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Transport of Benzenesulfonic Acid Derivatives through the Rat Erythrocyte Membrane

Shuji Kitagawa, Hiroshi Terada, and Fujio Kametani

Faculty of Pharmaceutical Sciences, University of Tokushima, Sho-machi, Tokushima, 770, Japan

Summary. Transport of benzenesulfonic acid derivatives through the rat erythrocyte membrane was studied. The transport properties, such as pH-dependence and effects of reagents reacting with amino-groups, were similar to those of anions like Cl^- through the human erythrocyte membrane. The rate of transport of anions through rat erythrocyte membranes is higher than through those of other mammals, such as guinea pig and bovine erythrocyte membranes. This relatively high rate of transport makes the rat erythrocyte membrane suitable for use in comparative studies on the transports of slowly penetrating substances, such as organic anions. The transport velocities of benzenesulfonic acid derivatives were compared with their physico-chemical properties. It was shown that the hydrophobicity has no effect on the transport, but the electronic property has a significant effect: the transport rate is mainly dependent on the e^- donor capacities. This feature is the inverse to the well-known inhibitory effect of these derivatives on other anion transport: the inhibition is mainly dependent on the e^- acceptor capacities. It is suggested that the transport is regulated by the binding capacity of anions to the transport site.

Key words benzenesulfonic acid derivatives · erythrocyte membrane · anion transport · structure-activity relationship

Introduction

Erythrocyte membranes show marked transport of anions. Many studies, mainly with human erythrocyte membranes, have shown that the transports of both inorganic and organic anions are mediated by band 3 protein, which spans the membrane (Rothstein, Cabantchik & Knauf, 1976). The transport of these anions through the human erythrocyte membrane exhibits characteristic properties, such as pH-dependence (Passow, 1969; Deuticke, 1970; Gunn, Dalmark, Tosteson & Wieth, 1973), temperature-dependence (Dalmark & Wieth, 1972; Brahm, 1977), and effects of reagents reacting with amino-groups (Knauf & Rothstein, 1971; Deuticke, 1977; Cabantchik, Knauf & Rothstein, 1978). Studies on anion transport through erythrocyte membranes of other mammals, although not detailed, have suggested that the mechanism of transport is the same as in human erythrocyte membranes (Gruber & Deuticke, 1973).

In this work we observed the transport properties of benzenesulfonic acid derivatives through rat erythrocyte membranes, and examined the relation of the transport rates of these derivatives with their physico-chemical properties. Since rat erythrocyte membranes seem to be more permeable to anions than those of other mammals (Gruber & Deuticke, 1973), they are useful for clarifying the transport of slowly penetrating compounds, such as benzenesulfonic acid derivatives. Transport velocities were determined by measuring the rates of hemolysis of the erythrocytes caused by net transport of the organic anions in isotonic solutions of their ammonium salts. This method was developed by Aubert and Motais (1975) measuring the degree of the osmotic hemolysis in isotonic ammonium salt solution based on the observation of Hedin (1897). The observed transport velocities are organic anions/HCO_3^- hetero-exchange velocities (Cousin, Motais & Sola, 1975). This method is very useful for measuring transport rates or for examining transport mechanisms, without the necessity of determining the amounts of anions transported.

Materials and Methods

Materials

Benzenesulfonic acid derivatives were purchased from Wako Pure Chemical Industries, Osaka, Nakarai Chemicals Co., Kyoto, and Tokyo Chemical Industry Co., Tokyo. The ammonium salts of benzenesulfonic acid derivatives were obtained by neutralizing the acids with ammonium hydroxide in a hot water bath. They were crystalized twice from water before use. SITS (4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid, Nutritional Biochemicals, Cleveland, Ohio), TNBS (2,4,6-trinitrobenzenesulfonic acid, Tokyo Chemical Industry Co.), FDNB (1-fluoro-2,4-dinitrobenzene, Wako Pure Chemical Industries) and ANS (1-

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anilino-8-naphthalenesulfonic acid, Sigma Chemical Co., St. Louis, Mo.) were used without further purification.

Cell Preparation

Fresh rat (Wistar) blood was drawn into ACD coagulant solution (2.2 % sodium citrate, 0.8 % citric acid, and 2.2 % dextrose). The plasma and buffy coat were removed and the erythrocytes were washed three times with isotonic phosphate buffer containing 10 mM dextrose by centrifugation at $1{,}000 \times g$ for 10 min. They were then resuspended in phosphate buffer at a hematocrit value of about 20 %. Guinea pig and bovine (Holstein) erythrocytes were obtained in the same way.

Measurement of Transport Velocity

The transport velocities of benzenesulfonic acid derivatives were measured essentially by the method of Aubert and Motais (1975) by determining the rates of hemolysis of erythrocytes in isotonic solutions of the ammonium salts of the acids. The rate of hemolysis is a measure of the relative rate of exchange of external organic anion (A^-) and internal HCO_3^- or OH^- [mainly HCO_3^- (Cousin et al., 1975)]. This method is very convenient for measuring and comparing the transport velocities of a series of compounds without direct hemolytic activities. None of the benzenesulfonic acid derivatives tested had any direct hemolytic effect. Since A^-/HCO_3^- exchange results in no osmotic change, it is concluded that the ammonium salts of these acids exert their hemolytic activities by penetrating through the membrane. In practice, about 5 µl of erythrocyte suspension were added to 3 ml of isotonic solution of the ammonium salt of the test benzenesulfonic acid derivative (165 mM), and the change in optical density associated with hemolysis was measured at 610 nm. The relative transport velocities of these compounds were determined by measuring the half-time of hemolysis ($t_{1/2}$), which was defined as the time required for half the total change of optical density, as shown in Fig. 1 for the results with the 2-NH_2 and 4-NH_2 derivatives of benzenesulfonate. The value of $1/t_{1/2}$ is taken as a measure of the anion transport velocity. In these experiments the isotonic solutions of ammonium salts were adjusted to pH 7.4 with 0.33 M NaOH or H_3PO_4 just before measurement, unless otherwise mentioned. The temperature was maintained at 34 °C.

Results

Inhibition by Reagents Reacting with Amino-groups

The solid line and the broken line in Fig. 1 illustrate the changes in the optical density of rat erythrocyte suspensions in the presence of the 4-NH_2 and 2-NH_2 derivatives of benzenesulfonate, respectively. The optical density decreased first slowly then rapidly. These changes corresponded to swelling and hemolysis, respectively, caused by penetration of the ammonium salts (Aubert & Motais, 1975). We examined the influence of reagents reacting with amino-groups such as SITS on the transport of benzenesulfonic acid derivatives through the membrane. As shown in Fig. 1, the changes in optical density were completely inhibited by 5×10^{-5} M SITS, indicating that SITS inhibited anion exchange

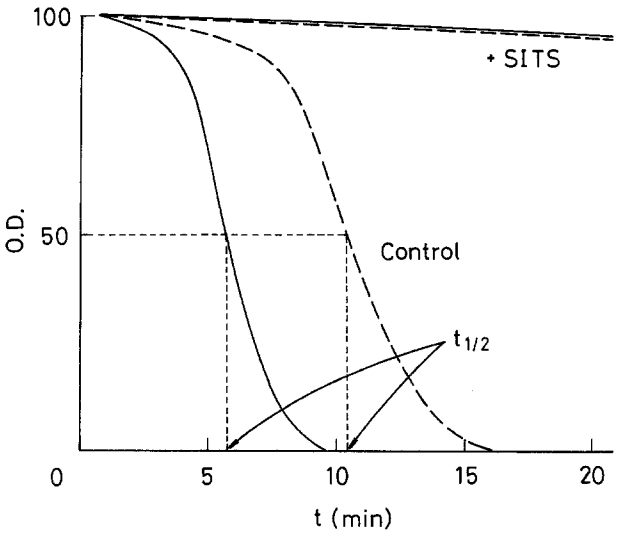

Fig. 1. Optical density changes of rat erythrocyte suspension associated with the penetrations of 2-amino- and 4-amino-benzenesulfonate and their inhibitions by SITS. Solid lines and broken lines show changes with 4-amino- and 2-aminobenzenesulfonate, respectively. The concentration of SITS in the medium was 5×10^{-5} M

diffusion of benzenesulfonic acid derivatives. Transport of these derivatives was also found to be inhibited by other reagents reacting with amino-groups such as FDNB and TNBS, and by the amphiphilic anion ANS; about 50 % inhibition was observed at 10^{-3} M FDNB, 2×10^{-3} M TNBS and 10^{-4} M ANS. These results are consistent with those on human (Knauf & Rothstein, 1971; Deuticke, 1977; Cabantchik et al., 1978) and bovine (Aubert & Motais, 1975) erythrocyte membranes.

pH-Dependence and Comparison with Other Mammalian Erythrocyte Membranes

Figure 2 shows the transport velocities of 4-aminobenzenesulfonate through erythrocyte membranes of three species of mammals determined at various pH's. At all pH values, the transport velocity was higher through rat erythrocyte membranes than through other mammalian erythrocyte membranes. The transport velocities are in the order rat > guinea pig > bovine. This order is the same as that of the passive transports of potassium and phosphate ions (Gruber & Deuticke, 1973; Kirk, 1977).

Figure 2 also shows the pH-dependence of the transport velocity of 4-aminobenzenesulfonate. The transport velocity of 4-aminobenzenesulfonate was higher at pH > 6.0, like the exchange rates of monovalent anions such as Cl^-. In most previous works Cl^- was used as a monovalent anion, and its penetration velocity through human erythrocyte

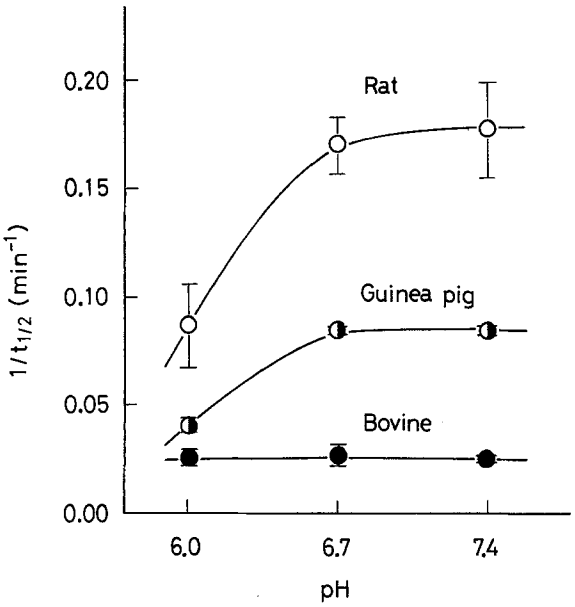

Fig. 2. Transport of 4-aminobenzenesulfonate through various mammalian erythrocyte membranes and its pH-dependence

Table 1. Transport rates of benzenesulfonic acid derivatives through rat erythrocyte membranes at pH 7.4, 34 °C

Substituents	$1/t_{1/2}$(min^{-1})
H	0.152±0.031
4-NH_2	0.178±0.023
4-CH_3	0.132±0.015
4-OH	0.089±0.008
4-Cl	0.069±0.014
4-C_2H_5	0.037±0.004
4-NO_2	0.024±0.002
2-NH_2	0.096±0.005
2-NO_2	0.022±0.003
2,5-CH_3	0.032±0.003
2-CH_3, 4-NH_2	0.169±0.014
3-CH_3, 4-NH_2	0.168±0.009

membranes was found to be maximal between pH 7 and 8 (Gunn et al., 1973). The transport velocities of Cl^- and benzenesulfonic acid derivatives, measured by determining hemolysis by their NH_4 salts, were also higher at pH > 6.0.

Relation of Transport Velocity to Structure of Benzenesulfonic Acid Derivatives

Table 1 summarizes the transport velocities of various benzenesulfonic acid derivatives. The transport velocities of unsubstituted benzenesulfonic acid, and the $-NH_2$ and monomethyl derivatives are high, whereas those of the $-NO_2$, $-C_2H_5$, and dimethyl derivatives are low. To determine the effect of the position of substitution, we compared the transport velocities of the 2- and 4- (or 3-) substituted derivatives. As shown in Table 1, the transport velocity of the 2-NH_2 derivative is considerably less than that of the 4-NH_2 derivative. However, the transport velocities of the 2-NO_2 and 2-CH_3 derivatives are not very different from those of the 4-NO_2 and 3-CH_3 derivatives, respectively.

Table 2. Structure-activity relationships of benzenesulfonic acid derivatives

Substituents	σ	π	E_s	$\log(1/t_{1/2})$
H	0.00	0.00	0.00	−0.82±0.09
4-NH_2	−0.66	−1.23	−0.61	−0.75±0.06
4-OH	−0.37	−0.67	−0.55	−1.05±0.04
4-CH_3	−0.17	0.56	−1.24	−0.88±0.06
4-C_2H_5	−0.15	1.02	−1.31	−1.43±0.05
4-Cl	0.23	0.71	−0.97	−1.17±0.09
4-NO_2	0.78	−0.28	−2.52	−1.61±0.03
3-CH_3, 4-NH_2	−0.73	−0.67	−1.85	−0.78±0.02

E_s in this Table is defined as $E_s(H)=0$. The values are 1.24 less than the original E_s defined as $E_s(CH_3)=0$.

To establish the relation of the physico-chemical properties of the derivatives with their membrane transport quantitatively, we examined the correlations between the transport velocities and three parameters of the 3- and 4- substituted derivatives: the electronic parameter σ (Hammett Constant), hydrophobic parameter π, and steric parameter E_s, as introduced by Hansch and Fujita (Hansch, Muir, Fujita, Maloney, Geiger & Streich, 1963; Hansch & Fujita, 1964; Hansch, 1969).

Values of the three parameters and the relative transport velocities, $\log(1/t_{1/2})$, are shown in Table 2. Values for σ and π for single substituents were taken from papers of Hansch's group (Leo, Hansch & Elkins, 1971) and McDaniel and Brown (1958). E_s values of single substituents were taken from the paper of Ungar and Hansch (1976). The relation between the transport velocities and these parameters are shown in Eqs. (1) through (7):

$$\log(1/t_{1/2}) = -0.50\sigma - 1.13 \quad (n=8,\ r=0.77,\ s=0.22) \quad (1)$$

$$\log(1/t_{1/2}) = -0.18\pi - 1.07 \quad (n=8,\ r=0.44,\ s=0.31) \quad (2)$$

$$\log(1/t_{1/2}) = 0.23E_s - 0.80 \quad (n=8,\ r=0.58,\ s=0.28) \quad (3)$$

$$\log(1/t_{1/2}) = -0.47\sigma - 0.05\pi - 1.13 \quad (n=8,\ r=0.78,\ s=0.24) \quad (4)$$

$$\log(1/t_{1/2}) = -0.42\sigma + 0.14E_s - 0.96 \quad (n=8,\ r=0.83,\ s=0.21) \quad (5)$$

$$\log(1/t_{1/2}) = -0.17\pi + 0.22E_s - 0.82 \quad (n=8,\ r=0.71,\ s=0.27) \quad (6)$$

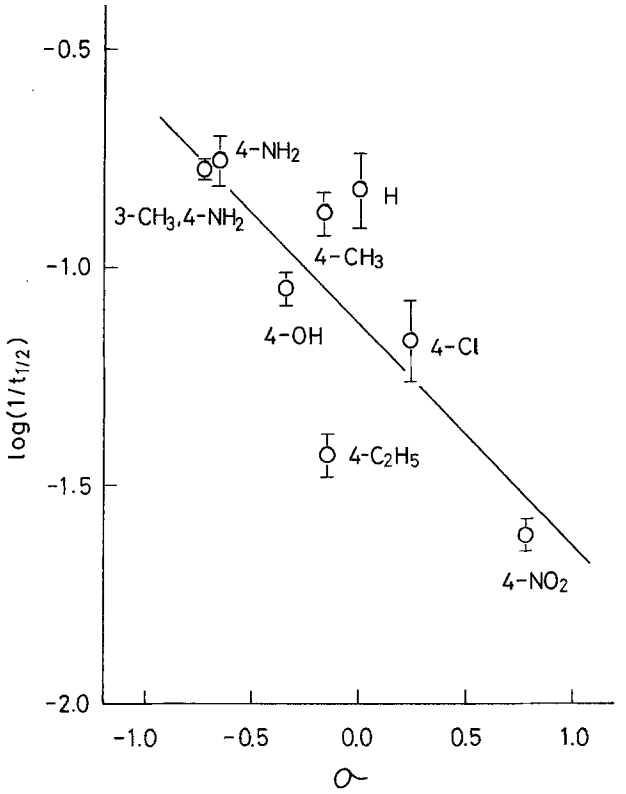

Fig. 3. Relation between transport velocities of benzenesulfonic acid derivatives and Hammett substituent constants, σ

$$\log(1/t_{1/2}) = -0.36\sigma - 0.07\pi + 0.14E_s - 0.95 \quad (n=8,\ r=0.84,\ s=0.23). \quad (7)$$

Here, n is the number of compounds tested, r is the correlation coefficient, and s is the standard deviation. From Eqs. (1) through (7), it is concluded that the electronic property of benzenesulfonate derivatives is of primary importance for the transport, and neither the hydrophobic property nor the steric factor alone has significant effect on the transport process. The negative sign of the electronic parameter σ in Eq. (1) indicates that the transport rate increases with increase in the electron releasing property of the substituents, i.e., the transport is governed by the e^- donor capacity of the compound. The reason why the correlation coefficient in Eq. (1) is not so great is that several artifacts (dependency on HCO_3^- levels, inhibitory effects of the anions tested on carbonic anhydrase, transient changes of transmembrane pH gradient, etc.) may affect the measurement of the transport rates in the method of ammonium salt lysis.

The relation between the transport velocities and σ is shown in Fig. 3. The transport velocity of unsubstituted benzenesulfonate is plotted above the regression line and that of 4-C_2H_5 derivative well below the line, suggesting that the steric factor somewhat affects the transport. The contribution of the steric factor to the transport process is also suggested in the relation of Eq. (5), where the addition of the steric factor slightly improves the correlation in Eq. (1), although the E_s term in Eq. (5) is not highly significant statistically.

Discussion

Properties of Organic Anion Transport through Rat Erythrocyte Membranes

Between pH 6.0 and 7.4, the rate of penetration of benzenesulfonic acid derivatives through rat erythrocyte membranes increases above pH 6.7. The penetration is remarkably inhibited by SITS, FDNB, TNBS and ANS. These results are consistent with those on the transport properties of monovalent anions, such as Cl^- through human erythrocyte membranes. With human erythrocyte membranes, the transport of monovalent anions such as Cl^- reaches a maximum between pH 7 and 8 (Gunn et al., 1973), and it is inhibited by reagents reacting with amino-groups, such as FDNB, TNBS and SITS, and also by amphiphilic substances, such as ANS (Fortes & Hoffman, 1974), 2,4-dinitrophenol (Deuticke, 1970), and local anesthetics, such as tetracaine (Deuticke, 1970; Gunn & Cooper, 1975). Our results suggest that organic anions, such as benzenesulfonate derivatives should transfer through rat erythrocyte membranes via a similar anion transport system to that in human erythrocyte membranes.

As shown in this report, the transport velocities of substituted benzenesulfonate anions through the erythrocyte membranes of three mammals are in the order rat > guinea pig > bovine. The same order has been observed for passive transport of ions, such as phosphate and potassium (Gruber & Deuticke, 1973; Kirk, 1977), and for compounds, such as glycerol, erythritol and acetic acid, which mainly penetrate through the lipid pathway (Deuticke, 1977). Therefore, the rat erythrocyte membrane is highly permeable to many substances, and this character makes this membrane suitable for use in studies on the transports of slowly penetrating substances like organic anions.

Mechanism Controlling Anion Transport through the Erythrocyte Membrane

According to several models for the anion transport (Gunn, 1973; Rothstein et al., 1976; Gunn &

Fröhlich, 1980; Rothstein, Ramjeesingh, Grinstein & Knauf, 1980; Wieth, Brahm & Funder, 1980), it has been considered that the transport of anions is mediated by a certain transporter. Probably a 95,000 dalton protein (band 3 protein) is a transporter itself (Rothstein et al., 1976). To obtain the information on the characters of this transporter, we observed the transport of benzenesulfonic acid derivatives. It is generally observed that biomembrane transport of various organic compounds which transfer through hydrophobic regions of membranes is governed by the hydrophobic property (π) of the organic compounds (Leo et al., 1971). However, in the case of benzenesulfonate derivatives, the hydrophobicity has no effect on the transport, but the electronic property (σ) has significant effect; the transport is mainly dependent on the e^- donor capacities. Thus, it is suggested that for the transport of benzenesulfonate derivatives the electrostatic interaction with a transporter protein is very important.

It is interesting to note that benzenesulfonate derivatives are reported to inhibit competitively sulfate exchange transport by the binding to a common transport site according to their e^- acceptor capacities (Barzilay & Cabantchik, 1979; Barzilay, Ship & Cabantchik, 1979). The inhibitory effect of benzenesulfonates inversely correlated with the transport rate. Such an inverse relation between the transport rates and inhibitory effects is also observed for halogen anions; that is, the order of the transport is $Cl^- > Br^- > I^-$ (Tosteson, 1959), whereas that of the inhibitory effect on sulfate exchange is the reverse: $Cl^- < Br^- < I^-$ (Wieth, 1970). From these results it is suggested that the binding of anions to the transport site is necessary both for the transport and the exhibition of the inhibitory effect. However, strong binding to this site is unfavorable for a high transport rate, and is favorable for strong inhibition. Therefore, anions should show an optimum binding capacity for a maximum transport rate. The binding of anions to the transport site controls the anion transport. However, we cannot exclude the possibility that binding of compounds like benzenesulfonate derivatives to a modifier site is involved in regulation of their transport rates (Dalmark, 1975, 1976).

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