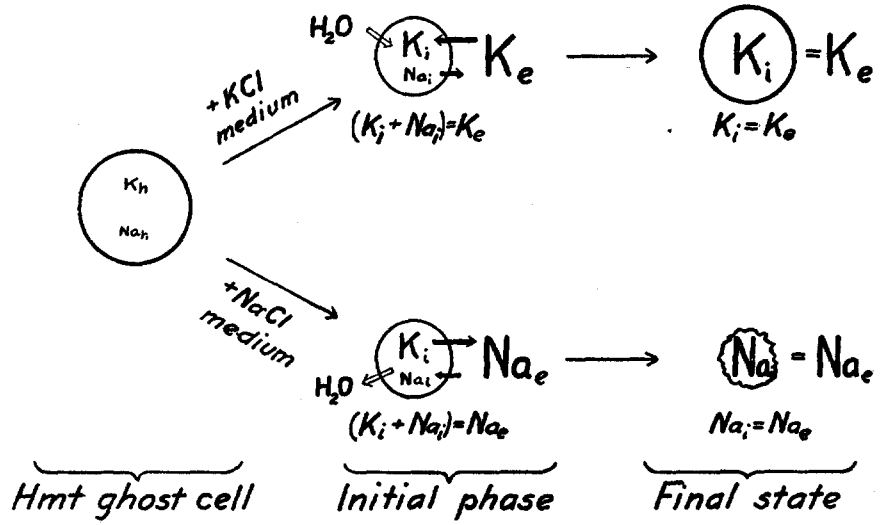

FIG. 8. Scheme of alkali ion exchange and volume changes of the ghosts (in equally hypertonic media of KCl and NaCl). The height of the (erect) ion symbols represents roughly the concentrations ("*i*" for the inside of the cells, "*e*" for the external medium). The medium first compresses the hemolysate ghosts (with expulsion of water, not indicated in the figure) to the conditions prevailing in the "initial phase" (= zero time). Now the "second, slow phase" starts with exchange diffusion of K and Na (the length of the filled arrows indicates roughly the diffusion velocities). In order to maintain isotonicity inside and outside, water (the open arrows) moves inwardly in the KCl medium and outwardly in the NaCl medium. In the final state the cells in KCl have *increased,* those in NaCl have *decreased* in volume.

3. Volume Changes Accompanying the Alkali Ion Permeation (the Second, Slow Phase).—The slow volume changes of the ghosts following the initial, rapid adjustment to the external osmotic pressure extended over about an hour, then rather constant states were attained. An inspection of Figs. 5 and 6 shows that there is a fundamental difference between the ghosts' behavior in KCl and NaCl media, with oleate present, in so far as the oleate conditions a marked shrinking of the cells when suspended in NaCl media, while they swell somewhat in KCl solutions. When oleate was omitted, the difference

between KCl and NaCl was less marked: cells in KCl swell slightly with time with about the same rate as in the presence of oleate, cells suspended in NaCl

TABLE IV

Potassium and Sodium Analyses of Ghost Cells and Supernatants, before and after Oleate Addition

K and Na in mN, volume V in per cent.

	K	Na	K + Na	K/Na	Cell volume V	
					Obs.	Calc.
Hemolysate (1 + 5)	13.4	7.0	20.4	1.92	0.78*	0.80‡
A. KCl medium $C_e = 57.9$						
Zero time (calc.)	37.5	19.6	57.1	1.92	0.55	0.49‡
5 min., before oleate	52.8	13.1	65.9	4.02	0.56	—
55 " , "	57.0	13.5	70.5	4.21	0.60	—
1 hr. after oleate	53.0	10.0	63.0	5.3	0.61	$V_\infty = 0.60$§
Supernatant (obs.)	51.0	5.9	56.9	8.6	—	—
Medium (calc.)	53.0	4.1	57.1	12.9	—	—
B. NaCl medium $C_e = 57.9$						
Zero time (calc.)	37.5	19.6	57.1	1.92	0.51	0.49‡
5 min., before oleate	23.1	37.7	60.8	0.61	0.51	—
75 " , "	19.1	40.6	59.7	0.47	0.53	—
1 hr. after oleate	3.2	51.1	54.3	0.06	0.36	$V_\infty = 0.35$§
Supernatant (obs.)	2.3	58.6	60.9	0.04	—	—
Medium (calc.)	0.7	56.4	57.1	0.12	—	—
C. NaCl medium $C_e = 31.8$						
Zero time (calc.)	20.6	10.8	31.4	1.92	0.73	0.66‡
5 min., before oleate	19.0	17.3	36.3	1.10	0.73	—
45 " , "	16.6	16.5	33.1	1.01	0.73	—
50 " after oleate	4.8	30.3	35.1	0.16	0.53	$V_\infty = 0.48$§
Supernatant (obs.)	2.0	33.7	35.7	0.06	—	—
Medium (calc.)	0.7	30.4	31.4	0.23	—	—

* Calculated on page 693.

‡ Calculated from equation (1) on page 680.

§ Calculated as V_∞ from equation (10) in the Appendix.

may retain a constant volume at least in solutions which are just isotonic and moderately hypertonic.

If attention is first focused on the oleate series, one may accordingly state that *cells in KCl swell, those in NaCl generally shrink.* This important difference can be explained by a hypothesis assuming that the ghosts' interior initially contains more potassium than sodium. In the absence of oleate these ions

diffuse out only very slowly after the cell bodies have been compressed. Under the influence of oleate, however, the relative cation impermeability of the cells is abolished and diffusion processes set in, tending to equilibrate the interior ion concentration with that of the medium. The higher "natural" diffusibility

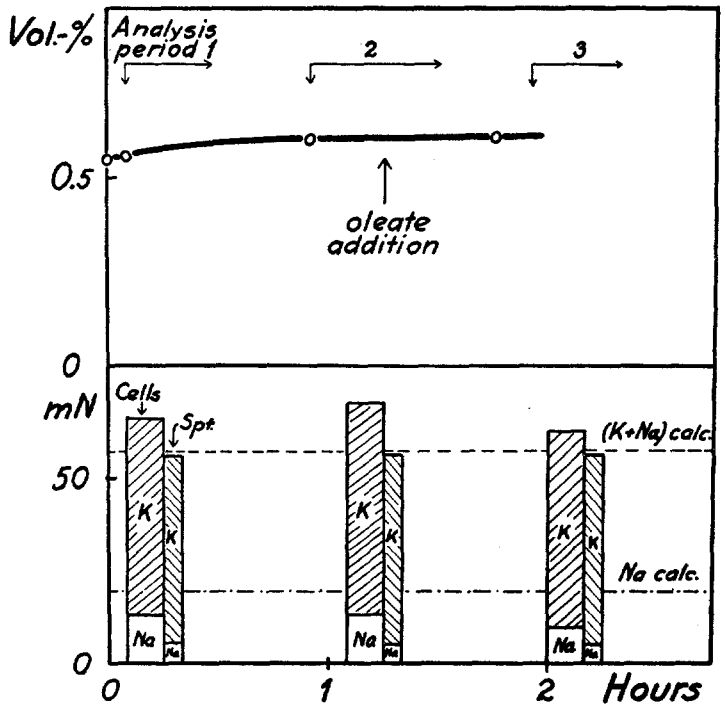

FIG. 9. An experiment with simultaneous volume and K and Na determinations of ghosts. 58 mN KCl medium (Table IV *A*). The separation of the cells in the centrifuges, etc. occupies an "analysis period." Here the oleate addition has no appreciable effect on cell volume or on alkali ion distribution.

of the K ions, as compared with that of the Na ions, accounts for the direction of the volume changes, because isotonicity inside and outside the membrane is always maintained. These changes are schematically represented in Fig. 8 and will be more closely considered in the Discussion. It is necessary first to analyze chemically the concentrations of the alkali ions in the systems in question.

4. The Ghost Volume Changes in Relation to the Alkali Ion Concentrations in Cells and Medium.—The contents of K and Na in the ghost cells were examined before and after the opening up of the cells for exchange diffusion processes by means of oleate addition. The procedure used was given in the technical section

(page 674). The results are collected in Table IV and graphically represented in Figs. 9 to 11.

(*a*) In the *KCl medium* (Table IV *A*, Fig. 9) very little happens, the ghosts swell slightly and are hardly influenced by the oleate (as already stated on

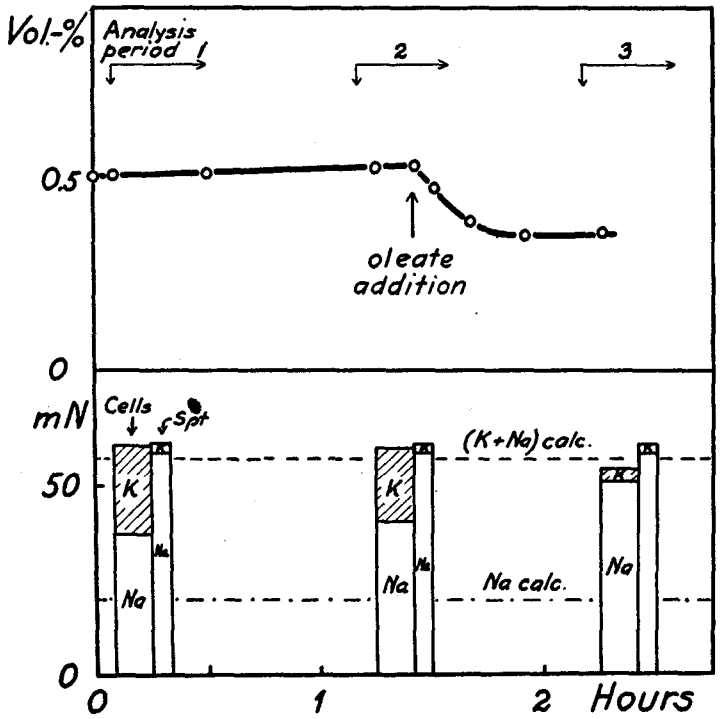

FIG. 10. An experiment with simultaneous volume and K and Na determinations of ghosts. 58 mN NaCl medium (Table IV *B*). Note the constancy of the higher K content (and low Na) of the cells before oleate addition. Oleate addition causes a shrinkage of the cells, a loss of K, and a gain of Na due to exchange diffusion.

page 682). The ionic picture as regards K and Na is roughly the same within the cells and in the supernatant solution and does not change significantly after the oleate addition. The somewhat higher total concentration (K + Na) in the cells is perhaps not beyond the limits of error of the rather complicated method of estimation, which may add up to about 10 per cent (*cf.* page 673). It should further be noticed that the *proportion* K:Na in the cells is of the same order as in the hemolysate cells, close to zero time values[3] (pictured in the diagram as *(K + Na) calculated* and *Na calculated*).

[3] The *calculated* zero time values of the K and Na concentrations of the cells are those of the hemolysate multiplied by the tonicity ratio; *i.e.*, the ratio between the

(*b*) In *NaCl media* (Table IV *B*, *C;* Figs. 10 and 11) several features are observable. In the first place one notices that *the ghost cell can maintain a considerable concentration difference between K and Na against the external*

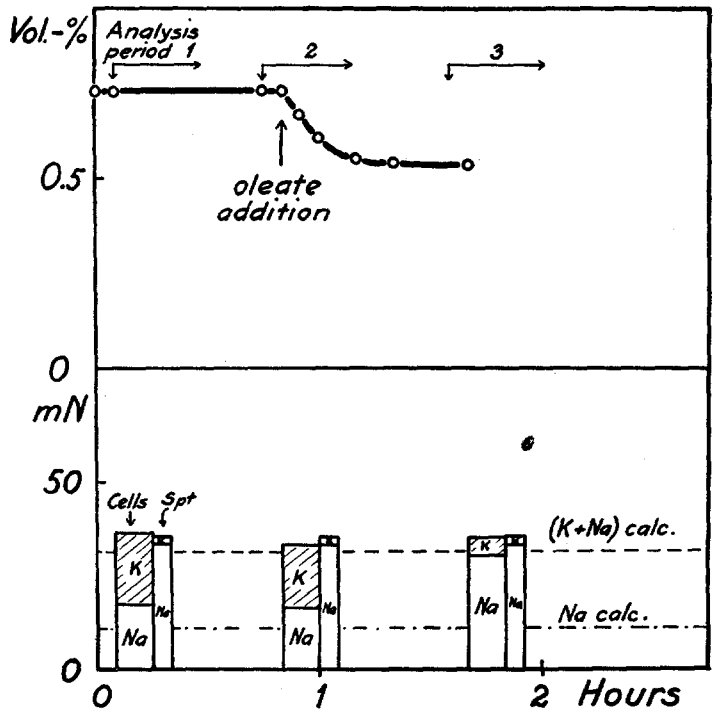

FIG. 11. An experiment with simultaneous volume and K and Na determinations of ghosts. 32 mN NaCl medium (Table IV *C*). Similar effects to those in Fig. 10, although more pronounced.

solution (supernatant), which remained practically unchanged for at least 1 to 2 hours until oleate was added. Secondly, after the oleate addition rapid and considerable shrinking takes place, accompanied by a loss of K from the cells and gain of Na leading to an approximate concentration equilibrium between the inside and outside.[4]

total concentration (C_e) of the medium and that of the (1 + 5) hemolysate (C_h). This calculation gives the alkali ion content in the ghosts under the assumption that the hemolysate cells behave as perfect osmometers, being semipermeable in the sense that only water readily passes the cell membrane.

[4] Old hemolysates, vigorous mixing, unsatisfactory aseptic procedure as to cleanness of glassware, presence of finger print fat are all conditions which give spontaneous volume changes and K loss as does added oleate.

The last column in Table IV contains volume values *calculated* in accordance with a theory for simultaneous water and ionic exchange processes, which will be presented in detail in the Appendix to this paper. The agreement between these figures and the experimental values is reasonably good.

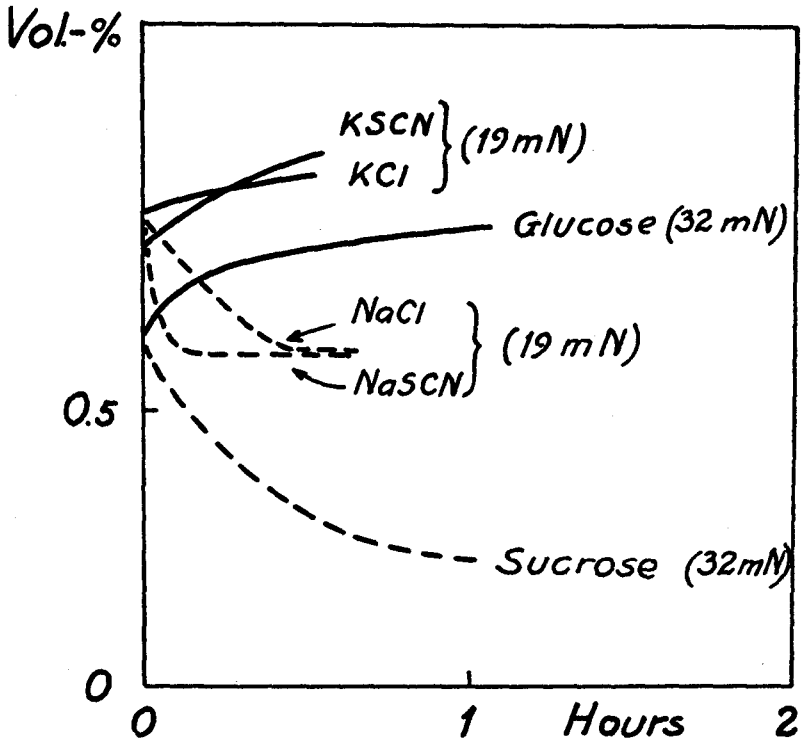

FIG. 12. The effects of KSCN, NaSCN, glucose, and sucrose compared with those of KCl and NaCl (with oleate). The K salts and glucose produce swelling and penetrate the ghosts well. The Na salts effect shrinking and are less penetrating. Sucrose causes marked shrinking and is almost non-penetrating.

Summarizing it may be stated that the slow volume change of the ghosts after oleate addition is the result of an *exchange diffusion of Na and K* (due to concentration differences across the ghost membrane) characterized by a simultaneous *maintenance of isotonicity of the cells* with regard to the medium. The significance of these conclusions will be discussed later.

5. Remarks on the Ghost Permeability for Other Alkali Salts and Some Non-Electrolytes.—Some orientation experiments have been performed with K and Na salts with anions other than chloride. Fig. 12 shows only the behavior of K and Na thiocyanate because they represent an extreme effect in the sense

that they give a much faster second phase volume change than the chlorides. The initial and final volumes do, however, approximately correspond to those of the chlorides. Also combined with SCN one notices that a K-containing medium produces swelling and a Na medium a shrinkage of the ghost cells. Lithium salts behave similarly to potassium salts, while calcium salts produce shrinking like sodium salts.

Experiments with glucose medium reveal a marked swelling and with sucrose a pronounced shrinking of the ghosts. This different behavior seems to indicate that *glucose penetrates the ghost membranes more rapidly* than the alkali chlorides; on the other hand, *the ghosts seem to be more or less impermeable to sucrose.*

DISCUSSION

The main results of the experiments described can be summarized as follows:—

The ghosts of human red blood cells, obtained by moderately hypotonic hemolysis, seem to have an intact semipermeable membrane, which allows very rapid passage of water but normally holds back K and Na ions (*i.e.* the ghosts behave as osmometers). Van't Hoff–Mariotte's law is applicable over a wide concentration (pressure) range of at least 40:1, if allowance is made for a "non-solvent volume" and for a small, constant internal pressure.

With small amounts of surface active material (oleate soap) it is possible to condition the ghost membranes to become permeable also to the alkali ions. Surface-conditioned ghosts still behave as good osmometers which always retain isotonicity with the suspending medium in spite of the in- and outflux of the alkali cations. During the exchange diffusion between the cell and the medium swelling or shrinkage may take place due to the fact that the diffusibility of K across the cell membrane is higher than that of Na.

These results will now be subject to some discussion, in which it is appropriate to consider first the methodological background.

1. On Reversal of Hemolysis and Related Optical Effects.—This investigation confirms the view of Bayliss (1924) and others that hypotonic hemolysis leaves the red cell membrane fairly intact after the loss of hemoglobin (which according to Davson and Ponder (1938) may take place through holes occupying 1/250 of the membrane surface). The membrane heals itself (*cf.* Barón (1928); Winokuroff (1929)). The claim of Brinkman and Szent-Györgi (1923) of a reversal of hemolysis was shown by Bayliss to be due to an optical effect of the salt addition, causing a "shrinkage of the corpuscles back to their normal size" thereby giving an increase in the refractive index difference (*cf.* Barón, who also cites the older literature in this field). No genuine uptake of hemoglobin has been demonstrated. A fixed amount of residual Hb inside the ghost attains however, a higher concentration after the shrinkage. None of the workers referred to has studied the quantitative behavior of volume changes,

although they were aware of them. Incidentally, Winokuroff, who cites a few data on the volumes of reversed blood cells, seems to deny their significance, for he states that "im Hämatokriten Scheinvolumina abgelesen werden und osmotische Einflüsse vortäuschen, die nicht vorhanden sind" (1929, p. 103).

From the previous works cited a question emerges which is worth discussing in view of the results presented in this paper. Is the hematocrit-light absorption method for volume determinations dependable? In other words, do the optical changes represent true osmotic effects?

As regards the light absorption method used here one has to remember that it is standardized by the hematocrit (*cf.* Fig. 2). As already pointed out (page 672) there is an appreciable scattering of the values obtained, particularly for the swollen ghosts. Offhand, it is impossible to decide which of these two methods best follows the changes of the average cell volume. With Winokuroff one might suspect that the hematocrit does not give appropriate packing of the ghosts, or that the form factor (see below) causes deviations. There are, however, some important features of our results, which promote some confidence in the present light absorption technique, at least as a relative method. First is the fact that it yields, over a wide range of volumes, values which conform amazingly well to a plausible quantitative expression, namely a modified van't Hoff–Mariotte law (*vide infra*). Secondly, in the Appendix of this paper it will be shown that the calculated kinetics of the volume changes during the slow phase agrees reasonably well with the experimental results (*cf.* the dotted lines of Figs. 5 *b* and 6 *b*).

A more direct argument can be obtained from the photomicrographs in Fig. 13, which clearly demonstrate, at least in NaCl medium, a shrinkage of the majority of the ghosts. The microscopic pictures reveal another important point, namely the differences among the cells as regards shape and probably size (*cf.* also Fig. 4 *b* to *d*). Apparently all results have to be considered as *averages* of a rather *heterogeneous population* of ghosts. In the darkfield microscope one gets the impression that the folds and crenations of the cells reflect, or diffract, the incident light strongly, hence causing the decrease in light transmission that is measured photometrically. Although these phenomena can all be due to decrease of the cell volumes, it may not necessarily be so. One might perhaps think of surface processes of various kinds giving rise to an uneven cell membrane without alteration of the ghost volume (*cf.* Ponder's discussion on the nature of crenation in his monograph (1948 *a*, page 92)).

As an indication of the possibility of membrane processes we might refer to the disc sphere transformations of intact erythrocytes described by Furchgott (1940) and by Ponder (1948 *a*, page 26). Similar shape transformations have also been observed during the present work with red cell ghosts. Details will be omitted here, but the photomicrographs (Figs. 13 *c* and *d*) demonstrate, however, a case in which a trace of surface-active material (skin fat) radically changes the surface structure of

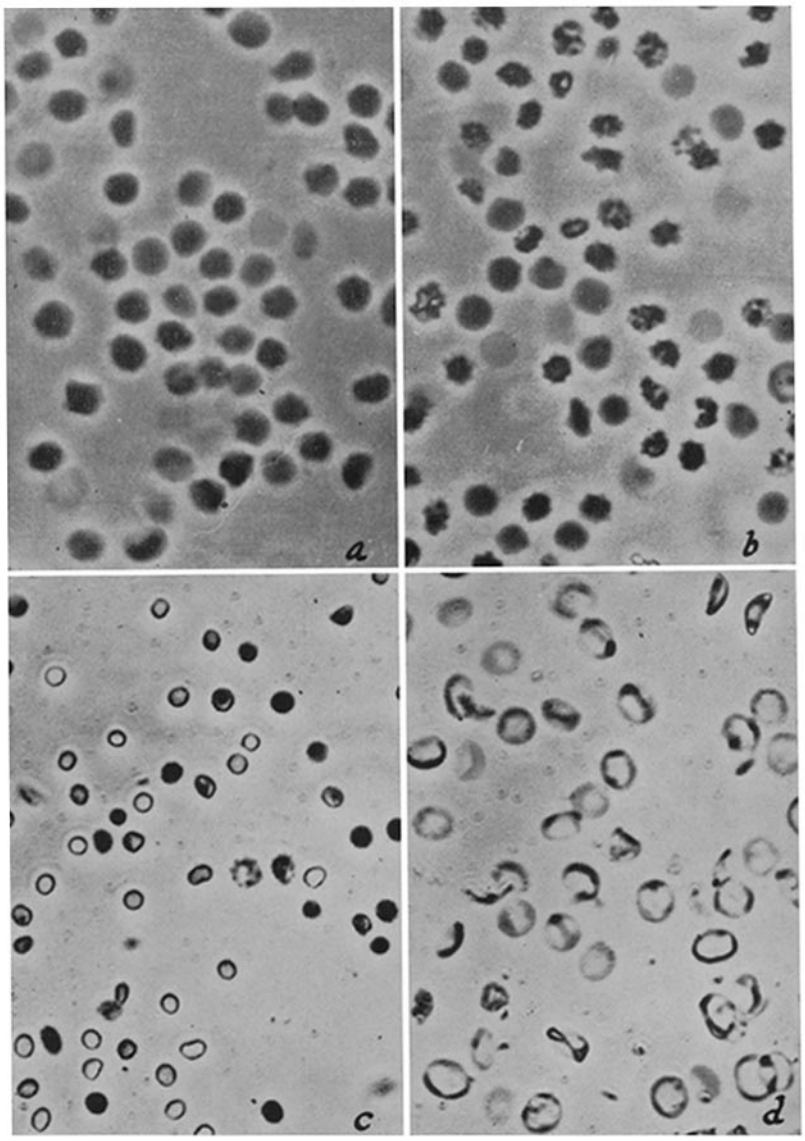

FIG. 13. Photomicrographs (phase contrast microscope). (*a*) Ghosts in a 58 mN KCl medium at pH 7.1 (+ oleate) after 30 minutes. (*b*) Ghosts in a 58 mN NaCl medium at pH 7.1 (+ oleate) after 30 minutes. Note the number of shrunken, often crenated cells as compared with the KCl case. (*c*) Ghost cells collected on the bottom of a *clean* quartz chamber (58 mN NaCl at pH 9.0). (*d*) The same as (*c*) but with minute traces of skin fat rubbed on to the bottom of the chamber. Note the transformation from compact spheroids of (*c*) to large discoid cells here.

the ghosts. Incidentally, it may be remarked that our findings are somewhat different from those reported by Ponder (*cf.* 1948 *a*, page 254).

When the morphological properties of the ghosts are considered, it may appear astonishing that the light absorption method really seems to measure, in the main, the osmotic volume variations.

2. Ghosts as Perfect Osmometers.—Now granting the accuracy and significance of the volume figures obtained, one may discuss the quantitative characteristics of the ghosts suspended in KCl or NaCl media of different concentrations, C_e. For the initial volume, V, it was found that an equation of the form

$$(C_e + a)(V + b) = c \tag{1}$$

gave an excellent representation of the observations when $a = +32$, $b = -0.060$, and $c = 38.5$ (*cf.* page 680 and Table III). The equation is a form of gas law, *pressure* × *volume* = *constant*, although for the pressure is substituted the proportionate concentration and the terms $+32$ and -0.060 represent correction terms.

In order to elucidate the possible significance of the correction terms it is instructive to study the more general gas law, that of van der Waals. This law is generally written

$$(P + \frac{a}{V^2})(V - b) = n \cdot RT \tag{2}$$

Here P and V are pressure and volume of n moles, R the gas constant (0.082 liter/atmosphere), T the absolute temperature, b the *Eigen* volume of the molecules, and a/V^2 the volume-dependent intermolecular attraction force (*Binnendruck*). Obviously our experimental Equation 1 is of the same form as Equation 2, although the *Binnendruck* term is put as a constant term ($+32$ units in the millinormality scale). The term b of equation (1) clearly has nothing to do with the *Eigen*-volume of the solutes. It seems rather to represent the *Eigen*-volume of the ghost; *i.e.*, the non-solvent volume (= dry substance volume).

The significance of the additive term, a, is more obscure. One is tempted to suggest that some form of internal pressure inside the ghost, or in its membrane, is the possible *formal* correspondence to van der Waals *Binnendruck* term. Actually there may exist a number of physical possibilities:

(*a*) In the first place, it is often proposed that there exist *elastic forces*[5] and *rigidity of form*, which oppose the osmotic swelling (*cf.* for instance Ponder (1944) and 1948 *a*, p. 83; Örskov (1946)). (*b*) Other hypotheses are concerned with a change of the free mass or activity of solvent water in the cell interior (bound water) (Örskov (1946); Ponder (1947, p. 386)). If these hypotheses were applicable, they would explain the deviations from unity of Ponder's empirical constant R (see below). (*c*) On the other

[5] Ponder (1944) estimated the bulk modulus to be of the same order as in gelatin.

hand, it seems obvious that the colloid osmotic pressure of interior components (proteins such as Hb or other non-penetrating substances) need not be taken into consideration for an explanation of the *positive a* term of equation (1). As the colloid osmotic pressure opposes the external pressure, it would, if acting alone, have yielded a negative sign of a. Besides, it appears less possible to have a colloid osmotic pressure of the high order corresponding to some tens of millimolarity. Related concepts dealing with a Donnan system may for similar reasons appear inapplicable, although the concentration of residual hemoglobin may rise appreciably in the highly compressed ghosts of the most hypertonic media. (*d*) The significance of the positive a term is, that a given external pressure gives *smaller* volumes than expected from the ideal van't Hoff–Mariotte law, even if allowance is made for the non-solvent volume of the cells. This fact cannot be reconciled with an assumption of an imperfectly semipermeable ghost membrane; *i.e.*, assumption of an ion leakage (*cf.* Ponder (1948 *a*, page 91)). If the K ions of, say a hypertonic KCl medium, were to enter the interior in appreciable amounts during the expulsion of the water taking place in the initial phase, the total amount of K ions would increase inside (by diffusion due to the direction of the concentration gradient), hence the pressure equilibrium would be attained at a *larger* cell volume than expected. This is, contrary to the experiments. In this connection, however, a reservation might be made with regard to certain effects that can follow from "diffusion against convection current" (*cf.* Wilbrandt, 1941, page 10). Pressure influences emerging from complicated ionic distributions are perhaps not yet exhausted. It might be worth while to enquire into the possible consequences of influences of an electrical membrane potential (Teorell 1935, 1937), or analyze the many cell-medium systems, which can be predicted from Jacobs' and Stewart's (1947) ingenious mathematical considerations. (*e*) The *hetereogeneity* of the ghost suspension (*cf.* page 689 and Fig. 13) need hardly lead to the appearance of the *positive* correction term a. Suppose the suspension to be a mixture of only two extreme types of cells, one being completely impermeable even to water, the other so highly permeable that not only water passes freely but also the ions inside and outside. In both extremes, and accordingly also in any mixture thereof, or of cells with intermediate properties, an external osmotic pressure excess would cause smaller volume changes, *i.e.* result in *larger* cells than expected from an ideal case, which again is contrary to the observations made here with the ghosts.

After having discussed qualitatively a number of possible reasons for the departure from the *simple, ideal* gas law we return to the quantitative side of the problem:

Ponder, who worked with intact red cells, expressed their osmotic behavior by the formula

$$V = RW\,(1/T - 1) + 100 \tag{3}$$

in which V is the volume in a medium of the tonicity T, W being the percentage of cell water and R an empirical constant introduced to reconcile observation with theory.[6] Ponder's formula is one form of the van't Hoff–Mariotte law; actually it can also be written $C_e(V - b) = C_o(V_o - b) =$

[6] R varies approximately between 0.5 and 1.0. A thorough discussion on the possible

constant, here C_o, V_o represent a reference state to which the tonicity unit $T = C_e/C_o$ is referred; furthermore $RW = (1 - b/V_o)$. It can now be seen that there is an important difference between our equation (1) and equation (3), the Ponder equation. Equation (1) has two corrective terms, one is in common with Ponder, *i.e.* b, which expresses the non-solvent volume (Ponder has instead the corresponding fraction of solvent volume W or RW), the other term which is missing in equation (3), is a, the internal pressure term. If the data of Table III, which fit so well in equation (1),[7] are inserted in the Ponder formula one finds that RW, instead of being constant, shows a marked systematic trend, varying from 0.9 in the most hypertonic medium to about 0.2 in the most hypotonic. Accordingly, it appears that *equation (1) is a more rational description of the osmotic behavior of the ghosts* (and perhaps also of intact red cells) *than equation (3)*.

3. The Ghost Volume of the Hemolysate.—The hemolysate, 1 volume of red cells + 5 volumes of H_2O, has an average (K + Na) concentration of 20 mN. Addition of 0.2 ml. hemolysate as in the procedure described to 4.1 ml. *isotonic* KCl or NaCl solution, would according to our equation (1) yield a *ghost* volume percentage of $V = 0.80$ [from $(20 + 32)(V - 0.060) = 38.5$]. If the red cell volume had remained intact during the hemolysis one would have expected a cell volume of $0.2 \times 1/6 \times 100/4.3 = 0.78$ per cent. Thus the *ghosts appear to have shrunk back to the original volume of the red cell*, as was assumed by Bayliss and by Ponder (1942, p. 259).[8]

During the actual phase of hemolysis, however, it is claimed that the red cell volume swells to a critical volume of about 170 per cent (*cf.* for instance Ponder (1948 *a*, p. 101); Guest and Wing (1942); Guest (1948)). Incidentally, one finds that the *maximal* ghost volume according to equation (1), taking $C_e = 0$, is $V = 1.26$, which is 162 per cent of the original red cell volume. The agreement between the volumes of the intact red cell and the ghost may perhaps support the view that the ghost retains many of the properties of the original cell. The shrinking back to the original volume after the hemolysis process is difficult to understand unless one assumes forces with the effect of something like elasticity.

4. Non-Solvent Volume of the Ghosts.—It has been concluded above that the

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significance of R is given in Ponder's monograph (1948 *a*, pages 83 to 114). *Cf.* also Ponder (1950).

[7] It may be added that a recalculation of equation (1) on *osmolar* basis (instead of on millimolar basis as before, assuming complete dissociation of the alkali salts) yields the same order of agreement between observations and theory. On osmolar basis equation (1) becomes $(\bar{C}_e + 48)(V - 0.062) = 60.8$, in which $\bar{C}_e$ denotes the milliosmolarity of the external medium, V as before the percentage total cell volume in the suspensions of the present experimental conditions.

[8] In his monograph Ponder (1948 *a*, page 254) instead states that the ghost dimensions are always smaller than those of the original cell.

corrective term b ($= -0.060$) of the concentration-volume formula equation (1), is likely to represent the non-solvent volume. As the ghost according to the preceding section has a total volume of 0.80 unit, one finds that *the non-solvent volume of the ghost occupies* $0.06/0.80 = 0.075$, *i.e.* 7.5 per cent, or equally well, that *the water content of the hemolysate ghost is approximately 92 per cent*, which seems to be a reasonable figure.

When the ghosts are "reversed," that is have shrunk in strongly hypertonic media, much of the water is expelled. For example, in the 843 mN medium of Table III the non-solvent volume was as high as 58 per cent, but yet the water occupied 42 per cent of the cell volume. The corresponding absolute water volumes are more impressive as measures of the osmotic effects: in the original ghosts the water content was 0.74 unit, in the highly shrunken ghosts only 0.04.

5. Conclusions as to the Osmotic Behavior of the Intact Ghost Cells.—The qualitative and quantitative considerations made above seem now to justify the following conclusions:

The *intact* ghosts, *i.e.* ghosts of a hemolysate suspension, shrink or swell as *perfect osmometers*, when due allowance is made for a constant *non-solvent volume* of about 7 per cent and for an approximately constant *inwardly* directed *internal pressure*, probably elastic or rigid in nature. This statement and the van der Waals type formula, equation (1), are valid for the initial phase immediately after the mixing of the ghosts into external media of any tonicity, hypertonic as well as hypotonic.

As to the nature of internal pressure it is difficult to make a decision with the evidence at hand. A mechanical elasticity or rigidity seems most probable, although it may be difficult to conceive of it as constant and independent of the degree of compression. Actually, the constant value of $a = +32$ is a product of the mathematical procedure employed; probably a formula with a volume-dependent a, would describe the observations as well as equation (1). Besides one must not forget that the complicated experimental technique may leave room for some systematic errors. The main argument in favor of something like elasticity, is the "shrinking back" after the process of hemolysis mentioned above and, in general, the absence of an unlimited swelling (*cf.* the hypothesis of colloid osmotic hemolysis of Wilbrandt (1941 *b*), also Ponder (1948 *b*)).

When emphasis is laid above upon intact ghosts, it is because of the following experimental observations regarding the *reversibility properties* of hypertonic and hypotonic ghosts: When hemolysate cells have been added to media which are *isotonic* or *hypertonic*, it is always possible by a further addition of salt externally to achieve shrinking to a still smaller value. Provided not too long a period (a few minutes) has elapsed since the addition of the hemolysate, one finds that the volume after the salt increase is nearly the same, as if the hemolysate had been initially mixed into a medium of the final total salt

content (*cf.* Table V, in which media of varying concentrations were all supplemented with KCl, etc. to the *same* final concentration and volume). On the other hand dilution with water has been found to produce a reswelling to about calculated values. The presence of oleate does not change the results.

As regards ghosts suspended in solutions, which have a considerably smaller total concentration than the hemolysate proper, *i.e.* in *hypotonic* media, one finds, as expected, swollen cells. On the addition of extra salt, however, these shrink far less than theoretically anticipated, which may be inferred from the

TABLE V

Experiments with Adjustment of Different Media to the Same Total Concentrations

	Medium concentration before adjustment (mN)					
	215	57.9	18.6	Hemolysate 20.4	8.8	5.5
	Ghost volume					
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
KCl, before*	0.155	0.435	0.771	0.80	1.15	1.31
", after‡	0.155	0.175	0.155	—	0.59	0.88
NaCl, before	0.145	0.360	0.765	0.80	1.22	1.31
" , after	0.145	0.150	0.155	—	0.54	0.79
Glucose, before	0.140	0.350	0.750	0.80	1.05	1.30
" , after	0.140	0.175	0.240	—	0.45	1.05
Sucrose, before	0.170	0.290	0.700	0.80	1.11	1.35
" , after	0.170	0.165	0.175	—	0.48	1.00

* "Before," 2 to 3 minutes after hemolysate addition to unadjusted medium.

‡ "After," 0.5 to 1 minute after adjustment to 215 mN.

experiments of Table V. These observations may justify the somewhat amplified conclusion that *ghosts behave as perfect and reversible osmometers only under conditions where they have been protected against swelling in excess of their original volume.* The fact, that the hypotonic ghosts are more or less irreversible, may be due to a rapidly developing ion permeability in the swollen state, resulting in a loss of their "osmometric" properties. A similar event certainly takes place during the swelling of the original red cell during the hemolysis, but in such a case repair is possible. Whether that is possible with the hypotonic, swollen ghosts has not been investigated. On the whole, it may be said that the behavior of hypotonic ghosts does not fit in very well with any of the explanations considered above. Stromatolysis and limitations imposed by optical conditions may, among other things, render futile any attempt to reconcile hypotonic and hypertonic properties of the ghosts.

6. Permeability to Potassium and Sodium.—In attempting to analyze the permeability properties of the ghosts for the alkali ions, it is appropriate to divide the discussion in two parts relating to the conditions before and after the addition of oleate.

(*a*) *Before oleate addition* it was found that the ghost cells could maintain a considerable concentration difference for both the K and Na ions against the surrounding medium (*cf.* the experiments shown in Figs. 9 to 11 and Table IV). In the case of NaCl media one is tempted to speak of potassium accumulation and a corresponding sodium impoverishment in analogy with the conditions of the intact red cells, because the concentration picture remains practically constant for long periods (here at least 1 hour). With the evidence available it is not possible to decide conclusively whether this state of affairs is a dynamic steady state due to *active transport* involving chemical processes (*cf.* for instance Flynn and Maizels), or whether it is simply a case of a *very low permeability* of the alkali ions in question (*cf.* also the discussion on false impermeability by Teorell (1949)). At any rate, it is likely that some ionic transfer does occur at the moment of mixing the hemolysate with the medium, *i.e.* in the initial phase, because the ghost K and Na concentrations are not those calculated for the ghost cells but somewhat intermediate between these figures and those of the external medium (*cf.* Figs. 9 to 11 and Table IV).

In order to obtain more information some *dialysis experiments* were performed:

The usual (1 + 5) hemolysate was placed in cellophane bags and dialyzed for 18 hours against large volumes of different solutions: (*a*) 100 mN KCl; (*b*) 20 mN KCl; (*c*) 20 mN NaCl; (*d*) Ringer's solution 1:7; (*e*) 14 mN KCl + 6 mN NaCl; (*f*) undialyzed hemolysate served as a control. When these dialyzed hemolysates were added to KCl and NaCl media ($C_e = 20$mN, with oleate addition) the following observations could be made with regard to the *second phase* behavior:

	KCl medium	NaCl medium
Swelling	(*a*) *c d e f*	—
No volume change	*b*	*c*
Shrinking	—	*a b d e f*

The absence of slow volume effects in the KCl medium on the hemolysate ghosts dialyzed against isotonic KCl (*b*), also in the NaCl medium with hemolysate dialyzed against isotonic NaCl (*c*), may indicate that the inside of the ghosts was brought into equilibrium with the outside; *i.e.*, that selective ionic distributions were absent.

The outcome of the dialysis experiments above may justify the assumption that *the ionic composition of the hemolysate ghosts is the result of a dialysis or diffusion equilibrium* between the original red cells and the five volumes of water added to promote hemolysis, rather than a manifestation of special active processes.

(*b*) *After oleate addition* there appeared a comparatively rapid transfer of the alkali ions, which is most simply explained by assuming that the oleate somehow opens the ghost membranes for these ions. The underlying mechanism remains, however, quite obscure. One may imagine some possibilities: some form of surface disintegration yielding holes thereby increasing the available area for ion diffusion; formation of surface complexes affecting the permeability properties (*cf.* Ponder (1949); Widdas (1951)) or a related idea, namely changes of the fixed charge density of the cell membranes, which may play a decisive role for the ion permeability (see below, the remarks on the water *versus* ion permeability, and Teorell, 1951). With respect to the effect of oleate, and some other fatty acid soaps, it is interesting to note that "lysins" of this and other types induce potassium leakage also on intact red cells (*cf.* Ponder (1951, 1948 *b*)).

As to the quantitative relation between the *rate of transfer of K and Na* in the oleate-conditioned ghosts, a rough estimate of the results of Table IV (Figs. 9 to 11) yields a mobility ratio Na:K of the order 0.4–0.8. This is in reasonable agreement with the ratio value of 0.5 used in the theoretical calculations of the best fitting curves of the KCl and NaCl experiments of Figs. 5 *b* and 6 *b* (see the Appendix V: k_2/k_1 = is *ca.* 0.5–0.7). The diffusion coefficient ratio Na:K could in the simplest case, neglecting the influence of membrane potentials and membrane fixed charges (*cf.* Teorell (1951)), be calculated as the ratio between Fick's diffusion constants (D for 1-1-valent electrolytes, u and v are the ionic mobilities in water)

$$D_{NaCl}:D_{KCl} = \left(\frac{2u_{Na}\cdot v_{Cl}}{u_{Na}+v_{Cl}}\right):\left(\frac{2u_{K}\cdot v_{Cl}}{u_{K}+v_{Cl}}\right) = \left(\frac{43\cdot 65}{43+65}\right):\left(\frac{65\cdot 65}{65+65}\right) = 0.8$$

Whether the experimental estimate of 0.5 compared with the calculated 0.8 signifies that the K ions have a higher mobility relative to the Na ions in the ghost membrane than in "free water" cannot be decided. Both experiment and theory are too approximate at present. It may be said, however, that there does not seem to exist in this case any indication of a pronounced selective K permeability in relation to that of Na (in the oleated ghosts).

7. Remarks on the Relations between Water Permeability and Ion Permeability.—The volume changes of the ghosts have been found to occur with great rapidity. During the initial phase volume adjustments for the securing of isotonicity, appreciable parts of the cell water were exchanged almost momentarily, while the amounts of ions exchanged were very much lower; *i.e.*, the ghost cell membrane behaves as an ion sieve. This fact, that water seems to move more easily than the alkali ions, is difficult to understand on the basis of the classical concepts of pore permeability, unless one regards the membrane as a sieve with holes of ionic dimensions. A perhaps better comprehension of an ion sieve permitting free passage of the water molecules is, however, possible on the basis of the fixed charge theory for membrane permea-

bility (Teorell, 1951). This theory shows that fixed electrical charges in the membrane (due to dissociation or to polar groups, etc.) may radically influence the transfer of otherwise freely moving *ions* while they do not influence *molecules* like those of water. In this particular case a retardation of the cation transfer, as observed before oleate addition, might be referred to a positive cell membrane. Any decrease of this positive charge (by means of oleate?) would then enhance the cation transfer.

Other mechanisms may also be possible, such as differential solubility in non-aqueous phases, etc. Apparently, much remains to be cleared up in the purely physicochemical field of permeability research, before final answers can be given as to the behavior of biological cells like the red cells or their ghosts.

SUMMARY

1. Erythrocyte ghosts from human blood were produced by gentle water hemolysis. The ghost-containing hemolysate (about 20 mN) was added to media of different composition (KCl, NaCl, glucose, sucrose, etc.) and varying concentration ranging from 8 to 840 mN. The volume changes of the ghost cells were followed by a light absorption method. The potassium and sodium concentrations were also analyzed in some representative cases.

2. The ghosts shrank, or swelled, in two stages. An initial phase with a momentary expulsion, or uptake, of water leading to an osmotic equilibrium, was followed by a second phase in which a slow swelling or shrinking proceeded toward a final constant volume.

3. The ghosts were semipermeable in the sense that water always passed rapidly in either direction so as to maintain isotonicity with the external medium.

The relation between ghost cell volumes (V) and the total concentration (C_e) of the suspension medium can be expressed by a modified van't Hoff–Mariotte law: $(C_e + a)(V - b) = constant$. Here a is a term correcting for an internal pressure and b is the non-solvent volume of the ghost cells. This means that the ghosts behave as perfect osmometers.

4. On the other hand appreciable concentration differences of the K and Na ions could be maintained across the intact ghost cell membranes for long periods. Whether this phenomenon is due simply to very low cation permeability or to active transport processes cannot be decided, although the first assumption appears more probable.

5. When the ghosts were treated with small concentrations of a lytic substance like Na oleate, the alkali ion transfer was greatly increased. This seems to be a simple exchange diffusion process with simultaneous, continued maintenance of osmotic equilibrium (= the second phase).

A simplified theory is also given for the kinetics of the volume variations and ion exchange during the second phase (*cf.* the Appendix).

6. Miscellaneous observations on the effects of pH, and of some other substances are discussed. Some shape transformations of the ghost cells are also described.

APPENDIX

The Kinetics of Exchange Diffusion with Simultaneous Maintenance of Osmotic Equilibrium.—I. Assumptions: Closed cells, with a non-elastic but non-rigid membrane of constant permeability properties containing only two solutes, say KCl and NaCl, are suspended in a large volume of a medium of the same salts. The water permeability is very high, whereby a *continuous osmotic equilibrium* between the cell content and the external medium is always maintained, even during the ion diffusion processes. For simplicity Fick's diffusion law is applied, *i.e.* the transfer rate of any constituent (here KCl and NaCl) is proportional to the concentration difference across the membrane (this implies neglect of the radical influences exerted by the electrical membrane potential as well as of the fixed charges of the membrane, *cf.* Teorell 1949, 1951).

II. Notations:

N_K, N_{Na}, N: amounts of K, Na, and (K + Na).

K, Na, C: concentrations (in the solvent space) of K, Na, and (K + Na).

Indices (h), (i), and (e) refer respectively to the original cells (before addition to the medium), the inside of the cells (after the addition), and the external medium.

V_h, V_0, V_t, V_∞: the cell volume (solvent space) of respectively the original cells (here hemolysate ghosts) before addition to the medium, the cells immediately after the addition ($t = 0$), the cells at the time t, and the cells in the final equilibrium state ($t = \infty$).

k_1, k_2: transfer constants of K and Na (here being proportional to the diffusion constants of KCl and NaCl).

III. Basic equations according to the assumptions made

Fick's law:

$$\frac{dN_K}{dt} = -k_1(K_i - K_e); \qquad \frac{dN_{Na}}{dt} = -k_2(Na_i - Na_e) \tag{1a, 1b}$$

$$\frac{dN}{dt} = -k_1(K_i - K_e) - k_2(Na_i - Na_e) \tag{2}$$

Isotonicity conditions:

$$\frac{dN}{dV} = C_e \because \frac{dN}{dt} = C_e \cdot \frac{dV}{dt} \tag{3}$$

$$C_e = K_i + Na_i = K_e + Na_e \because (Na_i - Na_e) = -(K_i - K_e) \tag{4}$$

van't Hoff–Mariotte law:

$$V_0 \cdot C_e = V_h \cdot C_h \because V_0 = \frac{V_h \cdot C_h}{C_e} \tag{5}$$

IV. Derivation of formulas: Combining (2) with (3) and (4), (2) is rewritten as

$$\frac{dV}{dt} = \frac{k_2 - k_1}{C_e} \cdot (K_i - K_e) \tag{6}$$

From (1 *a*) and (6)

$$\frac{dN_K}{dV} = - \frac{k_1 \cdot C_e}{k_2 - k_1} \tag{7}$$

Integration of (7) between the initial volume V_0 and the intermediate volume V_t, observing that the initial *amount* of cell K is $(K_h/C_h) \cdot C_e \cdot V_0$, yields after dividing through by V_t

$$K_i = C_e \cdot \left[\frac{K_h}{C_h} \cdot \frac{V_0}{V_t} + \frac{k_1}{k_2 - k_1} \cdot \left(\frac{V_0}{V_t} - 1 \right) \right] \tag{8}$$

Equation (8) expresses *the cell potassium concentration in terms of the volumes* V_0 *and* V_t. The cell Na is $(C_e - K_i)$. The V_0 is given in known quantities by (5). V_t can be defined by equation (9), derived by substitution of K_i from (8) in (6) and by appropriate integration as

$$t = \frac{C_e}{(k_1 \cdot Na_e + k_2 \cdot K_e)} \cdot \left[(V_0 - V_t) + 2.3 \cdot V_\infty \cdot \log \frac{V_0 - V_\infty}{V_t - V_\infty} \right] \tag{9}$$

in which

$$V_\infty = V_0 \cdot \frac{C_e}{C_h} \cdot \frac{(k_1 Na_h + k_2 K_h)}{(k_1 Na_e + k_2 K_e)} \tag{10}$$

V. The relation between the Na and K permeability can be determined with the aid of (11), derived from (10) as the ratio k_2/k_1.

$$k_2/k_1 = - \frac{Na_h - \frac{V_\infty C_h}{V_0 C_e} \cdot Na_e}{K_h - \frac{V_\infty C_h}{V_0 C_e} \cdot K_e} \tag{11}$$

Note that $(V_0 C_e)/C_h = V_h$. Application of (11) to the experimental data recorded in Table IV (page 683) yielded for the relation k_2/k_1: *Experiment A* (KCl) *0.69; Experiment B* (NaCl) *0.56; Experiment C* (NaCl) *0.58.*

The *absolute* values of k_1 and k_2 can be determined from the slope dV/dt at zero time according to (6) when k_2/k_1 is known.

VI. A comparison between the theory and the actual observations has been made on the experimental data recorded in Fig. 5 *b* (KCl) and Fig. 6 *b* (NaCl) (pages 678 and 679). In these figures the dotted curves were evaluated according to the principles outlined above (with $k_2/k_1 = 0.5$). The V_0 was calculated from the *modified* van't Hoff–Mariotte equation (page 680), not from its simplified version (5) in order to obtain a better fit. For simplicity, however, the V_t was calculated with neglect of the correction terms (*cf.* page 691), which involves no serious error. With the exception of the highly hypotonic media, the agreement can be regarded as satisfactory with respect to the approximate nature of the comparison made.

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