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On the mechanism of transport of salicylate and *p*-hydroxybenzoic acid across human red cell membranes

M. M. JOY AND D. J. CUTLER*

Department of Pharmacy, University of Sydney, Sydney 2006, Australia

The pH-dependence of efflux of salicylate and *p*-hydroxybenzoic acid (PHB) from human red cells indicates that the un-ionized species penetrates the membrane. No effect of the anion channel blocker 4,4′-diisothiocyano-2,2′-disulphonic stilbene was observed. The temperature-dependence of efflux suggests that the energy barrier to transport of salicylate and PHB is the transfer of the acids from water into the membrane, rather than transport through the membrane interior. Intracellular binding of both acids was found to be pH-dependent.

A number of studies have been conducted to investigate the mechanism of uptake of salicylate by red blood cells. Schanker et al (1964) found that the rate of uptake of a series of structurally related acids, including salicylate, roughly followed their chloroform–water partition coefficients. In view of the rapid uptake observed, and the small proportion of molecules in the un-ionized form (salicylate is more than 99·99% ionized at physiological pH), those authors proposed a mechanism of ion transport according to which the ionized group passed through an aqueous pore, while the hydrophobic part of the molecule passed through the lipid membrane phase immediately adjacent to the aqueous pore. Dalmark & Wieth (1972) studied the pH-dependence of salicylate efflux from red cells and found an almost ten-fold decrease in efflux rate when the extracellular pH was increased by one pH unit and concluded that salicylate is transported in the un-ionized form. That study was conducted at a salicylate concentration of 120 mM, about 100 times therapeutic levels (Flower et al 1980). Such high concentrations are known to have membrane stabilizing effects (Kalbhen et al 1970) and to cause morphological changes in red cells (Fujii et al 1979). Furthermore, at 120 mM salicylate was the major contributor to the osmolarity of the system which suggests the possibility of serious artifacts due to disturbance of the normal electrolyte balance.

Aubert & Motais (1975) studied the uptake of salicylate and *p*-hydroxybenzoic acid (PHB) into bovine red cells by determining the time to haemolysis following the addition of the ammonium salt of the organic acid. The absence of inhibition in the presence of the anion transport blocker SITS (4-acetamido-4′-isothiocyano-2,2′-disulphonic stilbene), or acetazolamide (a carbonic anhydrase inhibitor which reduces the amount of intracellular hydroxyl ions available for exchange with anions entering the cell), led to the conclusion that the undissociated acid penetrated the membrane. Because of the high concentrations used (sufficient to cause lysis), and the stabilizing effects of both salicylate and PHB against lysis, this conclusion may not be relevant to salicylate within the therapeutic range.

More recently Crandall et al (1982) used a stopped-flow rapid reaction apparatus to study the influence of salicylate on chloride–bicarbonate exchange. It was observed that in the presence of salicylate protons were lost from the extracellular fluid. This was attributed to the transport of the undissociated acid across the membrane. The anion transport inhibitor DIDS (4,4′-diisothiocyano-2,2′-disulphonic stilbene) did not prevent the pH change in extracellular fluid, which also persisted in the absence of chloride ions. These observations suggest that ion exchange is not associated with salicylate transport, supporting the proposal that the undissociated acid is the species transported.

In contrast is the conclusion of Nishihata et al (1984) that salicylate is transported in the ionized form, while PHB is transported as the undissociated acid. This was based on the observation that the anion transport inhibitor DIDS, at increasing concentrations, caused a progressive decrease in the uptake of salicylate by red cells, while PHB uptake was not altered. In a washout study DIDS retarded the efflux of salicylate.

Thus the evidence relating to the transport of salicylate across the red cell membrane is contradictory, with experimental support for both the ion and

* Correspondence.

the non-ion as the penetrating species. The aim of this study was to shed further light on the transport mechanism by studies on the efflux kinetics at therapeutic concentrations of salicylate and at comparable concentrations of PHB. The pH and temperature dependence of efflux kinetics and intracellular binding, and the effect of the anion transport blocker DIDS have been studied with both salicylate and PHB.

MATERIALS AND METHODS

Rates of efflux were studied by first equilibrating the red cell suspension with the acid at the required temperature at a concentration in the range 1–2 mM, diluting with buffer, then measuring the appearance of the acid in the extracellular fluid. Cells were separated by rapid filtration, by the method of Dalmark & Wieth (1972). These studies were conducted with haematocrits of about 5%. Haematocrits were determined by the spectrophotometric method of Brown (1983). In preliminary studies the acid was assayed in both the buffer and the filter (which contained the retained cells). Since adequate recovery was demonstrated, later studies involved measurement of acid in the buffer only. The acids were assayed by HPLC (precolumn: Sepralyte C18, 40 μm, analytical column: Nucleosil C18, 10 μm, detector: Jasco Uvidec-100-III [at 254 nm for PHB, 280 nm for HSal], mobile phase: methanol–water–acetic acid (1:1:0·02, flow rate 1·5 mL min^{-1}. Retention times: PHB 25 min, HSal 5·2 min, benzoic acid [internal standard] 4·4 min). For the PHB assay benzoic acid was added as internal standard, the solution was heated in a boiling water bath for 1 min and protein precipitate removed by centrifugation. The resulting aqueous phase was injected into the HPLC. For the salicylate assay this procedure resulted in interference in blank samples: protein precipitation with methanol and trichloroacetic acid (Maulding & Young 1980) proved satisfactory. Standard curves were constructed for each day's assay. Standard curves were linear with correlation coefficients greater than 0·99.

Equilibrium binding studies with intact cells were conducted by allowing the red cell suspension containing the acid to come to equilibrium (about seven half-lives, as judged from the kinetic studies), separating the cell and buffer fractions by centrifugation (Eppendorf 5414S) followed by assay of the acid in the buffer. Equilibrium binding to lysed cells was determined by equilibrium dialysis (Dianorm), with measurement of the acid in both compartments after equilibrating for 3 h. Membrane binding was also determined by equilibrium dialysis. The membrane fraction was prepared by the method of Schwoch & Passow (1973).

In studies to examine the potential blocking effect of DIDS, cells were incubated for 30 min at 37 °C with DIDS at a concentration of 0·1 mM for a 50% haematocrit before efflux or equilibrium binding studies. The incubation conditions were those reported by Funder et al (1978) to produce maximal irreversible blockade of chloride transport.

Materials

p-Hydroxybenzoic acid (PHB) and salicylic acid (BDH Chemicals, England) were recrystallized before use (m.p. 213–215 °C and 157–159 °C, respectively). DIDS (4,4′-diisothiocyano-2,2′-disulphonic stilbene) was obtained from Sigma Chemical Company, USA. Other chemicals were reagent grade. Red blood cell concentrate was obtained from the Red Cross Blood Bank (either healthy volunteers or persons returning from malaria endemic regions) and used within 35 days of donation. Cells were washed four times with buffer before use, removing residual plasma and surface cells at each washing. Most studies were conducted in phosphate buffered saline; a small number of studies were conducted using 10 mM HEPES (*N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulphonic acid), made isotonic with sodium chloride.

RESULTS AND DISCUSSION

Efflux rates

Efflux followed first order kinetics. The data were fitted to the equation

$$B = B_\infty + (B_0 - B_\infty)\, e^{-kt} \qquad (1)$$

where B is the buffer concentration at time t, B_0 is the buffer concentration at time 0, B_∞ is the corresponding equilibrium concentration and k is the apparent efflux rate constant. Non-linear regression was used to determine B_∞ and k (veng Pedersen 1977).

The pH-dependence of the efflux rate constant for PHB and salicylate is shown in Fig. 1. The pH shown in this Fig. is that measured in buffer (pH_b) while the pH of major significance for efflux is the intracellular pH (pH_c). These are related by the equation (Funder & Wieth 1966)

$$pH_c = pH_b + \log_{10}(3{\cdot}094 - 0{\cdot}335\, pH_b) \qquad (2)$$

which applies over the range 6·3 to 8·5. For qualitative interpretation of the data of Fig. 1 it is sufficient to note that a change in buffer pH (e.g. relative to 7·4) is accompanied by a change in

intracellular pH which is in the same direction but of reduced magnitude.

It is clear that conditions favouring the ionized species (high pH) resulted in a reduced apparent efflux rate constant, suggesting that it is the un-ionized species of both acids which penetrates the membrane. Also shown in Fig. 1 are the results of studies carried out in the presence of the ion channel blocker DIDS. No change in the apparent efflux rate constant for PHB or salicylate occurred due to DIDS. This agrees with the result obtained by Nishihata et al (1984) for PHB but contrasts with their result for salicylate. Possible reasons for this discrepancy are presented later.

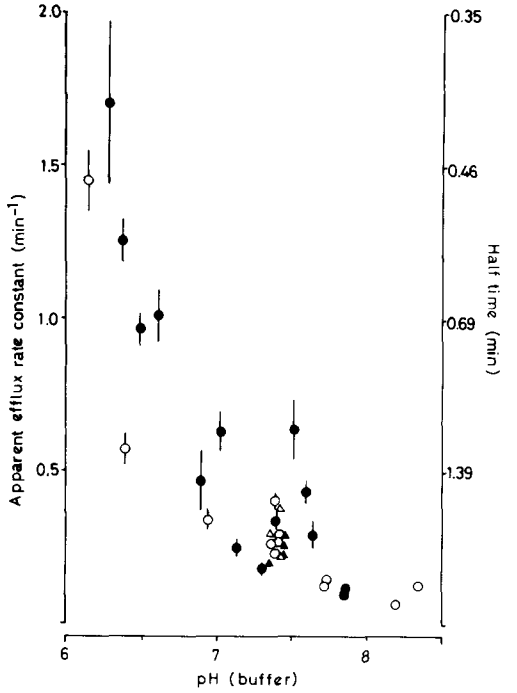

FIG. 1. Dependence of the apparent efflux rate constant on buffer pH for salicylate at 2 °C (●) and PHB at 15 °C (○) and influence of the anion channel blocker DIDS: salicylate + DIDS at 2 °C (▲), PHB + DIDS at 15 °C (△).

While the data of Fig. 1 on the pH-dependence of the efflux rate constant appear to suggest that the un-ionized species is transported, there are other possible explanations for these observations, consistent with ion transport. One explanation is that the observed pH-dependence is due to a pH effect on the red cell membrane. If this is so it could be expected to operate through one of two mechanisms. One possibility is a direct and specific effect on the anion channel. This is not consistent with the observations of Gunn et al (1973) who showed that the rate of transport of chloride through the red cell anion channel increased with pH over the range 5·5 to 9·0, rather than decreased as seen here. It is therefore unlikely that the pH-dependence of efflux is due to an effect on an anion channel, unless the anion channel for salicylate and PHB differs from that for chloride.

The other likely effect of pH on the membrane is a change in the electrical potential across the membrane, with the observed effect on transport possibly attributable to pH-dependent changes in the electrochemical potential. The effect of pH on the transmembrane electrical potential has been studied by Glaser (1979) who showed that the potential (inside–outside) decreased with increasing pH. According to the Nernst-Planck equation (Schultz 1980) a decrease in transmembrane potential should increase the rate of efflux of an anion, contrary to the observations. Thus, the pH-dependence of efflux cannot be accounted for by changes in electrical potential.

Intracellular binding

A further possibility is that the reduction in the efflux rate constant with increasing pH is the consequence of increased binding, independent of the species transported across the membrane. The pH-dependence of the equilibrium binding was studied to investigate this possibility. Fig. 2 shows the pH-dependence of the binding of salicylate and PHB to lysed cells. These studies were conducted at 37 °C for comparison with the study by Nishihata et al (1984), discussed below. The data indicate that binding to the cell contents increases significantly with decreasing pH. The trend is the opposite of that required to explain the efflux kinetic data in terms of decreased binding with decreasing pH. Some binding of the ionized species occurs since binding is apparent at the highest pH at which only a very small proportion of the acids are present in the un-ionized form. However, this ion binding cannot explain the observed pH-dependence of efflux if the ionized acid is the transported species.

Nishihata et al (1984) claim that binding of salicylate, but not PHB, to intact red cells is due to intracellular accumulation; they claim that the binding of PHB is due to an interaction with the red cell membrane. The evidence for this interpretation is that osmotic conditions which cause cell shrinkage reduced salicylate uptake but caused no change in PHB uptake. To investigate this proposal binding studies were conducted with isolated red cell mem-

branes. Fig. 2 shows the binding of salicylate to isolated membranes as a function of pH. The extent of binding was very low at pH 7·4 but increased at lower pH values. This indicates that the salicylate non-ion partitions readily into the membrane but that this is not quantitatively significant at pH 7·4 because of the small fraction in the un-ionized form. Contrary to the proposal of Nishihata et al (1984), no binding of PHB to membranes could be detected at any pH. We conclude that the uptake of both salicylate and PHB by red cells is almost exclusively due to intracellular accumulation at physiological pH.

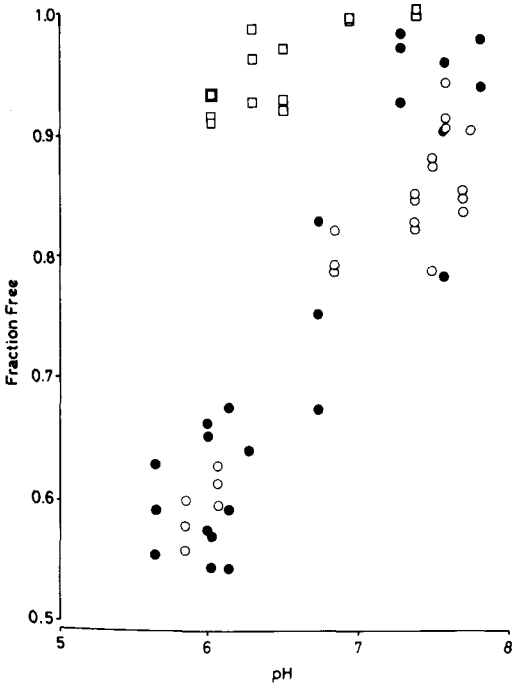

Fig. 2. Dependence of the fraction free on pH for binding to lysed cells and the membrane fraction: salicylate at 37 °C to lysed cells (●), PHB at 37 °C to lysed cells (○), salicylate at 37 °C to isolated membrane fraction (□).

An objection to the use of lysed cells to study intracellular binding is that the binding sites experience a somewhat different environment following lysis. Among other effects, the medium is more dilute than the intracellular environment of the intact cells. For this reason, further experiments were conducted to study the intracellular binding using intact cells. These were carried out at the same temperatures as the efflux rate studies. The data, expressed as the equilibrium distribution ratio (cell concentration/buffer concentration) are shown in Fig. 3; they indicate an increase in binding at lower pH values. As with the lysed cell data, the pH-dependence of binding cannot explain the pH-dependence of efflux if the ion is the transported species.

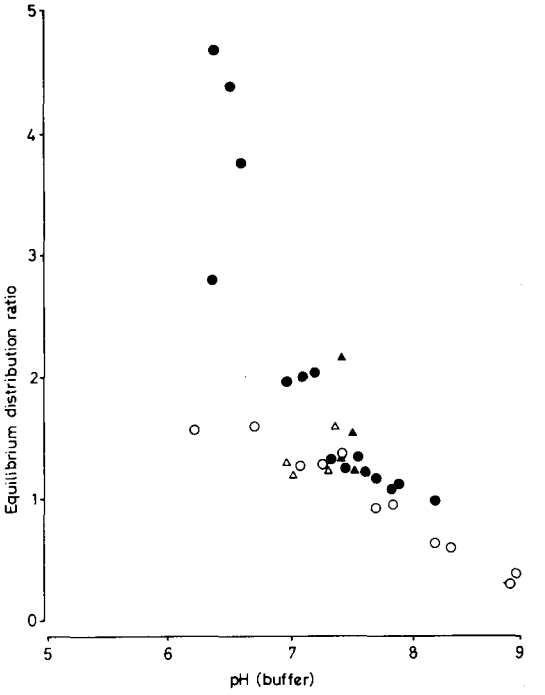

FIG. 3. Dependence of the equilibrium distribution ratio (cell concentration/buffer concentration) on buffer pH for salicylate at 2 °C (●), PHB at 15 °C (○), and the influence of the anion channel blocker DIDS: salicylate + DIDS at 2 °C (▲), PHB + DIDS at 15 °C (△).

Fig. 3 also shows that the equilibrium distribution ratio was not altered by the addition of DIDS for either acid. The replacement of phosphate buffered saline with isotonic HEPES as the suspending buffer caused no significant change in the binding to intact cells (data not shown).

Temperature dependence of efflux

The studies on salicylate uptake were carried out at 2 °C rather than 37 °C because rates of uptake are too rapid at the higher temperature to be directly measured. Our conclusion that ion transport does not contribute significantly may be valid at 2 °C, but not at physiological temperatures. This could be the case if the temperature-dependence of ion transport were much greater than the temperature-dependence of non-ion transport. The apparent activation energy of 120–155 kJ mol^{-1} reported by Dalmark & Wieth (1972) for ion transport supports this possibil-

ity. However, they observed an apparent activation energy of only 55 kJ mol^{-1} for salicylate. This difference in apparent activation energy between salicylate and the ions which were studied provides further evidence against the salicylate anion as the transported species.

Since the data of Dalmark & Wieth (1972) were obtained at salicylate concentrations about 100 times therapeutic concentrations, we investigated the temperature-dependence of the apparent efflux rate constant at therapeutic levels. Our results agree with those at the higher concentration. We obtained an apparent activation energy of 47 ± 7 (s.d.) kJ mol^{-1} in good agreement with the value 55 kJ mol^{-1} reported by Dalmark & Wieth (1972). This value is much smaller than the activation energies observed for ion transport systems. It is similar to, or smaller than, the values obtained for other neutral species transported across the red cell membrane (e.g. acetic acid, erythritol and acetazolamide enter the red cell by non-ionic diffusion with activation energies 92, 113 and 57 kJ mol^{-1}; Holder & Hayes 1965; Deuticke 1977) and is too small to allow for a marked increase in the contribution of ion transport at higher temperatures.

The temperature-dependence of efflux of PHB yielded an apparent activation energy of 130 ± 2 (s.d.) kJ mol^{-1}, much higher than for salicylate and close to the value for ion transport. We feel that this is coincidental, in that other evidence against ion transport for PHB is strong. Inspection of the structures of PHB and salicylate suggest that the removal of a molecule of PHB from an aqueous environment requires the breaking of 5 hydrogen bonds, while only 3 hydrogen bonds need to be broken for salicylate (due to intramolecular hydrogen bonding). A wide range of values have been reported for the energy of a hydrogen bond. Taking Pauling's (1960) value of 20 kJ mol^{-1} provides 60 kJ mol $^{-1}$ for salicylate and 100 kJ mol^{-1} for PHB, which are of the correct order of magnitude. This would be consistent with the dominant energy barrier to transport being transfer of the acid from water into the membrane phase. The two molecules have a similar minimum cross-sectional area, and it would be surprising if the apparent activation energy differences were due to differences in frictional resistance in the membrane interior.

Conclusions

The present studies indicate that transport of PHB and salicylate across the human red cell membrane is due to passive diffusion of the non-ion across the lipid interior of the membrane. This is in agreement with the studies of Dalmark & Wieth (1972), Aubert & Motais (1975), and Crandall et al (1982), but conflicts with the conclusions of the recent study by Nishihata et al (1984) who claim that the ionized species of salicylate is transported via the anion channel. The Nishihata study was conducted at 30 °C; a two-fold dilution in ice-cold saline was used with the aim of terminating uptake. This was followed by centrifugation and two further washes with ice-cold saline before assaying the acid present in the red cells. It is evident from the present studies that very considerable losses would occur from cells, before assay, with this procedure. The change in suspending medium, from buffer to saline, would alter the distribution of salicylate and PHB due to a change in intracellular pH. Loss of intracellular bicarbonate would occur, lowering intracellular pH and increasing the efflux rate and intracellular binding. In the presence of DIDS the mechanism of adjustment of intracellular pH when the pH of extracellular fluid varies will be impaired, so the intracellular pH will be higher than when DIDS is absent. The higher intracellular pH would reduce the efflux rate and reduce the equilibrium distribution ratio which could be misinterpreted as blockade of the transport of the anion. A further complication is the presence of SCN^-, known to be an anion channel blocker (Dalmark & Wieth 1972), at a concentration of 40 mM in the suspending buffer. We conclude that the evidence apparently favouring ion transport of salicylate is an experimental artifact.

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