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pH dependence of phosphate transport across the red blood cell membrane after modification by dansyl chloride

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Dansylation of the red blood cell membrane inhibits monovalent anion transport as measured by means of ^{36}Cl and enhances divalent anion transport as measured by means of $^{35}SO_4$ (Legrum, Fasold and Passow (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 1573–1590 and Lepke and Passow (1982) J. Physiol. (London) 328, 27–48). In the present work the effect of dansylation on phosphate equilibrium exchange was studied over the pH range where the ratio between monovalent and divalent phosphate anions varies. At high pH, phosphate equilibrium exchange was enhanced; at low pH, exchange was inhibited. The pH maximum of phosphate equilibrium exchange, seen at pH 6.3 in untreated ghosts is now replaced by a plateau. The inverse effects of dansylation on the rates of exchange at high and low pH suggest that both monovalent and divalent phosphate anions are accepted as substrates by the anion transport protein. A tentative attempt to obtain a quantitative estimate of the ratio of monovalent and divalent phosphate transport indicates that in the untreated red cell membrane over the pH range 7.2–8.5 the transport of HPO_4^{2-} is negligible compared to the transport of $H_2PO_4^-$.

Introduction

The band 3 protein mediates transport across the red blood cell membrane of both monovalent and divalent inorganic anions. Nevertheless, their pH dependences differ. The equilibrium exchange of Cl^- increases with increasing pH until, above about pH 7.0 a plateau (at 0°C) or a flat maximum (at more elevated temperatures) is attained [1,2]. The equilibrium exchange of SO_4^{2-} passes through a maximum around pH 6.3 [3,4]. The increase of SO_4^{2-} flux in the low pH range up to the maximum has been related to the deprotonation of a modifier site [5]. The decrease above the maximum is believed to reflect the decrease of protonation of an additional amino acid residue that participates in the co-transport of a proton with the divalent anion species [6]. The pH dependence of phosphate equilibrium exchange also shows a maximum around pH 6.3. In this pH range both monovalent and divalent phosphate anions are present (pK 7.2 at physiological ionic strength and 25°C). This raises the question about the relative contributions of the two anion species to the observed penetration rate. Schnell and associates [7] found considerable variations of the apparent V_{max} with pH while the apparent K_m is nearly pH independent. They concluded that the transport system accepts monovalent and divalent anion species equally well. On the other hand, Runyon and Gunn [8] derived from an analysis of the net exchange of intracellular chloride for ex-

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Abbreviations: APMB, 2-(4-amino-3-sulfophenyl)-6-methyl-7-benzothiazolsulfonic acid; H_2DIDS, 4,4′-diisothiocyanato-1,2-diphenylethane-2,2′-disulfonic acid.

tracellular phosphate as measured at different external pH values the conclusion that only the monovalent $H_2PO_4^-$ is transported.

The present paper deals with the effects of modification of the red cell membrane by dansyl chloride [9]. In contrast to a gamut of other chemical modifiers [10] this agent produces different effects on monovalent and divalent anion transport. The former is slightly inhibited, the latter considerably enhanced. When the dansylation is carried out in the presence of the potentiating agent APMB, the enhancement may exceed a factor of one thousand [11].

Dansyl chloride penetrates easily across the red blood cell membrane and is capable of reacting with amino acid residues carrying amino, imidazole, phenolic OH and SH groups [12]. Thus, it labels many amino acid residues in band 3 and other membrane proteins in addition to the amino acid residues whose dansylation is responsible for the effects on anion transport. This has prevented, so far, the identification of these amino acid residues. Nevertheless, the striking difference of the effects of dansylation on the equilibrium exchange of monovalent and divalent anions offers a means for studying the capacity of monovalent and divalent phosphate anions to serve as a substrate for the band 3 protein. If both types of anions could be transported, then after dansylation one would expect to find an inhibition at low pH where the monovalent form prevails and an acceleration at high pH where the divalent form predominates.

Materials and Methods

Anion equilibrium exchange was measured in red cell ghosts prepared as described by Schwoch and Passow [13]. The hemolysis ratio was 1 : 50 and the hemolysis medium contained 4 mmol/l $MgSO_4$, 0.5 mmol/l acetic acid; the temperature at hemolysis was 0°C. Resealing and subsequent flux meaurements were carried out in a 'standard medium' containing 1 mmol/l sodium phosphate, 20 mmol/l EDTA and 130 mmol/l NaCl. During resealing the pH was 7.4.

Dansylation was carried out in the dark in the standard medium at pH 6.6, 37°C, for 30 min at a hematocrit of 5%. Dansyl chloride was applied as a cyclodextrin complex [9]. At the end of the incubation period, the non-reacted and hydrolysed dansyl chloride was removed by washing with standard medium, pH 6.6. When dansylation was performed in the presence of APMB, the washing procedure included 3 incubation periods at 37°C of at least 20 min each. This served to ensure that any APMB that had previously penetrated into the cells was completely removed. All controls were treated similarly, except that no dansyl chloride was present.

Flux measurements were performed after loading the ghosts with $^{32}PO_4$ at pH 6.6 (45 min, 37°C). The ghosts were then washed free of extracellular ^{32}P in cold media of the appropriate pH and resuspended in these media at a hematocrit of 1% and a temperature of 30°C. Control experiments have shown that the presence of HCO_3^- from the air ensures the pH equilibration between ghosts and medium during the washes provided the hematocrit is less than about 2 percent. The appearance of ^{32}P in the supernatant was followed and rate constants were calculated as described by Legrum et al. [9]. Sulfate flux was measured as described above except that the standard medium contained 1 mmol/l Na_2SO_4 in place of the sodium phosphate. Cl^- equilibrium exchange at 4°C was measured in standard medium containing 1 mmol/l Na_2SO_4 by means of the filtration technique of Dalmark and Wieth [14]. In the experiments at 0°C, the inhibitor stop method as described by Ku et al. was used [10]. Parallel experiments were run where the media contained 10 or 25 μM H_2DIDS. The rate constants presented in the figures represent the values obtained after deduction of the small corresponding rate constants for the H_2DIDS insensitive flux. Over the pH range 8.5–6.8 the ghost volume is independent of pH. There is a tendency of an increase in volume at pH values below this range, which amounts to maximally 15–20% at pH 5.5. The rate constants given in the text and in the figures were corrected for this effect. The correction does not influence the results of the calculations presented on p. 285 which pertain to high pH values and only to ratios of rate constants measured at equal pH values.

Results and Discussion

For technical reasons, in previous work, the effects of dansylation on chloride transport had

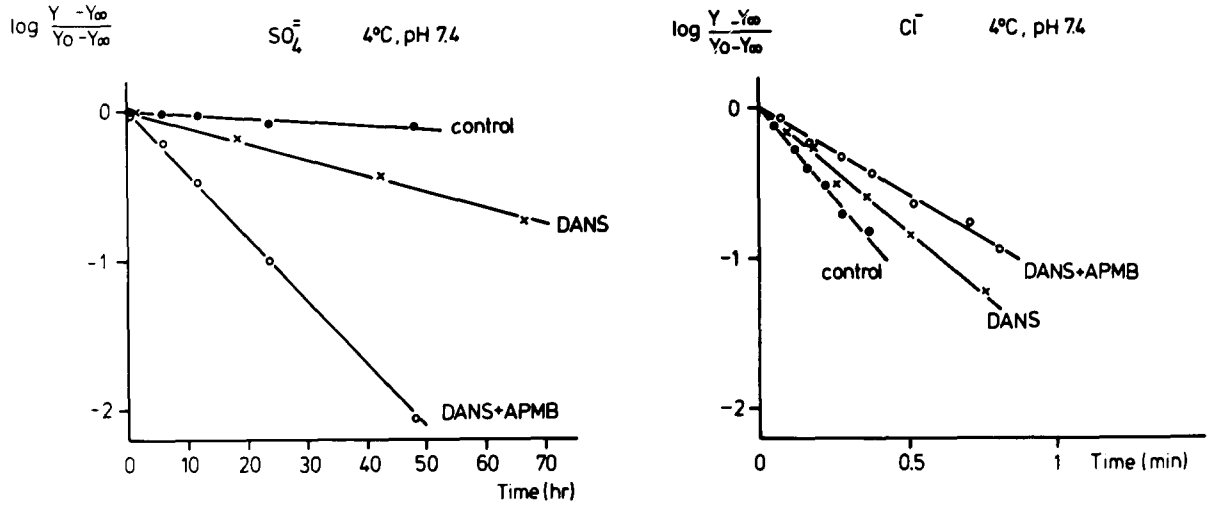

Fig. 1. Sulfate and chloride equilibrium exchange is untreated (control) and dansylated ghosts. Dansylation at 0.125 mM dansyl chloride in standard medium in the absence (DANS) or presence (DANS + APMB) of 5 mM APMB. On the ordinate, Y, Y_0, and Y_∞ represent, respectively, the radioactivity in the supernatant at times t, $t = 0$ and $t = \infty$. The efflux was measured in standard medium at pH 7.4, 4°C. Abscissa: time in minutes (Cl^-) or hours (SO_4^{2-}).

been measured at 0°C, those on sulfate transport at either 30 or 37°C [11]. Fig. 1 shows that even if both sulfate and chloride flux are measured at the same temperature inverse effects can be demonstrated: Cl^- flux is inhibited, SO_4^{2-} flux is enhanced. The relationship between the two changes is linear [15].

Fig. 2a illustrates the effects of dansylation on the pH dependence of sulfate equilibrium exchange. It shows that after dansylation the maximum of the pH dependence of sulfate transport is in fact replaced by a plateau that extends from about pH 7.1 to at least pH 8.5. Fig. 2b shows the effect of dansylation on the pH dependence of phosphate equilibrium exchange. As may be seen phosphate transport is considerably enhanced at high pH (7-times at pH 8.5) where HPO_4^{2-} prevails and partially inhibited at low pH (60% at pH 6.4) where $H_2PO_4^-$ predominates. The decrease of inhibition of the modified phosphate transport at pH values below the maximum is not unexpected. A qualitatively similar decrease is seen for Cl^- equilibrium exchange after dansylation, where the inhibition is nearly pH independent down to about pH 6.0 but disappears completely at pH 5.0 (Fig. 3). The enhancement and inhibition of phosphate equilibrium exchange at high and low pH, respectively, leads to the nearly complete disappearance of the original pH dependence of phosphate equilibrium exchange.

The results suggest that in the untreated red blood cells: (1) at low pH a significant fraction of phosphate transport is accomplished by the monovalent species $H_2PO_4^-$ and (2) at high pH a fraction is transported as the divalent species HPO_4^{2-}.

The qualitative conclusions drawn above raise some questions about the quantitative relationships between the effects of dansylation on phosphate and sulfate transport. The enhancement of phosphate transport at pH 8.5 is about 7-fold. At this pH, about 95% of the phosphate exists in the divalent form. One would expect, therefore, a response to dansylation more similar to that of sulfate transport which, at this pH, amounts to an about 1600-fold enhancement (Fig. 2a). The enormouse discrepancy is, however, not unreasonable if one takes into account the different rates of penetration of monovalent and divalent phosphate ions and the different effects of dansylation on these two rates. If the small fraction of $H_2PO_4^-$, present at pH 8.5, would carry most of the phosphate across the membrane, then a several thousand-fold increase of HPO_4^{2-} transport would enhance total phosphate transport only a little.

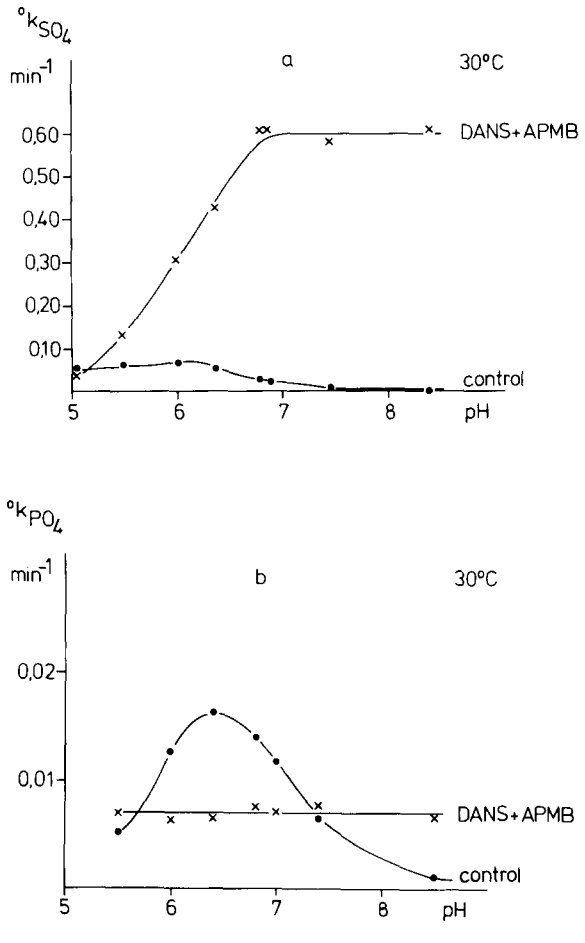

Fig. 2. (a) pH dependence of sulfate equilibrium exchange in untreated ghosts (control) and in ghosts dansylated in standard medium in the presence of 5 mM APMB (DANS+APMB). After removal of excess dansyl chloride and APMB, equilibrium exchange of sulfate was measured in standard medium at 30°C and at the pH indicated on the abscissa. The rate constant of the band 3-mediated H_2DIDS-sensitive efflux indicated on the ordinate was calculated by subtracting from the efflux observed in the absence of H_2DIDS, the efflux observed in the presence of 25 μmol/l H_2DIDS. Note that the maximum of the control at pH 6.2 is less pronounced at the low sulfate and high chloride concentration chosen in these experiments than in experiments at high SO_4/Cl ratios usually employed in the literature (see, for example, Ref. 4). (b) pH dependence of phosphate equilibrium exchange in untreated ghosts (control) and ghosts dansylated in standard medium in the presence of 5 mM APMB (DANS+APMB). Flux measurements were performed at 30°C and the pH indicated on the abscissa after removal of APMB and excess dansyl chloride. Ordinate: rate constant for H_2DIDS-sensitive phosphate efflux.

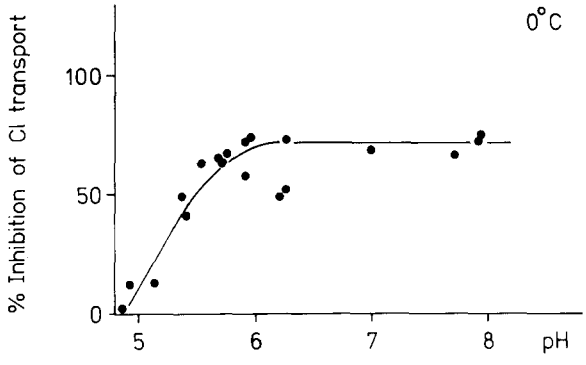

Fig. 3. pH dependence of inhibition of Cl^- equilibrium exchange in ghosts dansylated in standard medium in the presence of 5 mM APMB. Flux measurements were performed in standard medium at 0°C at the pH indicated on the abscissa after removal of APMB and excess dansyl chloride. Ordinate: Inhibition of chloride equilibrium exchange in dansylated ghosts.

In fact it would seem plausible to use the different results obtained with sulfate and phosphate as a basis for a tentative and semiquantitative estimate of the relative rate of penetration of $H_2PO_4^-$ and HPO_4^{2-}. As shown in the appendix on the plausible but as yet unproven assumption that the pH dependence in both untreated and dansylated red cells for $H_2PO_4^-$ and HPO_4^{2-} are essentially similar to the respective pH dependencies of Cl^- and SO_4^{2-} transport, one can calculate that at pH 8.5 the monovalent phosphate transport is about 5000-times faster than the divalent phosphate transport. For other pH values different ratios are obtained since according to the assumption made above the pH dependence of monovalent and divalent anion transport is different. The results can be summarized by the statement that at pH values between pH 6.0 and 8.5 phosphate is essentially transported as the monovalent form. At low pH, up to the maximum in Fig. 2b, the flux is determined by both pH dependent changes of the rate constant and changes of the fraction of phosphate that exists in the monovalent form. Above the maximum, where the rate constant for monovalent anion transport becomes pH independent, the observed flux would be essentially determined by the decrease of the concentration of the $H_2PO_4^-$.

Although the calculation of the ratio k_1/k_2 * involved the use of data on the effect of dansylation, the numerical value obtained pertains to the untreated, intact red cell membrane. The calculated ratio of the penetration rates of monovalent and divalent phosphate ions is about 2 orders of magnitude lower than the ratio $^{\circ}k_{Cl}/^{\circ}k_S$ for which, in the untreated red cell, at pH 8.5, we calculated a value of about $3.26 \cdot 10^5$. Iodide penetrates at a rate which is about 2 orders of magnitude lower than the rate of penetration of Cl^- [14]. Thus, the ratio k_1/k_2 is likely to be of the same order of magnitude as the ratio $^{\circ}k_I/^{\circ}k_S$.

The result of our calculations is, therefore, not implausible and agrees with the notion that divalent phosphate is accepted as a substrate by the band 3 protein, but that its rate of transport is negligible compared to the rate of transport of the monovalent anion species. Although the interpretation of the effects of dansylation on monovalent and divalent phosphate transport on the basis of the effects on Cl^- and SO_4^{2-} transport is suggestive, it is clear that independent evidence is required for a definitive solution of the long standing issue of the relative ratio of monovalent vs. divalent phosphate transport.

Appendix

If it is assumed that phosphate flux J_{12} across the untreated membrane, as measured by means of ^{32}P, is equal to the sum of the fluxes of $H_2PO_4^-$ and HPO_4^{2-}, one obtains:

$$J_{12} = k_{12} \cdot [\bar{P}] = k_1 \cdot [H_2PO_4^-] + k_2 \cdot [HPO_4^{2-}] \quad (1)$$

The corresponding equation for dansylated ghosts reads:

$$J_{1122} = k_{1122} \cdot [\bar{P}] = k_{11} \cdot [H_2PO_4^-] + k_{22} \cdot [HPO_4^{2-}] \quad (2)$$

The symbols have the following meaning:

$[\bar{P}]$, sum of $[H_2PO_4^-]$ and $[HPO_4^{2-}]$; k_{12}, k_{1122}, experimentally determined rate constants for total phosphate flux in untreated and dansylated ghosts, respectively. k_1, k_{11}, k_2, k_{22}, rate constants for monovalent (index 1, 11) and divalent (index 2, 22) phosphate transport in untreated (index 1, 2) and dansylated ghosts (index 11, 22).

The ratio j_{12}/j_{1122} is equal to:

$$\frac{k_{12}}{k_{1122}} = \frac{k_1 + k_2\left(\frac{K}{H^+}\right)}{k_{11} + k_{22}\left(\frac{K}{H^+}\right)} = q \quad (3)$$

where $K/H^+ = [HPO_4^{2-}]/[H_2PO_4^-]$.

We assume $k_{11} = \alpha k_1$ and $k_{22} = \beta k_2$, where for any given pH, α and β are constants. Thus α and β are measures of the effect of dansylation on the monovalent and divalent anion transport, respectively. Inserting into Eqn. 3 yields:

$$\frac{k_1}{k_2} = \left(\frac{K}{H^+}\right)\left(\frac{q\beta - 1}{1 - q\alpha}\right) \quad (4)$$

For the calculation of k_1/k_2 it is necessary to estimate the numerical values of α and β. Assuming the effects of dansylation on monovalent and divalent anions to be independent of the chemical nature of the anion species considered, α and β can be inferred from the inhibition of Cl^- transport and the augmentation of SO_4^{2-} transport.

The transport of Cl^-, SO_4^{2-} and phosphate in untreated and dansylated ghosts has been measured in media containing Cl^- at 130 mmol/l and either phosphate or sulfate at 1.0 mmol/l. For this condition we obtained for the transport of sulfate, phosphate and chloride at pH 8.5, the following rate constants: $0.035 \cdot 10^{-2}$, $0.10 \cdot 10^{-2}$, and 114 min^{-1}, respectively, for undansylated ghosts. The former two values have been determined by ourselves, the latter value has been derived from data published by Brahm [2] *. After dansylation, the corresponding values are $57.69 \cdot 10^{-2}$, $0.68 \cdot 10^{-2}$ and 34.2 min^{-1}, respectively. The latter value ($^{\circ}k_{Cl}$) was calculated on the assumption that the inhibition by dansylation measured at 30°C is

* The symbols introduced in this paragraph have the following meaning: k_1, k_2, $^{\circ}k_{Cl}$, $^{\circ}k_I$, $^{\circ}k_S$ = rate constants for efflux of $H_2PO_4^-$, HPO_4^{2-}, Cl^-, I^- and SO_4^{2-}, respectively, all pertaining to the untreated red cell membrane.

* In Table IIa of his paper, Brahm gives 142 min^{-1} for $^{\circ}k_{Cl}$ at pH 7.2. In the text on page 290 he states that $^{\circ}k_{Cl}$ decreases by 25% when the pH is increased from 7.2 to 9.0. By linear interpolation we obtain for pH 8.5 a decrease of about 20%.

equal to the inhibition measured at 0°C. From these values we derived $\alpha = 0.3$ and $\beta = 1648$. Inserting these values that pertain to pH 8.5 (where 95% of the phosphate exists in the form of HPO_4^{2-}), we calculated $k_1/k_2 = 5050$; i.e. monovalent phosphate ions penetrate about 5000-times faster than the divalent phoshate ions. For other pH values different results are obtained since it was stipulated that the pH dependence of the transport of $H_2PO_4^-$ is similar to that of Cl^- and that of HPO_4^{2-} similar to that of SO_4^{2-} and hence that α and β vary with pH.

It should be noted that the numerical results obtained for k_1/k_2 pertain to the specific set of conditions existing in the present experiments. In particular they have been calculated on the assumption that in the presence of the high and constant concentration of Cl^- and at the low concentration of phosphate and sulfate used, the rate constants of monovalent and divalent phosphate and of sulfate do not change with the concentrations of $H_2PO_4^-$, HPO_4^{2-} and SO_4^{2-}, respectively. Unpublished experiments show that this is not strictly true, especially in dansylated ghosts. Nevertheless, the deviations are small enough not to invalidate the conclusions drawn above.

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