I. A STUDY OF PASSIVE POTASSIUM EFFLUX FROM HUMAN RED BLOOD CELLS USING ION SPECIFIC ELECTRODES

II. QUANTITATION OF HUMAN RED BLOOD CELL FIXATION BY GLUTARALDEHYDE

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ABSTRACT

I. A STUDY OF PASSIVE POTASSIUM EFFLUX FROM HUMAN RED BLOOD CELLS USING ION SPECIFIC ELECTRODES

It is shown that, by using ion specific electrodes, the small potassium leakage induced by ouabain in human erythrocytes can be measured continuously and precisely.

Upon addition of isotonic sucrose solution to a suspension of red cells in physiological saline the passive (ouabain-insensitive) potassium efflux is directly proportional to the chloride ratio. (i. e., an exponential function of the total membrane potential). The same result is obtained upon addition of hypertonic sucrose solution, suggesting that neither osmolarity nor intracellular concentrations have any influence on the passive potassium efflux.

The independence of the potassium efflux and osmolarity can be verified by addition of glucose to the cell suspension. Glucose penetrates the red cells leaving the intracellular volume, and thus the membrane potential as well as the intracellular concentrations, unchanged.

Adding water or hypertonic sodium chloride solution to red blood cell suspensions shows that the potassium efflux increases slightly in more concentrated salt solutions. Inasmuch as this effect can be interpreted as a pure ionic strength effect, the experiments corroborate the hypertonic sucrose solution experiments in demonstrating no dependence of the potassium efflux upon intracellular concentrations.

The results of this investigation as well as other studies (LaCelle

and Rothstein 1966, Donlon and Rothstein 1969, Cotterrell and Whittam 1971) show that the passive permeability of the human red blood cell to potassium depends uniquely on the membrane potential near physiological conditions, while it depends on parameters such as pH or concentrations for values of the membrane potential over 40mV. This suggests that two different mechanisms of transport might be involved: one would control the permeability under normal conditions; the other would represent a leak through the route normally used by anions and become important only under extreme conditions.

ABSTRACT

II. QUANTITATION OF HUMAN RED BLOOD CELL FIXATION BY GLUTARALDEHYDE

The uptake of glutaraldehyde by human red blood cells has been measured as a function of time by a freezing point osmometer. The rate of attachment of glutaraldehyde to the cell proteins is high over the first hour, declining to zero over a period of a few days. The number of glutaraldehyde molecules cross-linking with each hemoglobin molecule is of the order of 200, in reasonable agreement with the calculated number of attachment sites. The cell membrane is immediately highly permeable to glutaraldehyde. Selective permeability to ions is lost during fixation. Ionic equilibrium is obtained only after a few hours. An optimum fixation technique for shape preservation is suggested.

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INTRODUCTION

Because it is readily available in great quantities and its structure is simpler than that of other cells, the erythrocyte is an object of choice for the study of cell membrane properties. The active pumping of sodium and potassium across the red cell membrane has been demonstrated for some time (Maizels and Patterson 1940) and it is probably now the best studied of all active transport processes.

A necessary lemma to the complete elucidation of active transport mechanisms in the red cell membrane is the explanation of the cells' passive transport properties. How does the red cell discriminate between ions and, above all, between anions and cations? What parameters determine the flux of each ion? How do the ionic fluxes depend on those parameters? Such questions must be answered if one is to explain the ionic steady state across the red cell membrane.

Presently, the most favored theory for the passive transport of ions through the red cell membrane is the so-called "fixed charge hypothesis". In this hypothesis the cell membrane is considered analogous to an ion exchanger membrane. The main supporting evidence for the theory comes from the study of penetration of polyvalent anions such as sulfate or phosphate across the cell membrane. While chloride, hydroxide and bicarbonate cross the membrane in matters of seconds or less, the polyvalent anions penetrate slowly enough to allow the successful use of radioactive tracers (Whittam 1964). A second hypothesis which could explain the data on passive membrane permeability in the

red cell is the carrier hypothesis according to which ions would combine specifically with mobile components of the membrane and so be "transported". A short review of these hypotheses and the supporting experimental evidence will be presented.

As shown in the Appendix, the Nernst-Planck equation for the flux of ions (I)

$$J_\alpha = - D_\alpha (\text{grad}\,[\alpha] + z_\alpha [\alpha] \frac{F}{RT} \text{grad}\,\psi) \qquad \text{(I)}$$

can be derived from irreversible thermodynamics theory under some simplifying assumptions, the most notable of which is to neglect the phenomenological cross-coefficients. Assuming steady state and a constant electrical field through the membrane, equation (I) can be integrated (Goldman 1943) to yield Goldman's constant field equation ((II) page 11) which has been used extensively to interpret experimental data. For example Rothstein et al., (LaCelle and Rothstein 1966; Donlon and Rothstein 1969) used Goldman's equation to explain the large cationic fluxes that they observed upon increase in the membrane potential. We shall see that there seems to be little justification for this application of Goldman's equation.

Rothstein and coauthors obtained their data by suspending the red cells in isotonic mixtures of sodium chloride and sucrose solution. The large salt effluxes that resulted were measured by a conductivity bridge. This method is only applicable when the ionic strength of the suspending medium is much lower than the normal physiological one. The results might be barely relevant to the normal functioning of the red blood cell. Donlon (1968) extended Rothstein et al., results toward

higher ionic strengths by measuring net potassium effluxes with a flame spectrophotometer. This method is cumbersome and not very accurate.

Cotterrell and Whittam (1971) used radioactive tracers to obtain their data. This method which measures only unidirectional fluxes is even more cumbersome than the use of a flame spectrophotometer and the interpretation of the data is not straightforward.

One of the purposes of this investigation is to test the feasibility of a new technique to follow net ionic fluxes through the red cell membrane. Using ion specific electrodes to measure the activities of various ions in the extracellular medium, it should be possible to monitor continuously the corresponding net ionic fluxes through the membrane. We shall see that the technique is mainly useful to measure potassium fluxes.

One has direct access to the extracellular medium in order to vary the experimental conditions. An indirect access to the intracellular medium is obtained through the osmotic properties of the red cells: intracellular concentrations can be increased or decreased by changing osmotically the red cell volume. These means of varying intracellular and extracellular concentrations will be used to study how the potassium efflux depends on parameters such as intracellular potassium concentration and membrane potential principally. As we want to limit this investigation to the study of passive fluxes, the red cells will be poisoned with ouabain, which has been shown to block specifically the active transport mechanisms (Post, Albright and Dayani 1967).

By increasing our knowledge of the detailed phenomenological aspects of the passive transport of potassium across the red cell membrane, we hope to throw some light on the transport mechanism itself.

BACKGROUND

The studies of penetration of water and non-electrolytes across the erythrocyte membrane suggest the existence of a predominantly lipid membrane containing pores of a maximum diameter of about 8 A° (Solomon 1968). This accounts for the high correlation between lipid solubility and permeability, for the sieve effect on hydrophilic solutes and for the characteristics of water penetration such as the differences between bulk and diffusional flow and the apparent activation enthalpy.

The most striking fact concerning the passive permeability of the red blood cell membrane to ions is their ability to discriminate between anions and cations. For example it has been shown that chloride crosses the membrane about a million times faster than potassium (Whittam 1964). Such behavior is the main basis for the development of the fixed charge hypothesis which attempts to explain the permeability characteristics of the erythrocyte membrane by supposing the existence of fixed cations in the aqueous channels within the membrane. In view of the demonstrated negative charge on the surfaces of the cells (Whittam 1964), the fixed cations are thought to be located inside the membrane, somewhere in the pores through which the hydrophilic ions are supposed to migrate. The model is then that of an ion exchanger membrane. The behavior of such membranes has been extensively studied (Helfferich 1962), theoretically and experimentally, and predictions can be made on which to test the model for the erythrocyte membrane.

First, the discrimination between ions should be mainly a function of the sign of their electric charge. It has been shown that in support to the hypothesis, the anions: sulfate, fumarate, lactate, succinate and phosphate (LaCelle and Passow 1966) behave qualitatively in the same way with regard to their diffusion across the membrane at equilibrium. Also, in experiments where red blood cells are suspended in low ionic strength media, it has been demonstrated that the permeability to cations depends only to a minor extent on the chemical nature of the diffusing ion (Rothstein 1968).

As no strong acid or basic group is known to exist in proteins, the fixed charge inside the membrane is most probably due to the protonation of weakly basic groups such as $R\text{-}NH_2$ or $R=NH$, etc. (Lehninger 1970). The fixed charge should then depend strongly on the pH.

Fig. 1 and Fig. 2 show the effect of pH on sulfate and potassium permeability respectively. The sulfate data were obtained by tracer technique at equilibrium - i. e., no net flux - and they are typical of anions. The potassium curve was obtained from experiments where the cells are suspended in low ionic strength media. Both are consistent with a decrease in the fixed positive charge with increasing pH, such as would happen with a weakly basic group. As the pH inside the membrane is not related to the extracellular pH in a simple way, these data cannot be directly interpreted in terms of an acidity constant.

Increasing the salt concentration on both sides of an ion exchanger membrane produces a loss of selectivity between co- and counter-ions

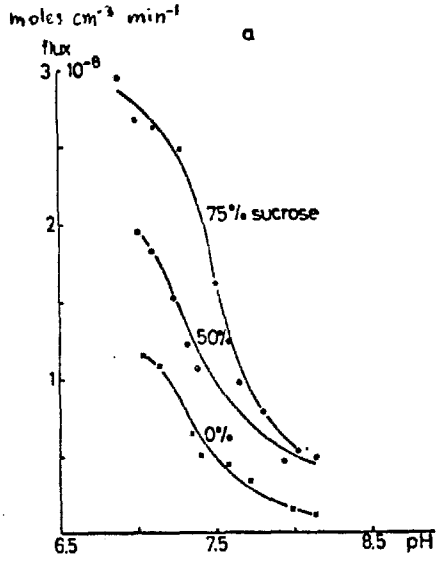

Fig. 1. pH dependence of SO_4^{--} permeability at Donnan equilibrium as measured at constant sulfate concentration and varying chloride concentrations in the medium (from Passow, 1969).

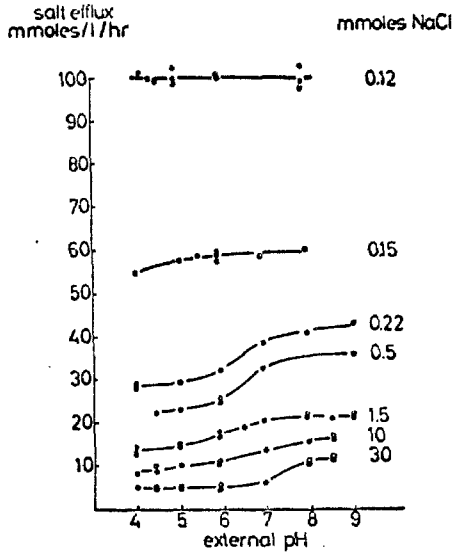

Fig. 2. The effect of external pH on the salt efflux at different external NaCl concentrations (from La Celle and Rothstein, 1966).

(Helfferich 1962). Such an effect is shown for the human erythrocyte in Fig. 3 where large potassium losses are induced by adding large amounts of sodium chloride to the suspending solution.

The dissociation of an acidic group such as $-NH_3^+$ increases with temperature and thus the fixed positive charge within the membrane should decrease. This effect should counteract the increment of ion flux with temperature and one would anticipate a rather low temperature coefficient for anion permeability. In contrast to this prediction an apparent activation energy of about 30 Kcal/mole has been found for sulfate and phosphate permeability across the erythrocyte membrane. To explain this discrepancy between the model and the reality Passow (Passow 1969) has suggested that a barrier other than the fixed charges could determine the rate of anionic diffusion through the red cell membrane. The fixed charges would only determine the concentration of anions within the membrane.

LaCelle and Rothstein (LaCelle and Rothstein 1966), in their experiments with low ionic strength red blood cell suspensions, found an apparent activation energy for cation permeability of about 12 Kcal/mole. This would be in excellent agreement with the enthalpy changes associated with the dissociation of amino groups (9 to 13 Kcal/mole).

If amino groups are responsible for the selectivity of the red cell membrane to anions over cations, it should be possible to block those groups and thus suppress this permselectivity. Such an effect is obtained in experiments with 1 fluoro 2-4 dinitrobenzene (DNFB) where large potassium losses are induced by the addition of small amounts of

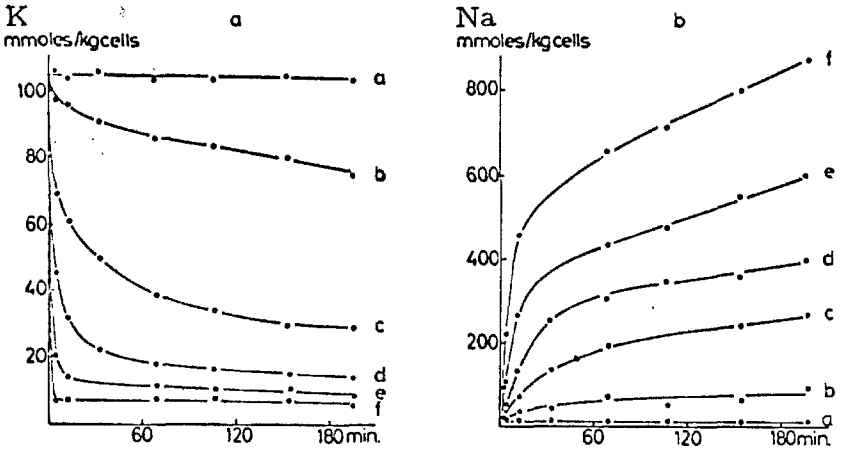

Fig. 3. Effect of hypertonic NaCl solutions on potassium permeability of human red blood cells. Temperature: 37°C, pH 7.4. Ordinate: (a) potassium content, (b) sodium content of cells, in mmoles/kg cells; abscissa: time in minutes. The sodium chloride concentrations of the medium amounted to a=0.166, b=0.995, c=1.33, d=1.66, e=1.99, f=2.66 moles/l (from Passow, 1969).

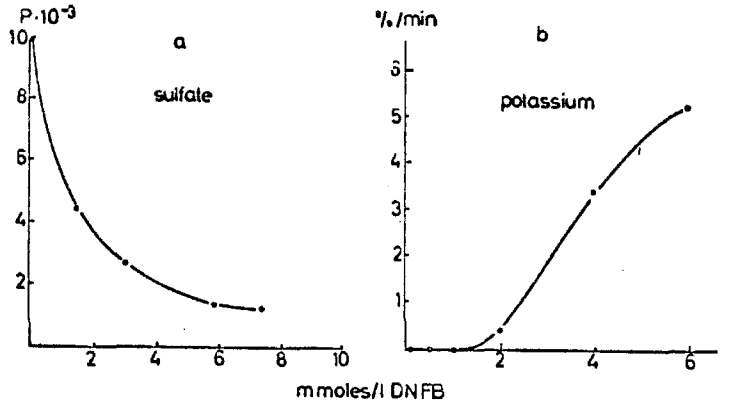

Fig. 4. Action of 1-fluoro-2,4-dinitrobenzene (FDNB) on sulfate and potassium permeability. (a) Ordinate: permeability constant min^{-1}. (b) Ordinate: rate of K net loss in percent of original K constant per minute. (from Passow, 1969).

the reagent (Fig. 4). Although DNFB is not completely specific for amino groups, negative results obtained with reagents specific to other groups suggest that amino groups are indeed the sought-for fixed cationic charges (Passow 1969).

All the experimental evidence presented above in support of the fixed charge hypothesis for the red blood cells is of a qualitative nature. The main body of quantitative evidence comes from Passow's experiments on sulfate permeability (Passow 1961, 1964, 1965, 1969). By tracer techniques, he measured the flux of SO_4 across the red cell membrane at equilibrium, varying the pH and the extracellular chloride and sulfate concentrations. He was able to reduce all his data to a single curve: "flux" versus "sulfate concentration within the membrane" (Fig. 5). This intramembrane concentration was computed from the extracellular conditions by hypothesizing a 2.5 M concentration of fixed groups within the membrane with a dissociation constant of 10^{-9}. Deuticke (Deuticke 1970) made similar experiments on phosphate diffusion and using the same hypothesis was able to reduce his data in the same way (Fig. 6). Both obtained an exponential dependence of the fluxes on the membrane concentrations of the anions, which is not predicted by the fixed charge hypothesis. Passow interpreted this result as he did for the temperature effect by suggesting mechanisms other than the fixed charge being the diffusion limiting barrier for ıions. Those other mechanisms are still at a completely hypothetical ge.

An attempt to obtain quantitative support for the fixed charge

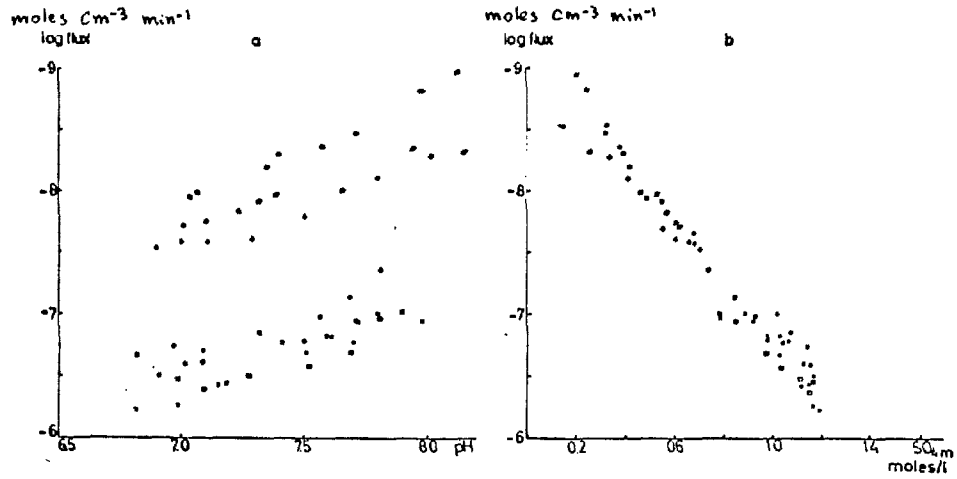

Figure 5

a. Sulfate ion fluxes under various conditions of pH, chloride concentration and sulfate concentration. (Upper set of points is the same as that of Fig. 1)

b. Data of Fig. 5.a plotted against the calculated sulfate concentration within the membrane.

(Taken from Passow, 1969)

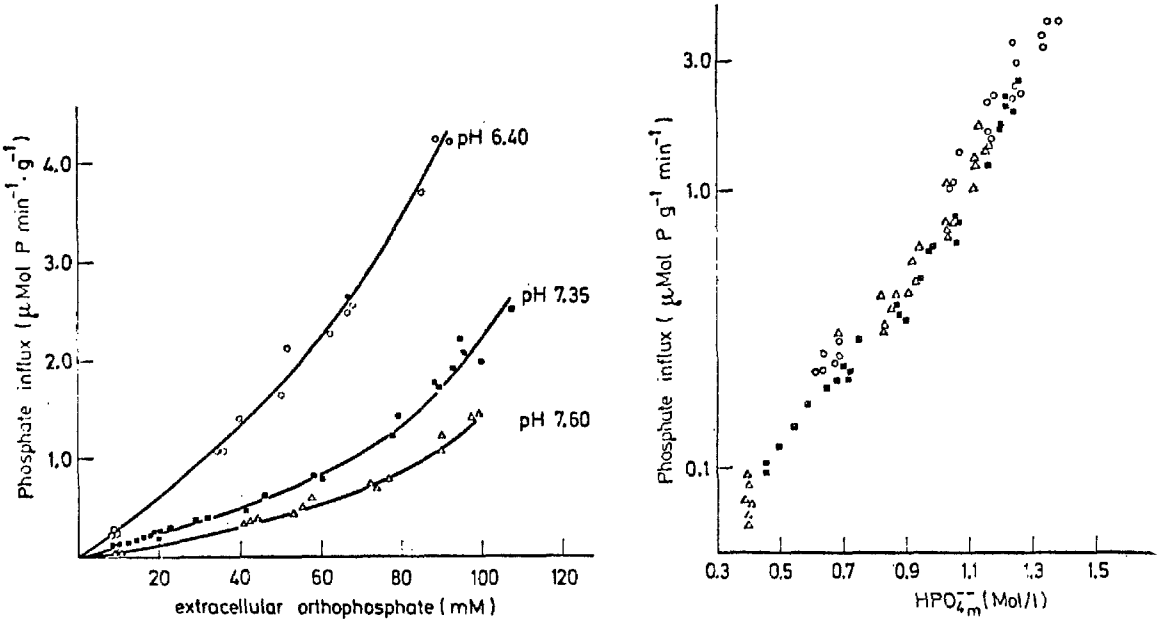

Figure 6

a. Phosphate influx under various conditions of pH and phosphate concentration.

b. Data of Fig. 6.a plotted against the calculated phosphate concentration within the membrane.

(Taken from Deuticke, 1970)

hypothesis by measuring cationic fluxes was made by Wilbrandt and Schatzmann (Wilbrandt and Schatzmann 1960) and then LaCelle, Rothstein and Donlon (LaCelle and Rothstein 1966, Donlon and Rothstein 1969, Rothstein 1968). By suspending red blood cells in low ionic strength media whose isotonicity was maintained by sucrose, they were able to induce a salt leak measurable by conductivity methods. Their main findings are shown in Fig. 7. The curve "rate of salt leakage" versus "logarithm of outside ionic concentration" is made up of three linear segments with markedly different slopes. The effect of osmolarity is also shown on the graph. The authors interpreted their results using Goldman's constant field equation for the flux of ions (see Appendix)

$$J_\alpha = -P_\alpha \cdot \frac{[\alpha]_i \cdot e^{-z_\alpha \cdot V_m} - [\alpha]_0}{e^{-z_\alpha \cdot V_m} - 1} \cdot V_m \qquad \text{(II)}$$

where J_α is the flux of the ion α; P_α is a permeability coefficient; z_α is the charge of the ion; $[\alpha]_i$ and $[\alpha]_0$ are the ion activities on each side of the membrane; V_m is the adimensional electrical potential across the membrane: $V_m = \frac{\psi F}{RT}$ (ψ = membrane potential; F = Faraday constant; R = gas constant; T = absolute temperature).

Taking the difference of potential V_m to be given by the Donnan ratio of the chloride ion:

$$V_m = \ell n \frac{[Cl]_i}{[Cl]_o}, \qquad \text{(III)}$$

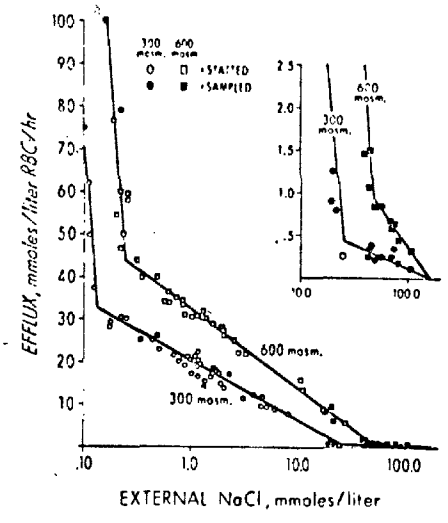

Fig. 7a

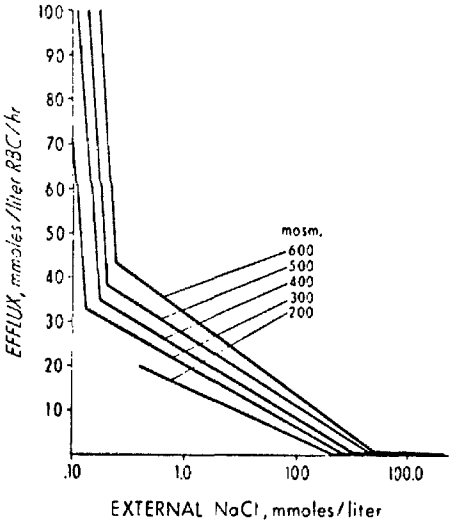

Fig. 7b

a. The effect of external NaCl concentration on the rate of salt efflux from red cells suspended on 300 and 600 mOsm media. An enlargement of the region of higher ionic strength is shown is the inset.

b. The effect of external NaCl concentration and osmolarity of the medium on the rate of salt efflux from red cells.

(From Donlon and Rothstein, 1969)

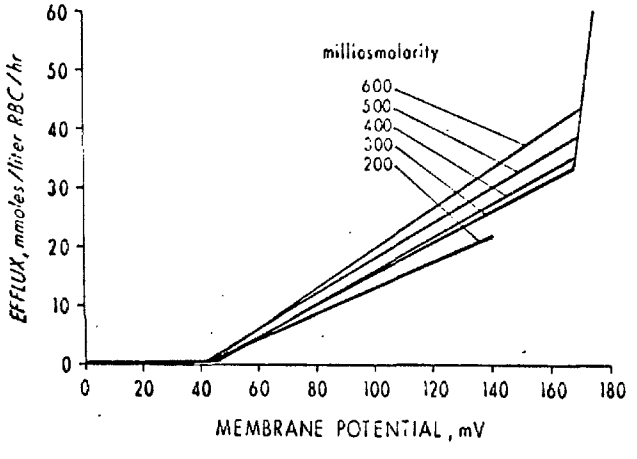

Fig. 8. The effect of the membrane potentials on salt efflux into low ionic strength media of various tonicities. Identical data as Fig. 7 (From Donlon and Rothstein, 1969).

the results can be replotted as shown in Fig. 8. The linear segments were then explained by the application of Equation (II) and a permeability coefficient could be computed for each of them.

Barr (Barr 1965) has shown that the Goldman equation for the diffusion potential across a membrane

$$V_m = \ell n \frac{\Sigma P_- [\alpha_-]_i + \Sigma P_+ [\alpha_+]_0}{\Sigma P_- [\alpha_-]_0 + \Sigma P_+ [\alpha_+]_i} \qquad \text{(IV)}$$

is valid in a number of cases where the field is far from constant. Equation (IV) reduces to (III) if Cl is the only anion and if P_{Cl} is much larger than the permeability coefficients of all the cations. (The permeability coefficients P_α used here are directly proportional to Goldman's "mobilities".) However, contrary to what Wilbrandt, Rothstein and coauthors seemed to imply, this does not justify in any case the use of Eqn.(II) for the fluxes of ions unless, as has been shown by Teorell (1953), the total concentrations of diffusible species are equal on both sides of the membrane. This is clearly not the case in experiments where cells are suspended in low ionic strength media. Furthermore, the difference of potential V_m which appears in the expression of the fluxes of ions across an ion exchanger membrane is the diffusion potential across the membrane and bears little relationship to the total membrane potential, V_{tot}, used by the authors to explain their data:

$$V_{tot} = V_i + V_m + V_0 \,. \qquad \text{(V)}$$

The potentials V_i and V_0 are developed across the boundaries of the membrane because of the Donnan exclusion due to the membrane

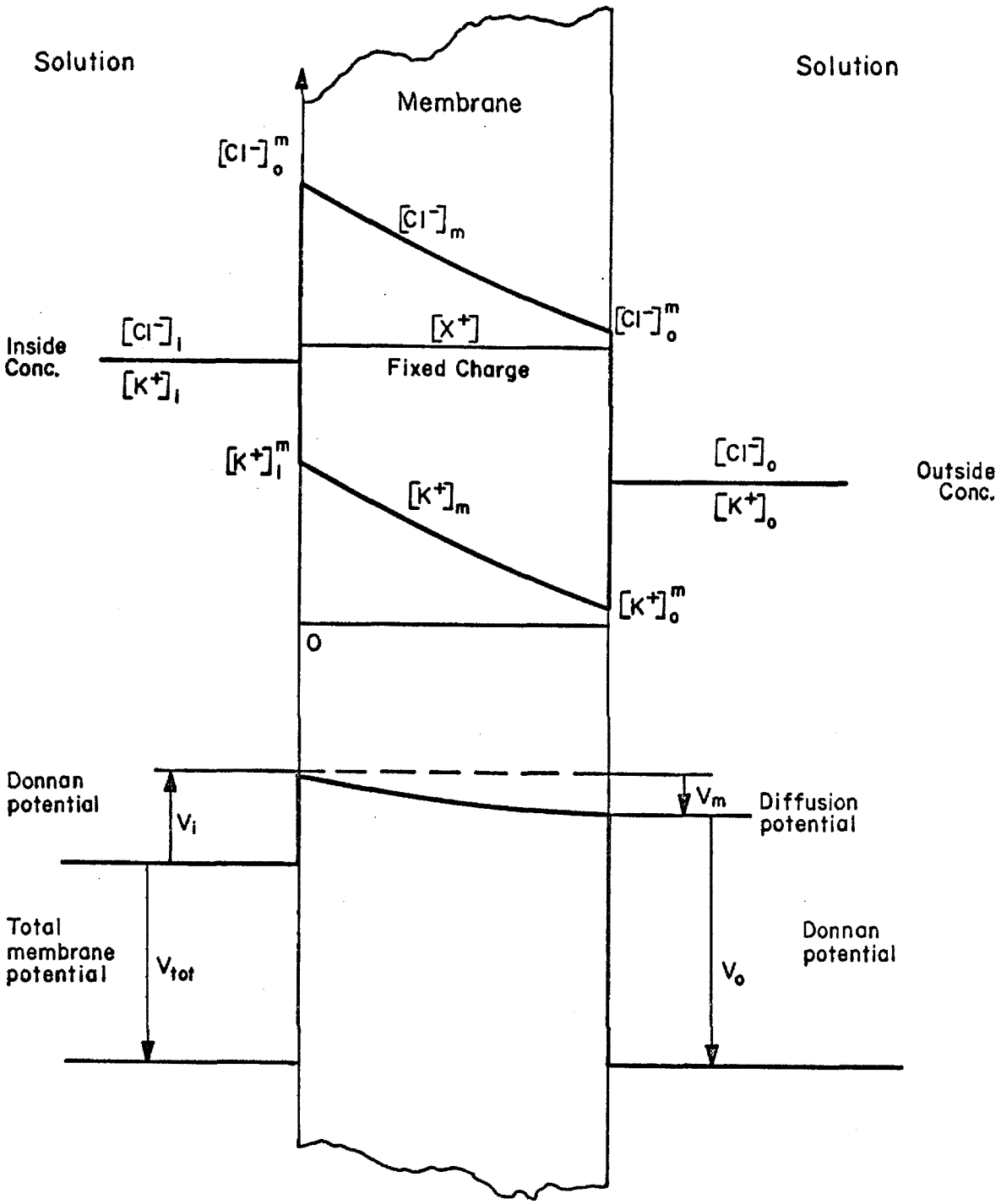

Fig. 9. Profiles of ionic concentrations and electrical potential for the diffusion of K Cl through an anion exchanger membrane.

charges. The potential V_m is developed across the interior of the membrane because of the differences in the self diffusion coefficients of the co- and counter-ions. Figure 9 depicts these various potentials. To obtain the diffusion potential V_m, Eqn. (IV) should be applied to the concentrations at the limits of the membrane, not in the bulk.

It is clear then that, in no way, can Wilbrandt, Rothstein, et al's results be taken as a verification of the fixed charge hypothesis. In fact, they demonstrate an unexpected and unique influence of the total electrical potential across the erythrocyte membrane on the cationic fluxes. This phenomenon was documented more thoroughly in a recent paper by Cotterrell and Whittam (1971). Using radioactive tracers, they studied the effect of substituting EDTA or citrate for chloride in the extracellular medium. They found little effect on active (ouabain-sensitive) cationic fluxes whereas the passive (ouabain-insensitive) fluxes of both sodium and potassium were markedly affected by an increase in the membrane potential as shown in Fig. 10. The authors concluded that the membrane potential affects the permeability of the red cell membrane in an asymmetric way with respect to influxes and outfluxes of cations. This explanation is even less satisfactory than the use of the Goldman equation as it does not even consider the possibility of changes in driving forces.

Further experiments are warranted to clarify the nature of the total membrane potential influence on the cationic fluxes. This is one of the questions that the present investigation will attempt to answer:

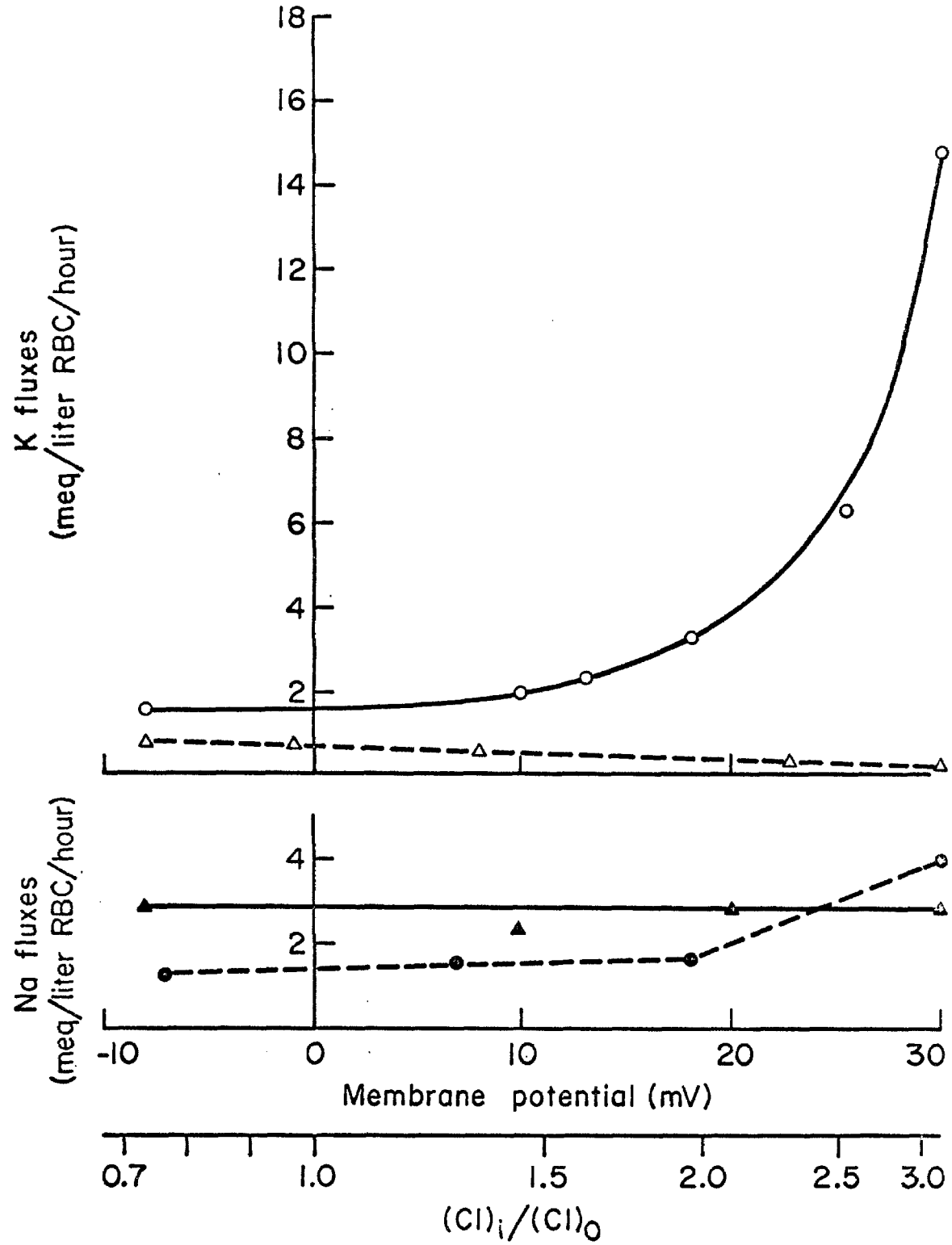

Fig. 10. Effect of replacing chloride by EDTA in the extracellular medium. o K efflux; Δ K influx; • Na efflux; ▲ Na influx. (from Cotterrell and Whittam, 1971).

how do the passive cationic fluxes depend on the concentrations and the total membrane potential?

A completely different way of explaining the passive permeability of the erythrocyte membrane to ions is to hypothesize carriers akin to those that have been demonstrated for the penetration of sugars. As they are hypothetical, these carriers may have any desired characteristics in order to satisfy the experimental data and especially the high selectivity of the red cell membrane for anions. This carrier hypothesis is not exclusive of the fixed charge hypothesis in as much as two distinct parallel routes could coexist in the membrane for ionic fluxes. The ions could both be diffusing through pores and be "transported" by macromolecules. Indeed the existence of such parallel routes is already accepted by the proponents of the fixed charge hypothesis who also consider the active transport to be performed by carriers.

No direct evidence for the participation of such carriers in the passive transport of ions has been found so far. Indirect evidence is almost as lacking although Whittam (Whittam 1964) reports some, such as the specific inhibition of the passive cationic fluxes by Digoxin. The main advantage of the carrier hypothesis is that it can explain readily the differences found between the transport characteristics in erythrocytes of different species. It allows for a genetic control of the passive transport of ions (mainly cations).

MATERIALS AND METHODS

General Set-Up

Figure 11 shows a diagram of the general experimental set-up. The red blood cell suspension is kept in the inner chamber of a 250 ml jacketed pyrex beaker. Water from a constant temperature bath (Radiometer VTS · 13c) circulates in the jacket. The temperature of the system is then maintained at 37°C ± 0.1°C and it is measured by a thermocouple (Yellow Springs Instruments 43 TA). A magnetic stirrer keeps the suspension well mixed. The electrodes are mounted on the cover of the beaker and dipped directly into the cell suspension. They are connected to a switching box (Orion - 605) and their output is read on a digital pH -mV meter (Orion - 801). A constant voltage regulator is interposed between the meter and the electrical outlet to insure maximum stability in the readings. Because of the high impedance of some of the electrodes, it was necessary to enclose the system in a Faraday cage.

Electrodes

Eight electrodes are usually used simultaneously: two reference, two potassium, one chloride, one pH, and two sodium electrodes.

Either of the two reference electrodes can be used in conjunction with any of the other six electrodes. One is a single fiber junction calomel electrode (Beckman 39410) filled with saturated KCl; the other one is a sleeve-type Ag/AgCl electrode (Orion 90-01) filled with a special solution (Orion 90-00-01).

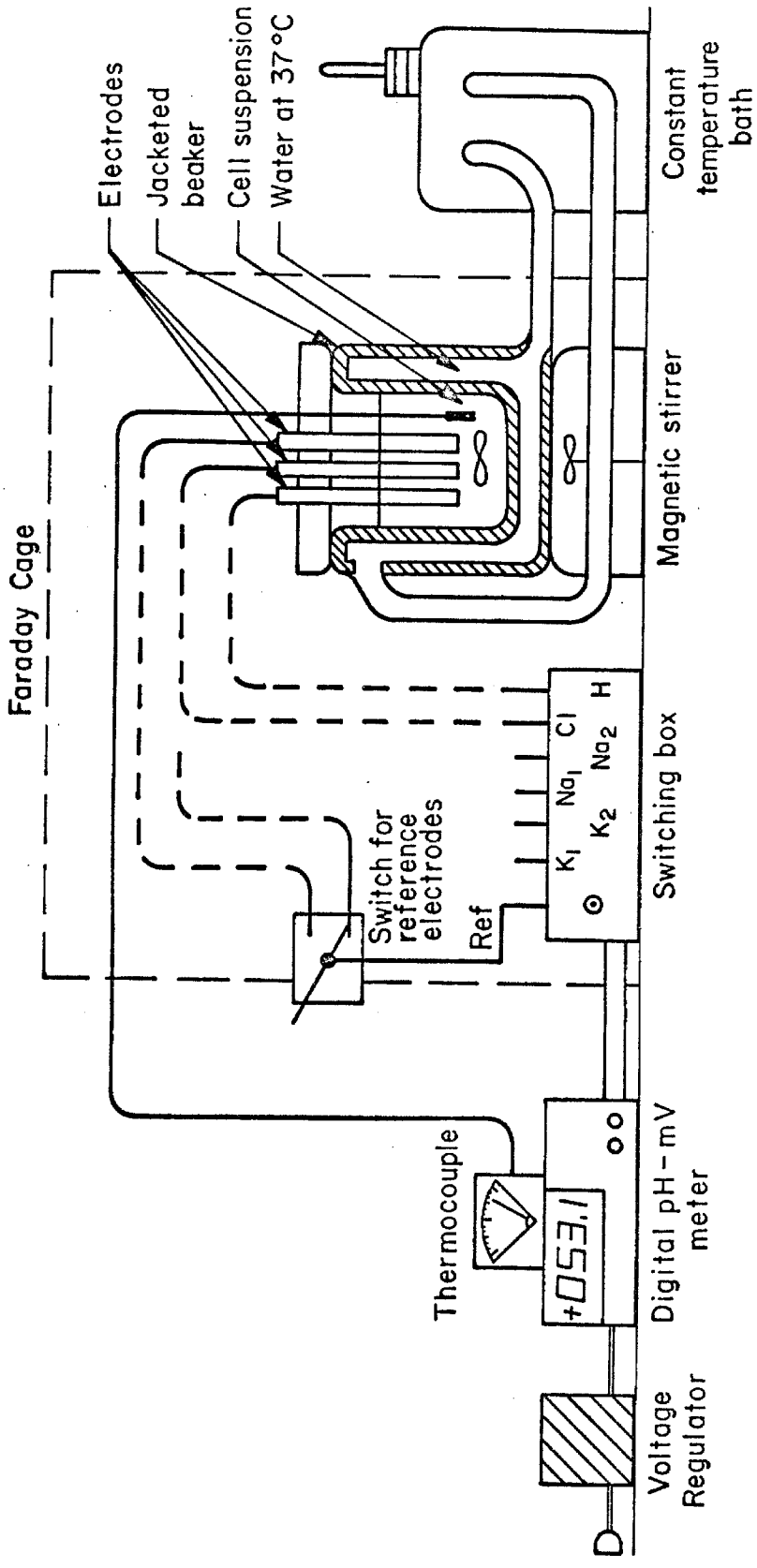

Fig. 11. Diagram of the experimental set up

Such reference electrodes will leak potassium ions into the measured solution. This leakage must be maintained to values much below the potassium outflux from the red blood cells in order to obtain meaningful measurements. Double junction reference electrodes can and have been used in some experiments but they tend to introduce a junction potential that drifts markedly. In any case it was found that in red blood cell suspensions, the potassium leakage from the reference electrodes is always negligible, probably as a result of partial clogging of the liquid junctions. This will be seen in the first part of Fig. 19: before the cells are added in the saline solution, the potassium readings are stable within the accuracy of the method. The use of the two electrodes simultaneously allows a better control of the drift they sometimes undergo.

The chloride ion electrode is a liquid ion exchanger electrode (Orion 92-17). It was found to yield much better results than silver-silver chloride metallic electrodes which tended to lose their silver chloride coating in a few hours. This was probably due to oxidation reduction reactions in the red cell suspension.

The sodium and the pH electrodes are glass electrodes (sodium: Corning 476210 and Beckman 39278; pH: Beckman 39301). Both sodium electrodes were found to have very low responses. As their function was merely to monitor any drift in the reference electrodes, the more stable one — which also happened to have the lower response (39 mV per decade) — was usually used for correction purposes.

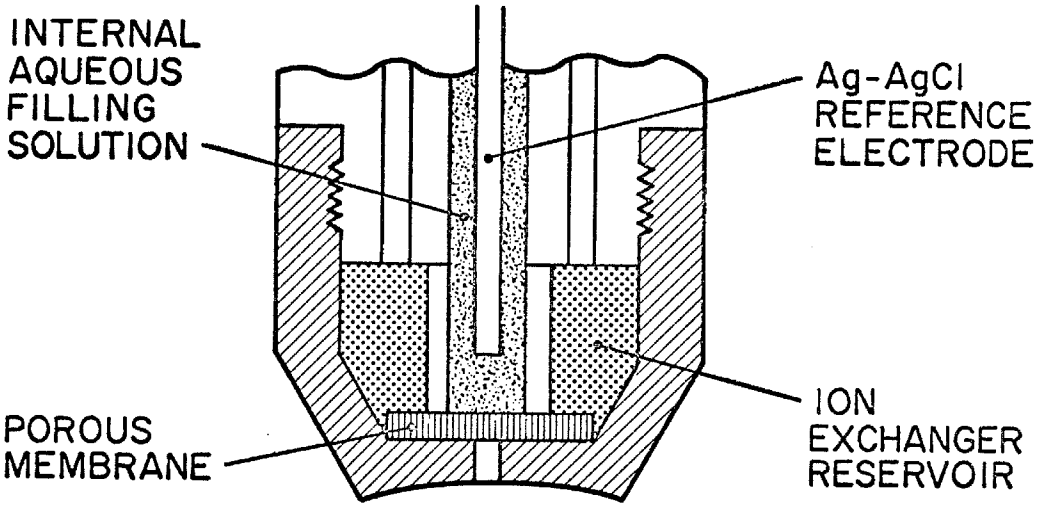

Fig. 12. Diagram of a liquid ion exchanger electrode

Potassium Electrode

The two potassium ion electrodes are identical liquid ion exchanger electrodes (Orion 92-19). The pertinent features are depicted in Fig.12. The liquid ion exchanger is a solution of valinomycin absorbed on a porous membrane. The internal filling solution is usually 0.1MKCl. These potassium electrodes were found to age rather rapidly (a few days to a few weeks). They, then, have a reduced response slope and tend to give erratic readings. This is the reason why two of them are used simultaneously: one is used as a back up for the other.

I. Calibration

A high selectivity for potassium over sodium of these electrodes is the main characteristic enabling one to measure small potassium concentration increments in a solution containing principally sodium chloride. This selectivity was found to be as high as the manufacturer claimed: about 10^4. A typical calibration curve for a new electrode is shown in Fig. 13. Note that the calibration was performed with an excess of sodium ions and that the slope is near the theoretical one (59mV instead of 61.5mV). As shown on the same figure, the response of the electrode is not ideal when the solution is diluted with water. This effect is not understood although it has been noted that the electrode readings tend to be increased when the sodium chloride concentration of the medium was decreased. The electrode resumes its near theoretical behavior if potassium chloride is again added after dilution of the system (dotted line).

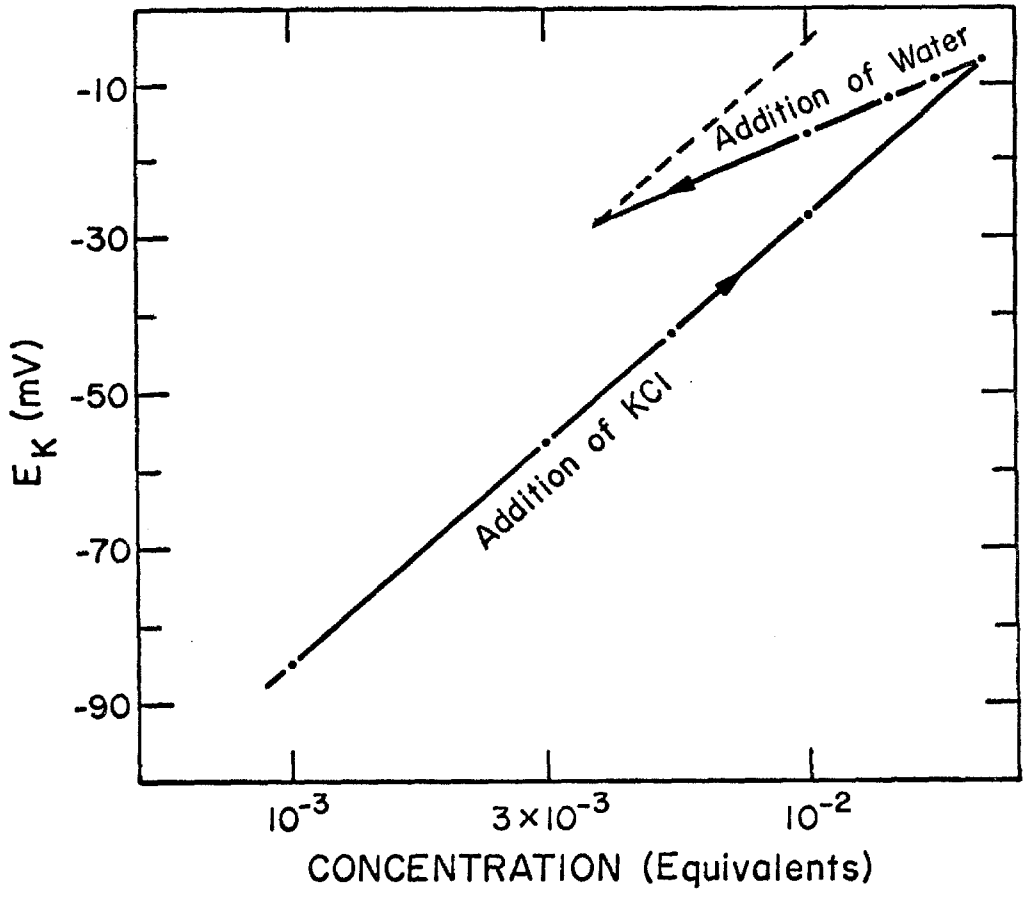

Fig. 13. Calibration curve of the potassium electrode in isotonic buffered saline.

The markedly nonideal behavior of the potassium electrode upon dilution of the solutes does not create special difficulties in our experiments since only increases in potassium concentration are measured.

Calibrations such as that of Fig. 13 were normally performed in the saline used for suspending the red blood cells. In one experiment, known additions of KCl were made in the presence of red cells in order to check the validity of the calibration curve obtained in saline for measuring potassium concentrations in red cell suspensions.

Table 1 shows some of the data from this experiment. The first column gives the time, the second one gives the potassium electrode readings, the third one gives the potassium concentration obtained from the calibration in saline (Fig. 14).

Upon addition of a volume V_{add} of saline with a potassium concentration $(K)_{add}$ to an extracellular volume V_0 with a potassium concentration $(K)_0$, the new extracellular potassium concentration $(K)'_0$ is given by

$$(K)'_0 = \frac{V_0(K)_0 + V_{add}(K)_{add}}{V_0 + V_{add}}$$

$$\therefore \quad \frac{(K)'_0}{(K)_0} = \frac{V_0 + V_{add}\,\frac{(K)_{add}}{(K)_0}}{V_0 + V_{add}}$$

For the first addition at time 150 minutes, we get

$$\frac{(K)'_0}{(K)_0} \simeq \frac{46.2 + 1.\frac{100}{2.95}}{46.2 + 1} \simeq 1.69.$$

Table 1

(The red cells were initially suspended in K free saline)

	Time (min)	E_K (mV)	(K) (mM)
V_T = 79 ml Hct = 41.5% $\therefore V_0 \simeq$ 46.2 ml	10	-100.5	0.55
	50	- 76.0	1.40
	100	- 64.1	2.20
	150	- 56.5	2.95
	+ 1 ml 0.1 M KCl saline		
V_T = 80 ml Hct = 41% $\therefore V_0 \simeq$ 47.2 ml	155	- 43.1	4.95
	160	- 42.8	5.00
	200	- 41.0	5.35
	240	- 39.0	5.80
	280	- 37.2	6.20
	+ Ouabain		
V_T = 80 ml Hct = 41% $\therefore V_0 \simeq$ 47.2 ml	230	- 36.7	6.30
	300	- 36.1	6.45
	350	- 33.1	7.25
	400	- 30.2	8.10
	+ 1 ml 0.1 M KCl saline		
V_T = 81 ml Hct = 40.5% $\therefore V_0 \simeq$ 48.2 ml	405	- 24.7	10.0
	410	- 24.5	10.1
	450	- 22.5	10.8
	500	- 21.5	11.5

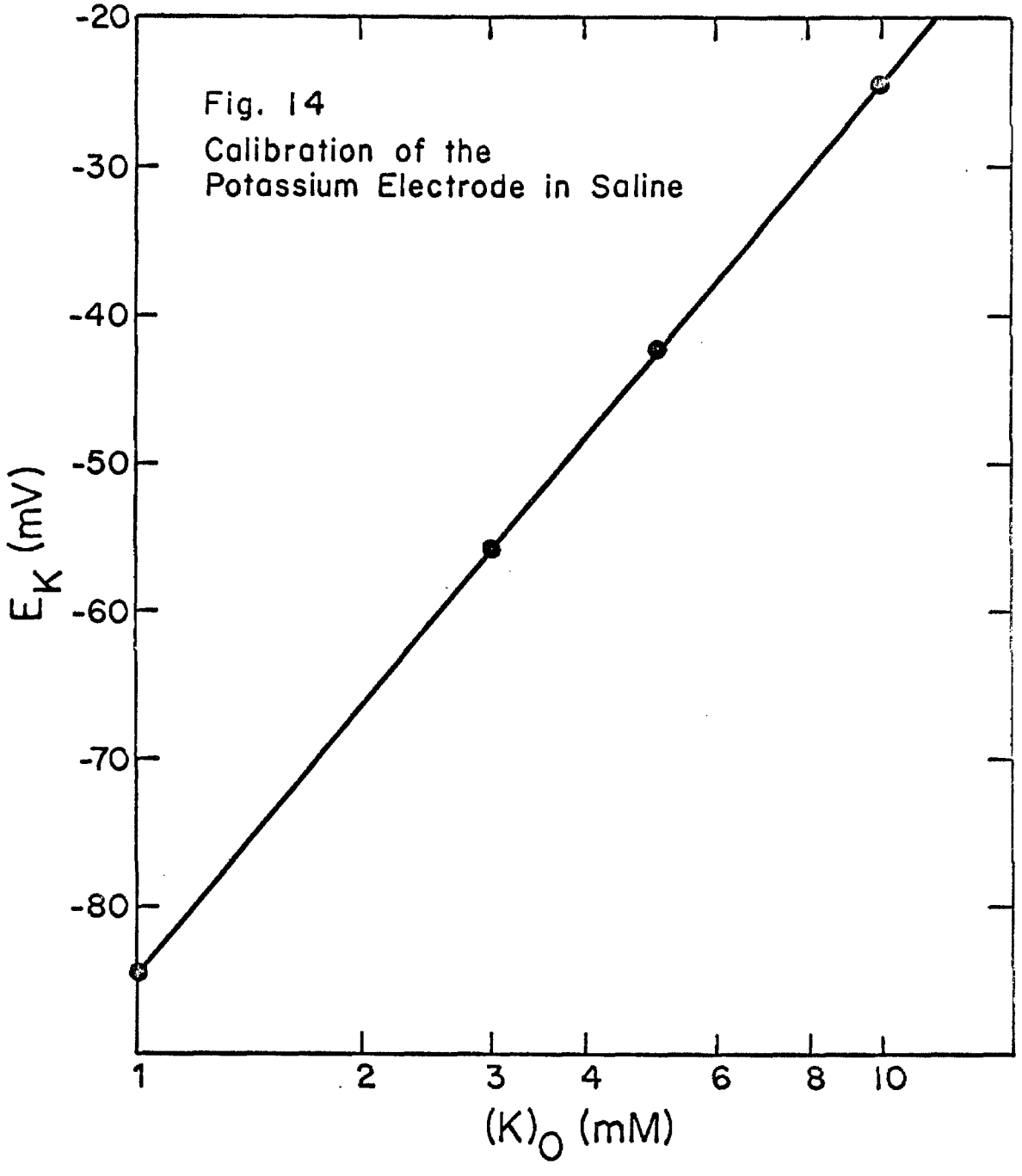

Fig. 14
Calibration of the
Potassium Electrode in Saline

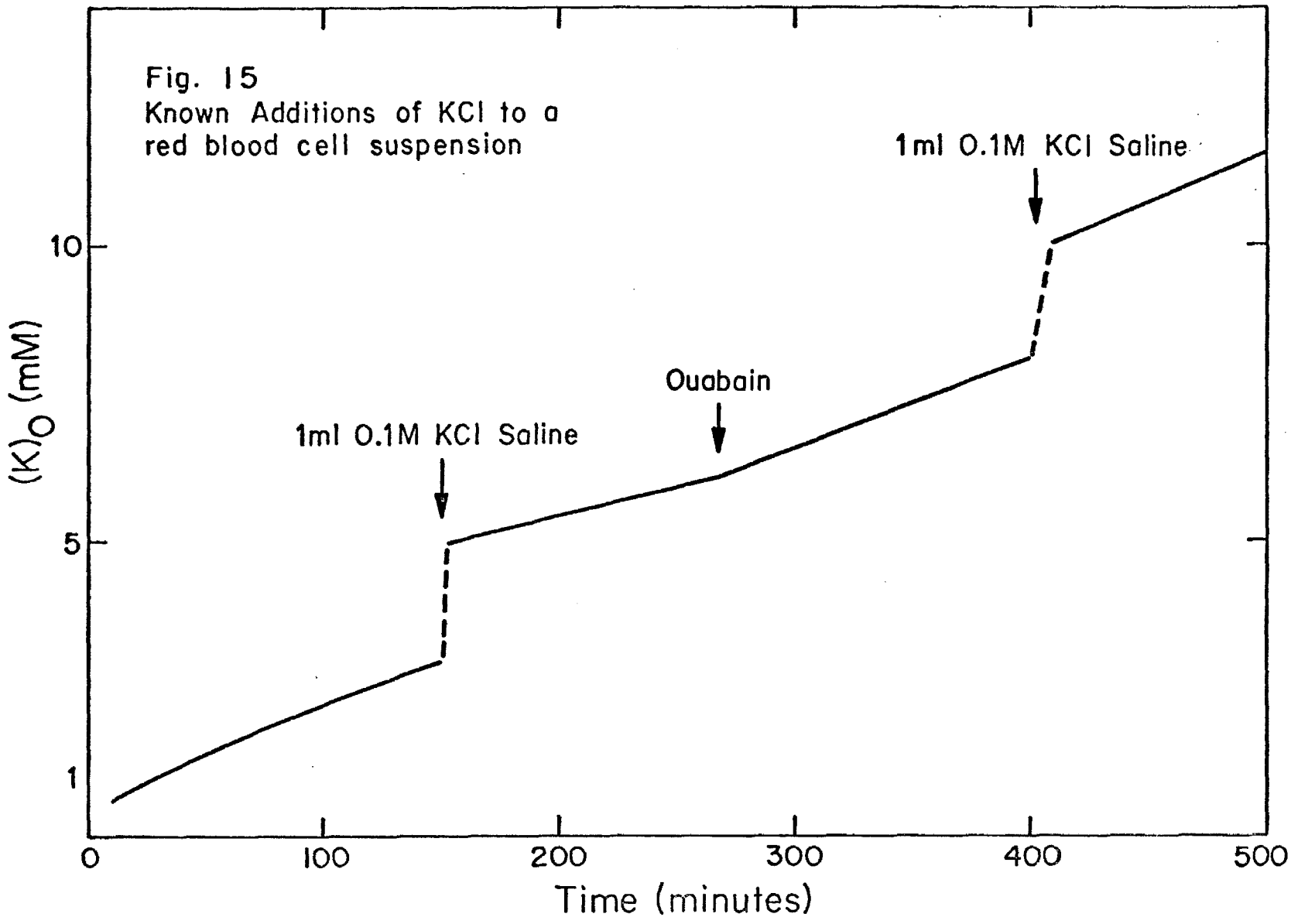
Fig. 15
Known Additions of KCl to a red blood cell suspension
1ml 0.1M KCl Saline
Ouabain
1ml 0.1M KCl Saline
10
5
1
$(K)_O$ (mM)
0
100
200
300
400
500
Time (minutes)

For an ideally behaving electrode such an increase in concentration should produce a jump in potassium electrode reading of 13.4mV, (Fig.18) which is precisely the value found experimentally.

For the second addition at time 400 minutes, we get

$$\frac{(K)'_0}{(K)_0} \simeq \frac{47.2 + 1.\frac{100}{8.10}}{47.2+1} \simeq 1.23 ;$$

this should produce a jump in potassium electrode reading of 5.5mV. Again this value is in precise agreement with the experimental one.

It can also be noted that, although the first potassium addition did reduce the measured rate of potassium efflux from the red cells — which is expected since the cationic pump was presumably working at that time —, the second potassium addition, performed in the presence of Ouabain, left the measured potassium efflux virtually unchanged. This can be seen on Fig. 15 and is consistent with ideal behavior of the electrodes in cell suspensions.

II. Stability.

Since the experiments always lasted several hours, it is important to ensure that the electrodes are reasonably stable during similar lengths of time.

Table 2 gives potassium and sodium readings in a 10mMK saline solution over a 20 hour period. Both readings shift to lower values but, as usual, their difference stays remarkably constant, suggesting that the drift comes from the reference electrode. By using

Table 2

Time (min)	E_K (mV)	E_{Na} (mV)	$E_K - E_{Na}$ (mV)
0	-13.7	+13.8	-27.5
60	-13.6	+13.7	-27.3
184	-16.0	+11.5	-27.5
253	-16.7	+10.7	-27.1
988	-20.8	+ 6.3	-27.1
1100	-20.7	+ 6.1	-26.9

Table 3

Time (hrs)	E_K (mV)	E_{Na} (mV)	$E_K - E_{Na}$ (mV)
12:10	-14.2	2.6	-16.8
13:30	-14.3	2.5	-16.8
15:00	-13.9	2.6	-16.5
16:30	-14.3	2.3	-16.6
18:00	-14.5	2.2	-16.7

the correction explained further in details, a maximum drift of 0.6 mV over a 20 hour period is obtained for the potassium electrode.

As red cells always leak some potassium, it is not possible to directly check the potassium electrode stability in cell suspensions. However, one can recalibrate the electrode in saline at the end of an experiment. Table 2 shows potassium and sodium electrode readings in saline solution at the start of each of a series of five experiments performed consecutively. Before correction the potassium readings are stable within 0.6 mV; after correction a stability of 0.3 mV for 6 hours is obtained.

The electrodes were not always recalibrated at the end of each experiment; however the electrodes were usually left overnight in the cell suspension so that the calibration at the beginning of the next experiment gives a good idea of the stability of the electrodes. Table 4 shows a typical series of readings for a given potassium electrode in 10 mM K saline over a 10 days period. Except for the last reading on March 14 where the electrode was discarded for lack of response, the variations of the corrected potassium readings are less than 1 mV in a day, comparable to that found by leaving the electrodes in saline as shown in Table 2.

III. Non-ideality upon dilution.

Since the potassium electrode is only used to monitor increases in potassium ion concentration in the red blood cell suspension, no systematic study of its nonideal behavior upon dilution of the medium was undertaken. However it has been verified that it is not an artifact

Table 4

		E_K	E_{Na}	$E_K - E_{Na}$
Different reference electrode	March 4	-25.6	+ 2.7	-28.3
→	March 5	-18.4	+ 9.5	-27.9
	March 7	-23.0	+ 4.1	-27.1
→	March 8	-13.7	+13.8	-27.5
	March 9	-22.1	+ 5.8	-27.9
	March 11	-24.6	+ 4.9	-29.5
	March 12	-24.1	+ 4.8	-28.9
→	March 14	-18.5	+ 4.8	-23.3

due to a rapid leakage from the reference electrode. Table 5 shows how the same low electrode response is obtained whether the electrodes are present or absent during the dilution of the medium.

It has also been verified that this nonideal behavior is not the result of a very slow electrode response. In Table 5, the second reading was taken 5 minutes after dilution and the last one about 1 hour afterwards. The potassium electrode reading is essentially unchanged. Five minutes was chosen empirically as a sufficient time to allow the electrode to equilibrate after dilution of the medium. This time is short enough not to affect the results of the experiments.

It is interesting to note that the electrode behaves ideally upon calibration in pure KCl solution (Fig. 16). This implies that changes in ionic strength are not the cause of its nonideal behavior upon dilution.

IV. Effect of Glucose and Sucrose

Table 6 shows that the potassium electrode is not affected by the addition of crystalline sucrose or glucose to a saline solution. It will be seen that addition of sucrose solution is indistinguishable from that of water (Fig. 21). These controls insure the validity of the potassium measurements after modification of the cell suspension by addition of glucose or sucrose.

V. Effect of Valinomycin

The presence of the valinomycin solution in the porous membrane of the potassium electrode could introduce a major artifact. The valinomycin could slowly dissolve into the aqueous phase and, from

Table 5

		E_K(mV)
(I)	40 ml of saline	-12.5
(II)	Addition of 10 ml water to (I) in the presence of the electrodes	-14.9
(III)	40 ml saline + 10 ml water prepared in the absence of the electrodes	-15.0
	(II) + (III)	-15.0

Table 6

	E_K(mV)
50 ml saline	-14.3
Addition of 1.5 g dextrose	-14.3
Addition of 1.5 g sucrose	-14.2

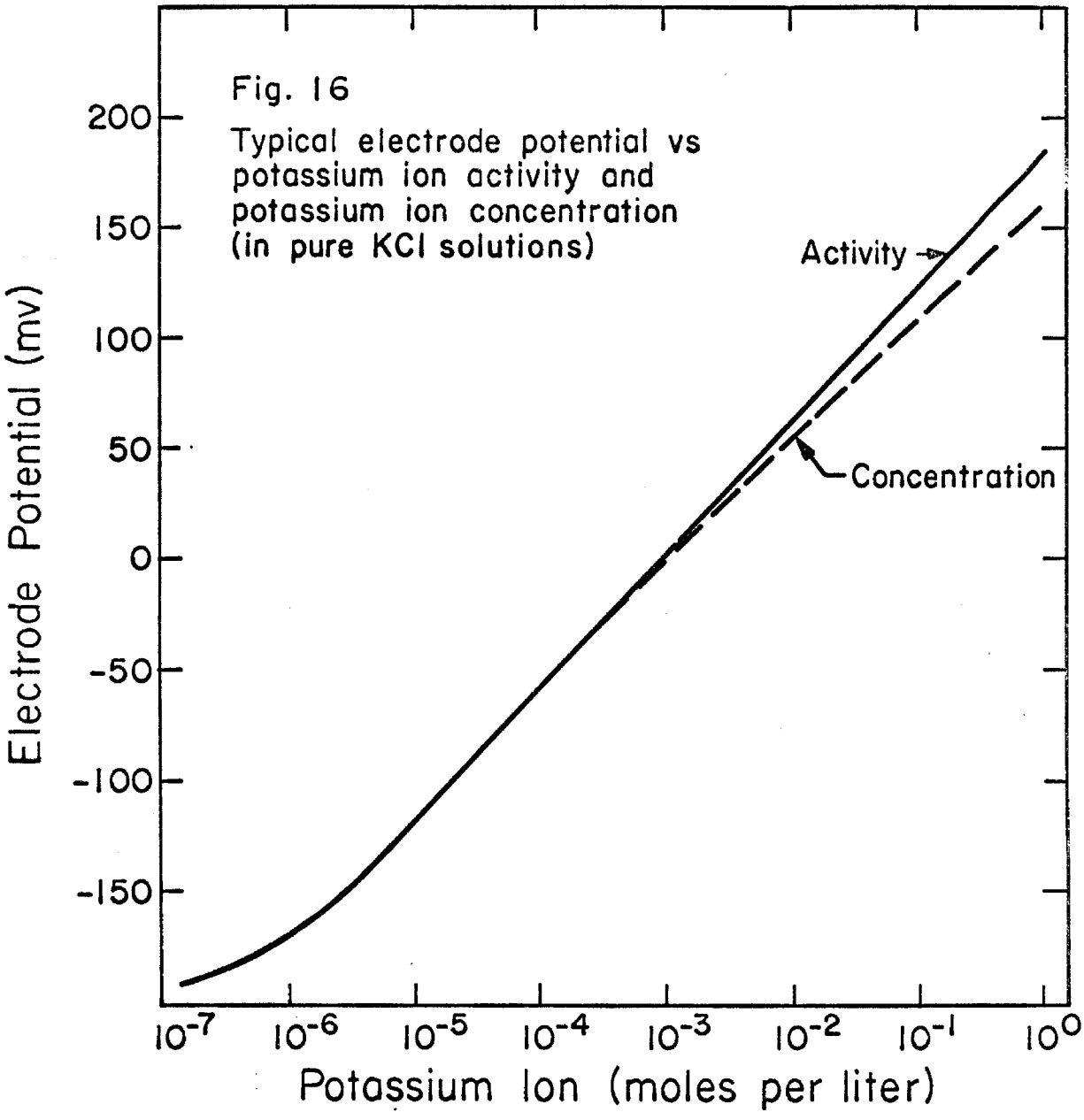
Fig. 16
Typical electrode potential vs potassium ion activity and potassium ion concentration (in pure KCl solutions)
Activity
Concentration
Electrode Potential (mv)
200
150
100
50
0
-50
-100
-150
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
Potassium Ion (moles per liter)

there, into the erythrocyte lipids. The potassium outflux would then be increased markedly as has been shown by Tosteson, et al (Tosteson, Cook, Andreoli, Tieffenberg, 1967). To verify that this effect is either too small or too slow to affect significantly the measurements, an experiment was performed where 0.5ml of valinomycin solution was added directly to the cell suspension. The organic solution was seen to be immiscible with the aqueous one and to float on top of it. Although the surface of contact between the two phases was increased by a factor of about 100 (from a few mm^2 in the membrane of the potassium electrode to some cm^2 on top of the suspension), thus facilitating the transfer of the valinomycin, it did not affect the red blood cells in any measureable way. This can be seen in Fig. 17. The failure to measure any effect was not due to some electrode defect, as addition of ouabain resulted in the usual increase in the slope of the potassium readings. (See Results.)

Osmolarity and Haematocrit

The osmolarity and the haematocrit of the cell suspension were measured when needed by withdrawal of 0.6ml samples. About 0.2ml were used for osmotic measurements with a freezing point depression osmometer (Osmette 2007-Precision Systems); the rest, sucked into haematocrit capillaries, was spun for six minutes at 13,000g in a microcapillary centrifuge (International Equipment Co. -Model MB). The packed cell volume was then read on an optical comparator (International Equipment Co. -Model CR). By taking the solid volume of the cells to be 30 percent of their total packed volume under isotonic conditions (Ponder 1948), the intracellular volume could be computed at all times.

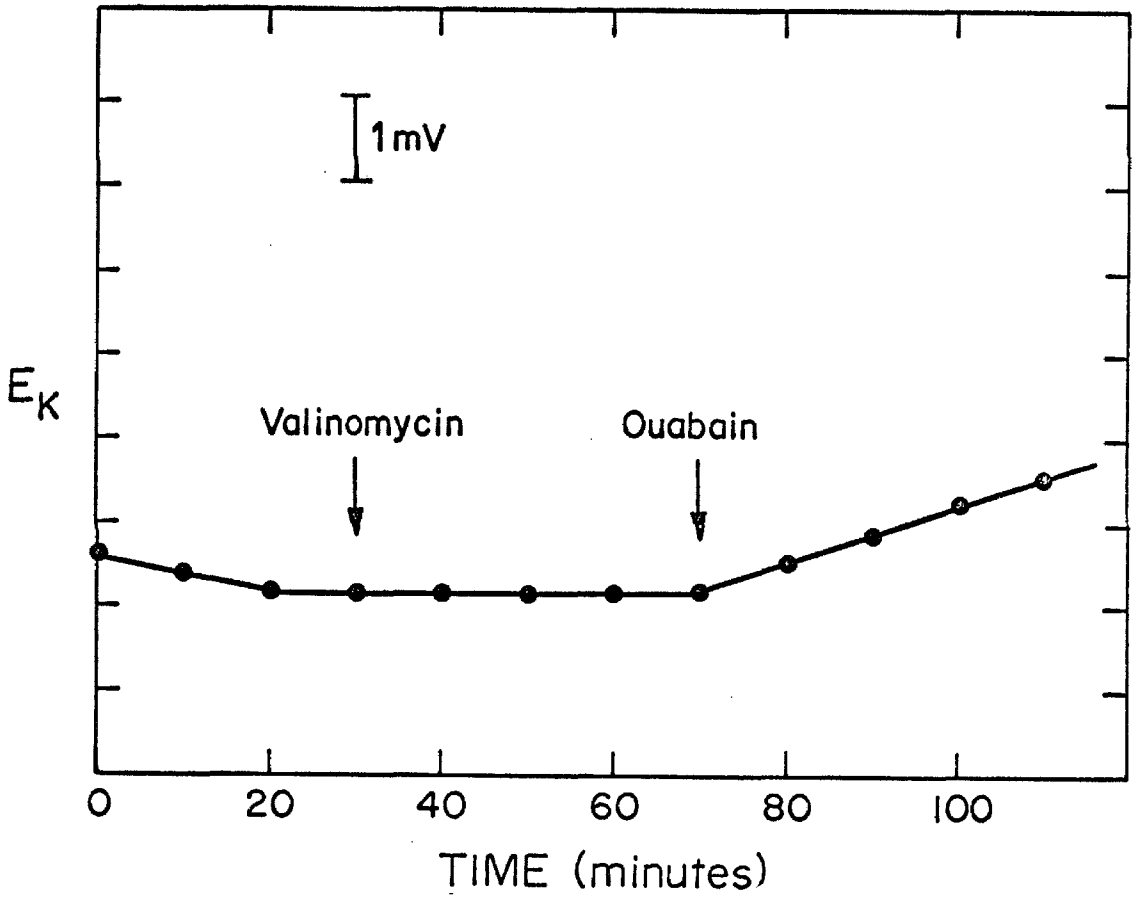

Fig. 17. Addition of 0.5 ml of potassium ion exchanger solution (valinomycin) to 70 ml of a 31% red blood cell suspension in physiological saline. No effect on the potassium readings is detected. Subsequent addition of ouabain produces the usual increase in the readings (see results).

Red Blood Cell Suspensions

O^+ type blood from healthy donors was obtained from the blood bank in standard 500 cc bags with 75 cc of acid citrate dextrose solution (ACD) as anticoagulant. For each experiment, 80 cc samples were incubated for three hours at 37° C with about 40 mg of Adenosine added to their plasma. The samples were then spun down at 3000 g for ten minutes. The plasma and the buffy coat were removed and the cells washed one time in a buffered saline solution (4.40 g NaCl, 0.68 g KH_2PO_4, 0.69 g $NaH_2PO_4 \cdot H_2O$, 15.2 g $Na_2HPO_4 \cdot 7H_2O$ in 1 ℓ of water). This preparation yielded about 30 cc of washed packed red cells that were added to 40 cc of the saline solution in which the electrodes were allowed to equilibrate. The pH of this suspension was usually very close to 7.0 and the isotonic haematocrit around 40 percent. Ouabain (Strophanthin G - Calbiochem) was usually added later to the cell suspension.

Correction for Drift; Computation of Ionic Fluxes and Precision

The concentrations present in the extracellular saline solution at the start of an experiment were the following:

$(K^+) \simeq 5$ meq; $(Na^+) \simeq 195$ meq; $(Cl^-) \simeq 75$ meq; (Phosphates) $\simeq 67$ meq.*

For a typical K^+, Na^+ exchange of 1 meq per liter of red blood cells, per hour, the actual changes in concentration should be of about $1 \times \frac{40}{60} \approx 0.67$ meq/liter of saline/hour. This is a 13 percent increase in

*Some experiments were performed in saline containing different potassium concentrations, NaH_2PO_4 being replaced by KH_2PO_4, for example.

potassium concentration and a 0.3 percent decrease in sodium concentration per hour. For ideally-behaving electrodes this would lead to about +3.1 mV and -0.07 mV changes per hour respectively. Given a precision in the readings of ±0.1 mV, it can be seen that in the same one-hour long experiment, the outflux of potassium can be easily measured, while an equal influx of sodium cannot. This great insensibility in the sodium measurements allows one to correct for drifts in the reference electrodes. Any drift that exceeds markedly the expected changes in the sodium ion electrode readings can be corrected for. The presence of two such electrodes allows one to verify that the drift is indeed due to the reference electrode and not to the sodium electrodes themselves.

A further refinement in this correction is given by the comparison of the sodium electrodes with the chloride one. As the slope of the chloride ion electrode is negative (the readings in mV decrease as the chloride concentration is increased) while all the others are positive, any similar variations in the sodium and chloride electrodes' responses have to be due to variations in the common reference electrode. Otherwise these variations would mean either similar identical drifts in independent electrodes or similar relative increase in sodium and relative decrease in chloride concentrations (or vice versa). Either of these coincidences is highly unlikely.

This is one of the advantages of using different electrodes simultaneously: widely unacceptable readings can be discarded or corrected for by cross checking between the different electrode outputs.

The changes in potassium concentration can be computed from the data using the Nernst equation:

$$E_K = E_K^{ref} + \lambda \cdot \log [K] \tag{1}$$

$$\therefore \quad \Delta E_K = \lambda \cdot \log \frac{[K]'}{[K]} \tag{2}$$

where E_K is the reading of the potassium electrode; E_K^{ref} is a reference value which depends on the choice of reference electrode and internal filling solution; λ is the slope of the potassium electrode (59mV per decade); $[K]$ and $[K]'$ are the potassium activities in the extracellular solution at the beginning and at the end of an experiment respectively. $\alpha = \frac{[K]'}{[K]}$ is given in Fig. 18 as a function of ΔE_K. At constant ionic strength, the ratio of the concentrations is equal to the ratio of the activities:

$$\frac{(K)'}{(K)} = \frac{[K]'}{[K]} = \alpha \tag{3}$$

The potassium flux J_K can then be computed:

$$J_K = V \cdot \frac{d(K)}{dt} \tag{4}$$

$$J_K \simeq V \cdot \frac{\Delta(K)}{\Delta t} \tag{5}$$

$$J_K \simeq V \cdot (K) \cdot \frac{\alpha - 1}{\Delta t} \tag{6}$$

where V is the extracellular volume and Δt the time elapsed during the experiment. Normalizing this flux to a constant number of red cells:

$$\overline{J}_K \simeq \frac{V}{V_{RBC}^{isotonic}} \cdot (K) \cdot \frac{\alpha - 1}{\Delta t} \; . \tag{7}$$

If the experiment is made under isotonic conditions,

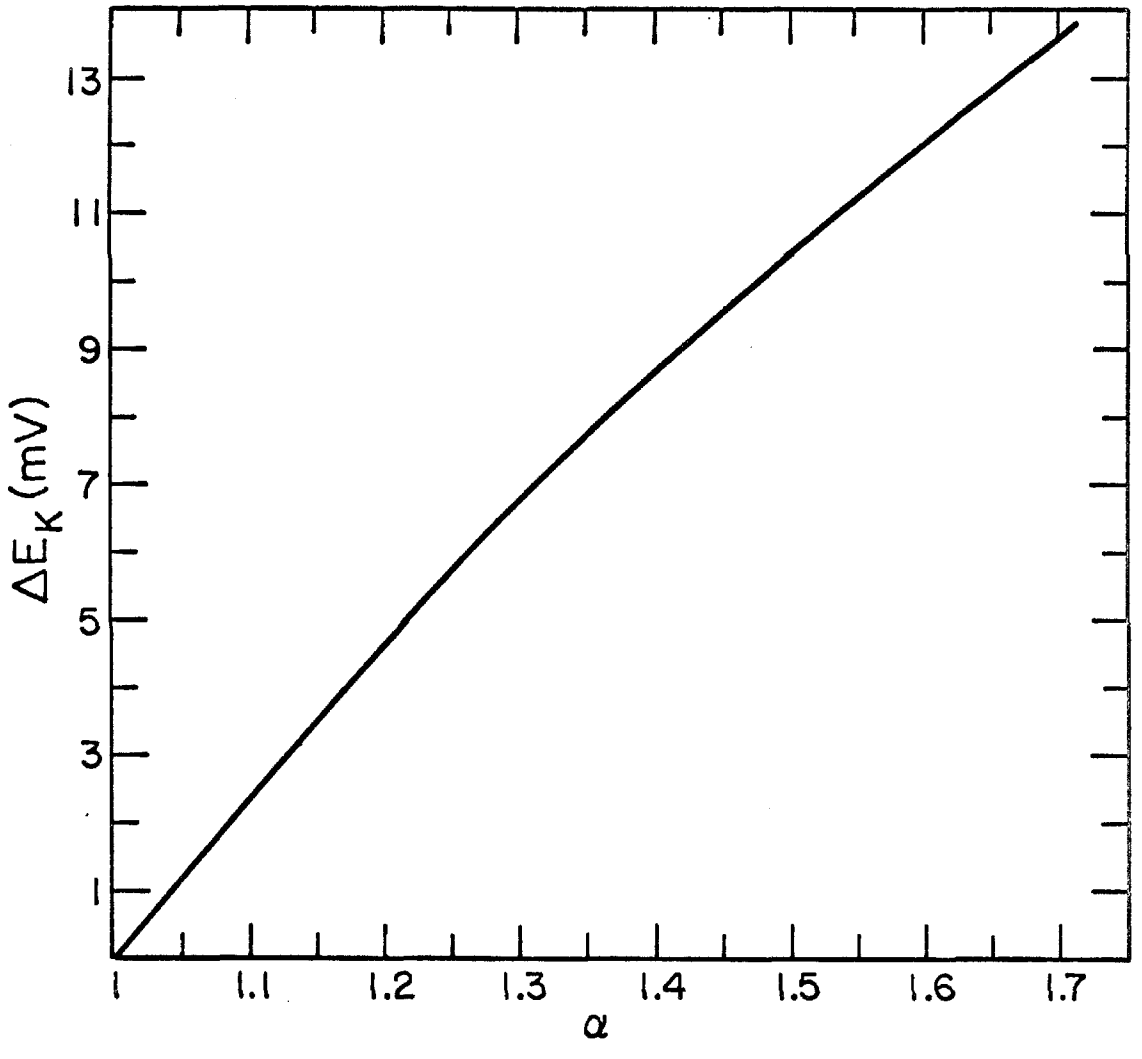

Fig. 18. $\alpha = [K]'/[K]$ computed as function of ΔE_K from Equation (2) with $\lambda = 59$mV.

$$\frac{V}{V_{RBC}^{isotonic}} = \frac{1 - H}{H}$$

where H is the haematocrit of the cell suspension. Therefore

$$\bar{J}_K \simeq \frac{1 - H}{H} \cdot (K) \cdot \frac{\alpha - 1}{\Delta t} \tag{8}$$

At the end of an experiment, another was often started after diluting the suspension (see Results). Upon sudden dilution, we shall see that the total amount of extracellular potassium remains unchanged:

$$V_2 \cdot (K)_2 = V_1 (K)_1' \tag{9}$$

Then, from (6):

$$J_{K,2} \simeq V_2 \cdot (K)_2 \cdot \frac{\alpha_2 - 1}{\Delta t_2} . \tag{10}$$

Substituting from (9):

$$J_{K,2} \simeq V_1 \cdot (K)_1' \cdot \frac{\alpha_2 - 1}{\Delta t_2} . \tag{11}$$

Using (3):

$$J_{K,2} \simeq V_1 \cdot (K)_1 \cdot \alpha_1 \cdot \frac{\alpha_2 - 1}{\Delta t_2} . \tag{12}$$

Normalizing:

$$\bar{J}_{K,2} \simeq \frac{1 - H_1}{H_1} \cdot (K)_1 \cdot \alpha_1 \cdot \frac{\alpha_2 - 1}{\Delta t_2} . \tag{13}$$

The recurrence is obvious:

$$\bar{J}_{K,n} \simeq \frac{1 - H_1}{H_1} \cdot (K)_1 \cdot \alpha_1 \cdot \alpha_2 \cdots \alpha_{n-1} \cdot \frac{\alpha_n - 1}{\Delta t_n} \tag{14}$$

Precision. Differentiating (8) and taking the absolute values, we obtain an upper limit on the error:

$$\frac{\delta \bar{J}_K}{\bar{J}_K} \leq \frac{\delta H}{H} + \frac{\delta H}{1-H} + \frac{\delta (K)}{(K)} + \frac{\delta \alpha}{\alpha - 1} + \frac{\delta (\Delta t)}{\Delta t} . \tag{15}$$

For $\delta H \leq \pm 0.5\%$, $H \simeq 40\%$, $\frac{\delta(\Delta t)}{\Delta t} \leq \pm 3.10^{-5}$

and $\frac{\delta(K)}{(K)} \leq \pm 0.5\%$, we get:

$$\frac{\delta H}{H} + \frac{\delta H}{1-H} + \frac{\delta(K)}{(K)} + \frac{\delta(\Delta t)}{\Delta t} \leq \pm 2.5\% . \tag{16}$$

Substituting (3) into (2), differentiating and taking the absolute values, we obtain the maximum relative error on α:

$$\frac{\delta \alpha}{\alpha} \leq \frac{\delta(\Delta E_K)}{\lambda} + \frac{\delta \lambda}{\lambda} \log \alpha . \tag{17}$$

For $\delta(\Delta E_K) \leq \pm 0.1$mV, $\lambda \simeq 60$mV, $\delta\lambda \leq \pm 0.5$mV and α between 1 and 1.7(i. e: $\Delta E_K \leq 13.5$mV), the maximum relative error on α varies almost linearly with α between 0.17% and 0.36%. By keeping α sufficiently large — say $\alpha \geq 1.08$ (i. e: $\Delta E_K \geq 2.0$mV)—, the error $\frac{\delta \alpha}{\alpha - 1}$ is minimized:

$$\frac{\delta \alpha}{\alpha - 1} \leq \pm \frac{0.20\%}{0.08} = \pm 2.5\% \tag{18}$$

Substituting (16) and (18) into (15) yields:

$$\frac{\delta \bar{J}_K}{\bar{J}_K} \leq \pm 5\% . \tag{19}$$

For consecutive experiments, the relative errors on α are cumulative as seen by derivation of Equation (14):

$$\frac{\delta \bar{J}_{K,n}}{\bar{J}_{K,n}} \leq \frac{\delta \bar{J}_K}{\bar{J}_K} + (n-1)\ \frac{\delta\alpha}{\alpha}\ , \tag{20}$$

where $\frac{\delta \bar{J}_K}{\bar{J}_K}$ is defined by Equation (15)(or 19)) and $\frac{\delta\alpha}{\alpha}$ by Equation (17). $\frac{\delta\alpha}{\alpha}$ increases with α; thus for $\alpha \leq 1.7$ (i. e: $\Delta E_K \leq 13.5\text{mV}$) we get:

$$\frac{\delta\alpha}{\alpha} \leq 0.36\% \ . \tag{21}$$

No more than six experiments were ever performed in series:

$$n \leq 6 \tag{22}$$

Substituting (19), (21) and (22) in (20) the worst possible error is obtained:

$$\frac{\delta \bar{J}_{K,n}}{\bar{J}_{K,n}} \leq \pm\ 7\% \ . \tag{23}$$

RESULTS AND DISCUSSION

Typical Experiments - Effect of Ouabain

Fig. 19 shows the results of a typical experiment. The graph consists of three distinct parts:

1) 0 to 20 minutes. The electrodes are first allowed to stabilize in 40cc of saline solution; all the readings are seen to become constant.

2) 20 to 50 minutes. When 30cc of packed red blood cells are added to the system the pH electrode reading jumps by about 24mV (pH7.4 to pH7.0). This is due to the acidity of the cells that have been stored in ACD. The hydrogen ion concentration then decreases as the extracellular phosphate buffer exchanges with the intracellular chloride. This is seen by a decrease in the chloride electrode reading (corresponding to an increase in Cl^- activity). The following rise in the hydrogen ion concentration is thought to be the result of metabolic activity of the red blood cell which produces lactic acid (Whittam 1964). The maximum pH is always reached between 20 and 40 minutes after addition of the cells into the saline solution. Coincidentally in this particular experiment ouabain is added at about the time the pH reaches its maximum. The sodium and potassium electrodes give stable readings slightly different from those of the saline solution.

3) 50 to 100 minutes. Addition of ouabain in the system has a unique effect of increasing the rate of change of the potassium readings. This is the expected result as the sodium-potassium pump is supposed to be poisoned. The amount of ouabain added is not critical as it is always in great excess of the minimum necessary to inactivate the

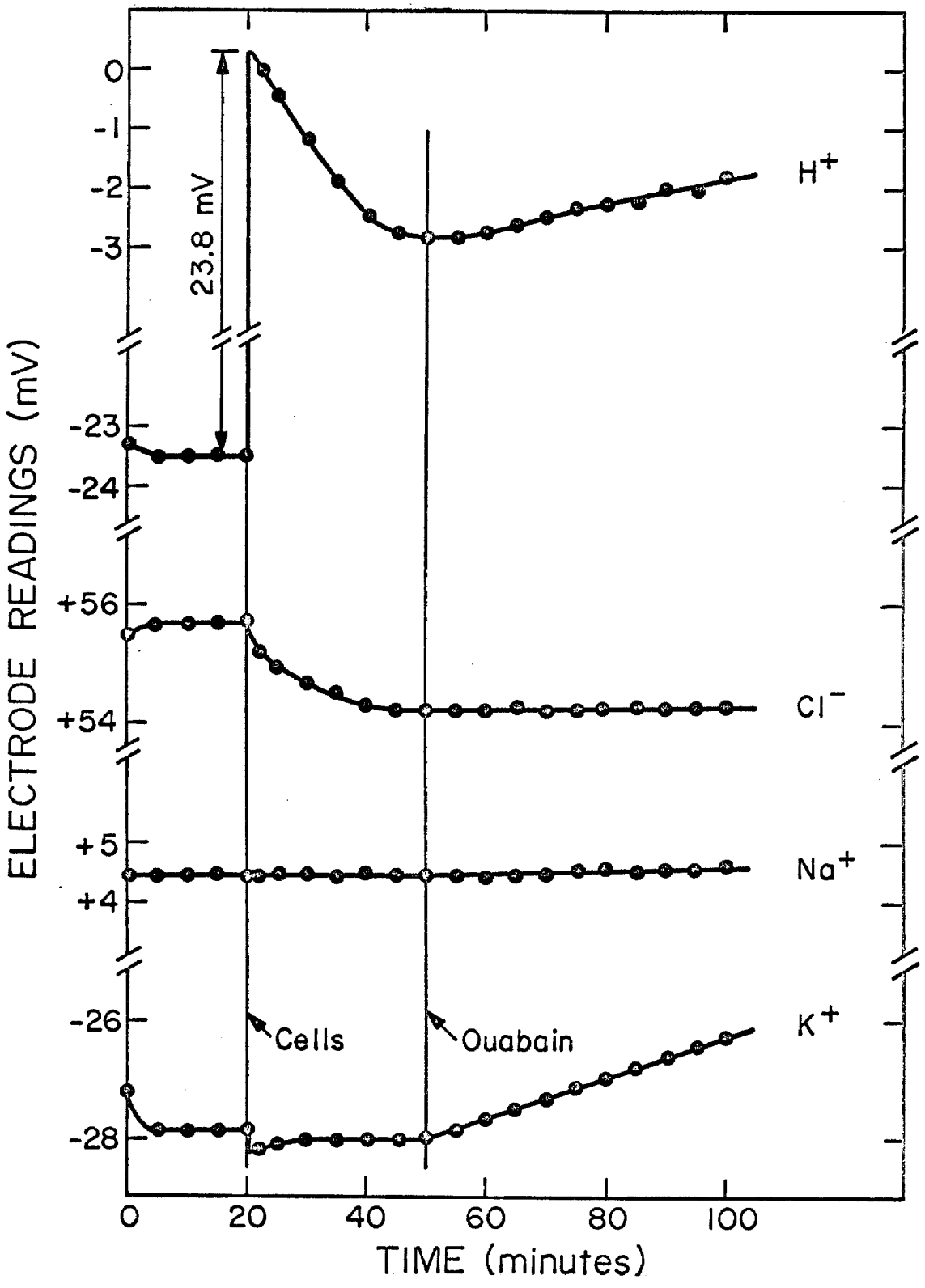

Fig. 19. Typical experiment. At time 20 minutes 31.5 cc of packed red cells are added to 40 cc of isotonic buffered saline, yielding a suspension of 39% haematocrit. At time 50 minutes 15 mg of ouabain are added to the system.

active transport: about 10^{-4}M compared to 10^{-7}M reported in the literature (Hoffman and Ingram 1968). Contrary to appearances, the potassium readings do not really fall on a straight line as the ordinate is a logarithmic scale for the concentrations. This can be verified when the changes in potassium concentration are sufficiently large as in Fig. 22.

Both sodium and chloride readings remain constant within the accuracy of the method. (The sodium reading increases by about 0.1mV in one hour).

Reproducibility

It turned out to be difficult to obtain reproducible results from blood to blood or from day to day on the same blood. Fig. 20 gives the outflux of potassium in ouabain-poisoned red blood cells as a function of the storage time. One blood sample had markedly high early readings that became more average as the storage time increased. No explanation was found for this behavior. It might correspond to some deficiency in this particular blood sample since healthy blood (the authors') gave low readings immediately after being drawn. The difficulty in the reproducibility of the results was alleviated by varying the experimental conditions on the same blood, in the same day, in the course of a unique experiment.

Dilution of the Red Blood Cell Suspension

In order to vary the experimental conditions, water or a sucrose solution were often added to the red cell suspension. Such an addition

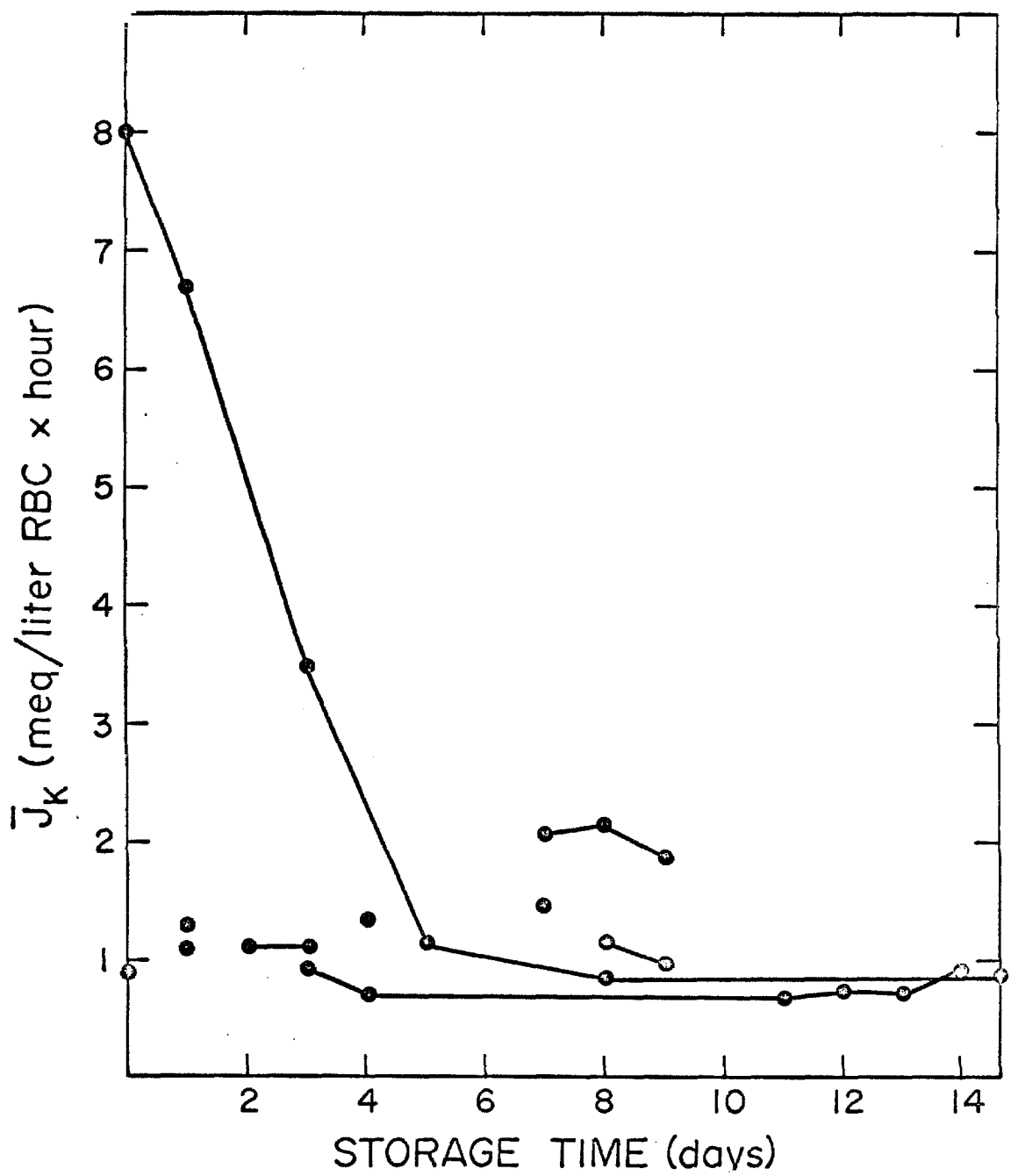

Fig. 20. Passive potassium efflux in function of the storage time for 0^+ human blood obtained from the blood bank. Connected points represent the data from a common blood sample.

dilutes the extracellular solution and, almost instantaneously, the sodium and potassium readings drop while the chloride reading jumps. Fig. 21 shows these fast reading changes as a function of the corresponding dilutions of the extracellular compartment. The lines are computed by taking slopes of 59mV, 39mV and 24mV per decade for the chloride, sodium and potassium electrode, respectively. The slopes chosen for the chloride and sodium electrodes are those obtained in their calibration. The low value of 24mV taken for the potassium electrode is the one found in Fig. 13 with the addition of water. All the experimental points are seen to follow rather closely the lines expected on the basis of the behavior of the electrodes.

According to Gary-Bobo and Solomon (1968), because of changes in the haemoglobin charge, a chloride exchange should usually accompany variations in cell volume. Such an exchange is probably the reason why the chloride readings jump somewhat more than expected. This effect is barely noticeable here as the pH is always about neutral and thus very close to the isoelectric point of haemoglobin.

As diluting the extracellular compartments yields similar results no matter whether the cells are shrinking, swelling or retaining a constant volume (depending on the tonicity of the added solution), water must cross the membrane free of ions. For example, if potassium accompanied the water that comes out of the cells upon addition of crystalline sucrose, the corresponding fast drop in potassium reading should be smaller than the value obtained, for a similar dilution of the extracellular medium, by adding isotonic sucrose. Figure 21 shows that this is not the case. The total ionic content of each compartment —

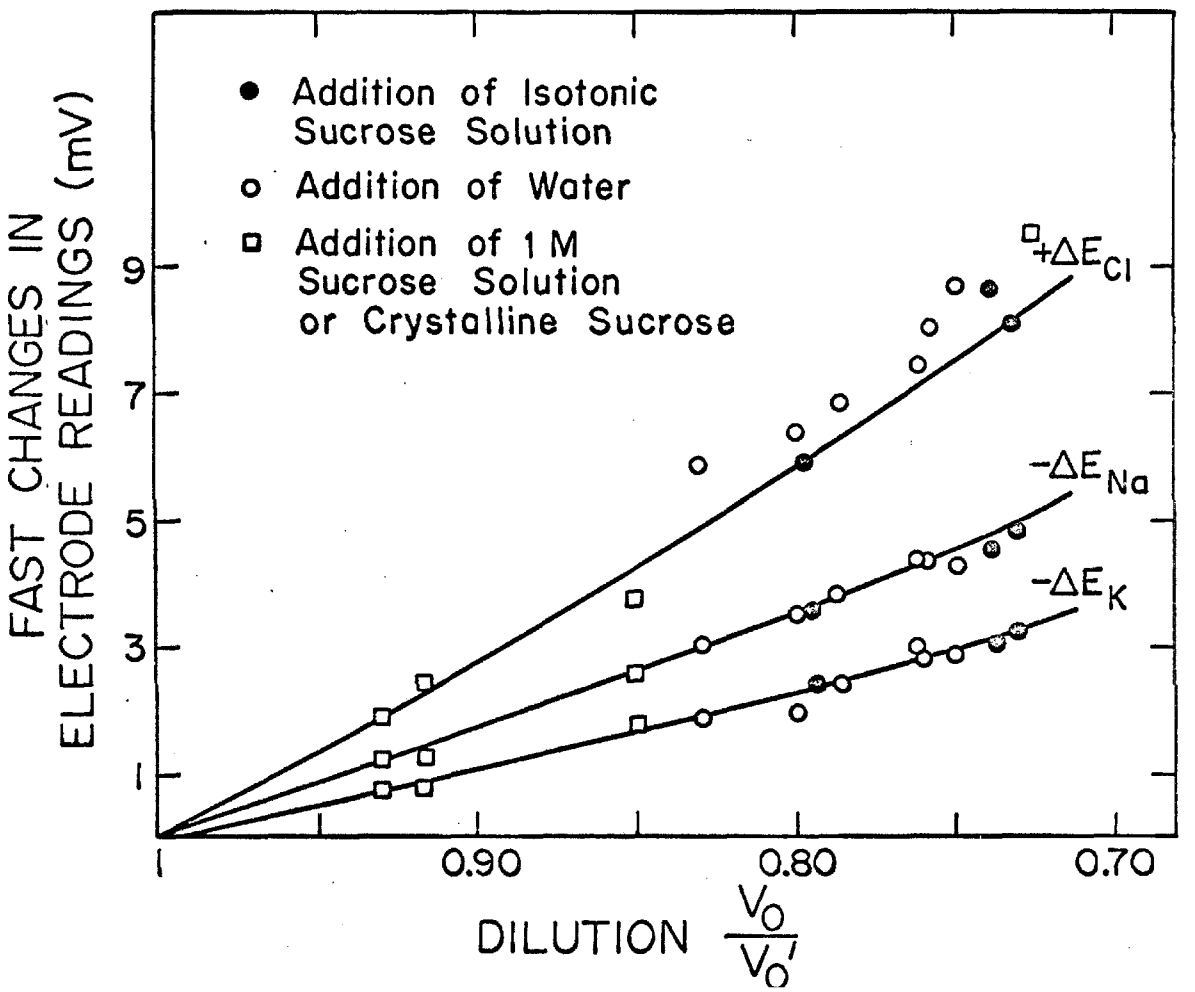

Fig. 21. Fast changes in electrode readings upon dilution of the extracellular medium (V_0 and V_0' are the measured extracellular volumes before and after dilution respectively).

intracellular and extracellular — will be considered constant upon addition of water or sucrose solution.

Addition of Isotonic Sucrose Solution

As the results obtained by Wilbrandt and then by Rothstein and coworkers were thought to be rather unexpected, a series of experiments was performed to try to extend their results to the physiological range. Fig. 22 shows such an experiment where given amounts of isotonic sucrose solution are added to the suspension of ouabain-poisoned red blood cells. Table 7 analyzes the results of this experiment. The outflux of potassium increases markedly as the sucrose solution is added. A plot of $\bar{J}_K/\bar{J}_K^{ref}$ versus $(Cl)_i/(Cl)_o$, from this experiment and other similar ones is given in Fig. 20. $\bar{J}_K^{ref}$ is the potassium outflux when the red cells are suspended in isotonic saline at pH = 7.0. The chloride ratio is then approximately 1. For the other cases $(Cl)_i/(Cl)_o$ is computed from the changes in intracellular and extracellular volumes: $(Cl)_i/(Cl)_o \simeq V_o/V_o^{ref} \times V_i^{ref}/V_i$. The curve $\bar{J}_K/\bar{J}_K^{ref}$ versus $(Cl)_i/(Cl)_o$ is seen to follow the identity line. Therefore, the potassium flux $\bar{J}_K$ is proportional to the chloride ratio $(Cl)_i/(Cl)_o$.

Addition of Crystalline Sucrose or Concentrated Sucrose Solution

In order to gain some insight into the preceding phenomenon, experiments were performed where concentrated sucrose solution or even crystalline sucrose was added directly to the cell suspension as is shown in Fig. 24 and analyzed in Table 8. The surprising result is

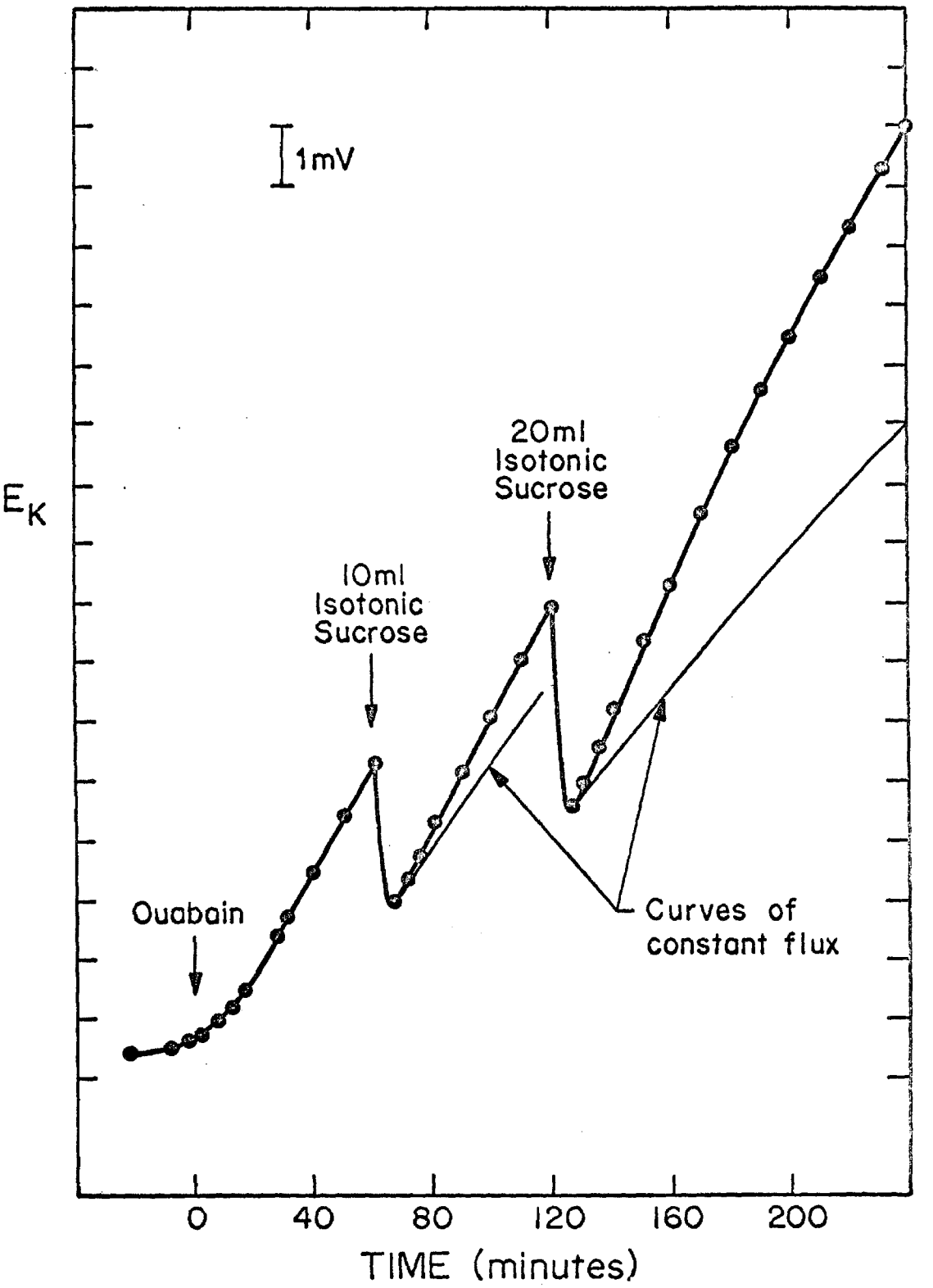

Fig. 22. Addition of isotonic sucrose solution to a red cell suspension: effect on the potassium efflux.

Time (minutes) = Δt	(Reference) 0 to 60	60 to 120	120 to 240
Haematocrit (%) = H	38.0	32.2	24.7
Osmolarity (milliosmols) = Ω	303	303	305
Total Volume (ml) = V_T	70	79.5	99
V_o/V_i (1)	2.38	3.05	4.51
ΔE_K(millivolts) (2)	5.3	5.6	12.1
α (3)	1.230	1.245	1.605
$\bar{J}_K$(meq/liter RBC × hour) (4)	2.03	2.69	4.15
$\bar{J}_K/\bar{J}_K^{ref}$	1	1.31	2.01
$(Cl)_i/(Cl)_o$ (5)	1	1.28	1.90

Table 7: Computation of the potassium fluxes from the results of Fig. 19. (Addition of isotonic sucrose solution; $((K)_1 \simeq 5.5$ meq)

(1) $V_o/V_i = \dfrac{V_T \cdot (1-H)}{V_T \cdot H - V_S}$ where $V_S = 0.7\ (V_T \cdot H)^{ref}$.

(2) ΔE_K is obtained on the graph by extrapolation of the steady part of the curve to the chosen times.

(3) α is obtained from ΔE_K using Fig. 18.

(4) $\bar{J}_K$ is computed according to equations (8) or (14).

(5) $(Cl)_i/(Cl)_o = V_o/V_i \cdot (V_i/V_o)^{ref}$.

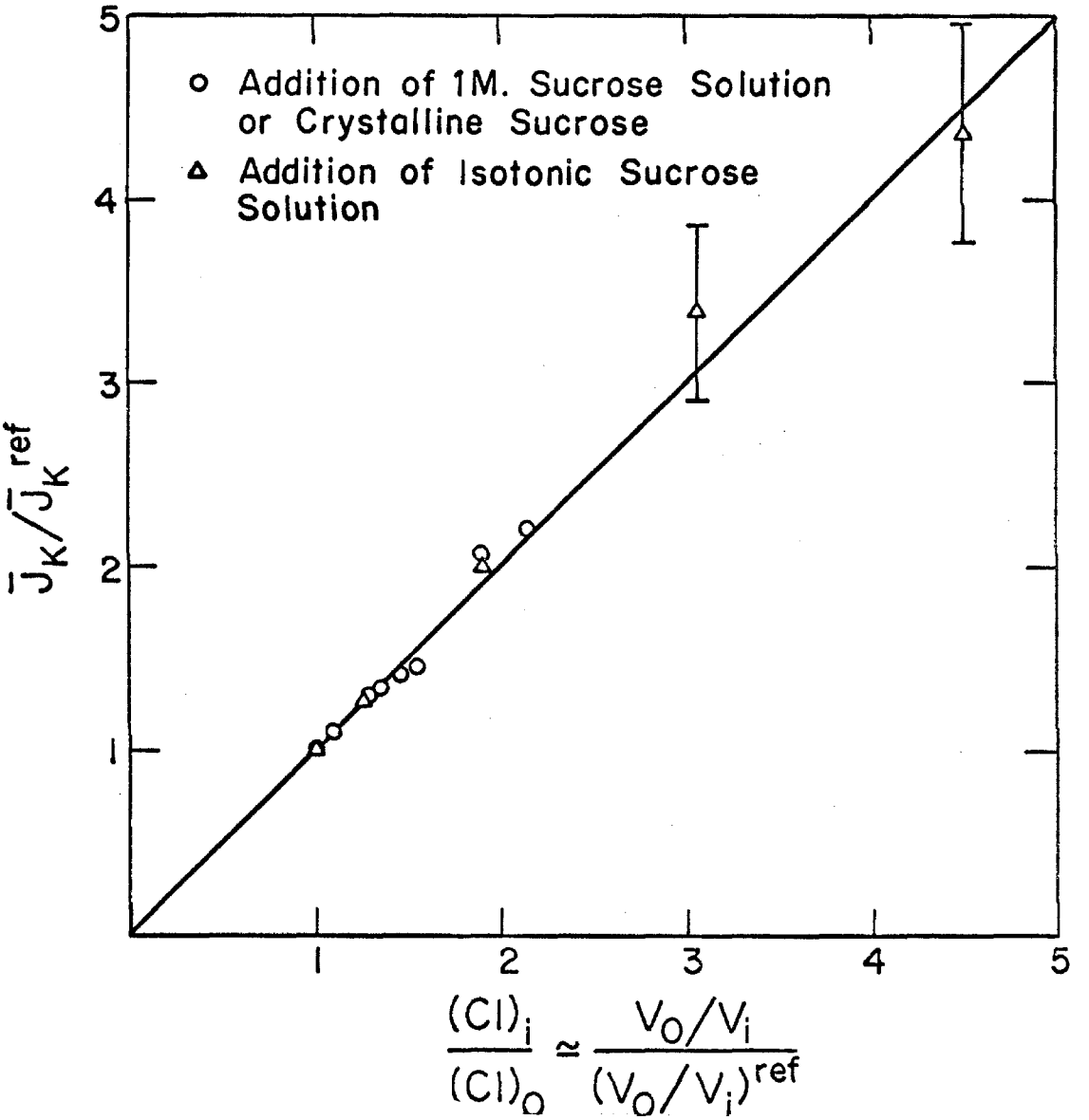

Fig. 23. Variations of the passive potassium efflux from red blood cells as a function of the chloride ratio. The osmolarity was increased up to 520 milliosmols in experiments where the chloride ratio was modified by addition of an hypertonic sucrose solution.

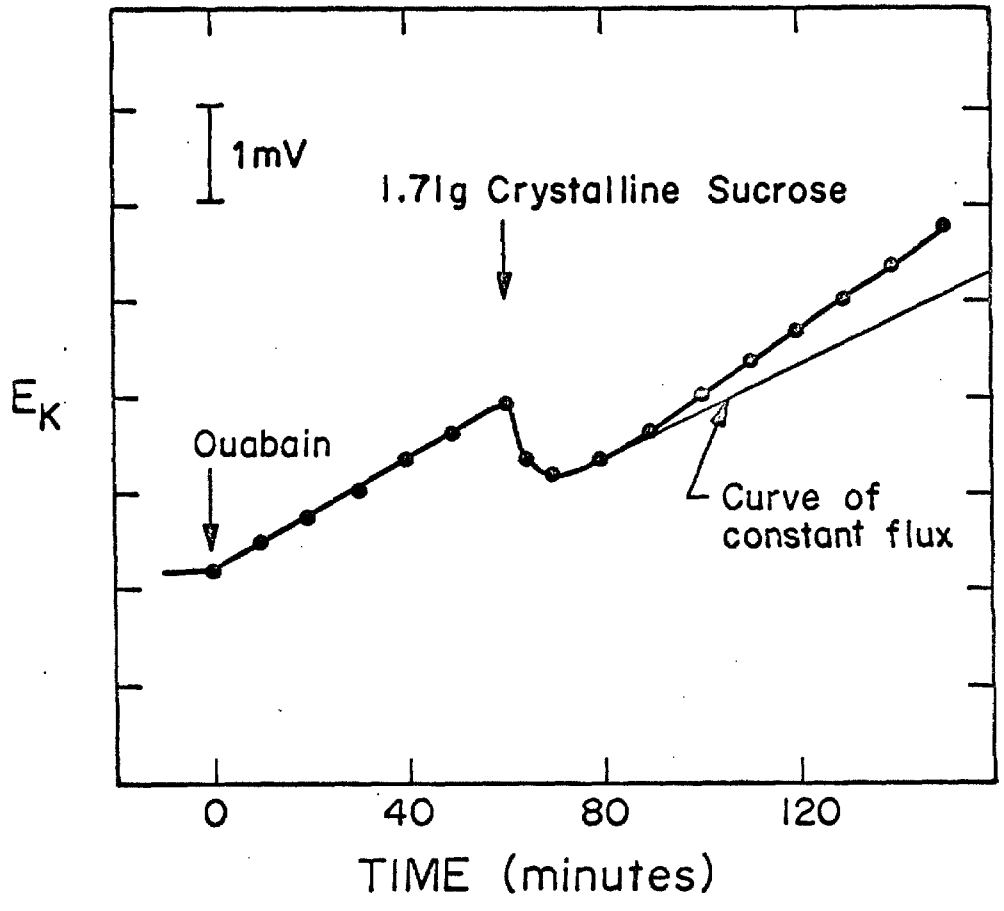

Fig. 24. Addition of crystalline sucrose to a red cell suspension: effect on the potassium efflux.

Time (minutes) = Δt	(Reference) 0 to 60		70 to 150
Haematocrit (%) = H	38.5		34.0
Osmolarity (milliosmols) = Ω	307		375
Total Volume (ml) = V_T	100		99.5
V_o/V_i (1)	2.28		2.91
ΔE_K(mV) (2)	1.7	0.3	2.7
α (3)	1.070	1.010	1.110
$\bar{J}_K$(meq/liter RBC x hour) (4)	1.12		1.43
$\bar{J}_K/\bar{J}_K^{ref}$	1		1.28
$(Cl)_i/(Cl)_o$ (5)	1		1.27

Table 8: Computation of the potassium fluxes from the results of Fig. 21. (Addition of crystalline sucrose; $((K)_1 \simeq 10$ meq)

(1) $V_o/V_i = \dfrac{V_T \cdot (1-H)}{V_T \cdot H - V_S}$ where $V_S = 0.7\ (V_T \cdot H)^{ref}$

(2) ΔE_K is obtained on the graph by extrapolation of the steady part of the curve to the chosen times.

(3) α is obtained from ΔE_K using Fig. 18.

(4) $\bar{J}_K$ is computed according to equations (8) or (14).

(5) $(Cl)_i/(Cl)_o = V_o/V_i \cdot (V_i/V_o)^{ref}$

that the increase in potassium outflux varies in the same way with the ratio $(Cl)_i/(Cl)_o$ as in the experiment where isotonic sucrose is added to the cell suspension. This is shown in Fig. 23 where the points obtained from both kinds of experiments are seen to follow the same identity line. Such a result is surprising since the addition of hypertonic sucrose solution shrinks the cells and thus increases the intracellular potassium concentration. (Up to 70% increase for experiments where the osmolarity was brought up over 500 milliosmols). There are two possible explanations for this apparent independence of $\bar{J}_K$ and $(K)_i$: either the increase in intracellular concentration has indeed no effect on the potassium outflux or its effect is balanced out by the changes in membrane permeability due to the increase in osmolarity.

Addition of Glucose

To test the second explanation one can study the influence of changes in osmolarity at constant intracellular concentration. The addition of glucose allows such a study as it penetrates the red cells leaving the intracellular volume unchanged. Fig. 25 and Table 9 present such an experiment. After each addition of glucose a transient fall in extracellular concentrations is obtained due to the shrinkage of the cells. A corresponding transient increase in potassium outflux is also seen. As glucose equilibrates on both sides of the cell membrane the potassium outflux returns to its original value. Fig. 26 summarizes the results of similar experiments: increasing the osmolarity by addition of glucose leaves the steady potassium outflux essentially unchanged.

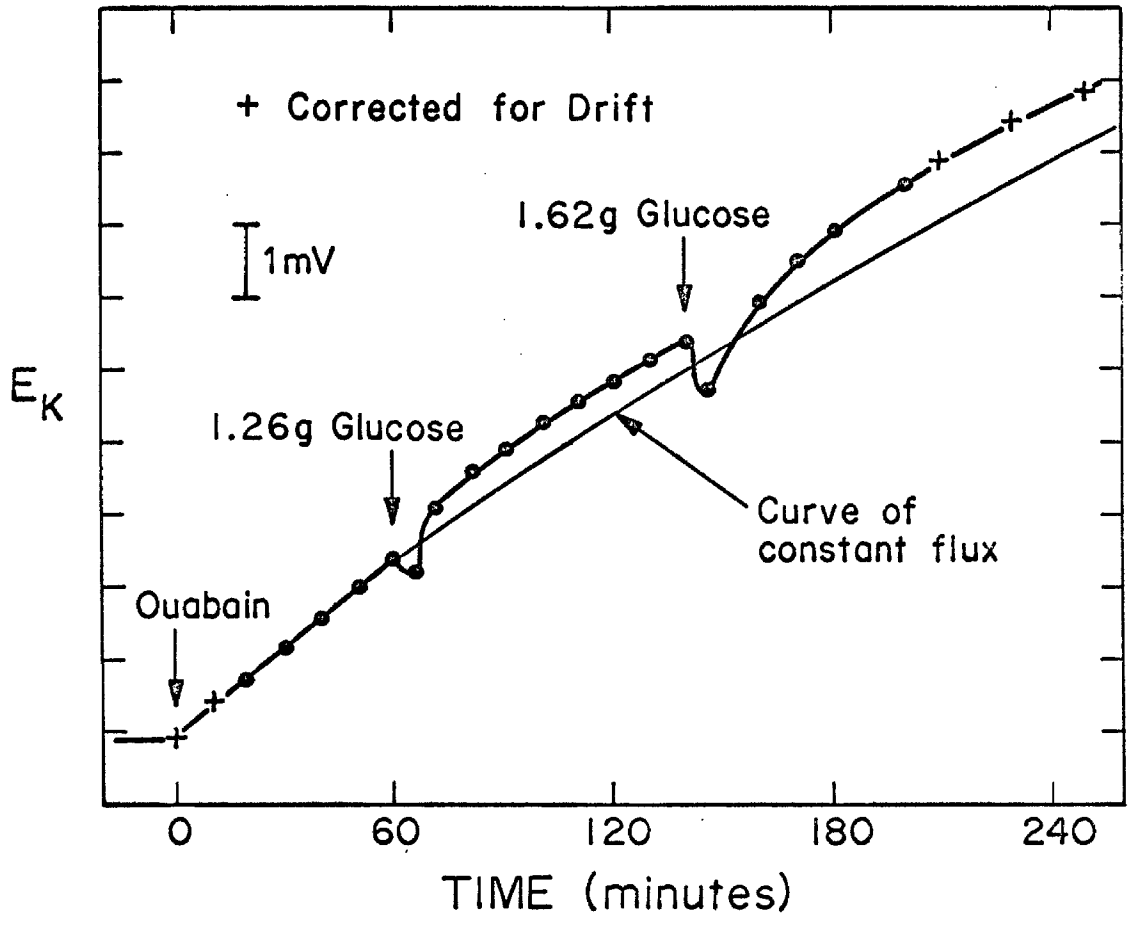

Fig. 25. Addition of glucose (dextrose) to a red cell suspension: effect on the potassium efflux.

Time (minutes) = Δt	(Reference) 0 to 60		70 to 140		180 to 250
Haematocrit (%) = H	34.5		34.7		35.0
Osmolarity (milliosmols) = Ω	310		420		565
Total Volume (ml) = V_T	70		69.5		69
ΔE_K(mV)(1)	2.4	0.7	2.4	1.5	2.0
α(2)	1.100	1.030	1.100	1.060	1.085
$\overline{J}_K$(meq/liter RBC x hr)(3)	0.35		0.92		0.91
$\overline{J}_K/\overline{J}_K^{ref}$	1		0.97		0.96

Table 9: Computation of the potassium fluxes from the results of Fig. 22. (Addition of glucose; $((K)_1 \simeq 5$ meq)

(1) ΔE_K is obtained on the graph by extrapolation of the steady part of the curve to the chosen times.

(2) α is obtained from ΔE_K using Fig. 18.

(3) $\overline{J}_K$ is computed according to equations (8) or (14).

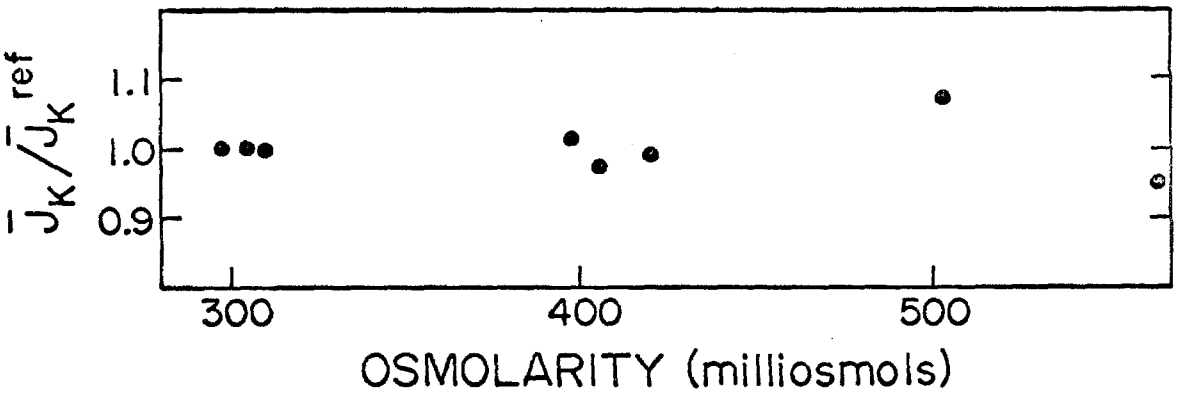

Fig. 26. Effect of osmolarity on the passive potassium efflux from red blood cells. The osmolarity is changed by addition of glucose.

Addition of Water or Sodium Chloride

From the results of the previous experiments, it appears that the potassium outflux in ouabain-poisoned red blood cells is independent of the intracellular potassium concentration and a unique function of the total potential difference across the cell membrane (i. e., the ratio $(Cl)_i/(Cl)_o$). An interesting test of this hypothesis would be obtained by varying the intracellular concentrations while maintaining a constant ratio $(Cl)_i/(Cl)_o$. Such a condition can be approximated by the addition of water or sodium chloride in the suspending medium. Although these additions change the ionic strength, it is hoped that its influence is small enough not to mask the expected result, i. e., no change in the potassium outflux. The experiments presented in Fig. 27 and summarized in Fig. 28 are not quite conclusive. Transient effects on the red cell membranes due to osmotic shock and experimental difficulties with the electrodes upon sudden changes in ionic strength probably concur in scattering the data. However, the results corroborate somewhat the hypothesis: varying the intracellular concentrations by ±33% does not change the potassium outflux by more than ±15%.

Discussion

Direct use of electrodes in red cell suspensions is likely to create artifacts such as those discussed in Materials and Methods. It is somewhat of an art to obtain reliable data. From time to time the electrodes simply fail and a whole series of experiments has to be discarded. However, as the results of a typical experiment show, the method seems

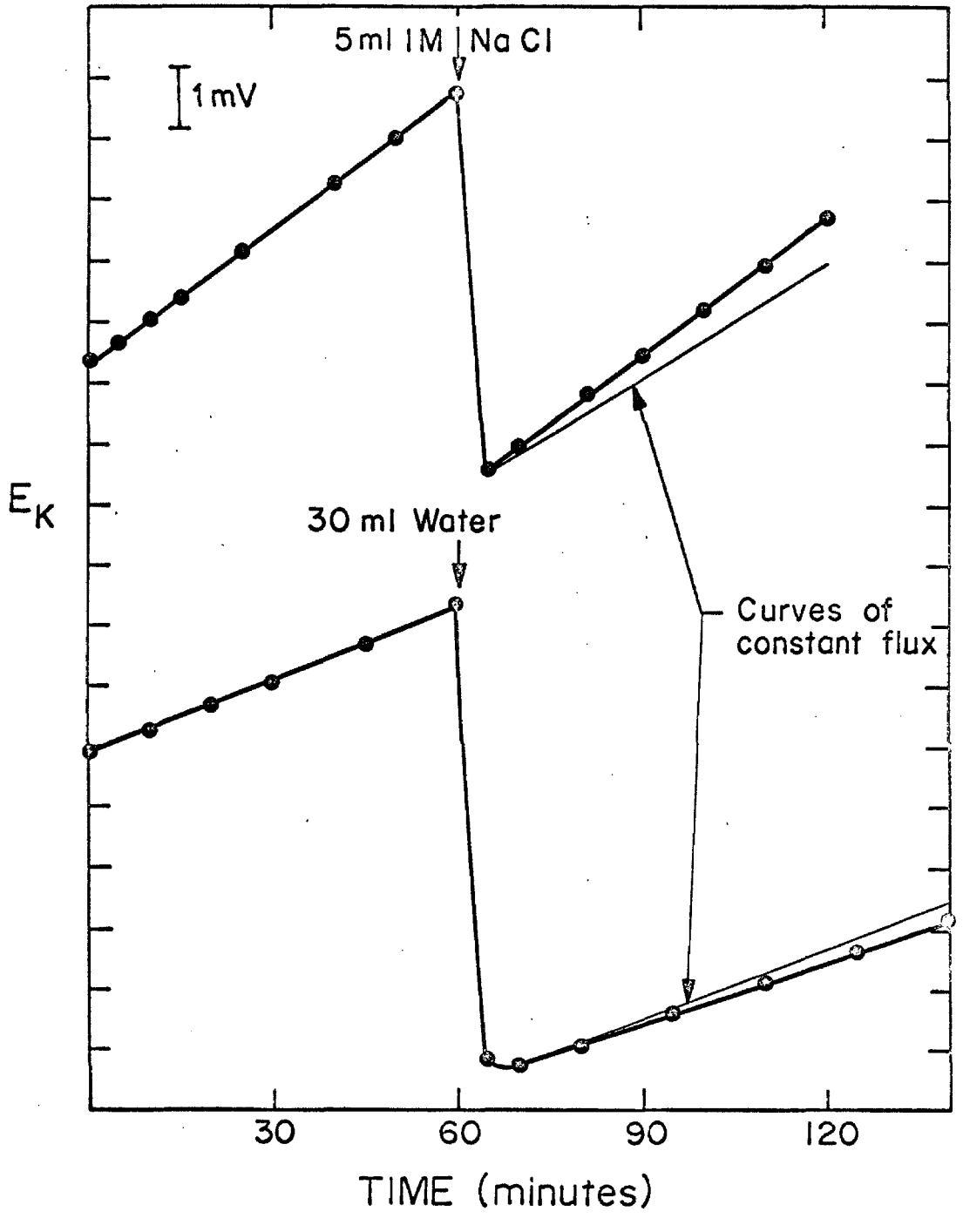

Fig. 27. Addition of NaCl (upper graph) or water (lower graph) to a red cell suspension: effect on the potassium efflux.

	I		II	
Time (minutes) = Δt	(Reference) 0 to 60	60 to 120	(Reference) 0 to 60	60 to 140
Haematocrit (%) = H	38.0	29.9	26	21.5
Osmolarity (milliosmols) = Ω	303	413	298	193
Total Vol. (ml) = V_T	71	75.5	63.5	93
ΔE_K(mV)(1)	4.4	4.5	2.4	2.8
α(2)	1.190	1.195	1.100	1.115
J_K(meq/lit.RBC x hr)(3)	1.74	2.12	3.42	3.25
$\overline{J}_K/\overline{J}_K^{ref}$	1	1.22	1	0.95

Table 10: Computation of the potassium fluxes from the results of Fig. 24. (I - upper graph: addition of Na Cl; $(K)_1 \simeq 5.6$ meq; II - lower graph: addition of water; $(K)_1 \simeq 12$ meq)

(1) ΔE_K is obtained on the graph by extrapolation of the steady part of the curve to the chosen times.

(2) α is obtained from ΔE_K using Fig. 18.

(3) $\overline{J}_K$ is computed according to equations (8) or (14).

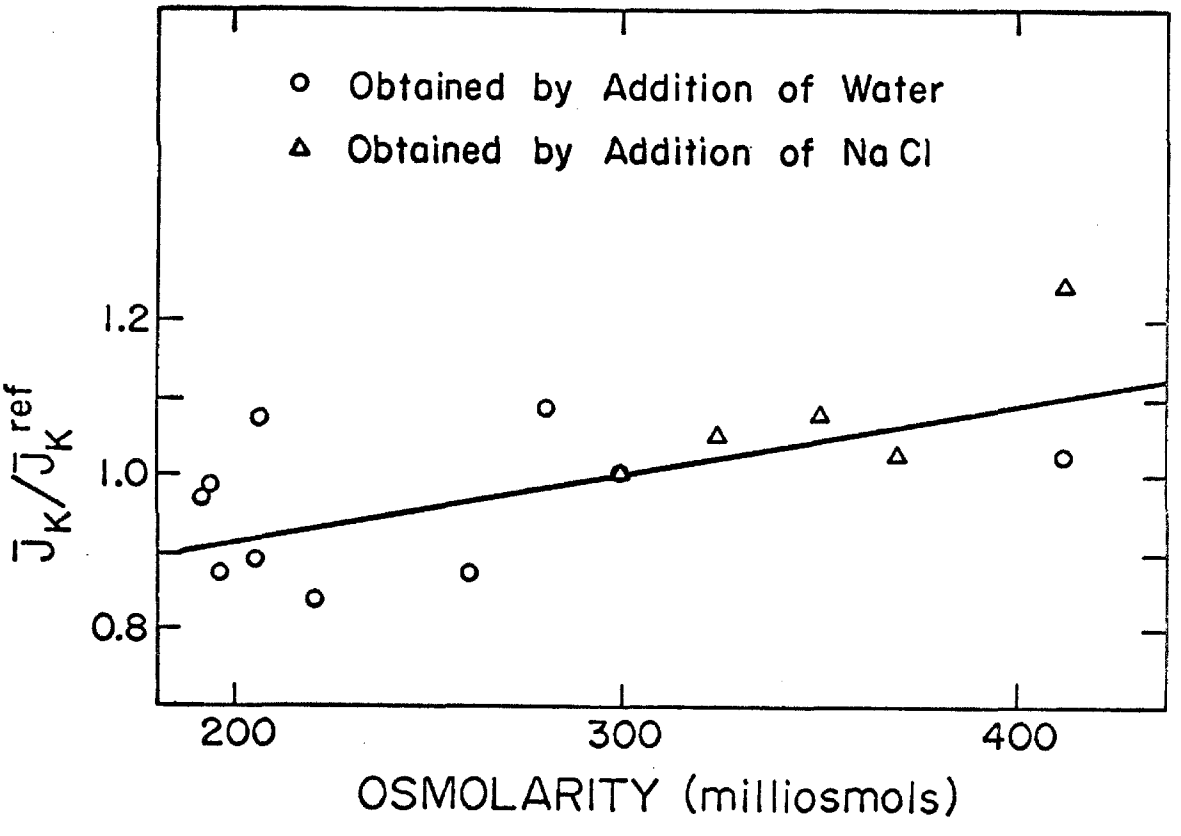

Fig. 28. Effect of changing the salt concentration on the passive potassium efflux from red blood cells.

to work as well as can be expected: sodium, chloride and hydrogen ions can be controlled continuously and the small potassium outflux induced by addition of ouabain can be measured with reasonable accuracy.

The experiments where isotonic sucrose is added to the cell suspension confirm the results of Rothstein and coworkers: the main parameter determining the rate of potassium efflux from red cells suspended in low ionic strength media is the potential difference across the membrane. However, Rothstein and Donlon claimed that, close to the normal ionic strength, the potassium outflux depended linearly on the membrane potential (Fig. 8). From our experiments, the flux seems to depend linearly on the chloride ratio and, thus, exponentially on the membrane potential. The ratio of the chloride concentrations is taken as good approximation for the ratio of the chloride activities (from which the membrane potential should be computed) since, in the range of ionic strength considered (0.15M to 0.25M), the activity coefficient of the K Cl varies by about 3%. (Butler 1964). Fig. 29 shows a plot of the actual data obtained by Donlon. It appears that the so-called linear dependence is more likely exponential. The line drawn on the graph corresponds to the equation that we obtained: $\bar{J}_K$ = constant $\times (Cl)_i/(Cl)_o$ taking an arbitrary point of reference among the data. Fig. 29 also corroborates the results we obtained in experiments with hypertonic sucrose solutions: no matter whether the sucrose solution is isotonic or hypertonic, the potassium outflux is a unique function of the total membrane potential.

Using the data of Fig. 10, one can plot the difference between the unidirectional potassium fluxes versus the chloride ratio as shown in Fig. 30. The net flux is found to be proportional to the chloride ratio

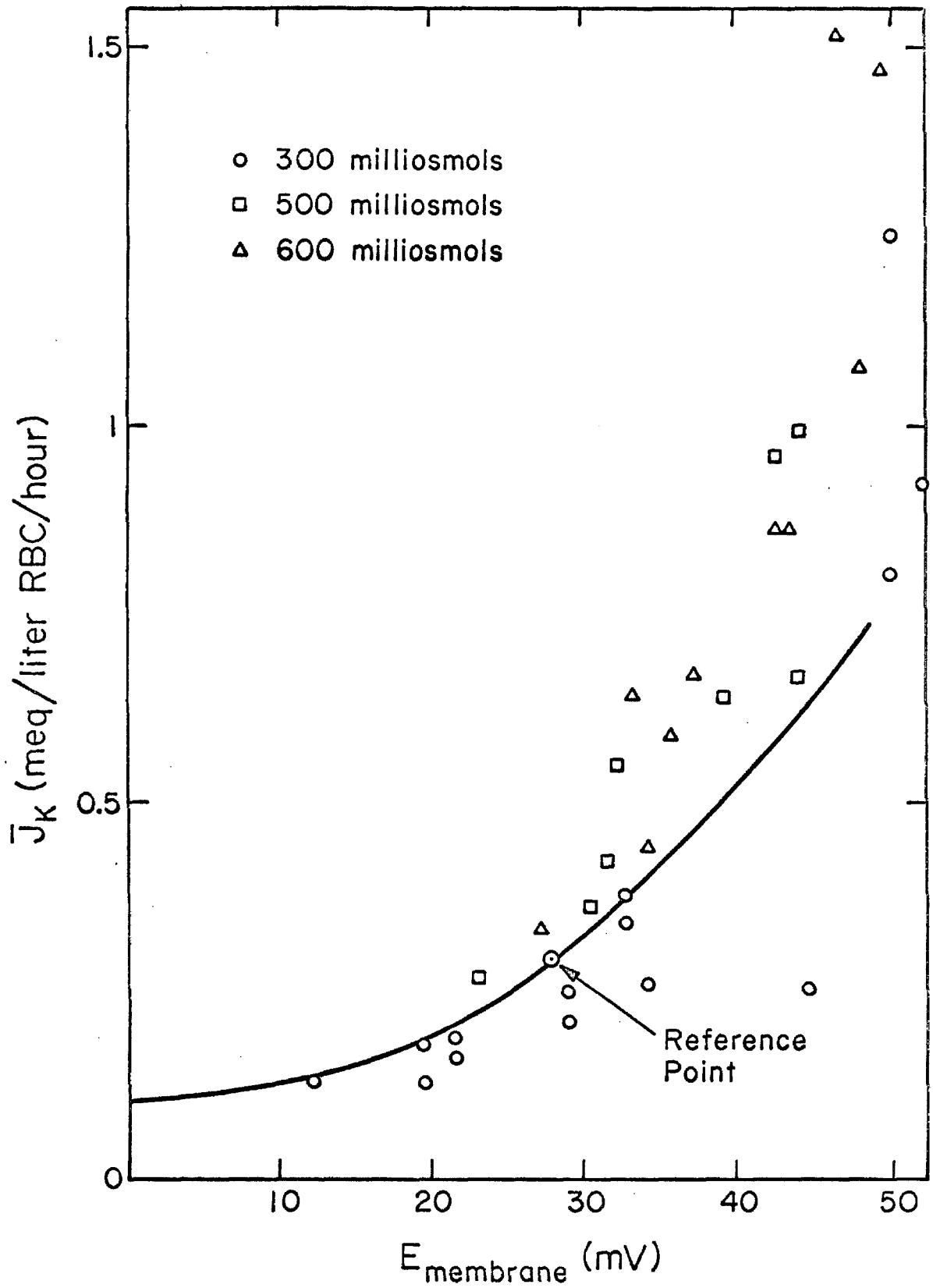

Fig. 29. The potassium efflux from red blood cells as a function of the membrane potential. (The membrane potential is modified by addition of a sucrose solution). Data are taken from Donlon (1968).

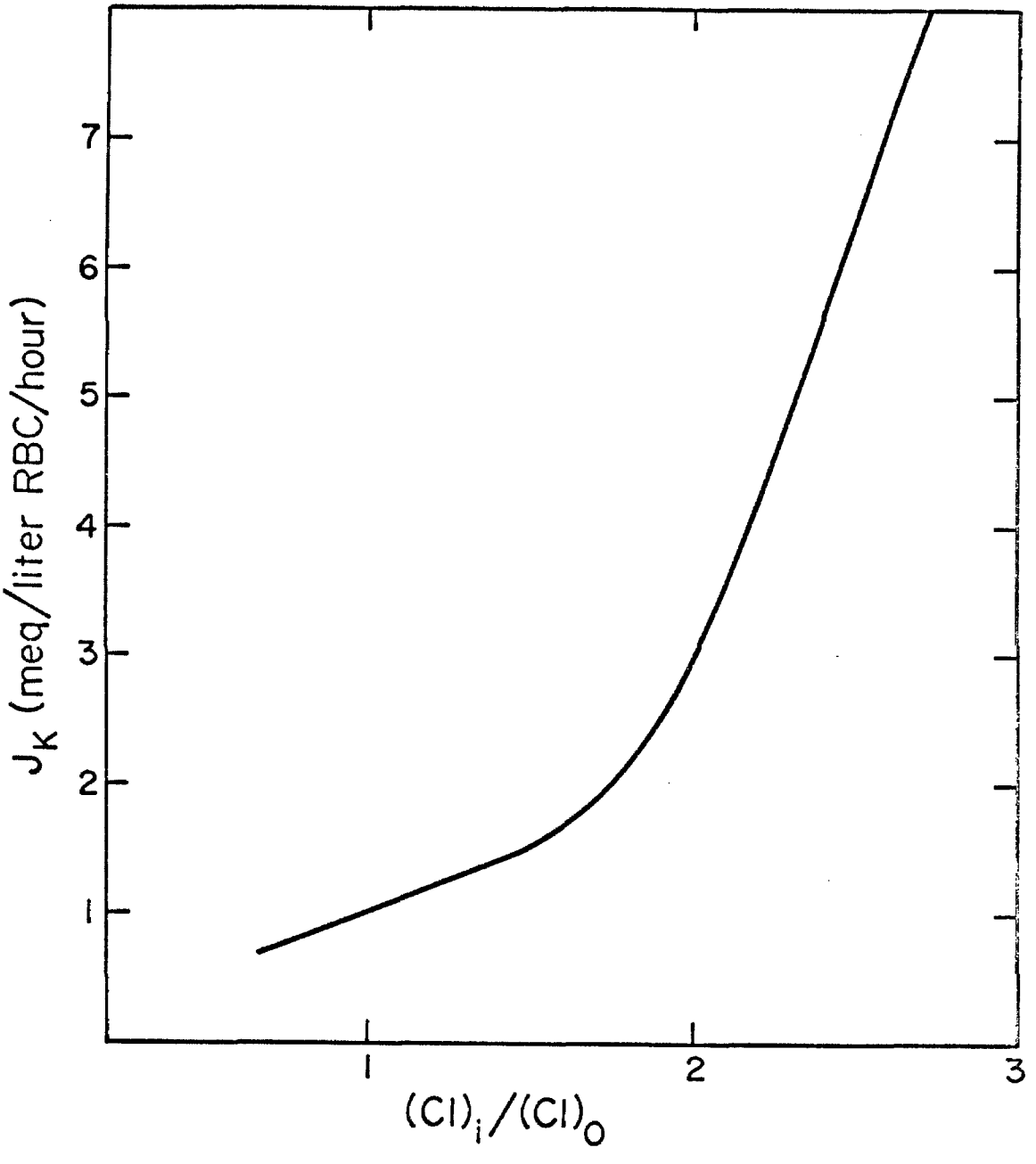

Fig. 30. The net potassium efflux from red blood cells as a function of the chloride ratio. (The chloride ratio is modified by replacing chloride with EDTA). The curve is obtained from the data of Fig. 10.

from $(Cl)_i/(Cl)_o = 0.75$ to $(Cl)_i/(Cl)_o = 1.5$. This is an especially interesting result as Cotterrell obtained the data by replacing the extracellular chloride with EDTA. The extracellular ionic strength was thus maintained constant, contrary to what happened in Donlon's experiments as well as those reported here. The possibility that changes in extracellular ionic strength could have accounted for the increase in potassium efflux has then to be discarded.

The lack of influence of osmolarity, per se, on potassium outflux demonstrated by the glucose experiments is in agreement with the results obtained by Donlon for the addition of glycerol which, like glucose, penetrates the red cell. Because of results with various sucrose solutions at ionic strength between 0.2 and 20mM, Rothstein and Donlon concluded that hypertonicity has a definite effect on membrane permeability to potassium. However, the addition of glycerol failed to demonstrate such an effect as can be seen in Fig. 31.

Although the experiments where sodium chloride or water are added to the cell suspension are not quite conclusive, the average result of the scattered data tends to show a slight increase in potassium outflux with increasing ionic strength. This would be in agreement with the results obtained at very high ionic strength and shown on Fig. 3. In as much as the ionic strength effect, per se, could explain the changes in potassium outflux, the data of Fig. 28 support the hypothesis that changes in intracellular potassium concentration do not affect the net flux of potassium.

By decreasing the extracellular pH, one can increase the chloride ratio across the red cell membrane (or vice versa) without changing any

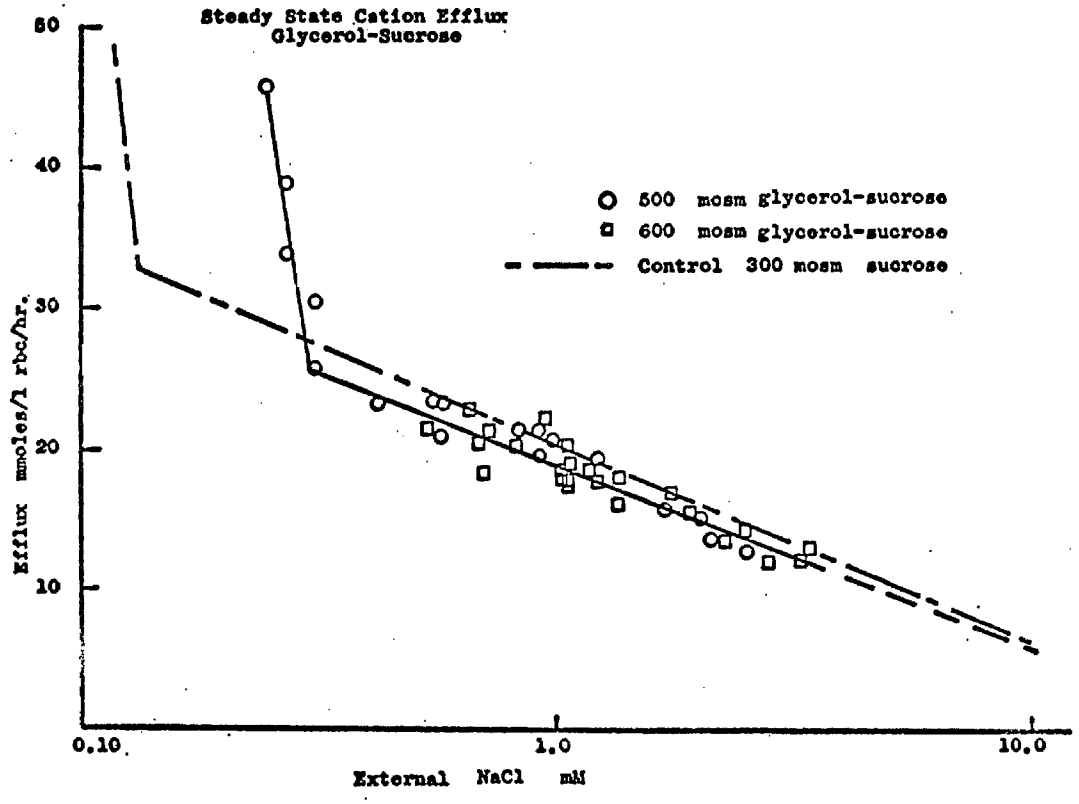

Fig. 31. Effect of adding glycerol to a suspension of red blood cell in low ionic strength media. (from Donlon 1969)

of the cationic concentrations. No experiment of this sort has been presented here because, in addition to modifying the chloride ratio, changing the pH is likely to affect any existing transport mechanism. The literature results on this subject are somewhat contradictory; the most accepted one is that changing the external pH from 6.0 to 8.0 has little effect on the potassium flux from red cells suspended in normal physiological saline. (Passow 1969 - Cotterrell and Whittam 1971). This could be due to a balance between a decrease in driving force (chloride ratio) and an increase in permeability (decrease in fixed cationic charge within the membrane, for example) as the pH increases.

The principal results of this investigation can be summarized as follows: The main parameter determining the outflux of potassium induced by ouabain in red cells near physiological conditions is the total membrane potential. The potassium outflux appears to be independent of intracellular concentrations and proportional to the chloride ratio. Neither changes in osmolarity or in ionic strength affect greatly this potassium outflux.

These results are in sharp contrast to the usual predictions of the fixed charge hypothesis. A saturation is predicted for the flux of counter ions when their bulk concentration is lower than the fixed charge concentration in the membrane, but not for the co-ion (Helfferich 1962). One way to reconcile the fixed charge hypothesis with the independence of the potassium flux and the intracellular potassium concentration is to consider as separate the rate determining barrier

for diffusion and the sites controlling the intramembrane concentration--much in the same way as Passow has suggested for anions. For example, Fig. 32 illustrates such a model. The negative charges on the surfaces of the membrane fix the total cation concentrations in the neighborhood of the membrane. The positive charges lining the pores control the diffusion of cations under fixed boundary conditions. It is not clear how such a model could account for the proportionality of the potassium flux and the chloride ratio: diluting the suspension with isotonic sucrose solution should have little effect on the boundary condition at the inner surface of the membrane. The potassium efflux should thus remain about constant upon addition of isotonic sucrose as long as the extracellular potassium concentration is much lower than the intracellular one. This is in disagreement with the experimental results.

Other mechanisms such as the mediation of a carrier or negatively charged pores can be saturated and make the potassium outflux independent of the intracellular concentrations. From the observed differences between the characteristics of the anionic and cationic permeabilities, it is quite probable that they correspond to different transport routes; for example specialized pores or carriers. The most compelling evidence in support of this hypothesis is the asymmetric effect of pH variations on cationic and anionic fluxes.

For membrane potentials between 40 and 160 mV, Rothstein et al., found that the observed effects of concentrations, pH and temperature on the cationic fluxes supported the proposition that cationic permeabilities of the red cell are controlled by fixed positive charges

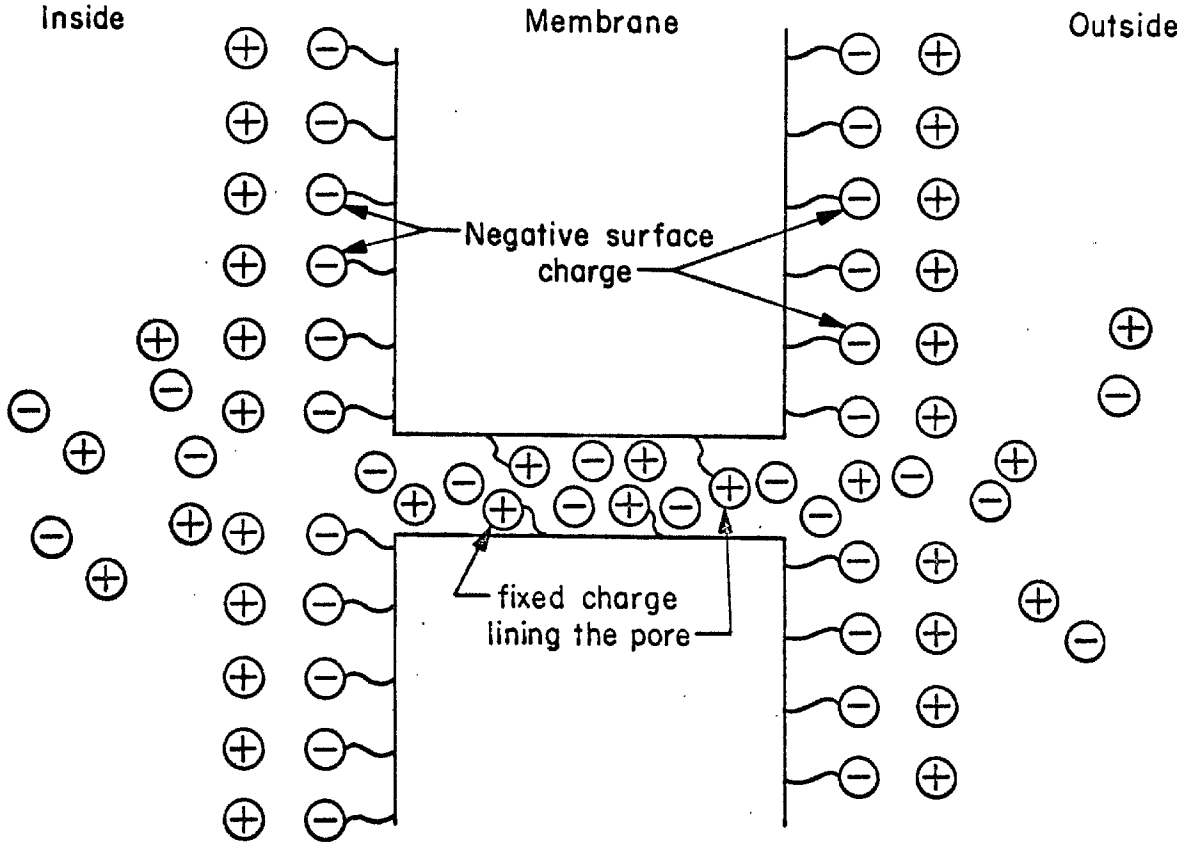

Fig. 32. Diagram of a hypothetical model for the red cell membrane.

such as $R\text{-}NH_3^+$. One can then postulate that for membrane potentials between 40 and 160 mV the main part of the cationic fluxes use the same pathways as those normally used by anions.

It then appears that two distinct mechanisms, say A and B, would be reponsible for the passive cationic fluxes. A would be the main route for cationic fluxes near physiological conditions. It would be saturated and its characteristics would be such that the potassium flux would depend exponentially upon the membrane potential and be relatively independent of pH or concentrations. B would correspond to a cationic leakage through the route normally used by anions and be comparatively small under physiological conditions. The sum of the two cationic fluxes mediated by A and B would yield such curves as those of Fig. 7 and Fig. 8. A would dominate for the two extreme parts ($V_m < 40$ mV or $V_m > 160$ mV) while it would be the opposite in the middle range. Fig. 8 would be, for example, the sum of an exponential and a straight line. Although direct extrapolation of the curve of Fig. 29 to potential around 160 mV yields values about twice as large as those actually observed, this could be explained by the effect of decreasing the extracellular ionic strength to values like 10^{-4} M.

The interpretation of Cotterrell's experiments is not quite straightforward since it is not clear what the effect of large concentrations of a strong complexing agent such as EDTA will be on the erythrocyte membrane. However, it can be simply postulated that, in addition to the membrane potential effect as it is observed in low ionic strength cell suspensions, EDTA will neutralize some of the positive charges controlling the anionic channels thus increasing cationic fluxes

through pathway B much more readily than in our experiments. This would explain why the linear dependence of the potassium efflux upon the chloride ratio breaks down for a membrane potential around 11 mV ($(Cl)_i/(Cl)_o \simeq 1.5$) in Fig. 30 while it seems to hold for potentials up to 30 mV in Fig. 29.

CONCLUSIONS

The experiments herein presented demonstrate the feasibility of using ion specific electrodes to measure ionic fluxes in red cell suspensions. The main results can be summarized as follows:

(1) The potassium efflux induced by ouabain in human erythrocytes near physiological conditions is independent of the intracellular concentrations of potassium (in the range 0.1M to 0.3M approximately);

(2) it is proportional to the chloride ratio for values from 1 to 5 (0 to 40mV);

(3) it is independent of osmolarity in the range 200 to 600 milliosmols;

(4) it increases slightly with ionic strength.

These results are in fair agreement with those obtained by Rothstein et al., using a conductivity method and with those obtained by Cotterrell and Whittam using radioactive tracers.

The fixed charge hypothesis, in its usual form, does not account for the above results. In order to explain all the experimental data available on the passive transport of potassium, two parallel routes for the efflux of potassium might be invoked: a special cationic pathway and a leakage through the channels normally used for anionic transport.

APPENDIX I

EQUATIONS OF IONIC DIFFUSION IN CHARGED MEMBRANES

In an isothermal system where the diffusing components do not undergo chemical reaction, the "flow" of an ionic species α can be written according to the irreversible thermodynamics theory:

$$J_\alpha = -\sum_\beta L_{\alpha\beta} \operatorname{grad} \tilde{\mu}_\beta \qquad (1)$$

where the subscripts α and β refers to the various solutes, $L_{\alpha\beta}$ are the phenomenological coefficients and $\tilde{\mu}_\beta$ represents the electrochemical potential of the species β. The flow J_α of the solute α with respect to the solvent reduces to the flux of α if there is no solvent flow.

In an isobaric system $\tilde{\mu}_\beta$ is given by:

$$\tilde{\mu}_\beta = \mu_\beta^0 + z_\beta F \psi + R T \ln [\beta] \qquad (2)$$

where μ_β^0 is the standard chemical potential of β, z_β is the charge of β, ψ is the electrical potential, $[\beta]$ is the activity of β, R, T and F have the usual meaning (see page 11).

Neglecting all the cross-coefficients, taking $L_{\alpha\alpha} = D_\alpha \cdot [\alpha]/R T$ for compatibility with Fick's law and considering the activity coefficients as constant throughout the system, (1) and (2) yield the usual Nernst-Planck equation:

$$J_\alpha = - D_\alpha \left(\operatorname{grad} [\alpha] + z_\alpha [\alpha] \frac{F}{RT} \operatorname{grad} \psi\right) \qquad (3)$$

The electric current is given as the sum of the ionic currents:

$$I = \sum_{\beta} z_{\beta} \cdot J_{\beta} \tag{4}$$

The Poisson-Boltzmann equation which interrelates space charge and electric field is usually replaced by the less rigorous electroneutrality condition:

$$X + \sum_{\beta} z_{\beta} \cdot (\beta) = 0 \tag{5}$$

where X represents the fixed charges in the membrane.

Various integrations of equations (3), (4), and (5) have been performed. (see for example, Teorell 1953 or Helfferich 1962). They usually suppose free diffusion, steady state, homogeneity of fixed charges and one dimensional geometry:

$$I = 0 \; ; \; J_{\alpha} = \text{constant} \; ; \; X = \text{constant} \; ; \; \text{grad} = \frac{d}{dx} .$$

A particularly simple solution is obtained for a 1:1 valent electrolyte $\alpha^{+} + \beta^{-}$:

$$J_{\alpha} = J_{\beta} = \frac{2}{\delta} \frac{D_{\alpha} D_{\beta}}{D_{\alpha} + D_{\beta}} \left[[\alpha]_i + [\beta]_i - [\alpha]_0 - [\beta]_0 - X V_m \right] \tag{6}$$

where δ is the thickness of the membrane $[\ ]_i$ and $[\ ]_0$ refers to the activities at the limits of the membrane, V_m is the adimensional diffusion potential across the membrane: $V_m = (\psi_i - \psi_0) F/R T$ and is given by:

$$V_m = \frac{D_{\beta} - D_{\alpha}}{D_{\beta} + D_{\alpha}} \cdot \ln \frac{D_{\alpha} [\alpha]_i + D_{\beta} [\beta]_i}{D_{\alpha} [\alpha]_0 + D_{\beta} [\beta]_0} \tag{7}$$

Not imposing any particular distribution of fixed charges but supposing a constant electrical field across the membrane, the conditions:

$$I = 0;\ J_{\alpha} = \text{constant};\ \text{grad} = \frac{d}{dx}\ ;\ \frac{d\psi}{dx} = \text{constant}$$

yield the Goldman equations (II) and (IV) (page 11 and 13). Under such conditions the electroneutrality equation (5) cannot be satisfied with an arbitrary distribution of fixed charges. It should be noted how much equations (II) and (IV) differ from equations (6) and (7) respectively.

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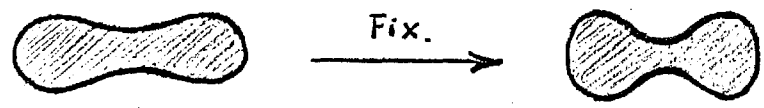
II. QUANTITATION OF HUMAN RED BLOOD CELL FIXATION BY GLUTARALDEHYDE

PROLOGUE

The following paper was published in the Journal of Cell Biology in January 1971.

An interest in red cell fixation for electron microscopy was developed from the idea that scanning electron microscope pictures could yield quantitative data on the geometry of the red cell under various conditions. As no satisfactory method of fixation for red blood cells was found in the literature, a fundamental study of fixation was undertaken. Several fixative agents were tried and glutaraldehyde was found to give the best results.

In effect this project did not succeed as the red cells were seen to deform upon the most careful chemical fixation. Microscopic optical observations showed typically the following geometrical changes in the cross section of the red blood cell:



The report presents the results of the study of red cell fixation by glutaraldehyde.

INTRODUCTION

Glutaraldehyde has been widely used as a fixative in electron microscopy since the demonstration by Sabatini et al., (14) that many enzymes retain activity (specificity) after glutaraldehyde treatment. Other advantages of glutaraldehyde include rapid cellular penetration and relative nontoxicity. Glutaraldehyde has also found use as a cross-linking agent in studies on crystalline enzymes (9, 10), and is being used by protein crystallographers for structure determinations(11).

This report uses the red blood cell (RBC) as a model object to study permeability changes to fixative and salts which occur during fixation. These results are used in an attempt to derive optimal conditions for preserving cell shape, in the hope that they will be applicable to other materials.

MATERIALS AND METHODS

Glutaraldehyde

Commercial glutaraldehyde was obtained from Polysciences, Inc., Rydal, Pennsylvania. This electron microscope grade glutaraldehyde is an aqueous unbuffered solution of 8% nominal concentration. 10-cc volumes are packaged in glass vials under an atmosphere of nitrogen. Upon receipt, the vials were stored at 4°C. Although the manufacturer stresses the purity and the monomeric nature of the product, the first 10 ampoules were markedly and uniformly polymerized as shown by an absorption peak at 235 mμ in the UV spectra (1, 3, 8). They will be referred to as glutaraldehyde sample No. 1. The second box of 10 ampoules (graciously offered in replacement of the first one) had a much lesser degree of polymerization and will be referred to as glutaraldehyde sample No. 2.

Preparation of Fixative Solutions

The fixative solutions were prepared as mixtures of glutaraldehyde solutions and buffered saline. Typically a stock solution was made up by adding 10 cc of aqueous glutaraldehyde solution to 10 cc of 600 meq ℓ^{-1} buffered saline (=13.40g NaCl + 1.35g KH_2PO_4 + 15.20g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter of water). Other solutions were then obtained by diluting the stock solution with a 300 meq ℓ^{-1} buffered saline (=4.40g NaCl + 1.35g KH_2PO_4 + 15.2g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter of water). Such preparations all have a final electrolyte concentration of 300 meq ℓ^{-1}, a pH of 7.4, and, of course, a variable concentration of glutaraldehyde.

Red Blood Cells (RBC)

Normal Packed RBC: Blood from healthy donors was obtained from the blood bank in standard 500 cc bags with 75 cc of acid citrate dextrose (ACD) as anticoagulant. The cells were spun down at 3000g for 10 minutes. The plasma, the buffy coat, and, as a precaution, the top layer of cells were then removed. The remaining packed cells, used without any further washing, are called normal packed RBC. Except in some control experiments, the cells were used within 3 days after withdrawal from the donor, and for those experiments involving critical examination of the shape (Figs. 5 and 6) they were always used within 24 hours.

Hemolyzed RBC: To obtain solutions of hemoglobin and cell membranes at the same concentration as found in packed RBC, normal packed RBC were frozen at -80°C by using a mixture of dry ice and ethanol, followed by thawing at room temperature. Phase-contrast microscopy demonstrated a high degree of hemolysis. The resulting solution will be referred to as hemolyzed RBC.

Washed RBC: One control experiment was performed with cells that had been washed four times with isotonic buffered saline, with the use of approximately 4 volumes of saline per volume of red cells (packed as above).

UV Spectrophotometry

Ultraviolet absorption was recorded at 24°C with a Beckman Ratio Recording Spectrophotometer Model DKA (Beckman Instruments Inc., Fullerton, California) with matched 1 cm quartz cuvettes

(Carl Zeiss, Inc., New York). The reference solutions were distilled water or phosphate-buffered saline solutions corresponding to those of the glutaraldehyde solutions. The samples and the references were diluted 10 times with distilled water.

Scanning Electron Microscopy

Fixed and water-washed red blood cells were prepared for scanning electron microscopy by air drying on an aluminum specimen mount, followed by the evaporation in vacuum of a conducting coating. A layer of carbon was first uniformly evaporated over the cell surface. Gold was then evaporated over the carbon. The carbon formed a thin, continuous conducting film over the cell surface, while the gold yielded a high secondary emission ratio. A Cambridge scanning microscope was used at 20 kv. Images were photographed on Polaroid 55-PN film.

Ion Measurements

A Na-sensitive electrode (Corning No. 476210) and a K-sensitive electrode (Orion No. 92-19) were used to measure the activities of sodium and potassium, respectively. The electrodes, together with a single junction reference electrode (Orion No. 20-01), were coupled to a digital pH/mV meter (Orion model 801) through a switching box. With adequate control of temperature and good grounding, precision better than 0.2mV was obtained. The pH and the selectivity constants were such that no correction for interference was necessary. Under such conditions, relative activities of the ions are readily obtained by

using the Nernst equation, and the accuracy achieved is of the order of 1%.[1]

Osmometry

The osmotic measurements were made with a freezing point depression osmometer.[2] Both 2 cc and 0.2 cc samples were used without any appreciable influence on precision or on absolute values. Commercial standards of 100 and 500 milliosmols (respectively, -0.1858°C and -0.9291°C) were systematically used for calibration before each series of measurements. The instrument is directly graduated in milliosmols. Although better precision can be achieved with special care, most measurements were reproducible to about ±2 milliosmols (±0.0037°C).

Tests showed that the presence of suspended RBC had no effects on osmotic readings. Therefore the red cells were left in the samples for osmotic measurements made during fixation.

In a typical experiment 1 ml of normal packed RBC was mixed rapidly in a test tube with 9 ml of fixative solution. The freezing point depression of the mixture was then recorded as a function of time by removing 0.2 cc samples for measurement at predetermined time intervals. The zero time was taken as the time of initial mixing, and the time of the measurement was recorded when pressing the seeding

[1] For theoretical and practical considerations on ion-sensitive electrodes, see Ion Selective Electrodes. R. A. Durst, editor. N. B. S. Special Publication 314, November 1969.

[2] Osmette 2007 precision systems.

button. To avoid any sedimentation of the cells, the tubes were closed with neoprene stoppers and kept in constant rotation. The experiments were carried out at a constant temperature of 24°C (±1°C). A sample of fixative solution was kept under similar conditions as the cell preparations and was measured as a reference.

RESULTS

Characterization and Calibration of the Solutions

The absorption spectra of glutaraldehyde samples No. 1 and No. 2 are shown in Fig. 1 and demonstrate peaks at 235 mμ and 280 mμ. In each sample all the vials gave reasonably similar results. It is seen that sample No. 1 absorbs more than sample No. 2 at 235 mμ, whereas the opposite occurs at 280 mμ. The spectra of the fixative solutions in which the buffered saline was added were also recorded and are similar to those shown in Fig. 1, the only difference being a slight upward shift of the 235 mμ peak.

Fig. 2 shows the result of a calibration curve for osmotic measurements of glutaraldehyde solutions. 10 different solutions were obtained by dilution of a common glutaraldehyde solution with distilled water. These solutions were then mixed with equal volumes of: (a) water for the lower curve; (b) 600 meq ℓ^{-1} buffered saline for the upper one, and then measured. As can be seen, both series of measurements lie on straight lines, but the lines are not parallel. Since the pure aqueous solutions of glutaraldehyde can be considered as nearly ideal, the corresponding freezing point depressions were taken as a direct measure of their concentrations: concentration in moles ℓ^{-1} = 0.5382 × freezing point depressions in °C. To obtain glutaraldehyde concentrations from osmolarity measurements of solutions containing the salts, one has to make a systematic correction given by the calibration curve.

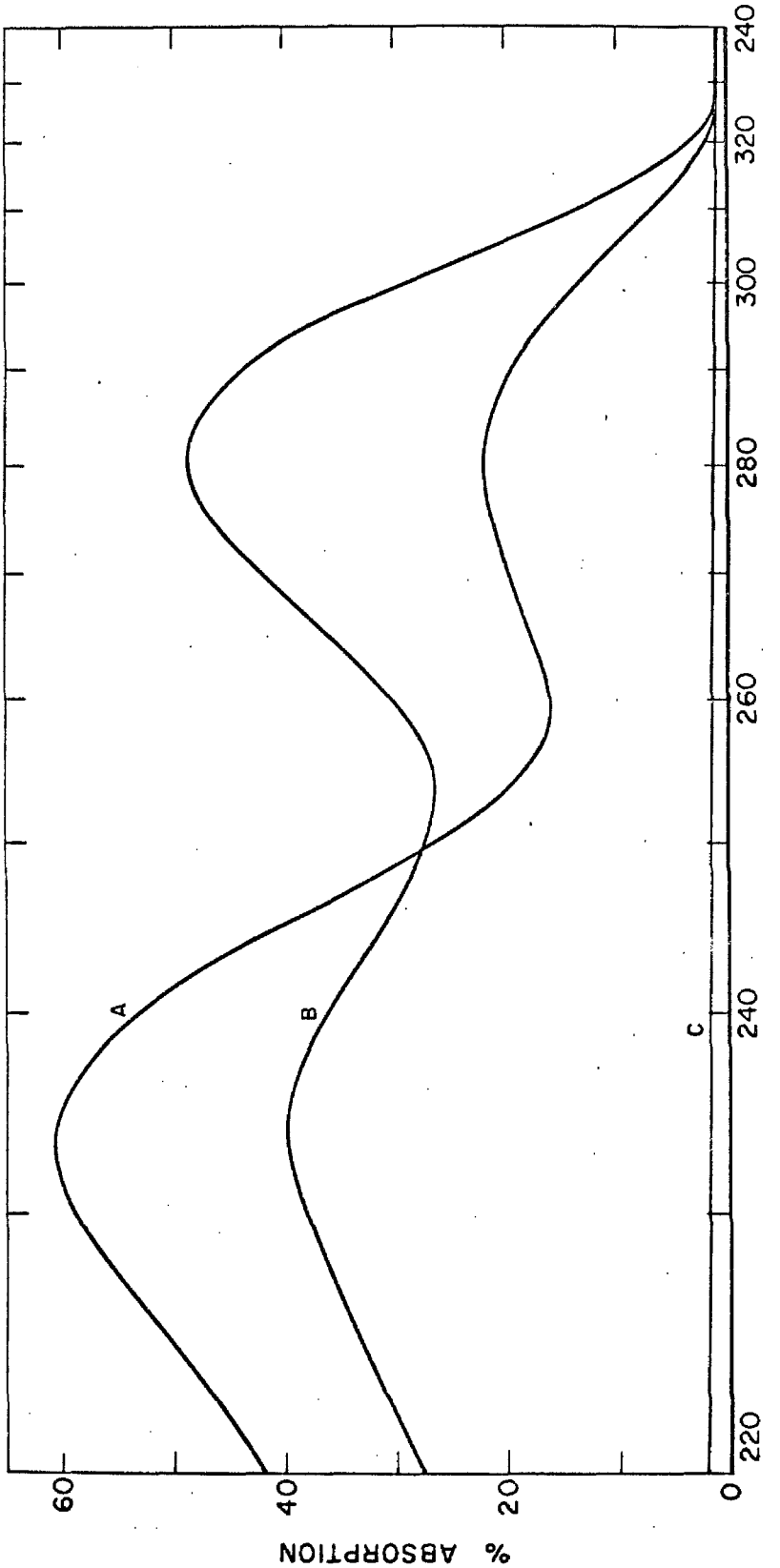


Figure 1 UV absorption spectra of glutaraldehyde samples. A, glutaraldehyde sample No. 1; B, glutaraldehyde sample No. 2; C, reference solutions.

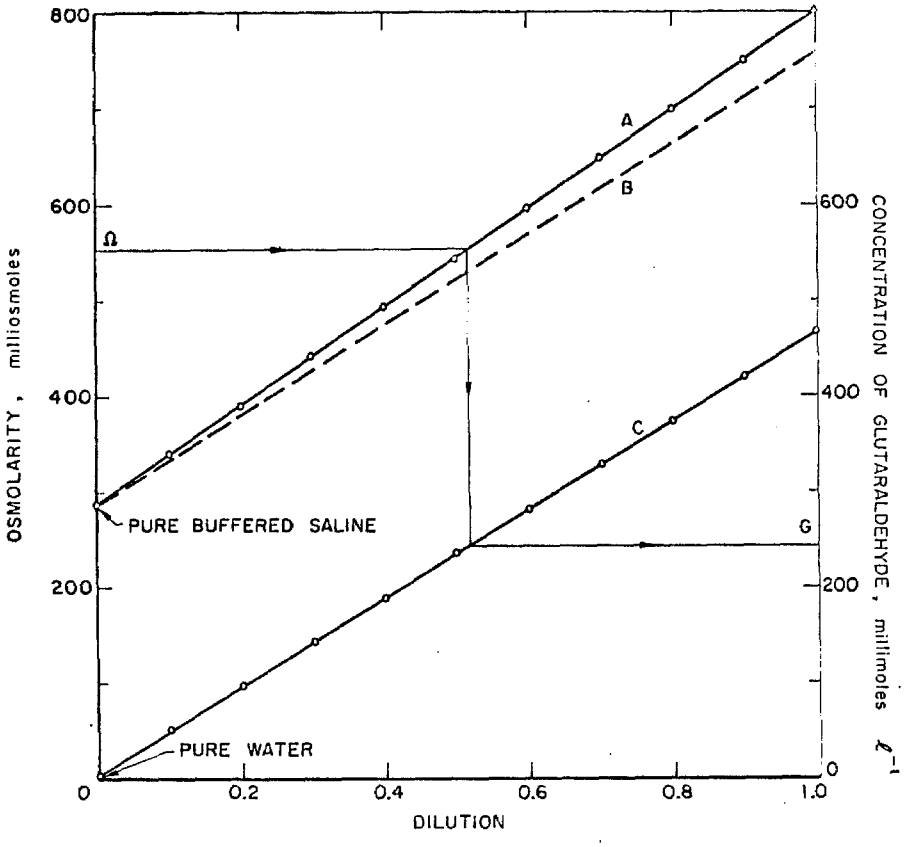


FIGURE 2 Osmometry of glutaraldehyde solutions. 10 different solutions were obtained by dilution of a common glutaraldehyde solution with distilled water. *A*, osmolarities of the 10 solutions mixed 1:1 with 600 meql^{-1} of buffered saline; *C*, osmolarities of the 10 solutions mixed 1:1 with distilled water; *B* has been drawn parallel to *C* in order to display the difference in slope between *A* and *C*. Arrows indicate the steps involved in deriving the glutaraldehyde concentrations (*G*) from the osmotic measurements of mixtures of glutaraldehyde and buffered saline (Ω).

Osmolarity as a Function of Time for Mixtures of Cells and Glutaraldehyde

Normal Packed RBC: Fig. 3 shows the results of three typical experiments in which various dilution factors and glutaraldehyde concentrations were used. An initial rapid drop in osmolarity over the first 30 minutes was followed by a slower rate of decrease in osmolarity over a period of 5-8 days. The influence of various parameters can be summarized as follows:

(a) Mixing did not seem to be a critical factor although it is clear that a good definition of the reference time depends on mixing. Similar curves were obtained whether continuous agitation was used or the tubes were shaken just before measuring.

(b) Experiments carried out with 10 day old blood gave identical results.

(c) Glutaraldehyde samples No. 1 and No. 2 gave results that were qualitatively similar but, as shall be seen, quantitatively different.

(d) The concentration of glutaraldehyde was an important factor; the most reproducible results were obtained with concentrations of the fixative solutions of 1.5% and over. Smaller concentrations gave results which were less reproducible.

(e) The dilution factor f = volume of fixative/volume of RBC = Vf/Vc, was also an important parameter. The

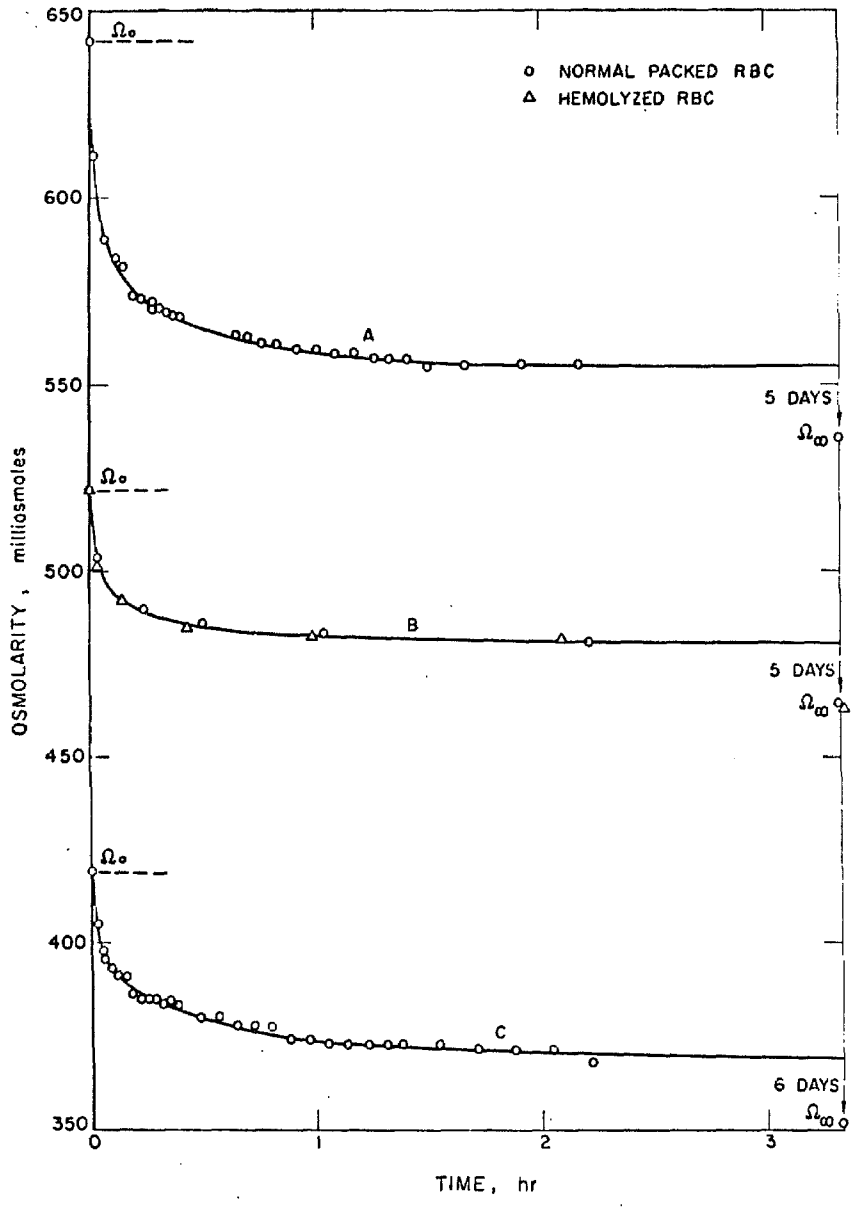


FIGURE 3 Osmolarity as a function of time for mixtures of cells and glutaraldehyde. *A*, 1 cc of normal packed RBC mixed with 9 cc of fixative solution (3.4% glutaraldehyde); *B*, 1 cc of normal packed and hemolyzed RBC mixed with 19 cc of fixative solution (2.2% glutaraldehyde); *C*, identical to *A* but 1.2% glutaraldehyde. Ω_0 are the osmolarities of the fixative solutions; Ω_∞ are the osmolarities of the mixtures after 5–6 days.

most consistent results were obtained with f values greater than 5. Values between 8 and 20 were usually used.

Hemolyzed RBC: Parallel experiments were carried out with the same fixative, the same dilution factor, and the same volume of RBC, but in one case the RBC were normal packed cells and in the other they were hemolyzed cells. The results of such parallel experiments are shown on the middle curve of Fig. 3 and can be said to be identical within experimental precision. It is worth noting that fixation of hemolyzed cells leads to the formation of a gel. For dilution factors up to 10, the entire mixture of cells and fixative solution is involved in the formation of this gel; gelation appears to be complete after about 10 minutes.

Washed RBC: Washing the cells did not qualitatively change the results. However, the recorded osmolarities were systematically lower (by 10-20 milliosmols) than the corresponding values for unwashed cells. The results of the experiments at low glutaraldehyde concentrations (< 100 millimoles ℓ^{-1}) were also more consistent when the washed RBC were used instead of the normal packed RBC. This can be seen in Fig. 4 (Fig. 4 is explained in the Discussion.)

Scanning Electron Microscopy

Fig. 5 illustrates the osmotic effect on the cells of a high percentage glutaraldehyde concentration in the fixative. The flat aspect of the cells is indicative of water loss. In contrast, the normal shapes of

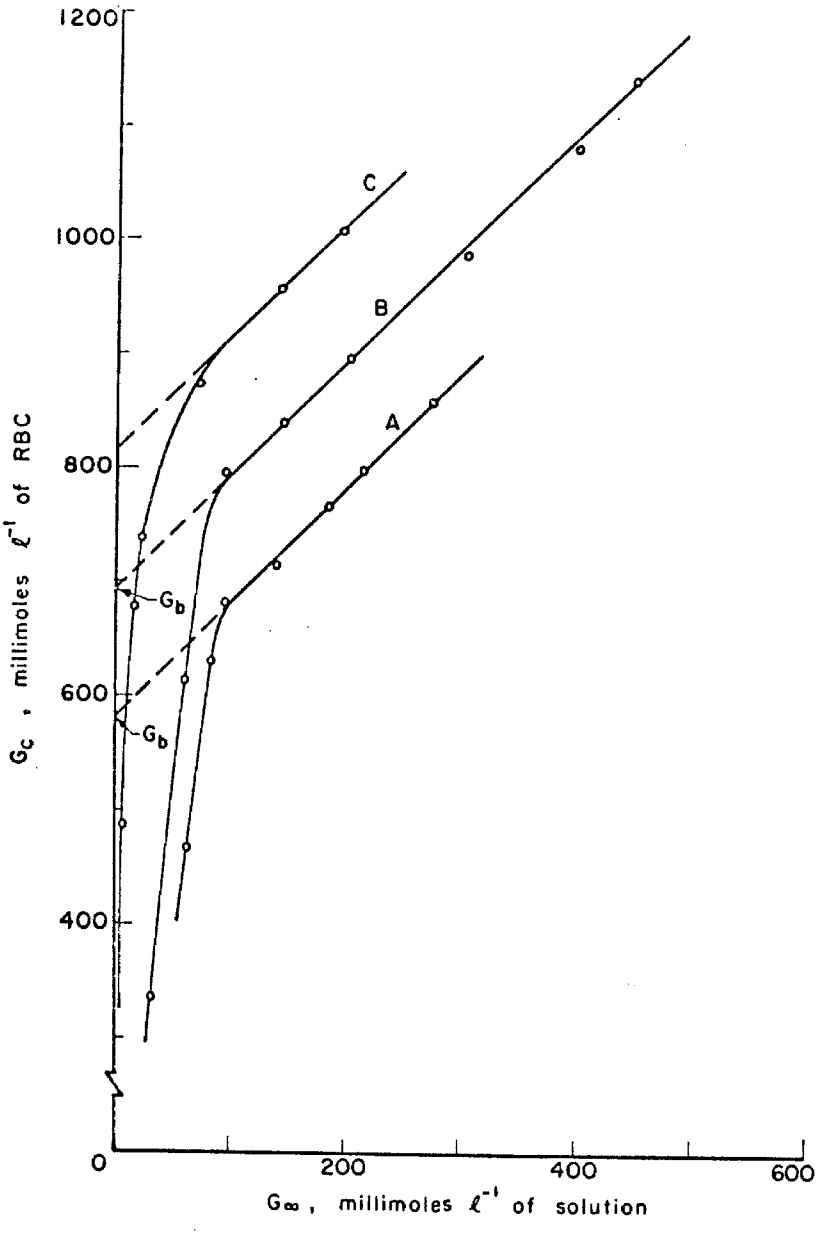


FIGURE 4 Equilibrium curves G_c *versus* G_∞. These curves are obtained by plotting the number of moles of glutaraldehyde G_c used up per liter of RBC *versus* the final glutaraldehyde concentration G_∞ in the solutions. *A*, normal packed RBC with glutaraldehyde sample No. 1; *B*, normal packed RBC with glutaraldehyde sample No. 2; *C*, washed RBC with glutaraldehyde sample No. 2. G_b is a measure of glutaraldehyde bound to red cell proteins.

RBC in Fig. 6 have been preserved by minimizing the osmotic imbalance due to glutaraldehyde during fixation. This has been achieved by adding 1% glutaraldehyde fixative solution, over a period of 15 minutes to RBC's in isotonic buffered saline.

Ion Measurements

28 cc of packed RBC were suspended in 47 cc of isotonic buffered saline and then fixed by addition of 30 cc of a 6% glutaraldehyde fixative solution (total volume = 105 cc). The temperature was maintained constant at 37.5°C (±0.1°C) and the activity of potassium and sodium ions continuously recorded. Table I gives results at some of the observed times, showing that leakage of ions took place during fixation and that an equilibrium was attained after some 2 hours.

Permeability of the Fixed RBC

Experiments were carried out on fixed cells (more than 5 days of fixation) to test the permeability of the membrane to solutes (see Table II). Fixed RBC that had been suspended in media of various compositions and osmolarities were mixed with solutions of various compositions and osmolarities. Assuming free exchange of solutes between the inside and outside of the cells and taking the fluid volume of the cell to be 70% and the packing efficiency of the hardened cells to be 60% (2), one can compute the expected osmolarities of the mixtures (Table II, footnote*). These computed values and the experimental osmolarities are shown in Table II and are seen to be within a few percent.

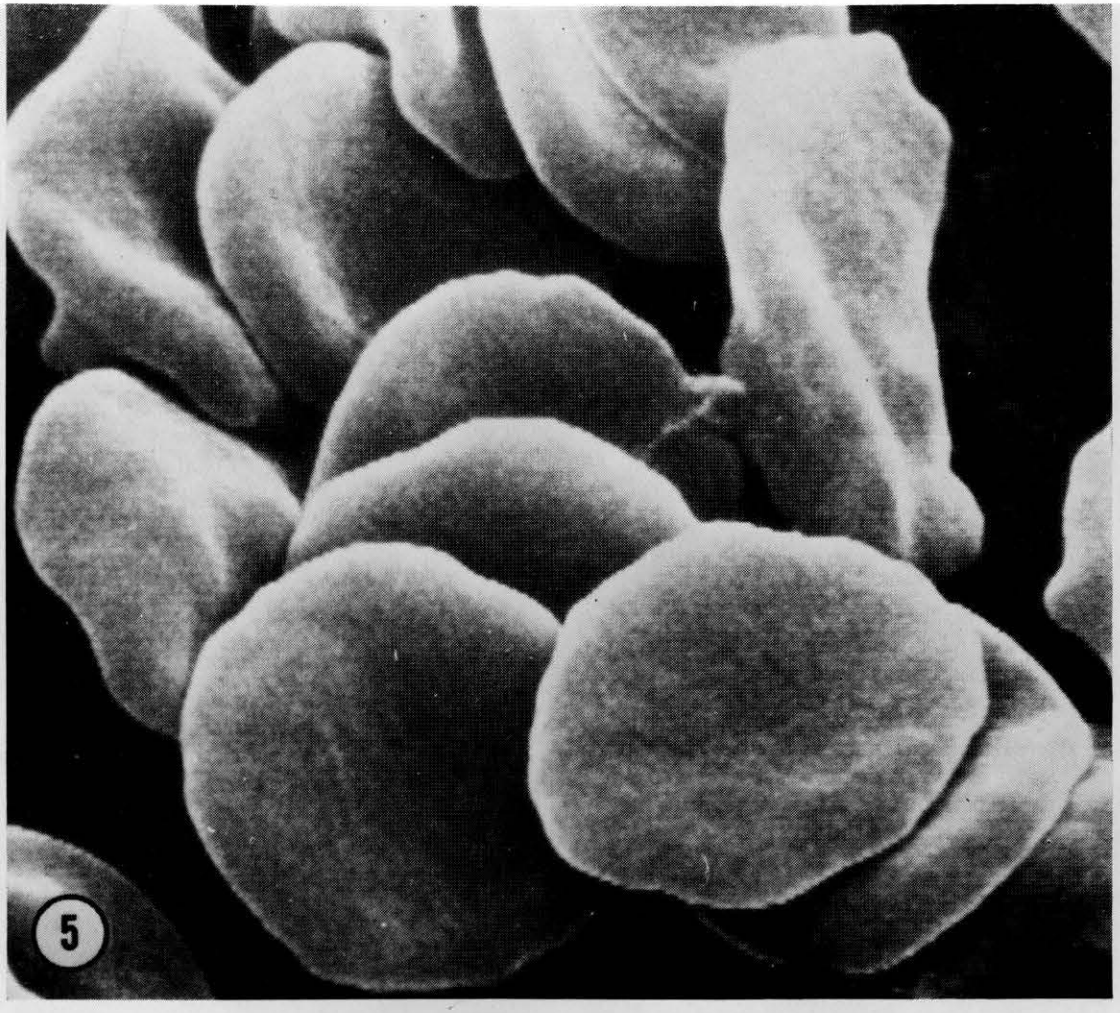


FIGURE 5 Scanning electron micrograph of RBC fixed for 2 hr by mixing the cells with a solution of high glutaraldehyde concentration (8%) and isotonic buffered saline.

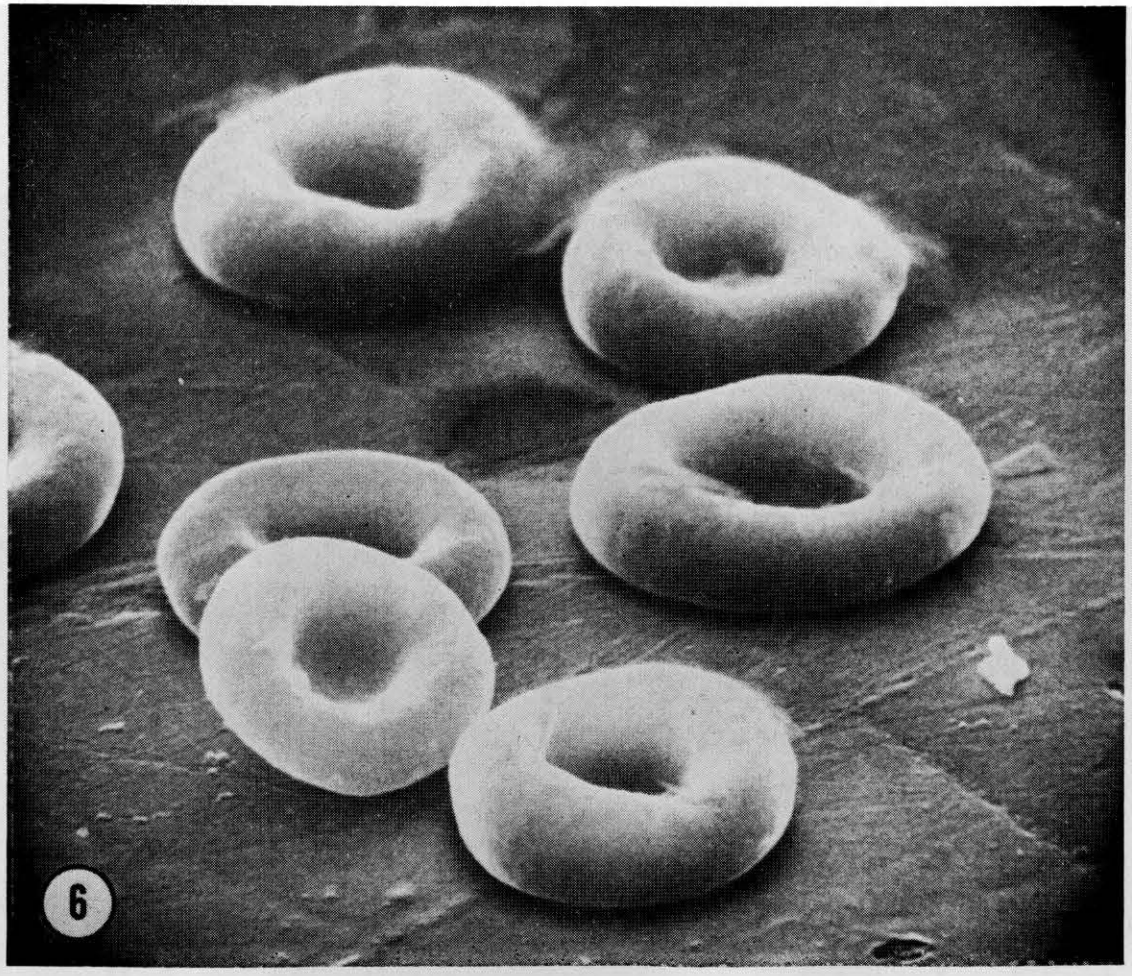


FIGURE 6 Scanning electron micrograph of RBC fixed for 2 hr in low glutaraldehyde concentration. A solution of glutaraldehyde (1%) and isotonic buffered saline was added to an isotonic suspension of cells over a period of 15 min.

TABLE I

Ion Activities in the Supernatant as a Function of Time

Time	K		Na	
	emf	Relative activity*	emf	Relative activity*
min	*mv*	%	*mv*	%
0	−6.0	100	+4.5	100
10	+2.7	138	+3.6	96.7
20	+11.5	192	+2.2	91.8
30	+17.2	238	+1.2	90.4
60	+23.5	302	+0.3	85.4
120	+25.2	322	+0.2	85.1
180	+25.7	327	+0.3	85.4

* The Nernst equation can be written: $E = E_0 + k \log(x)$ where E is the measured emf, E_0 is a reference value, x is the activity of the ion, and k is a constant ($k \approx 61.5$ at 37.5°C). Then one obtains a relative value with respect to any activity (x_1) by:

$$\frac{E-E_1}{k} = \log \frac{(x)}{(x_1)}$$

TABLE II

Permeability of Fixed RBC

Fixed RBC				Solutions			Mixtures	
		Supernatant						
Volume	Hct‡	Composition§	Osmolarity	Volume	Composition§	Osmolarity	Experimental osmolarity	Computed* osmolarity
cc			*millios-mols*	*cc*		*millios-mols*	*millios-mols*	*millios-mols*
1.05	0.92	BS and G	363	1.00	W	7	168	172
1.05	0.92	BS and G	363	1.00	BS	289	327	324
1.05	0.92	BS and G	363	1.00	BS and G	492	431	431
1.05	0.92	BS and G	363	1.00	BS	582	492	480
1.13	0.92	W	7	1.00	BS and G	492	264	257
1.00	0.80	W	7	1.00	KCl	385	231	222
1.00	0.80	W	7	1.00	NaCl	500	234	230
1.00	0.80	W	7	1.00	E	348	196	192

* These values are obtained in assuming (*a*) free exchange of solute; (*b*) fluid volume of the cells = 70%; (*c*) packing efficiency for Hct measurement = 60%. Then the solid volume of the cells is 18% of the packed cell volume (volume of cell × Hct).
Computed osmolarity of mixture

$$= \frac{\text{vol. RBC} \times (1 - \text{Hct} \times 0.18) \times \text{osmolarity of supernatant} + \text{vol. soln.} \times \text{osmolarity of soln.}}{\text{vol. RBC} \times (1 - \text{Hct} \times 0.18) + \text{vol. soln.}}$$

‡ Hct, hematocrit. It is here defined experimentally as the ratio of observed packed cell volume and total volume using a microcapillary centrifuge (6 min, 13,000 *g*).
§ BS, buffered saline; G, glutaraldehyde; W, water; E, ethanol.

DISCUSSION

In comparing the results of UV absorption of the glutaraldehyde samples with similar curves published in the literature (1, 3, 8), it appears that both samples either are partially polymerized or contain some impurities as demonstrated by the existence of the 235 mμ peak. However, in contrast to the pH of untreated commercial solutions which is usually about 2 or 3 (1, 3), the pH of both samples was neutral without added buffer. Therefore, the 235 mμ absorption peaks observed with our samples are probably due to polymerization of the glutaraldehyde solutions after their purification and not to impurities, in agreement with Robertson and Schultz (13). In other words, both samples can be considered as fairly pure mixtures of monoglutaraldehyde and various oligomers, sample No. 1 having a greater degree of polymerization than sample No. 2.

The two straight lines obtained for the calibration curves by plotting concentration against osmolarity are not parallel; the osmolarity of the solution containing both glutaraldehyde and buffered saline is more than the sum of the osmolarity of the individual solutions. This is theoretically predicted by cross effects between the salts and the organic solution because of their different polar characteristics with respect to water (reference 7, p. 531). The buffered saline "salts out" the glutaraldehyde. It decreases its solubility, i. e., increases its chemical activity. Reciprocally, glutaraldehyde salts out salt. This cross effect between the solutes can also be described with an osmotic coefficient different from 1.

Such an increase in the activity of glutaraldehyde can be expected to increase its polymerization. This is probably the reason for the observed upward shift of the 235 mμ absorption peak. However, this effect is certainly small compared to the direct effect on the freezing point depression and does not appreciably change the osmotic values observed.

The information given by the curves of Fig. 3, osmolarity versus time, can be divided into three parts related to (a) the first osmotic drop; (b) the overall shape of the curves; (c) the final osmotic values reached after a few days.

(a) The results obtained with normal packed RBC suggest that the initial drop in osmolarity is inversely proportional to the dilution factor. This initial drop could be due to a rapid entrance of glutaraldehyde into the cell and its immediate partition between the fluid inside the RBC and the fixative solution. Confirmation of this hypothesis is given by the results of the parallel fixation of normal packed cells and hemolyzed cells. Whether the hemoglobin is enclosed in the membrane or freely available for reaction with the fixative makes no difference in the osmotic drop. The dilution of the fixative solution by the cell volume as well as the rate of the reaction are the same in both cases; thus glutaraldehyde enters the cells readily. This explains why glutaraldehyde shows little or no osmotic activity with respect to the RBC and, thus, why it is necessary to use an isotonic concentration of electrolytes in the fixative solution. Any other concentration creates an osmotic imbalance that changes the volume of the cells while they are being fixed.

It is important to note that the diffusion of glutaraldehyde across the membrane, although fast, is not so fast as water diffusion and, in using very high concentrations of glutaraldehyde, one can demonstrate in the final fixed shape of the RBC the transient osmotic imbalance. This is illustrated in Fig. 5 where one can see the dehydrated aspect of the cells despite the salt's isotonicity in the fixative solution.

(b) The kinetics of the reaction of glutaraldehyde with the RBC do not appear to be simple. However, curves like those of Fig. 3 can be quite precisely fitted up to a few hours by equations of the type: $1/(\Omega - \Omega_1) = k \cdot t + \text{constant}$, where Ω is the osmolarity of the solution at time t and Ω_1 is a reference value corresponding to the osmolarity of the solution after 5-10 hours of fixation. Since Ω is a linear function of the glutaraldehyde concentration, it seems that such a fit implies a pseudo-second-order type of reaction. The later part of the reaction, from a few hours to a few days, cannot be fitted in the same way and might correspond to a different mechanism. It could be polymerization of the glutaraldehyde, although the control fixative solution did not show such a decrease in osmolarity.

(c) An interesting equilibrium curve is obtained by plotting the number of moles of glutaraldehyde G_c used up per liter of RBC versus the final glutaraldehyde concentration G_∞ in the solution. The osmolarity Ω_0 of the fixative solution before mixing with the cells allows the glutaraldehyde concentration G_0 of the fixative to be computed by using the calibration curves (Fig. 2). In the same way, the osmolarity Ω_∞ of the suspending solution after 5-8 days gives G_∞. Then: G_c = number

of moles of glutaraldehyde used up per liter of cell = $(G_0 - G_\infty)V_f/V_c$. (V_f = volume of fixative, V_c = volume of RBC).

Fig. 4 shows the equilibrium curves G_c versus G_∞, obtained with normal packed RBC for glutaraldehyde samples No. 1 and No. 2. It is seen that, for concentrations greater than 100 millimoles ℓ^{-1}, the curves are straight lines of slope 1. Such a slope suggests that the glutaraldehyde taken up by the cells is made up of two parts: (a) a fixed amount corresponding to the saturation of the cell proteins by the glutaraldehyde. Its value G_b is the intercept of the straight line with the vertical axis; (b) an amount of free glutaraldehyde in solution inside the cell and equal in concentration to the glutaraldehyde in the external medium.

This interpretation has been corroborated by the experiments testing the permeability of the membranes of the fixed cells. In fact it has been shown that, after 2 days of fixation, electrolytes as well as glutaraldehyde are freely exchangeable between the cells and their bathing solution, and that the concentrations inside the cells are identical to those in the solution. The results obtained on the ionic leakage across the membrane show that this increase in membrane permeability to salt occurs progressively over a period of approximately 2 hours.

The values G_b of bound glutaraldehyde are found to be of the order of 600 and 700 millimoles ℓ^{-1} for the samples No. 1 and No. 2, respectively. Since the amount of hemoglobin available for reaction is much larger than the amount of membrane proteins or of plasma proteins trapped between the cells, one can consider that the

glutaraldehyde reacts effectively with a 5 millimoles ℓ^{-1} hemoglobin solution, i. e., 120-140 molecules of glutaraldehyde saturate 1 molecule of Hb. If both aldehyde groups of each glutaraldehyed molecule react with a hemoglobin site, then there are 240-280 such sites. If, on the contrary, glutaraldehyde molecules react at one end with a hemoglobin site and at the other end with another glutaraldehyde molecule, then there might be 120 or even fewer sites. Thus, depending upon the glutaraldehyde polymerization that accompanies such a reaction, it can be considered that between 100 and 300 sites in each hemoglobin molecule react with glutaraldehyde molecules. This result is in agreement with the structure of hemoglobin and the known sites of reaction of glutaraldehyde with proteins (6, 12).

The difference observed between the samples in the value of G_b is thought to be qualitatively significant. It is probably due to a greater efficiency of the longer oligomers in cross-linking the proteins. Monomers might often be too short to be able to react at both ends, and the cross-linking is achieved by polymerization of those monomers. Thus more molecules of the less polymerized sample No. 2 are needed to saturate the proteins than of sample No. 1. This result is to be compared with that of Robertson and Schultz (13) who claimed that fixatives containing a high proportion of dimer fixed brain tissue better than purified glutaraldehyde containing a high proportion of the monomer.

Fig. 4 also shows an equilibrium curve obtained for washed RBC. It is seen that the curve is higher and follows the vertical axis more closely than the curves obtained for unwashed cells. The presence or

absence of plasma proteins probably plays a key role in these differences, mainly in introducing a systematic bias in the osmometry of unwashed cell solutions. Nonetheless, the overall shape of the curve is the same and the differences are small enough not to modify the above discussion.

Some optimal methods for cell fixation by glutaraldehyde have been published in the literature (4, 5). It is clear from the preceding discussion that the presence of an electrolyte at isotonic concentration is a sine qua non condition for shape preservation during the fixation of the red cells. The problem, then, is to determine the glutaraldehyde concentration and the dilution factor that will produce minimal distortion while allowing complete fixation of the cells. The curves of Fig. 4 gives the minimum amount of glutaraldehyde necessary to saturate the binding sites. A large dilution factor (10 or greater) will minimize the osmotic stress due to glutaraldehyde during the initial mixing. A still better way is to use a step-wise technique: the cells are suspended in an isotonic buffer, and drops of fixative solution (glutaraldehyde in isotonic buffer) are slowly added to the system. Fig. 6 illustrates this method of fixation.

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