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The Effect of Quaternary Ammonium Salts on the Transport of Selected Nonelectrolytes Across the Red Cell Membrane

INTRODUCTION

The compounds applied in this study belong to quaternary ammonium salts (V-n) and are surfactants with fungicidal, algicidal and bactericidal properties [1,2]. The results of previous studies indicate that the compounds we study, as well as similar ones, affect cell membranes and model membranes in the first place [3–6], this confirming the observation by Hotchkiss [7] that the cell membrane was the first target for some (other then ours) surface-active quaternary ammonium salts.

In our further studies we showed that the compounds we study affect the kinetics of ion permeation through liposome membranes [8–10], their fluidity [11–13], electric conductivity and the membrane potential of algae [14–15], mechanical strength of phospholipid membranes and haemolysis of the red blood cells [16–19]. The effect of similar compounds on haemolysis and cell shape were also performed by Isomaa [20]. It can be found that the compounds studied affect the lipid phase of the membrane in the first place, disturbing many of its biological functions. Our studies of the transport kinetics of labelled ions across model phospholipid membranes modified with the V-n compounds reveal such changes in the membranes' properties that the character of the permeation process changes drastically. Studies of the ion permeation process across the erythrocyte membrane by using the isotopic tracer method faced serious dificulties, however. The application of modifier concentrations lower than the haemolytic ones had no effect on the passive permeation of ions, whereas the application of higher concentrations resulted in smaller or greater haemolysis and made it difficult or impossible to measure the parameters characteristic of the permeation. For this reason we have attempted to study the process of nonelectrolyte permeation through the red blood cell modified with the V-n compounds by using the turbidimetric method and concentrations lower than the haemolytic ones. The aim of the study was to find the answer to the question whether such low modifier concentrations affect the kinetics of nonelectrolyte per-

meation through the erythrocyte membrane, and possibly explain the mechanism of the process as well as the mechanism of membrane-quaternary ammonium salts interaction.

MATERIALS AND METHODS

Fresh, heparinized pig blood was used in the experiments. It was washed, stored and modified in a phosphate buffer of pH 7.4. For modification of erythrocytes there was used solution of analytically pure glycine ester derivatives (V-n) of the general formula:

$$(CH_3)_3N^+CH_2COOR + Cl^-,$$

where $R=C_8H_{17}$, $C_{10}H_{21}$, $C_{12}H_{25}$, $C_{14}H_{29}$, $C_{16}H_{33}$. The letter n in the compound's symbol (V-n) indicates the number of carbon atoms in the alkyl chain.

The transport of the following nonelectrolytes across red blood cells was studied: glycerol, m-erythrite, DL-arabinose, D-saccharose, polyethylene glycol "400".

PREPARATION OF SAMPLES

Erythrocytes were separated from the plasma, washed 4 times in a phosphate buffer solution and centrifugated. From the condensed suspension of erythrocytes 6 portions were taken, 0.4 ml each; five of them for modification and one was the control sample. The latter was suspended in a pure phosphate buffer, whereas the former five in solutions of different concentrations of the modifier. The samples (of 4% haematocrit) were incubated for an hour at 37°C. After that they were centrifugated and supernatant was removed. Condensed samples thus obtained had 50% haematocrit.

TURBIDIMETRIC MEASUREMENTS

The samples thus prepared were next studied turbidimetrically; i.e., recording changes in transmittancy of the erythrocyte suspension caused by the transport of water and selected nonelectrolytes across the erythrocyte membrane. The measurements were performed by using the photoelectric spectrophotometer Spekol, equipped with a properly adapted TiMi attachment. 600 nm light was used. Changes in transmittance were recorded on a recorder of 0.5 cms^{-1} pass. The measurements were taken at 37°C.

The turbidimetric measurements were performed as follows: in a cuvette 2.99 ml of the studied nonelectrolyte solution was placed during continuous stirring, and 100% transmittance was set. Then 0.01 ml of modified erythrocytes was quickly injected into the solution and variation in transmittance of the suspension was followed in time. Changes in transmittance of the suspension illustrates the changes in its clarity.

In the course of the variation one can distinguish two main phases of the transport process (Fig. 1):

1) a rapid fall in the transmittance on injection of erythrocytes caused to their contraction due to water influx;

2) an increase in transmittance caused by the influx of water and the substance transported into the erythrocytes during their expansion.

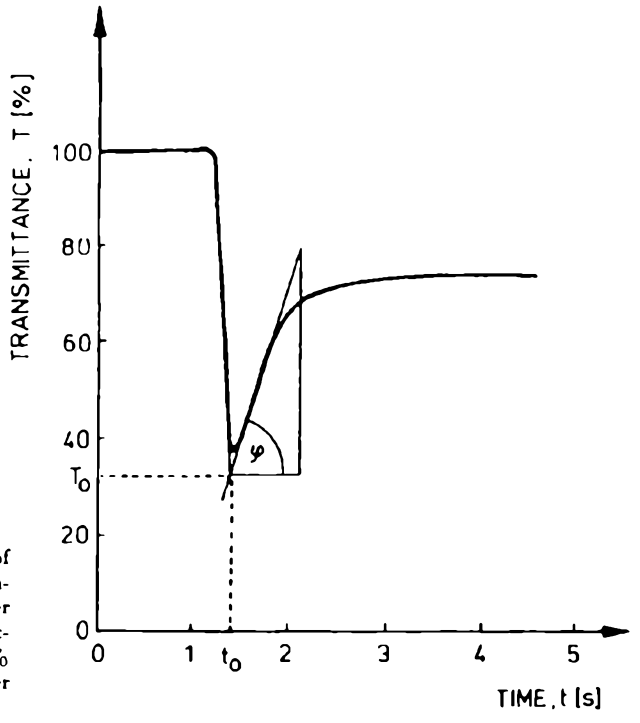

Fig. 1. Time course of transmittance of a suspension of erythrocytes after their injection to a nonelectrolyte solution. Symbols T_0 and t_0 are explained further in the text

The measurements were performed with all blood samples injected to the respective isotonic solutions of nonelectrolytes of 300 mM concentration.

The curves recorded represent changes in the suspension's transmittance during the process of nonelectrolyte permeation, and allow to determine the rate of change in transmittance $(\frac{dT}{dT})_{t_0}$ from the slope (φ) of the tangent at the start t_0 of the expansion (Fig. 1).

Knowing this rate and the minimum value of the transmittance T_0, which corresponds to the transition from one phase of the transport to another, one can in turn calculate the permeation constant of the dissolved substance. By using the relationship between the change in the erythrocytes' volume at the initial stage of expansion and the corresponding change in transmittance [22]:

$$\left(\frac{dV}{dt}\right)_{t_0} = K\left(\frac{d(\frac{1}{E})}{dt}\right)_{t_0} = \frac{K}{2,303} \cdot \frac{1}{T_0 ln^2 T_0} \cdot \left(\frac{dT}{dt}\right)_{t_0}, \quad (1)$$

where V — volume of erythrocytes; E — absorption of suspension; T — transmittance of suspension ($E = \log \frac{1}{T}$): K — proportionality coefficient characteristic of a suspension (dependent on the kind of suspension)
and knowing that:

$$\left(\frac{dV}{dt}\right)_{t_0} = PS\Delta c, \tag{2}$$

where P — permeation constant; S — surface of erythrocytes; Δc — initial concentration difference of the substance transported, we obtain finally:

$$P = \frac{K}{S\Delta c} 2,303 \frac{1}{T_0 ln^2 T_0} \left(\frac{dT}{dt}\right)_{t_0} \tag{3}$$

In order to illustrate the effect of consecutive concentrations of the modifier on transport properties of the membrane a relative permeation constant P/P_n has been introduced, which can be calculated using the formula:

$$\frac{P}{P_n} = \frac{T_{n0} ln^2 T_{n0}}{T_0 ln^2 T_0} \cdot \frac{\left(\frac{dT}{dt}\right)_{t_0}}{\left(\frac{dT_n}{dt}\right)_{t_0}}, \tag{4}$$

where P_n, T_n and T_{n0} refer to unmodified blood.

INHIBITION OF THE TRANSPORT PROCESS

In the experiments carried out to investigate the influnce of the inhibitors on the transport of nonelectrolytes across the erythrocyte membrane the following inhibitions were used: 5,5'dithiobis-(2-nitrobenzoic acid) — DTNB (water transport) and 4,4'-diisothiocyano-2,2-disulfonic stilbene — DIDS (anion transport), in view of the fact that nonelectrolytes permeate partly through the same route as anions and water. The inhibitors were used at concentrations corresponding to almost total inhibition of anion and water transport, i.e. DIDS — $1 \cdot 10^{-4}$ M, DTNB — $4 \cdot 10^{-4}$ M. The erythrocytes modified with the V-n compounds where incubated for 40 minutes at 37°C in an inhibitor solution. After this second incubation the erythrocytes were washed and centrifuged, like it has been during modification.

RESULTS

There has been studied the effect of V-n compounds, with $n = 8, 10, 12, 14$ and 16, on nonelectrolyte transport across erythrocyte membranes modified with solution of the compounds of various concentrations, which were of the following magnitudes:

V-8	10^{-3}M
V-10	10^{-4}M
V-12	10^{-5}M
V-14	10^{-6}M
V-16	10^{-6}M

The concentrations used did not exceed the haemolytic concentration. The experiments were performed at isotonic solutions of the nonelectrolytes. The results of measurements are presented in Figs. 2, 3 and 4.

Fig. 2 illustrates due to the example of V-14 a typical for all modifiers course of the relation between the permeation constant and concentration of the modifier. As seen in the figure, for each modifier there is a maximum, and the maxima for the respective nonelectrolytes are shifted relative to each other.

The results obtained indicate also that the value of $(P/P_n)_{max}$ and thus the modifier's transport effect increases with the number of carbon atoms in the alkyl chain of a compound. This relationship is shown in Fig. 3. Moreover, from the plots in Fig. 4 one can conclude that $(P/P_n)_{max}$ depends on the size of the permeating nonelectrolyte molecule. The following cross-section radii have been assumed of the nonelectrolytes molecules:

glycerol	— 0.27 nm
erythrite	— 0.36 nm
arabinose	— 0.40 nm
saccharoze	— 0.53 nm
polyethylene glycol "400"	— 0.55 nm

The experiment with inhibitors showed the following effect on the relative permeation constant:

Inhibition	$\frac{P}{P_n}$	
	Unmodified erythrocytes	Modified erythrocytes
without inhibitor	1.00	3.30
DIDS (10^{-4}M)	0.76	2.90
DTNB ($4 \cdot 10^{-4}$M)	0.78	2.70

The values of the relative permeation constant obtained in the present work were calculated by using formula (4) and values of T_0 and $(\frac{dT}{dt})_{t=0}$ determined from the plots of transmittance of the erythrocyte samples studied (Fig. 1). The maximum error $\Delta(P/P_n)$ was $\pm 12\%$.

DISCUSSION

The change in the value of the relative rate constants for nonelectrolyte transport across the red cell membranes modified with selected quaternary ammonium salts V-n suggests that the compounds induce changes in the properties of the membranes. Those changes in the membrane function may be responsible for disturbances in the cell metabolism and may account for the biological activity of

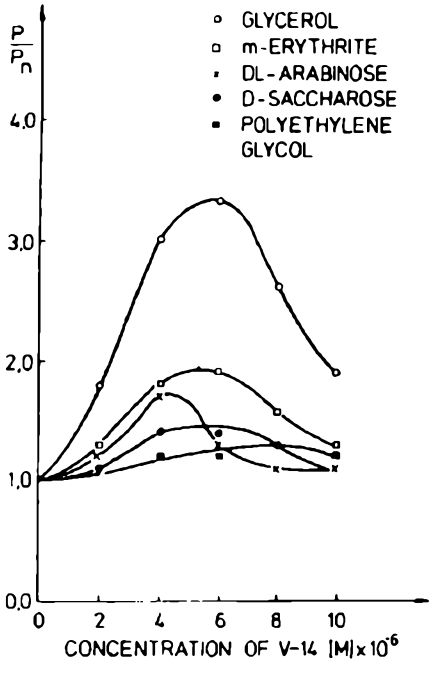

Fig. 2. The relative permeation constant of selected nonelectrolytes for their permeation through the red blood cell membrane modified with the V-14 compound versus its concentration

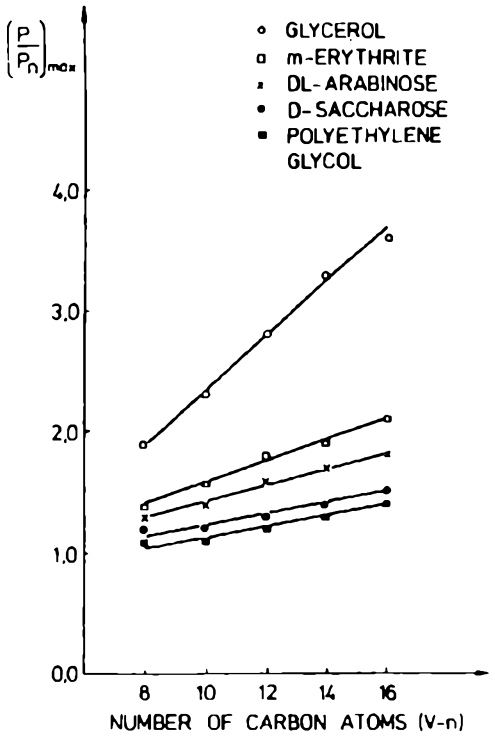

Fig. 3. The maximum value of the relative permeation constant of selected nonelectrolytes through the red blood cell membrane modified with the V-n compounds versus the modifier's alkyl chain length

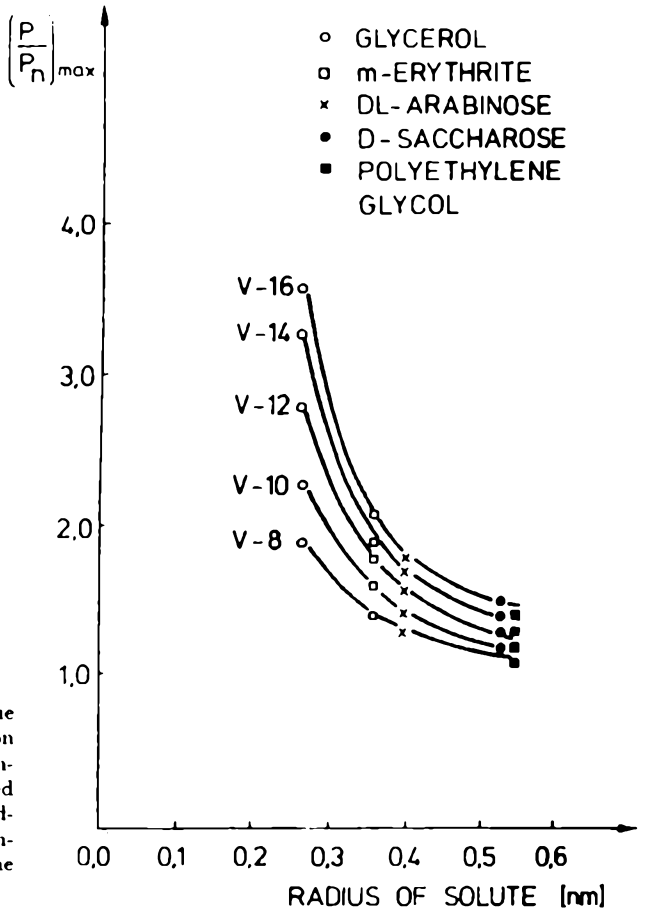

Fig. 4. The maximum value of the relative permeation constant of selected nonelectrolytes through the red blood cell membrane modified with the *V-n* compounds versus size of the nonelectrolyte molecule

the compounds, which is similar to the activity of some other compounds, of detergent and herbicide type, that directly affect the membrane structure [1]. The molecular mechanism of the action is, however, not known in detail. Therefore, one can ask the question what mechanisms of permeation or ways of permeation can be at all considered. Apparently, three kinds of transport paths can be considered (excluding carrier transport) for the passive nonelectrolyte permeation. The first is the diffusive mechanism, in the lipid phase of the membrane, first put forward by Overton [22]. Though the subsequent investigators ([23] for instance) introduced some corrections to that idea, especially with regard to the so-called hydrophilic nonelectrolytes, however solubility of nonelectrolytes in the lipid phase and the possibility of nonelectrolyte transport across the lipid phase are undeniable. The other possibility is permeation through aqueous channels (water-ionic), favoured by Solomon [24], among others. The third possibility is based on the assumption that permeation of nonelectrolytes can proceed simultaneously through the lipid phase and water-ionic channels.

What is the situation in the light of our investigations?

In the case of the first concept (transport across the lipid phase) one can notice that modification of the lipid phase with the compounds studied results in altered surface tension, this in turn accounts for changes in activation energy of the permeation process. The compounds studied induce changes in surface tension of the monomolecular lipid phase, which was found in [25]; this effect being dependent on the alkyl chain length of the modifier molecule: the longer the chain, the more decreases the surface tension. A decrease in surface tension results in decreased energy of activation of the permeation process; since, as shown in paper [26], activation energy E_a can be expressed approximately as

$$E_a = 4\pi r^2 \gamma,$$

where r the radius of the transported molecule assumed to be a sphere, and γ — surface tension.

It should be noticed that with increasing size of the permeating molecule the activation energy increases with the resultant decrease in the permeation constant, which we have also observed in our experiments. The permeation constant can also be affected by other factors. In particular, increased size of the permeating molecule results in decreased diffusion coefficient, which is expected to decrease the permeation constant still further.

The concept presented is in line with the results of our experiments which indicate that the permeation constant of the nonelectrolytes studied increases with the alkyl chain length of the modifier molecule (Fig. 3), and decreases with increasing size of the molecules transported across the erythrocyte membrane (Fig. 4).

The increased permeation induced by the modifiers studied can also, additionally, be explained by the effect the compounds have on membrane fluidity. As we showed previously [11, 13], the modifiers we use induce a slight increase in the liposome membrane fluidity, which should also affect the rate of diffusion of the permeating molecules.

The relationship found between the rate constant and modifier concentration exhibits a maximum (Fig. 2). A similar effect was also pointed out by Kozubek [27], when studying, using a different method, the permeation of nonelectrolytes across erythrocyte membranes modified with different compounds. It seems that one of the possible explanations of the effect follows from consideration contained in the paper [28]. Namely, if above a certain concentration of the modifier some structures develop in the outer solution, e.g. micelles above the CMC, they can compete with the erythrocytes for monomers of the modifier. The $V-n$ monomers will incorporate both into the erythrocytes and micelles; an increased number of micelles will result in a decreased number of monomers incorporating into the erythrocytes, this leading to decreased modification and permeation constant.

In the second case we assume a different mechanism; namely transport of nonelectrolyte molecules through aqueous-ionic channels. A first doubt that appears in this case follows from our previous experiments, which showed that at concentrations of the modifier lower than the haemolytic ones the compounds do not affect

the passive transport of labelled ions across pig cell membranes. In the present experiments, at concentrations of the modifier lower than the haemolytic ones, all the compounds studied markedly affect the nonelectrolyte transport. Moreover, one could expect that, in case the molecules permeate through membrane channels only (practically through the band-3 protein), the modifiers studied should rather inhibit transport, and not accelerate it. This follows from our previous studies (carried out on other objects — on algae) which showed that our modifiers are able to block ionic channels [16]. If, however, our compounds did not inhibit transport through the channels of the erythrocyte membrane, this transport should have been suppressed by the band-3 protein inhibitors DIDS and DTNB [24]. Our experiments show that DIDS and DTNB inhibits transport of the nonelectrolytes we studied only slightly. This may suggest that although some of the permeants go through channels, the major part of transport takes place in the lipid phase.

Hence follows the third concept, according to which the transport occurs both in the lipid phase of the membrane and in channels. At small concentrations of the *V–n* compounds the decisive effect on permeation has the lowering of the activation energy of the permeation process and the increase in membrane fluidity. The inhibitory effect of a small number of modifier's molecules on the channels is, may be negligible. With increasing concentration the inhibitory effect of the *V–n* compounds, independently of the effects connected with possible micelle formation, may result in a decrease in the resultant permeation constant (above a certain concentration of the modifier, which corresponds to the maximum in Fig. 2).

Concluding, one can say that nonelectrolyte molecules probably permeate both through the lipid phase and the band-3 protein channels. In order to make the hypothesis credible, further studies are needed.

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