The anion-transfer system of erythrocyte membranes

N-(7-Nitrobenzofurazan-4-yl)taurine, a fluorescent substrate-analogue of the system

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The fluorescent probe Nbd-Tau [*N*-(7-nitrobenzofurazan-4-yl)taurine] was synthesized and evaluated as a potential substrate of the anion-transport system of human erythrocyte membranes. The probe inhibited Cl^- exchange in a competitive manner from either surface of the membrane, displaying K_i values in the mM range at the inner surface and in the μM range at the outer surface. Inhibition from within cells was via interaction with Cl^- -transport sites, whereas from without it was via interaction with sites of unidentified nature. Nbd-Tau efflux from cells was monitored fluorimetrically in a continuous mode by a novel method that circumvents separation of the cells from the medium. Using this method, it is shown that Nbd-Tau efflux fulfils the following criteria of a substrate of the anion transport system: (a) susceptibility to classical and specific inhibitors of the system; (b) competitive inhibition with Cl^- for anion-transport sites; and (c) temperature coefficient comparable with that of Cl^- exchange. The fluorometric method is highly sensitive, versatile, and kinetically informative. With minor modifications it can be used for measuring anion transport across 'ghost' and isolated membrane vesicles.

The anion transport system of human erythrocytes has been extensively studied in terms of kinetic properties (Cabantchik *et al.*, 1978; Knauf, 1979; Gunn, 1979), physiological relevance (Wieth & Brahm, 1980), and structural organization of the functional polypeptides in the membrane matrix (Cabantchik *et al.*, 1978; Steck, 1978; Tanner, 1978; Guidotti, 1980). Various attempts have also been made at its functional isolation (Wolosin *et al.*, 1977) and reconstitution (Rothstein *et al.*, 1975; Ross & McConnel, 1978; Cabantchik *et al.*, 1980; Cabantchik & Loyter, 1980). However, major technical difficulties were found in studying transport in reconstituted systems, which were due to the rapidity of fluxes that take place in systems of high surface to volume ratios (e.g., vesicles) and the need for quick and efficient separation of vesicles from medium at defined times. Furthermore, there were limitations in the use of radioactive labelled substrates in terms of available specific activities relative to the small trapped volume of vesicles.

Abbreviations used: Nbd-Tau, *N*-(7-nitrobenzofurazan-4-yl)taurine; Nbd-Cl, 4-chloro-7-nitrobenzofurazan; DNDS, 4,4'-dinitro-2,2'-stilbenedisulphonic acid.

In an attempt to provide a sensitive marker for the activity of the red blood cell anion transporter, we developed a method of continuous monitoring of transport by fluorescence (Eidelman & Cabantchik, 1980). The method relies on the fluorescent anion analogue Nbd-Tau (Fig. 1) whose excitation and emission spectra overlap substantially with the absorption spectrum of haemoglobin. The selection of Nbd-Tau as a potential substrate was based primarily on our previous observations that the 2-nitro-4-azidophenyl derivative of taurine can serve as substrate of the system (Cabantchik *et al.*, 1976; Knauf *et al.*, 1978) and on structure-activity considerations of

Nbd-Tau

Fig. 1. Chemical structure of Nbd-Tau

potential anion-transport inhibitors (Barzilay *et al.*, 1979). As fluorescence originating from intracellular Nbd-Tau is mostly quenched, the egress of the analogue could be followed fluorimetrically in a continuous fashion, circumventing the need for separating cells from medium. In this work we demonstrate that Nbd-Tau can serve specifically as a substrate of the anion transport system of human erythrocytes. We base this claim on the following criteria: mutual competition between Cl^- and Nbd-Tau for transport sites, common susceptibility to specific inhibitors of the system, and similar temperature dependence. The method was sensitive with respect to time as well as with respect to the number of cells required, versatile (inasmuch as various systems can be monitored in parallel in an environmentally controlled fashion) and kinetically informative. The method used here to measure anion transport across intact erythrocytes was also adapted for studying anion transfer across white 'ghosts' or vesicles by replacing haemoglobin with a complexation quencher of Nbd-Tau (Darmon *et al.*, 1980).

Materials and methods

Source of materials

Chemicals were obtained from the following sources: Nbd-Cl, from Molecular Probes, Plano, TX, U.S.A.; phloretin and DNDS from ICN (K & K) Chemicals; 2-aminoethanesulphonic acid (taurine) from Sigma; Amphotericin B (type 1) from Squibb & Sons, Inc. All other chemicals were of either analytical or the best available grade. The covalent reagent 4,4'-di-isothiocyano-2,2'-stilbene-disulphonic acid was prepared and used as previously described (Cabantchik & Rothstein, 1972).

Synthesis and properties of Nbd-Tau

A solution of taurine (0.4M) in Na_2CO_3 (1M, 10ml) was treated with Nbd-Cl (0.5M) in ethanol (10ml) for 120min at 45°C. The product was evaporated to dryness, extracted with diethyl ether, dissolved in distilled water, and dialysed against distilled water at 4°C (Spectrapor-2 dialysis bag) to eliminate polymerized material. The diffusate was evaporated to dryness and extracted with ethyl acetate in a Soxhlet apparatus. The residue, which consisted of the sodium salt of Nbd-Tau, was extracted with ethanol in the same apparatus and crystallized from ethanol overnight at -10°C. The acid form of Nbd-Tau was obtained by dissolving the sodium salt in water and passing the solution over Dowex 50W (H^+ form) resin. The solution was evaporated to dryness, washed with diethyl ether and subsequently with light petroleum (b.p. 60–80°C), and kept desiccated at -10°C. The final yield was 65%.

Fig. 2. Spectral properties of Nbd-Tau. Excitation (b) and emission (c) spectra of Nbd-Tau (0.1 μM) in phosphate-buffered saline (pH 7.4, 25°C) are shown in full lines. The absorption spectrum of the compound (100 μM) in the same medium is shown (a) (broken lines).

The new compound had λ_{max} in aqueous solution (pH 4–8) 473 nm ($\epsilon = 23\,200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$), a fluorescence excitation maximum at 483 nm and a fluorescence emission maximum at 550 nm (Fig. 2). (Found: C, 28.2; H, 3.25; N, 16.5; S, 9.8; loss at 100°C, 10. Calc. for $\text{C}_{20}\text{H}_{18}\text{N}_4\text{S}_2 \cdot 2\text{H}_2\text{O}$: C, 29.4; H, 3.7; N, 17.1; S, 9.8; H_2O , 11%). The compound gave a single spot on t.l.c. plates, showing an R_F of 0.75 in propan-1-ol/ NH_4OH /water (6:3:2, by vol.) and an R_F of 0.2 in butan-1-ol saturated with water.

Spectroscopic techniques

Absorption spectra were determined with a Gilford spectrophotometer. Fluorescence excitation and emission spectra and time-dependent changes in fluorescence were monitored in a Perkin-Elmer MPF-4 spectrofluorimeter equipped with a four-cuvette turret and a thermostated unit connected to a constant-temperature bath-circulator (Haake).

Preparation of erythrocytes at different Cl^- concentrations

Erythrocytes withdrawn from healthy donors into heparinized tubes, or from recently outdated blood obtained from a blood bank (Hadassah Medical Center), were washed several times in iso-osmotic phosphate-buffered saline (NaH_2PO_4 , 20 mM; NaCl , 150 mM; titrated with NaOH to pH 7.4, or as indicated) to remove the buffy coat. The cell Cl^- concentration was modified by varying the extra-

cellular Cl^- concentration in the presence of Amphotericin B. This method is a modification (Schnell *et al.*, 1977) of the Nystatin technique described by Dalmark (1976). Briefly, cells (10% haematocrit) were washed with solutions containing 100–500 mM-KCl/25 mM- KH_2PO_4 [titrated to pH 7.0 (or as indicated)]/30 mM-sucrose and 5 μg of Amphotericin B/ml, and incubated in this medium for 90 min at 37°C. For solutions containing concentrations of KCl lower than 100 mM, cells were washed first with 150 mM-KCl in the presence of Amphotericin B and subsequently diluted by slow addition of a 25 mM- KH_2PO_4 /30 mM-sucrose solution (pH 7.0) until the final Cl^- concentration was reached. The Cl^- -loaded cells were washed thrice with the appropriate medium (same Cl^- concentration as reached in the cells) and incubated for another hour in the same medium.

Uptake of Nbd-Tau by erythrocytes

For measurements of the time dependence of Nbd-Tau uptake, cells (10% haematocrit) in iso-osmotic buffered saline, pH 7.0, were incubated at 37°C in media with different Nbd-Tau concentrations. Samples (1 ml) were withdrawn and transferred to 30 ml of pre-chilled (4°C) medium, centrifuged, and the supernatant was discarded. Cell pellets were washed and centrifuged once again. The final pellets were resuspended in 1 ml of distilled water to haemolyse the cells. An aliquot was used for the spectrophotometric determination of haemoglobin at 410 nm. A second aliquot was deproteinized with trichloroacetic acid [5% (w/v) final concn.] and the absorptivity of Nbd-Tau was read at 473 nm. The haemoglobin content per unit of cell water space was determined as previously described (Cabantchik & Ginsburg, 1977).

Loading of erythrocytes with Nbd-Tau

Erythrocytes, either loaded with different Cl^- concentrations by the Amphotericin B method or washed with iso-osmotic buffered saline, pH 7.0, were incubated with 5 mM-Nbd-Tau (or as indicated), for 5–7 h at 37°C with gentle shaking. The final volume of solution was 0.3–1 ml and the haematocrit was 10%. The intracellular concentration of Nbd-Tau was determined as shown above.

Loading of cells with $^{36}\text{Cl}^-$ and Cl^- fluxes

Cells previously loaded with the appropriate Cl^- and/or Nbd-Tau concentrations were washed twice with the appropriate buffer (i.e. same Cl^- concn. as under the loading conditions) at 0°C and incubated at 30% haematocrit with $^{36}\text{Cl}^-$ (The Radiochemical Centre) (0.3–1.0 $\mu\text{Ci}/\text{ml}$) for 20 min, at 4°C. Aliquots (approx. 80 μl) were transferred to polyethylene tubes and centrifuged for 3 min in a Beckman Microfuge at 4°C. The supernate was

aspirated and the end of the tube was cut off. The cells were sucked into a short length of polyethylene tubing attached to a 10 ml syringe pre-filled with 5 ml of flux medium. Flux was started by flushing the cells with the above 5 ml of medium into a jacketed vessel containing an additional 25 ml of medium at constant stirring and constant temperature (0°C) maintained with a Hetofrig controlled temperature circulator (Heto, Birkerød, Denmark). Samples were withdrawn at appropriate intervals by aspirating 0.8–1.0 ml through a combined prefilter/1.2 μm filter unit locked in 25 mm diam. holders (Sartorius GmbH) and connected to 5 ml Luer-lock syringes (Mawe & Hempling, 1969; Dalmark & Wieth, 1972). At the end of the experiment, 0.5 ml of each filtrate (i.e. cell-free medium) was added to 0.1 ml of aqueous trichloroacetic acid (30%, w/v), centrifuged, and 0.4 ml aliquots were transferred for counting of radioactivity with Hydroluma scintillation fluid (3.5 ml, Lumac AC). Samples were counted in a Packard PL scintillation counter. Similarly, 0.5 ml of the total suspension (time infinity, ∞) were treated with trichloroacetic acid, centrifuged and counted.

For equilibrium exchange conditions, the isotope exchange is given by the equation (Gardos *et al.*, 1969)

$$\ln\left(\frac{C_\infty - C_t}{C_\infty}\right) = -k \cdot t \quad (1)$$

where C_t and C_∞ are the radioactivities of $^{36}\text{Cl}^-$ appearing in the external medium at time t and time infinity (∞) (i.e. time of full equilibration) respectively, and k is the corresponding rate constant. Values of k were obtained from the slope of the natural logarithm of the fractional radioactivity remaining in cells $\{\ln[(C_\infty - C_t)/C_\infty]\}$ plotted against time t (Cabantchik & Ginsburg, 1977). For Cl^- fluxes studied in the presence of either extracellular or intracellular Nbd-Tau, the resulting rate constants of Cl^- exchange are designated k_i , as compared with k_0 which designates k in the absence of inhibitors.

Continuous monitoring of Nbd-Tau efflux by fluorescence

Cells (100 μl , 10% haematocrit) loaded with Nbd-Tau at the appropriate Cl^- concentrations were transferred to 1 ml of ice-cold iso-osmotic sucrose buffered with 30 mM-Tris/citrate and centrifuged for 10 s in an Eppendorf microcentrifuge. The cell pellet was washed with an additional 1 ml of the sucrose medium, suspended in 100 μl of ice cold medium, and filtered through a column (0.5 ml) of Dowex AG1 (X8, 25–50 mesh, citrate form) at 0°C. The suspensions of cells in ice-cold sucrose/citrate medium facilitates the complete and fast removal of

extracellular permeating anions without causing any measurable salt leakage from cells. To initiate fluxes, a 50 μ l aliquot of filtrate (approx. 1.5×10^6 cells) was injected into a cuvette of the spectrofluorimeter containing 2 ml of efflux medium at the indicated temperature. The course of Nbd-Tau egress from cells was monitored fluorimetrically either in a continuous mode on a single sample or in a discontinuous mode on four samples run in parallel in four different cuvettes. Settings were as follows: excitation wavelength, 475 nm (5 nm slit); emission wavelength, 540 nm (5–10 nm slits). Details of the method are given elsewhere (Eidelman & Cabantchik, 1980).

Analysis of kinetic data

All kinetic data were evaluated either by linear regression analysis (Dahlberg, 1948; Brownlee, 1965) or by non-linear regression analysis (Marquandt, 1963) using a Wang PCSII desk computer. Kinetic parameters are given with the calculated S.E.M.

Results

Loading of Nbd-Tau into cells

Erythrocytes take up Nbd-Tau in a concentration-dependent manner, reaching transmembrane equilibrium of probe (Donnan equilibrium) after several hours of incubation at 37°C, pH 7.0–7.4 (Fig. 3). For extracellular concentrations lower than 5 mM, 6–7 h of incubation were sufficient for attaining equilibrium. At higher concentrations, the requisite times of equilibration were very long. The results shown in Fig. 3 suggest that, in addition to back-flux of probe from cells, the time of equi-

Fig. 3. Time course of Nbd-Tau uptake into intact erythrocytes

Uptake was measured at 37°C as described in the Materials and methods section. The concentrations of Nbd-Tau in the loading medium were as follows: ■, 1; ▲, 2; □, 5 and △, 10 mM.

bration is affected by a self-inhibiting effect of the probe on its own transport.

The probe taken up by the cells was chemically identical to original Nbd-Tau in spectroscopic as well as chromatographic properties. This indicates lack of deterioration or intracellular metabolic conversion of the probe in the above experimental conditions. Most of the intracellular Nbd-Tau (>85%) was in the free form, as judged from the experimental Donnan ratios of 0.8–1.0 found at pH 7.0–7.4 (37°C). The probe was quantitatively retained in cells after washing the loaded cells with ice-cold medium. Dilution of the suspension of loaded cells with ice-cold medium and incubation for up to 20 min at 0°C resulted in no significant loss of intracellular material (results not shown). Similarly, no penetration of Nbd-Tau into cells was detected either at 0°C using untreated cells or at 37°C using cells chemically modified with the covalent reagent 4,4'-di-isothiocyano-2,2'-stilbene disulphonic acid (Cabantchik & Rothstein, 1972).

Inhibition of Cl⁻ exchange by Nbd-Tau

In order to assess whether or not Nbd-Tau is a substrate of the anion-transport system, it needs to be shown that it inhibits competitively the transport of the natural substrate (i.e. Cl⁻) from either side of the membrane. Therefore, the inhibitory effects were measured on Cl⁻ equilibrium exchange at 0°C with the probe present either inside or outside the cells. In these conditions (i.e. 0°C), the permeation of Nbd-Tau through the erythrocyte membrane is minimal (see above), while that of Cl⁻ is easily measurable.

The inhibition of Cl⁻ exchange can occur either by interaction with Cl⁻ transport sites, Cl⁻ modifier sites, or non-specific sites (Dalmark, 1976; Cabantchik *et al.*, 1978; Knauf, 1979). As shown before (Cabantchik *et al.*, 1978; Barzilay & Cabantchik, 1979a), the inhibition of Cl⁻ exchange by interaction with a single class of sites can be described in terms of the modified Dixon plot (Webb, 1963):

$$k_0/k_i = 1 + \frac{[I]}{K_i^n(1 + [S]/K)}$$

for a competitive inhibitor

and as $k_0/k_i = 1 + [I]/K_i$ for a non-competitive inhibitor, where k_0 and k_i are the respective rate constants of Cl⁻ efflux in the absence and in the presence of inhibitor, [I] and [S] are the concentrations of Nbd-Tau and Cl⁻ respectively, K is either the Michaelis constant for the transport site (i.e. K_s) or the constant of inhibition of Cl⁻ on its own transport via binding to modifier sites (i.e. K'_s), and K_i is the inhibition constant of Nbd-Tau. Thus, with the aid of the modified Dixon plot, it is possible to distinguish between competitive and non-competitive inhibition. Furthermore, for the case of

competitive inhibition, a replot of the x -intercepts, which are equivalent to the apparent $K_1(K_{1,app.})$

$$K_{1,app.} = K_1(1 + [S]/K)$$

against $[S]$, allows the determination of K_1 as well as of K . The calculated K value can be compared against the known values of K_s and K'_s , and thus the nature of the site of inhibition can be defined.

Inhibition from inside

In order to assess whether intracellular Nbd-Tau affects anion exchange, cells loaded with different Cl^- concentrations were also loaded with Nbd-Tau to different intracellular concentrations. Extracellular Nbd-Tau was removed by centrifugation and washing, and subsequently $^{36}\text{Cl}^-$ was incorporated by a 20 min incubation at 0°C . Efflux was measured in an Nbd-Tau-free medium at 0°C containing Cl^- at the same concentration as in the loading medium. The inhibition of Cl^- exchange by Nbd-Tau is shown in Fig. 4 (upper) in terms of the modified Dixon plot, and in Fig. 4 (lower) as a replot of $K_{1,app.}^{\text{in}}$ (the x -intercept of the Dixon plot as a function of $[\text{Cl}^-]$). The variation of $K_{1,app.}^{\text{in}}$ with $[\text{Cl}^-]$ is indicative of competitive inhibition. The extrapolated $K_{1,app.}^{\text{in}}$ at $[\text{Cl}^-] = 0$, K_1^{in} is 3.5 ± 1.5 mM and the value of K is 53 ± 34 mM.

Since the K_s for Cl^- exchange at 0°C is 65 mM, whereas the K'_s is 335 mM, it is suggested that Nbd-Tau competes with chloride for anion-transport sites at the internal surface of the membrane. Similar results were obtained with resealed 'ghosts', which had one-tenth the intracellular concentration of haemoglobin.

Inhibition from outside

The inhibitory effect of Nbd-Tau on Cl^- exchange was considerably more pronounced from without than from within cells. A 200-fold difference in K_1^{out} versus K_1^{in} indicates a marked asymmetry in the nature of the sites with which the probe interacts at the two surfaces. Regarding interactions at the outer surface, the modified Dixon plots for various $[\text{Cl}^-]$ (Fig. 5, upper part) were apparently linear over the range 0–0.1 mM-Nbd-Tau, indicating that inhibition resulted primarily from interactions with a single class of inhibitory sites. These interactions were of an apparent competitive nature (Fig. 5, lower part), displaying a K_1^{out} of 7.3 ± 1.4 μM and a K of 165 ± 60 mM. At this stage it would be premature to exclude interactions also with additional inhibitory sites, inasmuch as these appear not to be manifested as deviations from linearity in the above Dixon plots (Fig. 5, upper part). If these interactions in fact occur, we can predict that they are of low affinity nature, displaying K_1 values in the mM range. From present kinetic data it is not clear whether the main inhibitory effect of Nbd-Tau on

Cl^- exchange resulted from interactions with either transport or modifier sites. The calculated K value was significantly different ($P < 0.001$) from the K_s value of 65 ± 5 mM reported by Brazy & Gunn (1976) or from the K'_s value of 335 mM reported by Dalmark (1976). However, as pointed out by Knauf *et al.* (1979), the reported values were subject to considerable error due to the scattering of data as well as to the simplifications used in their calculations (Dalmark, 1976). Thus, the nature of the Nbd-Tau inhibitory sites cannot be specified.

Fig. 4. Inhibition of Cl^- exchange by internal Nbd-Tau. Erythrocytes were equilibrated by the Amphotericin method to the following chloride concentrations: Δ , 75; $\blacktriangle$, 150; $\blacksquare$, 300 and $\square$, 500 mM. The cells were subsequently loaded with Nbd-Tau to different final concentrations and incubated with Na^{36}Cl for isotope equilibration. The efflux of $^{36}\text{Cl}^-$ from cells into Nbd-Tau-free and radioisotope-free media was measured at 0°C . The calculated rate constants of efflux in the presence and absence of inhibitor, are k_1 and k_0 respectively. Upper graph, relative Dixon plot of k_0/k_1 versus Nbd-Tau concentration; lower graph, the x -intercept values obtained from the above graph, $K_{app.}$, are plotted against Cl^- concentration. The intercept on the y -axis yields $K_1 = 3.5 \pm 1.5$ mM and the intercept on the x -axis yields $K = 53 \pm 34$ mM ($r^2 = 0.98$). Bars denote s.d. values.

Fig. 5. Inhibition of Cl^- exchange by external Nbd-Tau. Cells were loaded with different Cl^- concentrations and equilibrated with ^{36}Cl as described in Fig. 3. Efflux was measured at $0^\circ C$ into media containing different Nbd-Tau concentrations. The upper graph is the modified Dixon plot of k_0/k_1 versus Nbd-Tau concentration obtained at different Cl^- concentrations. Symbols are as follows (in mM): (○) 25, (●) 50, (+) 100, (□) 150, (■) 200, (△) 300 and (▲) 500. The $K_{1,app}$ calculated for the various systems as shown in Fig. 4 were plotted against the Cl^- concentration (lower graph) yielding the following parameters: $K_1 = 7.3 \pm 1.4 \mu M$ and $K = 165 \pm 60 mM$ ($r^2 = 0.97$).

Continuous monitoring of anion transport by fluorescence: Nbd-Tau efflux from cells and its susceptibility to inhibitors of anion transport

The efflux of Nbd-Tau was followed in a spectrofluorimeter by continuous monitoring of the fluorescence of a suspension of cells loaded with probe without separation of medium from cells (Fig. 6). The instantaneous rise in fluorescence after

addition of cells $[F(0)]$ stems primarily from scattering of light by cells (intracellular fluorescence is mostly quenched by haemoglobin). It is followed by a time-dependent increase in fluorescence originating from Nbd-Tau molecules that have emerged from cells. Evidence for the latter is provided by a parallel experiment in which cell-free supernatants obtained by filtration were measured for fluorescence at indicated times (filled circles in Fig. 6). When the fluorescence reaches a plateau level (i.e. at equilibrium) $F(\infty)$, an aliquot of concentrated Nbd-Tau was added to the cell suspension in order to calibrate the fluorescence intensity scale. Fluorescence was linear up to $2 \mu M$ -Nbd-Tau. In order to prevent self-inhibition, flux experiments were run always under conditions in which extracellular Nbd-Tau at $t = \infty$ never exceeded $0.5 \mu M$.

The rate constant (k) of Nbd-Tau efflux was obtained by taking at least 20 experimental points along the fluorescence tracing (Fig. 6) and fitting them to the exponential relationship:

$$F(t) = F(\infty) - [F(\infty) - F(0)] \exp(-kt)$$

by a non-linear regression program based on the Lavenberg-Marquardt algorithm (Marquardt, 1963). The various F values represent the fluorescence intensity at the respective times t , 0 and ∞ . The non-linear least squares analysis gave an exponential fit virtually indistinguishable from the experimental tracing ($k = 0.061 \pm 0.001$, $r^2 = 0.99$). The corresponding k for data obtained with the filtration method was 0.062 ± 0.002 ($r^2 = 0.98$). The efflux profile of Nbd-Tau was virtually the same in media containing any of these buffers (at 20 mM): phosphate, citrate, Hepes[4-(N-2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], Tris, and in media containing any of these cations: Na^+ , K^+ , choline or Tris.

The method of monitoring transport by fluorescence was particularly useful for screening the effects of inhibitors (or other factors) which affect anion transport. Traces of fluxes of four different systems run in parallel are shown in Fig. 7. Although the fluxes were monitored discontinuously, they were sufficiently informative for calculating the requisite kinetic parameters of Nbd-Tau transport. These were obtained for two types of inhibitors, DNDS, a specific, non-penetrating inhibitor, and phloretin, a dipole potential modifier (Deuticke, 1977). Both compounds gave linear inhibition profiles to at least 95% inhibition in the modified Dixon plots (Fig. 8, inserts), yielding $K_{1,app}$ of $13.9 \pm 6.6 \mu M$ for DNDS and $7.1 \pm 0.5 \mu M$ for phloretin. These values are comparable with those observed for inhibition of either Cl^- exchange or sulphate exchange (Deuticke, 1977; Cabantchik *et al.*, 1978; Knauf, 1979). Nbd-Tau fluxes were also sensitive to chemical

Fig. 6. *Efflux of Nbd-Tau from cells*

Cells loaded with Nbd-Tau (4 mM) and filtered at 0°C through a Dowex AG 1 (X8; citrate form) minicolumn were injected into a thermostated vessel containing 25 ml of phosphate-buffered saline (pH 7.0), at 37°C, to give 1×10^6 cells/ml of suspension. A 2 ml aliquot of suspension was quickly transferred to a cuvette in the spectrofluorimeter, and the fluorescence intensity was followed at 37°C in a continuous fashion (—). In parallel, samples were withdrawn from the vessel, filtered through Sartorius filters (1.2 μ m mean pore diameter) as described in the Materials and methods section, and the fluorescence intensity of the filtrates were measured (●). Data analysed by the non-linear least squares method gave the following rate constants: $k = 0.061 \pm 0.001$ ($r^2 = 0.99$) for the method of continuous monitoring and $k = 0.062 \pm 0.002$ ($r^2 = 0.98$) for the method of sampling.

Fig. 7. *Comparative efflux profiles of Nbd-Tau*

The efflux of Nbd-Tau from cells (see Fig. 5) was fluorimetrically measured at 37°C in Tris/saline buffered media (pH 7.0), containing either 0, 10, 15 or 20 μ M-DNDS. The discontinuous traces of four parallel systems were obtained by rotating the multicuvette turret at given time intervals. All systems contained the same number of cells (4×10^5 /ml).

Fig. 8. Inhibitory effects of DNDS and phloretin on Nbd-Tau efflux

Nbd-Tau efflux was measured spectrofluorimetrically in media containing different concentrations of inhibitors [I]. The fractional flux k_i/k_0 is plotted against the corresponding concentration of inhibitor. The modified Dixon plots (see Fig. 4) given in the insets were constructed from same data but for ranges of [I] where k_0/k_i values were lower than 20 (i.e., 95% inhibition). Linear regression analysis of the linearized plots gave the following $K_{i,app}$ (μM) values: 13.9 ± 6.6 for DNDS and 7.1 ± 9.5 for phloretin.

modification of cells by 4,4'-di-isothiocyano-2,2'-stilbene disulphonic acid, a covalent reacting analogue of DNDS (Cabantchik & Rothstein, 1972) which binds irreversibly to the anion transporter (results not shown).

Fig. 9. Temperature dependence of Nbd-Tau efflux. Efflux of Nbd-Tau from cells (Fig. 6) was monitored at different temperatures in phosphate-buffered saline (pH 7.4) (upper graph). The calculated initial rates normalized to the same number of cells were plotted according to the Arrhenius scheme. The energy of activation calculated was 23.4 ± 1.4 kcal/mol (98 ± 6 kJ/mol).

Effect of temperature and of Cl^- concentration on Nbd-Tau efflux

Like halide and sulphate exchange, Nbd-Tau efflux was temperature-dependent (Fig. 9). An Arrhenius plot constructed from efflux profiles obtained in the 15–35.5 °C range gave an energy of activation of 23.4 ± 1.4 kcal/mol (98 ± 6 kJ/mol). This value is similar to that obtained for Cl^- exchange (Brahm, 1977) and is less than that described for sulphate (Passow, 1969).

The effect of Cl^- on Nbd-Tau efflux was measured in cells loaded with different Cl^- concentrations, using the Amphotericin technique. Most of the Amphotericin was apparently removed from the cells by washing, inasmuch as Nbd-Tau efflux was virtually the same in Amphotericin-treated or untreated cells. The symmetrical increase in Cl^-

Fig. 10. Effect of Cl^- concentration on Nbd-Tau efflux

Cells were loaded with Cl^- to the indicated concentrations and with Nbd-Tau (4 mM) in phosphate-buffered medium (pH 7.4) (see Fig. 4). The cells were washed of external probe and jetted into media containing the same Cl^- concentrations as in the loading media. The rate constant (k) of Nbd-Tau efflux is plotted against the Cl^- concentration. The inset describes the Dixon plot of the same data. $\circ$, $\blacksquare$, $\triangle$ and $\bullet$ denote ranges of rate constants obtained in four separate experiments; ($\blacktriangle$) denotes rate constants normalized for volume changes observed in cells at Cl^- concentrations different from 150 mM. The lines connecting through the points are the best fits obtained either by non-linear least squares method (broken line), which yielded $K_i = 49 \pm 12 \text{ mM}$ ($r^2 = 0.87$) for Cl^- inhibition of Nbd-Tau efflux, or by the linear least-squares method (full line, inset) which yielded $K_{i, \text{app}} = 15 \pm 12 \text{ mM}$ ($r^2 = 0.92$).

concentration outside and inside the cell produced a progressive inhibition of Nbd-Tau efflux (Fig. 10). Analysis of this inhibition, either by non-linear least squares analysis of k versus $[\text{Cl}^-]$ or by linear least squares analysis of $1/k$ versus $[\text{Cl}^-]$ (i.e. a Dixon plot) (Fig. 10, inset), showed that, at 37°C , Cl^- inhibited Nbd-Tau efflux by interaction with a single class of sites whose K_i was $49 \pm 12 \text{ mM}$ ($r^2 = 0.87$). Since this value is comparable with the K_s observed for Cl^- transport at 37°C by Brahm (1977), we conclude that Nbd-Tau and Cl^- share a common transport site at the inner surface of the membrane.

Discussion

In the present work we introduce Nbd-Tau as a fluorescent substrate of the anion-transport system and the method of continuous monitoring of anion transport by fluorescence, which rests on the use of the above fluorescent substrate.

The selection of the fluorescent nitrophenyldiazole derivative of taurine (Nbd-Tau) as a potential substrate of the anion transporter was based primarily on the previous work of Cabantchik *et al.* (1976) and Knauf *et al.* (1978), who showed that the 2-nitrophenyl-4-azidophenyl derivative of taur-

ine could serve as a substrate of the system. The two compounds were found to share structural elements which in previous structure-activity relationship studies have been shown to be essential for the interaction of various organic anions with the anion transporter, that are an anionic moiety and an aromatic hydrophobic counterpart carrying chemical groups of demonstrable e^- -acceptor capacity (Barzilay *et al.*, 1979). Specifically, the compounds share the tauryl group on the one hand, and the phenyl moiety substituted either with nitro, diazole or azido groups, on the other hand. Support for the rationale was found in preliminary screening studies which led to the finding that the 2,4-dinitrophenyl derivative of taurine could also serve as a substrate of the system (D. Jones & Z. I. Cabantchik, unpublished work) and to the finding that the fluorescent nitrophenyldiazole moiety showed marked e^- -acceptor potential (Lancet & Pecht, 1977). The Nbd-group was selected primarily because its excitation and emission spectra overlap largely with the absorption spectrum of haemoglobin. This ensures that in dilute cell suspensions fluorescence originating from intracellular Nbd-Tau will be mostly quenched by the absorbing haemoglobin, whereas fluorescence from extracellular Nbd-Tau will be minimally quenched. Since

Nbd-Tau shows very limited permeation across red cell membranes at 0°C, it was possible to load cells with the probe at 37°C, separate loaded cells from the extracellular probe at 0°C, and follow the egress from cells at 37°C into probe-free media. This egress was recorded fluorimetrically as a progressive increase in signal (Fig. 6). In that manner transport was monitored in a continuous fashion, circumventing the need for stopping transport, separating cells from medium and processing for counting for radioactivity at different time intervals.

In this work we attempted to demonstrate that Nbd-Tau can serve specifically as a substrate of the anion-transport system of human erythrocytes cells. First, we showed that under conditions of minimal penetration (i.e. at 0°C), Nbd-Tau placed intracellularly inhibited Cl⁻ exchange in a competitive fashion (Fig. 4). The site of inhibition was apparently the Cl⁻ transport site, as judged by the similar values of K obtained from competition kinetics (53 ± 34 mM) (Fig. 4) and the reported K_s of Cl⁻ exchange of 65 ± 5 mM (Brazy & Gunn, 1976). These results are analogous to those reported for the 2-nitro-4-azidophenyl derivative of taurine, which also interacts with anion-transport sites at the inner membrane surface (Knauf *et al.*, 1978).

Secondly, Nbd-Tau efflux, as measured spectrofluorimetrically, was fully sensitive to classical inhibitors of anion exchange such as DNDS and phloretin (Fig. 8), and the bimodal reagents 4,4'-diisothiocyano-2,2'-stilbene disulphonic acid and its dihydro analog, to the diuretics furosemide and MK-196 (Merck, Sharp & Dohm), and to the anti-inflammatory niflumic acid (results not shown). This reinforces the view that Nbd-Tau exits the cells via the anion transporter of erythrocytes. However, the observed $K_{1,app}$ of DNDS on Nbd-Tau efflux (measured at 37°C) was 4–6-fold higher than the $K_{1,app}$ on SO₄²⁻ exchange (measured at 25°C) (Barzilay & Cabantchik, 1979a) or was 1.5-fold higher than $K_{1,app}$ on Cl⁻ exchange (measured at 0°C) (Barzilay & Cabantchik, 1979b; M. Barzilay & Z. I. Cabantchik, unpublished work). This apparent discrepancy in the susceptibility of the transport system to a particular inhibitor might reflect a fundamental difference in the transport mechanism of the various anions; however, it might likely reflect, at least in part, the temperature coefficient of the inhibitor effect. Such negative temperature coefficient for $K_{1,app}$ is compatible with the recent work of Cousin & Motais (1979) which shows that the inhibitory potency of niflumic acid on anion exchange was reduced 10-fold when the temperature was raised from 0°C to 30°C.

Thirdly, the temperature dependence of Nbd-Tau efflux (range 15–35.5°C) gave an activation energy of 23.4 ± 1.4 kcal/mol (98 ± 6 kJ/mol). This value is similar to that published for Cl⁻ exchange

measured in the same range of temperatures (Brahm, 1977), but it is about 30% lower than the activation energy of sulphate or oxalate exchange (Passow, 1969; Cousin & Motais, 1979). The cause of these differences needs to be explored further.

Finally, the most important criterion on which the present work rests is that of competition between Nbd-Tau and Cl⁻ for common transport sites at the inner membrane surface. From the kinetic analysis of Nbd-Tau fluxes measured at different chloride concentrations we obtain that the $K_{1,app}$ of Cl⁻ is 49 ± 12 mM, a value not significantly different from that reported for Cl⁻ exchange (Dalmark, 1976; Brazy & Gunn, 1976; Gunn & Frohlich, 1979). Since the K_s for Cl⁻ varies little with temperature, and since modifier effects at 37°C are very small up to 300 mM-Cl⁻ (Brahm, 1977), it would be safe to conclude that the competitive inhibition of Cl⁻ on Nbd-Tau efflux resulted from interactions with Cl⁻ transport sites. Taken together, the above kinetic evidence is consistent with the idea that intracellular Nbd-Tau is transported via, if not exclusively by, the anion transport system of human erythrocytes.

The interactions of Nbd-Tau with the anion transport system at the two membrane surfaces are markedly asymmetric: at the inner surface, it acts on transport sites with mM apparent affinity (Fig. 4), but on the outer surface it acts on undefined sites with μ M affinities (Fig. 5). These asymmetric effects are also reflected in the transport properties of Nbd-Tau which show much higher transport rates from inside than from outside (Figs. 3 and 6). A possible explanation for this observed asymmetry is that Nbd-Tau interacts with transport sites on both surfaces, inasmuch as it is transported in both directions but it also interacts with high affinity sites at the outer surface. This latter interaction might also lead to self-inhibition of Nbd-Tau uptake, as observed in the loading experiments (Fig. 3).

Unfortunately, the kinetic nature of Nbd-Tau inhibitory sites could not be resolved in the present series of studies. The fluorescent Nbd-Tau is chemically similar to the photochemically reactive 2-nitro-4-azidophenyl derivative of taurine, which has also been shown to interact rather specifically with the anion transport system of erythrocytes (Cabantchik *et al.*, 1976; Rothstein *et al.*, 1977). In a recent study, Knauf *et al.* (1978) have shown that the probe had a marked asymmetric effect on Cl⁻ exchange. This effect was attributed to the fact that the probe interacted competitively with transport sites at the inner surface, showing affinity at the mM range, but interacted with 'modifier' sites at the external surface, showing approx. 20-fold higher affinity. The two probes also show similar kinetics in their inward and outward transport rates (Knauf *et al.*, 1978).

Based on the spectral properties of Nbd-Tau and

of the erythrocyte system, as well as on present evidence that Nbd-Tau is a substrate of the anion-transport system, we are confident that the method of continuous monitoring of transport by fluorescence can be used as a convenient indicator of anion transport in intact cells. The method, which is sensitive down to 10^5 cells/ml with normal instruments, is able to resolve fluxes in the minute range with standard mixing techniques and in the second range with appropriate rapid mixing devices. We have recently demonstrated (Darmon *et al.*, 1980) that the method is also applicable to anion transport measurements in white 'ghosts' and in isolated, erythrocyte-derived, vesicles which contain no haemoglobin. In this case, however, purified anti-(Nbd-albumin) antibodies were used to quench Nbd-Tau fluorescence in the outer medium.

At the present time it is not clear whether this method can be used to study anion transport in other types of cells. Nevertheless, with appropriate design of fluorescent analogues of substrates, it should be possible to apply the method of continuous monitoring of transport by fluorescence to study other transport systems of erythrocyte membranes (e.g., nucleoside, glucose) and hopefully of other cell membranes as well.

Note Added in Proof (Received 13 March 1981)

Recent studies (O. Eidelman, M. Zangvill & Z. I. Cabantchik, unpublished work) have shown that the competitive inhibitor DNDS was approx. 6-fold more potent on Ndb-Tau transfer in sulphate medium than in chloride medium (both measured at 30°C, pH 7.0). These results are in line with previous observations made on DNDS inhibition of inorganic anion self-exchange (Barzilay & Cabantchik, 1979a).

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