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Effects of Phlorizin on Net Chloride Movements Across the Valinomycin-Treated Erythrocyte Membrane

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Summary. The rate of valinomycin-induced KCl efflux from human red cells and ghosts was measured in the presence and absence of phlorizin. Extracellular phlorizin accelerated the KCl efflux. The effect depended on the phlorizin concentration and showed half saturation at about 0.4 mM phlorizin. Hunter's procedure was used to calculate Cl permeabilities (P_{Cl}) by means of the Goldman equation from rate constants of K^+ loss in valinomycin-treated ghosts. For saturating phlorizin concentrations a 20-fold increase, approximately, of P_{Cl} was calculated. The observed increase in P_{Cl} is in contrast to the almost total inhibition of Cl^- equilibrium exchange. Similar to the effects on anion exchange permeability the effect on P_{Cl} is only observed when phlorizin is present at the outer surface of the erythrocyte membrane while internal phlorizin is without effect. A similar asymmetry was observed in the stimulation of valinomycin-induced K^+ exchange at identical K^+ concentrations on both sides of the membrane. The effects of phlorizin were only observed if net KCl flow was out of the cells but not if it was in the opposite direction. The effect of phlorizin on net KCl movements and sugar transfer were unaltered when the phlorizin was subjected to several consecutive purifications. This indicates that the observed effects are due to the glycoside and not to contaminations with its aglycone.

The penetration of anions across the erythrocyte membrane can be followed under two different experimental conditions (Harris & Pressman, 1967; Scarpa, Cecchetto & Azzone, 1970; Hunter, 1971): (1) in the absence of cation movements where one anion species from the red cell interior exchanges against another in the medium, and (2) by rendering the membrane permeable for cations and then following the net salt movements which include the movements of the accompanying anion. The results of the two types of measurements showed that exchange permeability for halides is many orders of magnitude higher than the net permeability. This was confirmed by comparisons of the permeabilities as measured by means of radioisotopes at Donnan equilibrium with electrical conductance measurements (Hoffman & Lassen, 1971; Lassen, 1972).

The described findings raise the question whether chemical modifiers of permeability have similar or different effects on anion transfer when measured under the two conditions. The present paper is concerned with this question. As modifier we chose phlorizin, which like its aglycone, phloretin, is known to be an effective inhibitior of anion equilibrium exchange (Schnell & Passow, 1967; Wieth, Dalmark, Gunn & Tosteson, 1973).

Phlorizin does not penetrate across the red blood cell membrane and hence the sidedness of its effects can be studied. It has been shown that in contrast to sugar permeability, which could be inhibited from either surface (for the sidedness of the action of the aglycone, *see* Beneš, Kolínská and Kotyk, 1972), the equilibrium exchange for halide anions (Schnell, Gerhardt, Lepke & Passow, 1973) and sulfate (Lepke & Passow, 1973) could only be inhibited if the agent was present on the outer surface of the membrane. In view of the demonstrated asymmetry of the effect of phlorizin on anion exchange we included into the present work an investigation of the sidedness of the effect of this modifier on net salt movements.

Materials and Methods

All experiments were performed with citrated blood from healthy donors. Before use the cells were washed three times in isotonic saline and the buffy coat was removed by aspiration. Erythrocyte ghosts which retain the low cationic permeability of the intact cell membrane were prepared as described by Bodemann and Passow (1972, p. 16, last paragraph) except that hemolysis was performed at pH 6.0 (Lepke & Passow, 1972).

For determinations of potassium and sodium movements, the packed cells were added to the incubating medium and the extracellular medium was sampled by the filtration technique of Dalmark and Wieth (1972), or by rapid centrifugation in an Eppendorf 3200 microcentrifuge. In the microcentrifuge sedimentation was complete after 30 sec, and 10 sec after commencement of spinning was taken as the sampling time. Na^+ and K^+ were measured in an Eppendorf flame photometer, $^{42}K^+$ in a Packard autogamma counter or in a Packard Tricarb liquid scintillation spectrometer where the Cherenkov radiation was determined. Measurements of sodium uptake were corrected for trapped extracellular sodium as described by Passow (1969).

All determinations of cellular K or Na contents refer to amounts in a fixed number of cells of a measured volume of suspension. Concentrations are calculated per gram of original cell weight. In ghosts the calculated concentrations are lower than those in the media with which they were equilibrated prior to the resealing process. This is partly due to the fact that the volume of the ghosts is smaller than that of the cells from which they were derived and partly to the presence of type III ghosts which had been depleted of either K^+ or Na^+ prior to the start of the measurements in Na^+ or K^+ media, respectively (*see* Schwoch & Passow, 1973). The presence of leaky type III ghosts is assumed to account for the high initial K^+ concentration in the total ghost population in uptake experiments as inferred from extrapolation to zero time (Fig. 5*b*). If this were so, the potassium concentration inside the type II ghosts should be nearly identical to the K^+ concentration in the medium in which they were resealed; i.e., less than 5 mM/liter.

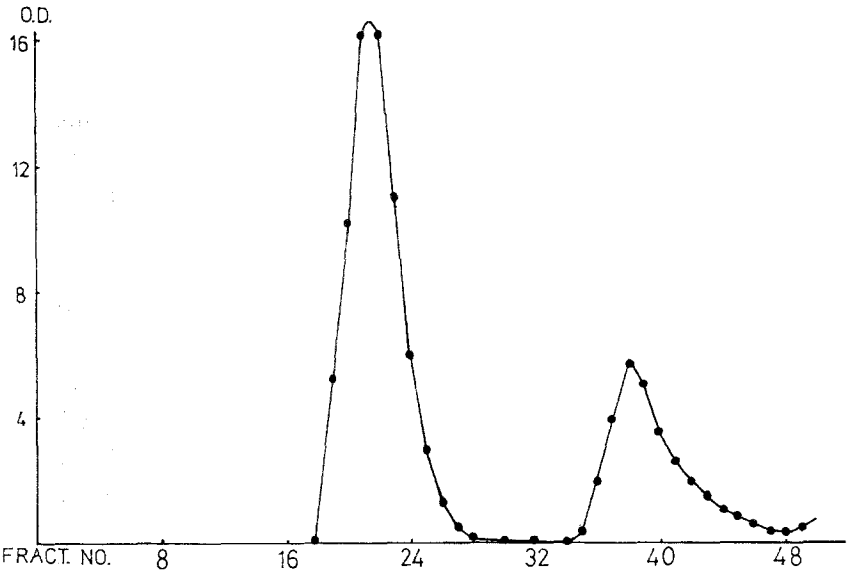

Fig. 1. Separation of a 1:1 (w/w) mixture of phlorizin and its aglycone. The second peak represents the phlorizin. Ordinate: optical density at 285 nm. Abscissa: fraction no. For details of elution procedure *see* text

The rate constants for efflux were obtained by dividing the initial linear portion of the curve relating the amount of radioactivity in the cells or the concentration in the medium to time by the amount of the permeating substance in the cells at zero time or the concentration in the supernatant at infinite time, respectively.

The experiments on D(+)-xylose transport were carried out at 0 °C, after equilibrating the cells for 30 min at 37 °C with a medium of 140 mM NaCl, 20 mM tris-HCl, 10 mM D-(+)-xylose, pH 7.2, containing a trace of the ^{14}C sugar. The equilibrated cells were washed once at 0 °C in tracer-free medium. The efflux of the labeled sugar into that medium was then determined by measuring the appearance of radioactivity in the supernatant.

Valinomycin (Calbiochem, Ltd.) was stored as a stock solution in ethanol (4.5×10^{-3} M) at 0 °C and all solutions containing valinomycin were prepared immediately prior to use. The alcohol concentrations in the experimental media were always 1%. In control experiments we found that alcohol concentrations up to 2% had no effect on the measured rate constants. Phlorizin and phloretin (from Roth Ltd.) solutions were also freshly prepared before us.

Phlorizin and phloretin were separated on Kieselgehl columns (Serva, 100 to 200 μ mesh) by elution with a mixture of chloroform/methanol/water (650:250:20, v/v). The efficiency of this separation procedure is shown in Fig. 1. For the purpose of the experiments described on p. 191 a sample from a lot of phlorizin which was found free of phloretin by thin-layer chromatography was run on a similar column. No trace of phloretin could be detected in the eluate. The glycoside obtained from this column, after evaporation under reduced pressure at 30 °C to remove solvents, was divided into two portions. One portion was retained and the other applied to a second column from which the further purified phlorizin was collected. The three batches of phlorizin, unpurified, after one column purification and after a second purification were used as described under Results.

Results

Determination of the Effect of External Phlorizin on P_{Cl}

If washed cells are suspended in buffered saline solutions (140 mM NaCl, 20 mM tris, pH 7.2) containing valinomycin, a loss of KCl occurs and the cells shrink. The effect of valinomycin at a given cell density is concentration-dependent and at high concentrations a limiting value of the rate constant for K^+ efflux is reached. A similar concentration dependence is also observed with ghosts (Fig. 2) although the absolute values observed in different experiments show some variation.

When packed red cell ghosts are added to buffered NaCl solutions containing phlorizin in addition to valinomycin, the rate of KCl loss is greatly accelerated, compared to controls without phlorizin. This effect is observed over the whole range of valinomycin concentrations between 5×10^{-7} M to 70×10^{-7} M. It depends on the phlorizin concentration and is saturable (Fig. 3). From these findings it is not clear whether phlorizin exerts its effects on K^+ or Cl^- movements or both.

Neither in the presence nor in the absence of phlorizin can the limitation of the rate of KCl efflux at high valinomycin concentrations be related to a

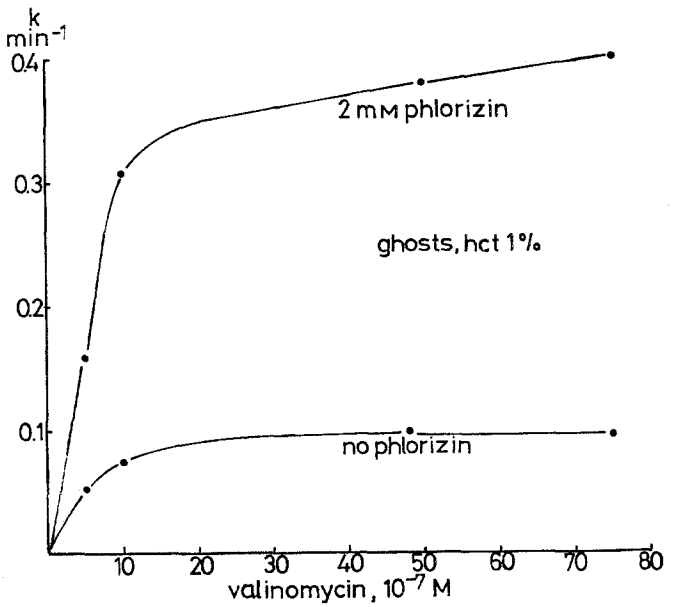

Fig. 2. Effects of increasing concentrations of valinomycin on rate constants for KCl efflux in the presence and absence of phlorizin. Temperature: 37 °C. The ghosts were resealed in a medium containing 140 mM KCl, 20 mM tris-Cl and suspended in a medium containing 140 mM NaCl, 20 mM tris-Cl and the valinomycin concentrations indicated on the abscissa. Phlorizin concentration: 2 mM

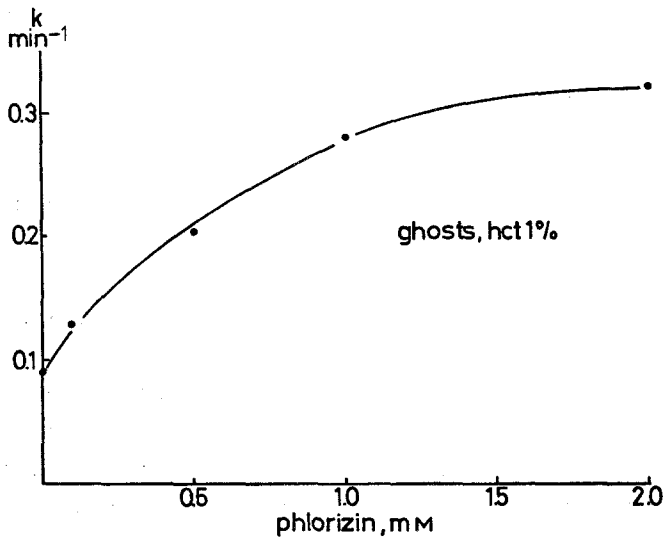

Fig. 3. Effects of increasing concentrations of phlorizin on rate constants of valinomycin-induced KCl efflux. Hematocrit: 1%. Temperature: 37 °C. Valinomycin concentration: 1×10^{-6} M. The ghosts were resealed in a medium containing 140 mM KCl, 20 mM tris-Cl and suspended in a medium containing 140 mM NaCl, 20 mM tris-Cl and the phlorizin concentration indicated on the abscissa

limitation of the cation efflux by the slow diffusion of the accompanying anion. Within the frame of the Goldman hypothesis, this may be seen as follows[1]:

$$J_K = P_K[K_i^+] \frac{\ln B}{B-1}$$

where

$$B = \frac{P_{Cl}[Cl_o^-] + P_K[K_i^+]}{P_{Cl}[Cl_i^-] + P_K[K_o^+]}$$

if $P_K \gg P_{Cl}$, $B = P_K/P_{Cl}$ and

$${}^{o}k_K = \frac{J_K}{[K_i^+]} = P_{Cl} \ln \frac{P_K}{P_{Cl}}.$$

In other words, the rate of KCl efflux will tend to increase as long as increasing the concentration of valinomycin in the membrane increases P_K. The saturation phenomena depicted in Figs. 1–3 represent, therefore, not primarily a limitation of cation efflux by a slowly migrating anion but some

1 The familiar notation is employed here, where the subscripts o and i denote extracellular and intracellular concentrations, respectively. J_K denotes the potassium flux and ${}^{o}k_K$ the rate constant for KCl efflux.

other effect presumably a saturation of the membrane with valinomycin (Hunter, 1973). These arguments apply when the extracellular medium is potassium-free. It can readily be shown from the Goldman equation that only in the presence of extracellular potassium, the rate constant for potassium efflux depends upon the ratio of intra- and extracellular potassium concentrations and is independent of P_{Cl}. At a particular valinomycin concentration a limiting value for the rate constant may then be reached dependent upon the potassium concentration ratio.

To see to what extent the Cl^- permeability of the membrane was increased by phlorizin, we employed the procedure used previously by Hunter (1971) for estimating P_{Cl} from measurements of the rate constants. For this purpose it was necessary to measure the rate of appearance of $^{42}K^+$ in extracellular media containing various levels of K^+. The experiments were carried out using ghosts containing 140 mM KCl, 20 mM tris-Cl, pH 7.2, with a small amount of $^{42}K^+$. The extracellular media contained 140, 80, 40 or 10 mM KCl with 20 mM tris-Cl; the remaining portions to 160 mM were made up with NaCl. The experiments were performed in the absence and presence of phlorizin in the medium. By knowing the internal and external concentrations of all ions and applying the Goldman (1943) equation in the form used by Hunter (1971), it is possible to calculate from the observed rate constants the changes of the permeabilities of Cl^- and K^+ due to the presence of the modifier.

Analysis of the data from such experiments yielded a permeability for K^+ of 0.026 min^{-1} (mean of three determinations) and for Cl^- of 0.006 min^{-1} (mean of three determinations) in the presence of 1×10^{-7} M valinomycin and in the absence of phlorizin. In the presence of 2 mM phlorizin both of these values were increased, to 0.301 min^{-1} (average of three determinations) for K^+ and to 0.117 min^{-1} (average of three determinations) for Cl^-. At a lower valinomycin concentration of 0.5×10^{-7} M, in the absence of phlorizin, P_K was reduced to three-quarters of the value at the higher valinomycin concentration. However, in accordance with Hunter's hypothesis little change of P_{Cl} was found. In the presence of phlorizin, at 0.5×10^{-7} M valinomycin, P_K is only one-third of P_K at 1×10^{-7} M, while P_{Cl} as calculated from the rates of KCl loss at the lower valinomycin concentrations remains nearly the same as at the higher valinomycin concentration (Table 1).

It should be borne in mind that the figures presented above are subject to inaccuracies inherent in the Hunter treatment of the experimental data. The results obtained with 1×10^{-7} M valinomycin show convincingly that P_{Cl} is increased by phlorizin by more than one order of magnitude. At the

Table 1. Measured rate constants of valinomycin-induced ^{42}K efflux (k_{obs}) compared with calculated rate constants (k_{calc}) which were obtained by assuming the P_{Cl} and P_K values indicated below [a]

Valinomycin (M)	Phlorizin (M)	K_o^+ mM	k_{obs}	k_{calc}	P_K	P_{Cl}
1×10^{-7}	2×10^{-3}	140	0.296	0.290	0.290	0.097
		40	0.186	0.197		
		10	0.167	0.157		
		0	0.120	0.142		
1×10^{-7}	2×10^{-3}	140	0.235	0.313	0.313	0.104
		40	0.219	0.212		
		10	0.160	0.169		
		0	0.123	0.152		
1×10^{-7}	2×10^{-3}	140	0.302	0.301	0.301	0.150
		80	0.256	0.255		
		40	0.199	0.218		
		10	0.157	0.185		
		0	0.186	0.173		
1×10^{-7}	0	140	0.0179	0.0218	0.0218	0.0073
		80	0.0172	0.0180		
		40	0.0147	0.0147		
		10	0.0118	0.0118		
1×10^{-7}	0	40	0.0176	0.0174	0.0304	0.0030
		10	0.0089	0.0108		
		0	0.0084	0.0078		
1×10^{-7}	0	140	0.0280	0.0267	0.0267	0.0067
		80	0.0220	0.0217		
		40	0.0160	0.0170		
		10	0.0130	0.0130		
0.5×10^{-7}	2×10^{-3}	140	0.086	0.086	0.086	0.135
		40	0.071	0.074		
		10	0.072	0.070		
		0	0.067	0.068		
0.5×10^{-7}	2×10^{-3}	140	0.084	0.084	0.084	0.135
		40	0.075	0.074		
		10	0.069	0.068		
		0	0.067	0.067		
0.5×10^{-7}	0	140	0.015	0.020	0.020	0.005
		80	0.018	0.016		
		40	0.012	0.013		
		10	0.011	0.010		
		0	0.006	0.009		

[a] Temperature: 25 °C. Hematocrit: 1%. For details *see* text. Rate constants and permeabilities are given in min^{-1}, potassium concentrations in the medium, K_o^+.

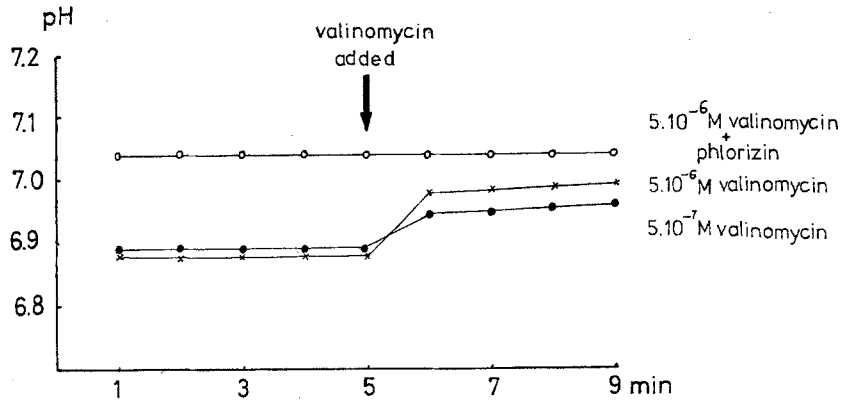

Fig. 4. Effects of phlorizin on valinomycin-induced pH change in the extracellular medium. 1% intact cells in 160 mM NaCl. Phlorizin concentration: 2 mM. Temperature: 20 °C (ambient)

lower valinomycin concentration, the evaluation procedure is quite insensitive to variations of P_{Cl}. Hence, it is only possible to demonstrate the compatibility of the results with those derived from the experiments at the higher valinomycin concentration rather than to provide independent support for the exact magnitude of the phlorizin-induced increase of P_{Cl}.

In the analysis of the data described above, it was assumed that valinomycin and phlorizin exert no other effect than to induce a net loss of KCl from the cells. Although the Na^+ movements (*see* Fig. 5*c*) were insignificant as compared to the KCl movements it was conceivable that H^+/K^+ exchange could account for some of the K^+ which appears in the extracellular medium. Such exchange would imply that phlorizin acts by increasing the permeability of the membrane to protons. If a sizable portion of the efflux of K^+ was in fact due to an exchange for H^+ then the pH of the suspension medium should rise. In the absence of phlorizin, valinomycin caused an increase in the pH of the extracellular medium (Fig. 4). Such an increase is to be expected since the increase of P_K should lead to the establishment of a diffusion potential which could provide the driving force for an uptake of H^+ or expulsion of OH^-. The volume of the external medium exceeds the volume of the cell water by about 150-fold. Hence, it can be calculated that the observed change of pH from 6.9 to 7.0 in the unbuffered medium could lead to the establishment of a H^+ concentration ratio between cell water and medium of about 40:1, which would be equivalent to an equilibrium potential of about -93 mV. However, since the cell interior is heavily buffered by hemoglobin, the actual concentration ratio should be much

smaller, possibly less than one would expect on the basis of the ratio P_K/P_{Cl} postulated to exist in valinomycin-treated red cells. Nevertheless, even though the observed change may be smaller than anticipated it is in the right direction. In the presence of 2.0 mM/liter phlorizin in the medium, K^+ loss is enhanced. Yet no change of pH can be detected. This cannot be entirely related to the fact that at pH 7.05 the agent exerts a strong buffering effect. Under the conditions existing in the experiment depicted in Fig. 4, the rate of K^+-efflux from the cells in 1 liter suspension should be 4×10^{-2} mM/min or 0.16 mM in 4 min (*cf.* Table 2). Since at the pH in our experiments about 50% of the phlorizin is in the undissociated form, about 1.0 mM phlorizin should provide the H^+ for an exchange against this quantity of K^+. This should lead to an easily measurable change of the external pH in the time period of experimental observation (> 0.1 pH units). Since no such change could be observed we conclude that net K^+/H^+ exchange does not make a major contribution to our findings. Experiments carried out by Dr. W. N. Aldridge and Mr. V. H. Parker confirm that phlorizin does not act as an uncoupler in mitochondria.

The Sidedness of the Effects of Phlorizin on P_{Cl}

In previous publications from this laboratory it had been shown that phlorizin can be trapped inside human red cell ghosts. The concentration inside the ghosts was found to be nearly identical to that in the medium with which the ghosts had been equilibrated prior to resealing. The incorporated agent was capable of inhibiting hexose equilibrium exchange but not anion equilibrium exchange. In control experiments it was shown that the absence of the effects on anion equilibrium exchange could not be related to a reduction of the effective concentration of the incorporated phlorizin by binding to the residual hemoglobin inside the ghosts (Lepke & Passow, 1973; Schnell *et al.*, 1973).

Using ghosts prepared by the same techniques as in the previous work, we studied the "sidedness" of the effects of phlorizin on the valinomycin-induced net KCl movements in red cell ghosts. Table 2 shows the effects of internal phlorizin as compared to external phlorizin. There is an acceleration of the KCl loss in the presence of external phlorizin, while internal phlorizin has little or no effect. The sidedness of the effect of phlorizin on the net loss of KCl is similar to that on the anion exchange at Donnan equilibrium as described earlier; but the effect on KCl net loss consists of an acceleration whereas that on equilibrium exchange consists of an inhibition.

Table 2. Asymmetric effect of phlorizin on the rate constant for valinomycin-induced K^+ efflux from human red cell ghosts [a]

Control, without phlorizin ($min^{-1} \times 10^{-3}$)	Phlorizin 2 mM outside ($min^{-1} \times 10^{-3}$)	Phlorizin 2 mM inside ($min^{-1} \times 10^{-3}$)	Incubation temperature (°C)
10.2	34.8	11.45	24.7
11.1	43.0	14.4	
52.8	137.0	64.8	37.0
36.4	120.0	38.2	
43.7	100.0	43.7	
38.3	93.8	38.8	

[a] Hematocrit: 5%. Valinomycin in concentration: 10^{-6} M. pH 7.2. The ghosts were resealed in a medium containing 140 mM KCl, 20 mM tris-Cl and suspended in a medium containing 140 mM NaCl, 20 mM tris-Cl.

Additional experiments were carried out to determine whether or not inward movements of KCl are also affected by phlorizin. In Fig. 5 are compared the effects of internal and external phlorizin on inward and outward K^+ movements. External phlorizin accelerates K^+ efflux (Fig. 5*a*) while internal and external phlorizin are without effect on K^+ influx from high K^+ media into sodium-loaded ghosts (Fig. 5*b*). In both situations Na^+ movements are little, if at all, affected by the presence of phlorizin and remain at the lower rates seen in the presence of valinomycin alone (Fig. 5*c*). However, the data show a second type of asymmetry; although external phlorizin accelerates the efflux of KCl into NaCl solutions, it does not affect the uptake of KCl from high K media into NaCl-loaded ghosts.

The Sidedness of the Effect of Phlorizin on P_K

The data in Table 1 show that in addition to net KCl loss, potassium equilibrium exchange in the absence of net movements is also enhanced by phlorizin. This extends former observations by Wieth *et al.* (1973) with phloretin and indicates that not only P_{Cl} but also P_K is increased. As can be seen in Fig. 6, extracellular phlorizin greatly accelerated the rate of appearance of $^{42}K^+$ in the extracellular medium, while phlorizin on the inner surface showed only a slight effect. Since valinomycin is generally considered to be a mobile carrier, the asymmetry of the effect of the nonpenetrating phlorizin observed under cation equilibrium exchange conditions strongly suggests the involvement of a structural asymmetry of the red blood cell membrane in the action of phlorizin on carrier kinetics.

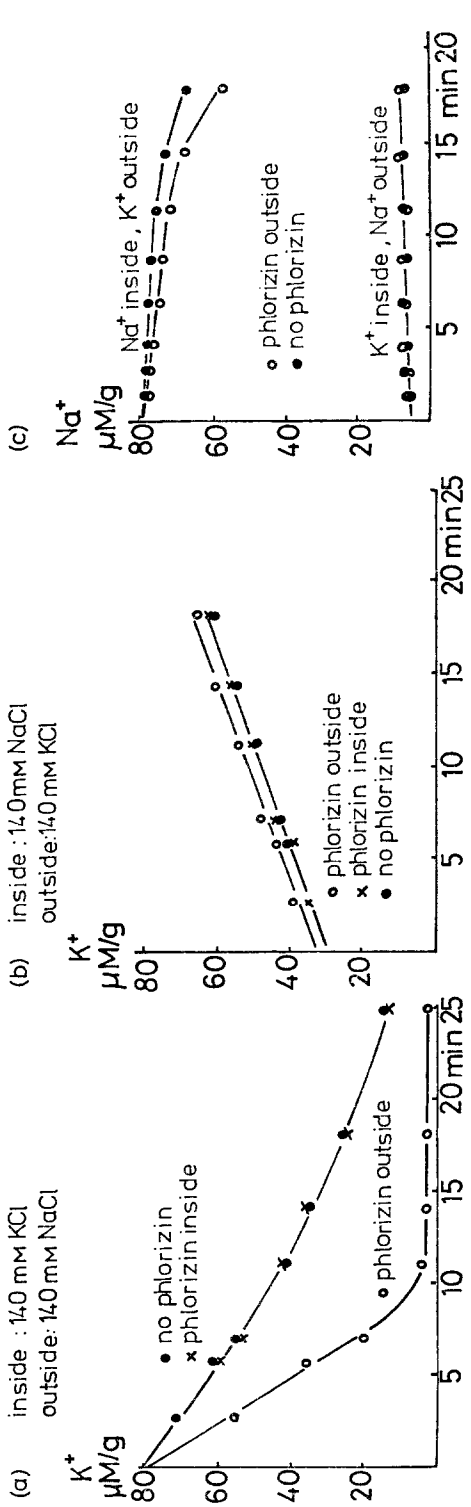

Fig. 5. Effects of phlorizin (2.0 mM) on (*a*) valinomycin-induced K efflux, (*b*) KCl influx, and (*c*) on sodium movements. Valinomycin concentration: 10^{-6} M. Internal and external solutions were buffered to pH 7.2 with 20.0 mM tris HCl. Red cell ghosts. The results indicated in the Figure are representative of four similar experiments. For details of calculation of internal concentration *see* p. 181. Hematocrit: 5%. Temperature: 37 °C

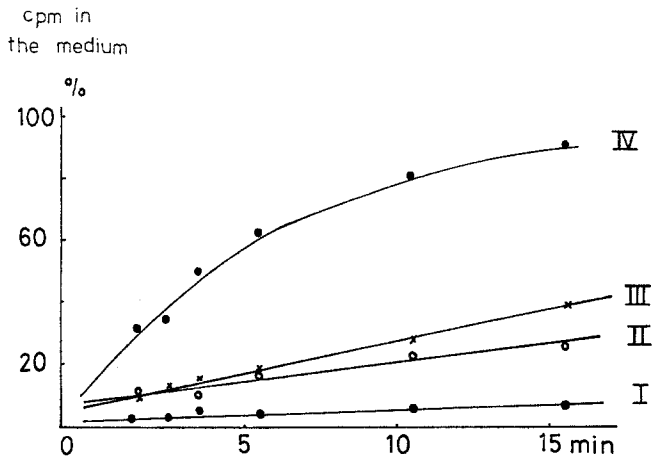

Fig. 6. Asymmetric stimulation of valinomycin-induced potassium equilibrium exchange in red cell ghosts. Hematocrit: 1%. Temperature: 25 °C. Ghosts and external medium contained 140 mM KCl, 20 mM tris-Cl, pH 7.2. I: control, neither valinomycin nor phlorizin present. II: 0.5×10^{-7} M valinomycin in the medium. III: 0.5×10^{-7} M valinomycin in the medium and 2 mM phlorizin inside the ghosts. IV: 0.5×10^{-7} M valinomycin and 2.0 mM phlorizin in the medium, no phlorizin inside the ghosts

The Influence of Contaminating Traces of Phloretin on the Effects of Phlorizin

The question has been raised to what extent the effects of phlorizin on sugar transport are due to contamination of the glycoside with small amounts of the more potent aglycone, phloretin (LeFevre & Marshall, 1959). This question is also pertinent in the context of the present work. Among various lots of phlorizin from the same manufacturer we found several which were heavily contaminated with phloretin. However, in one lot we could not detect, by thin-layer chromatography, any trace of the aglycone. To make sure that the biological effects observed with this lot were actually due to the glycoside and not to undetected contaminations with phloretin, we employed a sequence of two chromatographic purifications (*see* Materials and Methods) and checked, after each purification, the constancy of the biological effect. Table 3 shows that the inhibition of D-(+)-xylose transport and the acceleration of valinomycin-induced KCl loss by the selected lot of commercial phlorizin are very close to that obtained after 1 or 2 purifications. Hence, our results refer to phlorizin and not to contaminating impurities of the aglycone. Moreover, it is clear that the glycoside itself is capable of inhibiting sugar permeability of the red cell.

Table 3. Effects of purification of phlorizin on the rate constant of (a) valinomycin-induced KCl loss, and (b) D-(+)-xylose equilibrium exchange in intact human red cells [a]

Exp.	Control	"Crude" phlorizin	Fraction I	Fraction II
Valinomycin-induced KCl loss	0.066	0.139	0.130	0.128
Sugar transport	0.186	0.038	0.039	0.041

[a] The figures represent rate constants in min^{-1}. Salt loss was measured in the presence of 5×10^{-7} M valinomycin at 25 °C, sugar penetration in the presence of 10 mM D-(+)-xylose at 0 °C. Phlorizin concentration: 0.43 mM. Composition of media: 140 mM NaCl, 20 mM tris-Cl, pH 7.2.

Discussion

The present experiments show that the rate of net KCl transfer across the membrane of valinomycin-treated red blood cells or ghosts may increase up to three- to fourfold in the presence of phlorizin. This suggests that phlorizin enhances net chloride movements. This is in contrast to the previously described, strong (90 to 95%) inhibition of chloride equilibrium exchange by phlorizin (Schnell *et al.*, 1973) or the even more complete inhibition by phloretin (Wieth *et al.*, 1973). Such results emphasize the need to study for each single modifier, the effects on net and exchange anion permeability separately.

The opposite effects of phlorizin on net and exchange anion permeability would be in keeping with the assumption that two different permeation mechanism are involved. One of these mechanisms has been described by the Goldman equation (Hunter, 1971). The validity of this description is supported by the similarity of the calculated values for P_{Cl} in the presence of two different valinomycin concentrations which establish considerably differing values of P_K. This success of the Goldman equation to evaluate P_{Cl} would be consistent with the idea that anion penetration across one of these pathways follows the laws of simple electrolyte diffusion. However, it should be borne in mind that the two different pathways are operationally defined by the method of measuring and calculating permeabilities. Hence, compatibility with the existence of such pathways does not prove their actual existence. Moreover, the applicability of the Goldman equation to one of these pathways also does not permit an unambiguous interpretation of the mechanism of ion movements across this pathway. For example, in very narrow pores where the independence principle does not apply, the Goldman equation may still be valid at low concentrations for the penetrating

substance where the probability for the simultaneous presence of more than one ion per pore is negligible (Finkelstein & Holz, 1973). Also, carrier-mediated net ion transfer may give rise to kinetics which are similar to those for the diffusion of the uncombined ions as described by the Goldman equation (Adrian, 1969, p. 356). P_K and P_{Cl} in an equation which is formally identical to the Goldman equation may then refer to an entirely different molecular mechanism.

Our experiments show that regardless of its presence inside or outside the ghost, phlorizin affects only net efflux of KCl but not net influx. This finding is not reconcilable with the assumption of a Goldman-type electrolyte diffusion across a homogeneous membrane. We are unable to offer an experimentally supported explanation for this rectification. One reviewer of this paper pointed out that it might be possible to explain the failure of phlorizin outside to increase net KCl influx (Fig. 5*b*) by assuming that with a positive inside membrane potential the phlorizin either does not bind or is relocated in the membrane so that it does not affect P_K and P_{Cl}. If this were true, it would indicate an important limitation of the applicability of the Hunter technique to studies with inhibitors.

In view of the problems associated with the use of the Goldman equation, the meaning of our estimate of the increase of P_{Cl} by saturating concentrations of phlorizin by a factor of about 20 must be considered with caution. Nevertheless, it is interesting to note that Wieth *et al.* (1973) observed a similar effect of phloretin on P_{Cl} of gramicidin-treated red cells. However, the estimate of Wieth *et al.* (1973) is based on studies in media of low ionic strength. According to these authors low ionic strength itself causes changes of P_{Cl}. Hence, it is not quite clear whether a direct comparison of the changes observed at high and low ionic strength is really meaningful. Moreover, in unpublished experiments we found certain differences between the effects of phloretin and phlorizin which make us doubt that a quantitative comparison of the effects of the glycoside and its aglycone is really useful without further elucidation of these differences.

Both exchange permeability and P_{Cl} are only affected by external but not by internal phlorizin. At first glance this would suggest some common step in the two penetration mechanisms. However, a completely unrelated ion transfer process, the valinomycin-mediated potassium exchange with identical potassium concentrations on both sides of the membrane, shows the same type of asymmetric modification by phlorizin as anion penetration. Hence, such an inference would not seem justified.

The finding that the effect of phlorizin on three different transfer processes shows the same type of asymmetry, would suggest that the action of

the agent is not related to combination with specific protein molecules in different pathways. Instead it seems more plausible to suspect that interactions with the lipids are involved. This idea is supported by the observation that carrier-mediated ion transfer across black lipid membranes can be modified by amphiphilic substances, including modifiers of anion permeability of the red blood cell membrane, such as salicylate (McLaughlin, 1973) and phloretin (Cass, Andersen, Katz & Finkelstein, 1973). The action of these substances does not seem to be due to direct combination with the carrier molecules but to the indirect consequence of modification of the interface between the lipid of the membrane and the aqueous phases. There is evidence to suggest that the phospholipid distribution between the two surfaces of the red blood cell membrane is asymmetric (Zwaal, Roelofsen & Colley, 1973). Such asymmetry could possibly account for asymmetries of phlorizin binding and different effects on the loading and unloading of valinomycin in the two surfaces.

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