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THE INTERACTION OF THE ANIONIC FLUORESCENCE PROBE, 1-ANILINONAPHTHALENE-8-SULFONATE, WITH HEPATOCYTES AND HEPATOMA TISSUE CULTURE CELLS

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Summary

The fluorescence probe 1-anilinonaphthalene-8-sulfonate (ANS) has been used to characterize the anion transport properties of normal hepatocytes and hepatoma tissue culture cells. Incubation of hepatocytes in the presence of ANS (20 μM) resulted in a 35-fold enhancement of fluorescence and a 50 nm blue shift. The time course of this process is biphasic. A rapid initial fluorescence enhancement suggests ANS binding to the plasma membrane, and a slower component reflects the uptake of ANS into intracellular compartments. Analysis of ANS uptake showed this latter process to be saturable, with a K_m of 10 μM, to be temperature dependent and to occur only in viable cells. The above observations suggest a carrier-mediated anion transport mechanism. Incubation of hepatoma tissue culture cells with ANS (20 μM) gave a fluorescence emission spectrum similar to that obtained from purified plasma membranes. The kinetics of this interaction only exhibited a rapid initial binding of ANS. The second slow component was now absent, suggesting that ANS transport by the malignant cell system was greatly reduced. Transport of ANS could, however, be stimulated in the presence of the local anesthetic tetracaine. The observed transport was now saturable, temperature dependent, and as in normal hepatocytes, required viable cells, again indicating a carrier-mediated transport system. These studies suggest a significant alteration in membrane function in hepatoma tissue culture cells resulting in a major defect in anion transport.

Introduction

Many properties of mammalian cells have been shown to be mediated by the cell surface through processes such as transport, hormone binding and cell-

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; HTC, hepatoma tissue culture.

cell interactions [1—5]. The study of plasma membranes derived from normal and malignant cells has been the subject of numerous investigations. Attempts have been made to correlate altered cell properties with alterations in cell surface structure and function [6—8].

Isolated hepatocytes have been shown to possess specific transport sites for amino acids [9], hexoses [10], cortisol [11], cholic acid [12], L-triiodothyronine [13] and organic anions such as bromosulfophthalein [14,15] and taurocholic acid [16]. In an effort to probe the nature of anion transport in normal and malignant hepatocytes, we have utilized the anionic probe, ANS, in conjunction with fluorescence spectroscopy [17]. ANS has been used to study membrane structure of several isolated membrane systems [18—24]. We have previously reported the interaction of ANS with purified membranes derived from hepatocytes and hepatoma tissue culture (HTC) cells [25]. ANS has also been shown to be an effective probe in the study of anion transport in erythrocytes and Ehrlich ascites cells [26,27]. Studies have suggested that ANS interacts with the anion transport sites on the plasma membrane of erythrocytes and can inhibit Cl^- and SO_4^{2-} transport. ANS also inhibits SO_4^{2-} exchange in Ehrlich ascites cells by competing with this anion for the membrane-associated transport components. In this study we describe the interaction of ANS with intact hepatocytes and HTC cells. A preliminary report of this work has been presented in abstract form [28].

Materials and Methods

Tetracaine · HCl and ANS were purchased from Sigma Chemical Co. ANS was recrystallized several times from a saturated $MgCl_2$ solution as previously described [29]. Collagenase was obtained from Worthington Biochemical Corp. All other reagents were of analytical grade.

Cell preparation. Suspensions of isolated hepatocytes were prepared using a collagenase perfusion technique as previously described [30] from male Sprague-Dawley rats (200—250 g) fed ad libitum. Isolated hepatocytes were incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, saturated with 95% O_2/5% CO_2 in the presence of 10 mM glucose, for 30 min at 37° C. Following this incubation, the cells were washed and resuspended in 0.25 M sucrose/5 mM Tris · HCl, pH 7.4.

HTC cells were grown as suspension cultures in Swim's 77 medium supplemented with 5% fetal calf serum and 5% calf serum as previously described [31]. The cells were washed with phosphate-buffered saline, twice with 0.25 M sucrose/5 mM Tris · HCl, pH 7.4, and finally resuspended in the sucrose/Tris buffer.

Cell viability was estimated by trypan blue exclusion. Unless indicated, all experiments were carried out on cell suspensions with viabilities of 85—90% for hepatocytes and greater than 95% for HTC cells. Non-viable (less than 10% viability) hepatocytes and HTC cells were prepared by incubating the viable cells with 0.154 M arsenate for 1 h and 2 h, respectively. Cells were then washed 3 times with sucrose/Tris buffer and resuspended in the same buffer.

Experiments were done within 2—3 h after cell isolation. The cells were kept in 0.25 M sucrose/5 mM Tris · HCl, pH 7.4, saturated with 95% O_2/5%

CO_2 at room temperature. The cell viability was always checked at the end of each experiment. A decrease of less than 2% in cell viability was found for hepatocytes, whereas HTC cells remained greater than 95% viable. The experiments have been performed at least twice for each cell preparation and have been carried out with 2 to 3 different cell preparations. Experiments where comparisons were made were always performed with the same cell preparation. Standard errors for the reported values were less than 5%.

Fluorescence measurements. The fluorescence measurements were performed on a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer at a 90° angle to the exciting beam, with 6 nm slit widths for both excitation and emission channels and a 390 nm cutoff filter. The excitation wavelength for ANS was 365 nm. The sample compartment was maintained at constant temperature using a thermostated cuvette holder. Aliquots of ANS were added to the cell suspensions ($1 \cdot 10^6$ cells/ml) in 0.25 M sucrose/5 mM Tris · HCl, pH 7.4, quickly mixed by cuvette inversion and the fluorescence intensity recorded at 470 nm. This procedure could be performed in less than 10 s. Light scattering effects were insignificant under the conditions used in these studied. The interaction of ANS with the cell systems was analyzed by double reciprocal plots to give apparent K_m values. The effect of tetracaine on ANS fluorescence in the presence of the cell systems was determined by the addition of the anesthetic 10 s prior to the addition of ANS. The effects of cell viability and tetracaine on the cellular distribution of ANS fluorescence on the two cell systems were also analyzed using a Zeiss Universal fluorescence microscope equipped with a polaroid camera. High speed polaroid land film, type 57, was used for the fluorescence micrographs.

Results

Interaction of ANS with intact hepatocytes. When hepatocytes ($1 \cdot 10^6$ cells/ml) were incubated with 20 μM ANS in 0.25 M sucrose/5 mM Tris · HCl, pH 7.4, for 40 min at 25°C, a 35-fold enhancement in ANS fluorescence was observed compared to that obtained in buffer alone. The fluorescence enhancement was accompanied by a significant blue shift with an emission maximum of 470 nm. These results are shown in Fig. 1. The increase in fluorescence intensity as a function of time is shown in Fig. 2. The time course of fluorescence enhancement is biphasic in nature with a rapid initial increase followed by a slower component which reaches a plateau in 35 min. The rapid initial phase of fluorescence enhancement suggests ANS binding to the plasma membrane, while the slower component reflects the influx of ANS and subsequent binding to intracellular components. Fluorescence microscopic examination of hepatocytes incubated in the presence of ANS exhibited a strong, uniform distribution of fluorescence across the face of the cell (Fig. 3a) suggesting intracellular localization of the probe.

The influx rate was calculated from the initial slope of the slow component. As shown in Fig. 4a, this process is dependent on the concentration of ANS and is saturable at 60 μM ANS. These results suggest that ANS uptake by intact hepatocytes is effected by a facilitated transport mechanism. A Lineweaver-Burk plot of these data gave a straight line from which an apparent K_m of 10

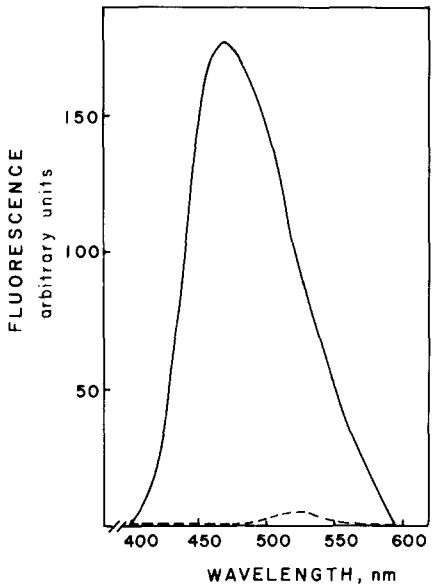


Fig. 1. Fluorescence emission spectrum of ANS in the presence of intact hepatocytes. ANS (20 μM) was incubated with hepatocytes (10^6 cells/ml) in 0.25 M sucrose/5 mM Tris · HCl, pH 7.4, at 25°C for 40 min (———). ANS in sucrose/Tris buffer (- - - - - -).

μM was calculated (Fig. 4b). To further define the mechanism of ANS uptake, measurements were made as a function of temperature. The process was shown to be temperature dependent (Fig. 5), where uptake was significantly higher at 37°C than at 25 and 4°C, again suggesting a facilitated transport process. In an effort to define the energy requirements for the ANS transport process, intact

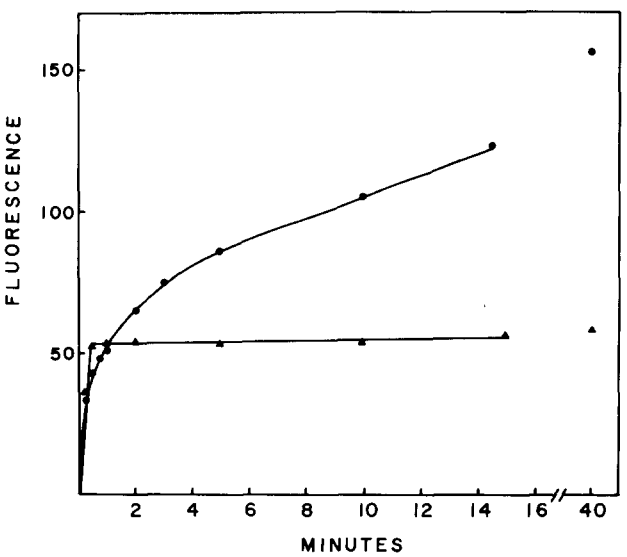


Fig. 2. Kinetics of interaction of ANS with intact hepatocytes. ANS (20 μM) was added to intact hepatocytes (10^6 cells/ml) at 25°C in 0.25 M sucrose/5 mM Tris · HCl (pH 7.4). Fluorescence emission was measured at various time intervals at 470 nm. ●———●, 85% cell viability; ▲———▲, 10% viability.

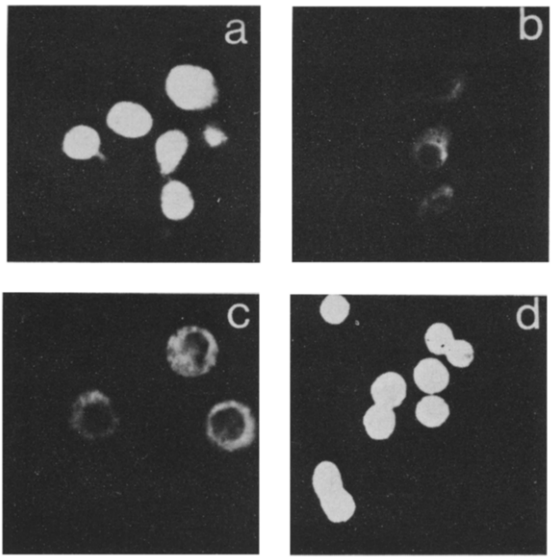


Fig. 3. Fluorescence micrographs of hepatocytes and HTC cells. Cells were incubated with ANS (20 μM) for 30 min at room temperature prior to examination. a, Viable hepatocytes; b, non-viable hepatocytes; c, viable HTC cells; d, viable HTC cells treated with 1 mM tetracaine.

hepatocytes were treated with 154 mM arsenate to give a cell suspension with 10% viability as assessed by trypan blue exclusion. No alteration in cellular morphology was observed by phase contrast microscopy (1000X). Extracellular proteins were measured before and after arsenate treatment. These studies showed that no intracellular proteins were released as a result of the arsenate treatment. As shown in Fig. 2, arsenate treatment resulted in a large decrease in the slow component of fluorescence enhancement. As shown in Fig. 3b, ANS fluorescence is also greatly diminished in intensity and is not distributed uniformly throughout the cell as compared to the viable hepatocytes. In addition, non-viable hepatocytes shocked in hypotonic solution (100 mosM) or in

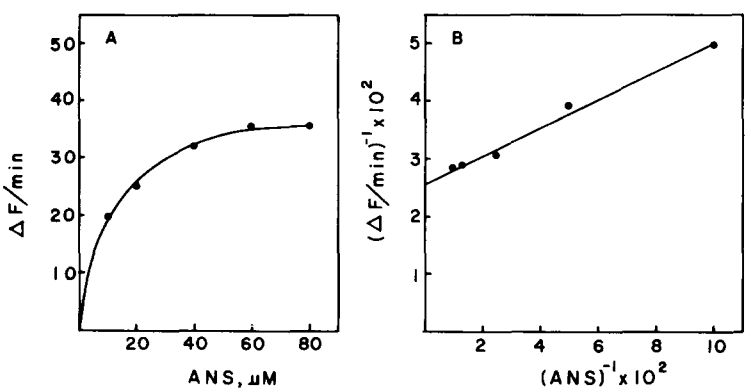


Fig. 4. A. Effect of ANS concentration on the rate of uptake into intact hepatocytes. Uptake was measured at 25°C at pH 7.4 in sucrose/Tris buffer. The rate is calculated from the initial slope of the slow component of fluorescence enhancement as shown in Fig. 2 (30—120 s). B. Double reciprocal plot of ANS uptake by hepatocytes indicates a K_m of 10 μM.

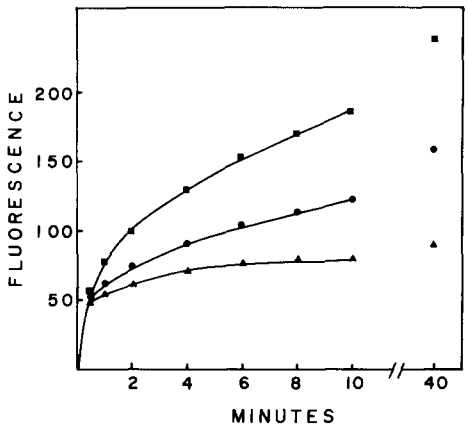
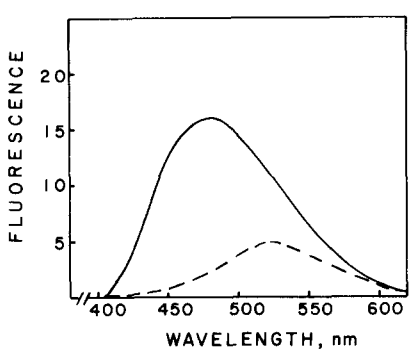


Fig. 5. The effect of temperature on the uptake of ANS by intact hepatocytes. ANS (20 μM) was incubated with hepatocytes (10^6 cells/ml) in sucrose/Tris buffer, pH 7.4. ▲———▲, 4°C; ●———●, 25°C; ■———■, 37°C.

a glass Dounce homogenizer (2—5 strokes) now showed a 70% increase in fluorescence over that observed for the unshocked non-viable cells, further supporting the idea that ANS binding to intracellular sites is greatly reduced. The above data suggest a significant reduction in ANS transport in non-viable hepatocytes.

Interaction of ANS with HTC cells. The addition of 20 μM ANS to a suspension of intact HTC cells (1 · 10^6 cells/ml) in 0.25 m sucrose/5 mM Tris · HCl, pH 7.4, for 40 min at 25° C, resulted in a 3-fold enhancement of fluorescence intensity over that observed in the absence of cells, and a significant blue shift



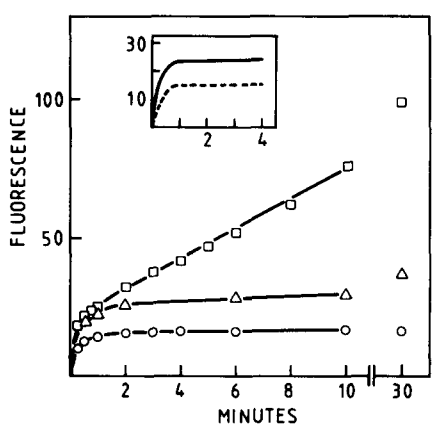


Fig. 6. Fluorescence emission spectrum of ANS in the presence of HTC cells. ANS (20 μM) was incubated with HTC cells (10^6 cells/ml) in 0.25 M sucrose/5 mM Tris · HCl, pH 7.4, at 25°C for 40 min (———). ANS in sucrose/Tris buffer (- - - - -).

Fig. 7. Kinetics of the interaction of ANS with intact HTC cells. Tetracaine was added 10 s before the addition of ANS (20 μM) to HTC cells (10^6 cells/ml). ○———○, HTC cells, 25°C; △———△, HTC cells + 1 mM tetracaine, 4°C; □———□, HTC cells + 1 mM tetracaine, 25°C. Inset: Time course of ANS interaction with isolated HTC cell plasma membranes in the presence (———) and absence (- - - - -) of 1 mM tetracaine.

with an emission maximum of 480 nm, as shown in Fig. 6. This spectrum is similar to that obtained from purified HTC cell plasma membranes [25]. The increase in fluorescence as a function of time is shown in Fig. 7. These results indicate a rapid initial binding of ANS to the plasma membrane which is completed in 15 s. The absence of a second slower phase, as observed in the normal hepatocytes, suggests that the influx of ANS into HTC cells is greatly reduced. Fluorescence microscopic examination of HTC cells incubated in the presence of ANS (Fig. 3c) shows a large decrease in fluorescence intensity and a non-uniform distribution as compared to viable hepatocytes, also supporting the above conclusion that ANS transport into the intracellular compartments is significantly reduced.

The addition of the local anesthetic, tetracaine (1 mM), resulted in a significant alteration in the time dependency of fluorescence enhancement (Fig. 7). Following a rapid initial fluorescence increase, a slow component is now observed, similar to that observed in the hepatocyte system, suggesting that the membrane perturbation by the anesthetic results in an increased uptake of the fluorescent probe. These results are also supported by fluorescence microscopic evaluation (Fig. 3d) where the ANS fluorescence is increased in intensity compared to the untreated HTC cells and is now uniformly distributed across the face of the cell, suggesting intracellular localization of the probe. In order to establish that the slow component is not caused by the time dependence of the anesthetic-ANS interaction with the membrane, the time course of ANS binding to purified HTC cell plasma membranes in the presence and absence of tetracaine is shown in Fig. 7 (inset). These results demonstrate that the fluorescence increase resulting from the presence of the anesthetic reaches a maximum value within 30 s. The increase in fluorescence in the rapid initial phase, when the anesthetic is added to the intact HTC cells (Fig. 7), can be attributed to the effect of the anesthetic on membrane-bound ANS. The stimulation of ANS uptake into HTC cells by tetracaine was also shown to be temperature dependent with a Q_{10} of 2.0 (Fig. 7), where at 4°C the slow phase

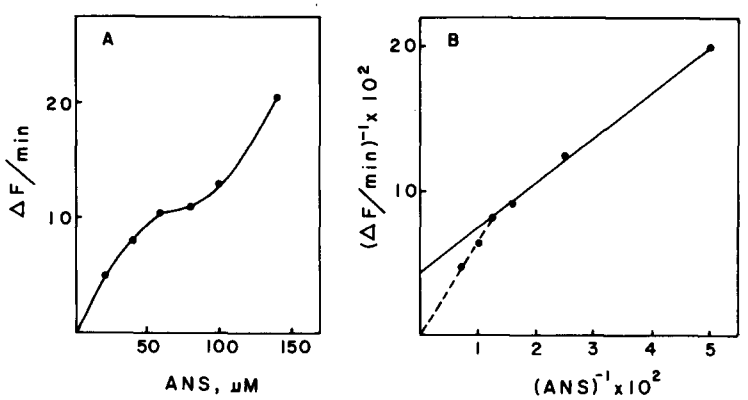


Fig. 8. A. Effect of ANS concentration on the rate of uptake into HTC cells in the presence of 1 mM tetracaine. The rate is calculated from the initial slope of the slow component of fluorescence enhancement as shown in Fig. 7 (15—60 s). B. Double reciprocal plot of ANS uptake by HTC cells in the presence of 1 mM tetracaine, indicating a K_m of 72 μM.

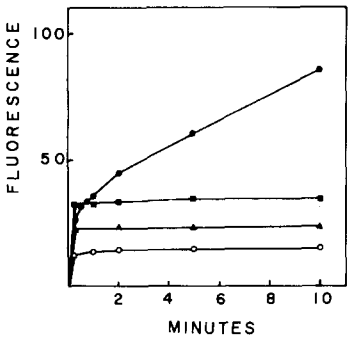


Fig. 9. Effects of tetracaine and cell viability on the kinetics of ANS uptake by HTC cells. Non-viable cells (10% viability) were prepared by treatment with arsenate as described in Materials and Methods. ANS (20 μM) was added to viable HTC cells (10^6 cells/ml) in the presence (●———●) and absence (○———○) of 1 mM tetracaine; ANS added to non-viable HTC cells in the presence (■———■) and absence (▲———▲) of 1 mM tetracaine. All studies were carried out at 25°C.

fluorescence enhancement was eliminated, suggesting that ANS was involved in a facilitated transport process. ANS uptake stimulated by tetracaine was shown to follow Michaelis-Menten kinetics at probe concentrations below 80 μM (Fig. 8a). Fig. 8b shows the Lineweaver-Burk plot of the influx data derived from the initial slope of the slow fluorescence enhancement component from HTC cells in the presence of 1 mM tetracaine (Fig. 7). This analysis gave an apparent K_m of 72 μM. At ANS concentrations higher than 80 μM, the plot deviates from linearity with an extrapolation of the data points passing through the origin, suggesting the involvement of a passive diffusion component.

In an effort to define the energy requirements for the ANS transport which is stimulated by tetracaine, intact HTC cells were treated with arsenate, to yield a cell suspension with a 10% viability value, as assessed by trypan blue exclusion. As shown in Fig. 9, non-viable HTC cells exhibited a similar time course fluorescence curve as that obtained from viable cells, both suggesting binding of ANS to the plasma membrane with a negligible transport component. In the presence of 1 mM tetracaine, which affects transport of ANS in viable cells, there appears to be no effect on transport in the non-viable cell system other than an increase in the initial burst of fluorescence enhancement which is caused by the effect of the anesthetic on ANS binding as previously described [25]. These results suggest that the perturbation of the HTC cell plasma membrane by the anesthetic results in the stimulation of ANS transport which is a facilitated transport process similar to that described for the normal hepatocyte system.

Discussion

In a previous report [25] we have investigated the interaction of ANS with purified plasma membranes derived from hepatocytes and from HTC cells. In the present study we have characterized the interaction of this fluorescent probe with the intact cell systems. The interaction of ANS with intact hepato-

cytes resulted in a marked increase in fluorescence intensity and a 50 nm blue shift. This increase in fluorescence was 35 times greater than that observed for ANS alone. The time course of fluorescence enhancement suggested an initial binding to the membrane followed by transport of the anionic probe into intracellular compartments. A similar biphasic time-course curve for ANS interacting with erythrocytes [26] and Ehrlich ascites cells [27] has been reported. Studies have suggested that ANS is transported into these cells and can act as a transport inhibitor of inorganic anions such as SO_4^{2-} and Cl^-. Other organic anion sulfonic acid derivatives, such as 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), which is nonpermeant [32,33], and *N*-(4-azido-2-nitrophenyl)-2-amino ethyl sulfonate [34], which is transported, have both been shown to be effective inhibitors of anion transport. SITS has also been shown to be effective in reducing ANS uptake [27] in Ehrlich ascites cells. Pyridoxal phosphate has been reported to be an effective inhibitor of anion transport whether or not it is fixed by $NaBH_4$ [35]. We have observed that 20 mM pyridoxal phosphate inhibits 70% of ANS uptake by hepatocytes (data not shown). The above studies suggest that ANS may thus be transported by the anion transport system in these cells. An anion transport protein from erythrocyte plasma membranes has been characterized using labeling, enzymatic and reconstitution techniques [32,34—38]. Recently, a high-affinity binding protein for the organic anion, sulfobromophthalein has been isolated from hepatocyte plasma membranes [39].

In viable hepatocytes (85% viability), ANS uptake was concentration dependent, reaching saturation at 60 μM ANS (Fig. 4a,b). The process was also shown to be temperature dependent (Fig. 5), with a Q_{10} of 2.2. These results suggest a carrier-mediated transport mechanism. Non-viable cells, however, exhibited negligible uptake of ANS (Fig. 2), strongly suggesting the requirement for metabolic energy for the functioning of the anion transport system. These results were corroborated by fluorescence microscopy (Fig. 3a,b). Similar uptake results in isolated hepatocytes have been reported for the organic sulfonate anions, bromosulfophthalein [14] and taurocholic acid [16], and the carboxylate anion, cholic acid [12]. In non-viable hepatocytes, the initial ANS fluorescence enhancement (Fig. 2), which is attributed to binding of the probe to the membrane, is greater than that observed in the viable cells. This increased fluorescence may be due to an alteration in the number of ANS binding sites or in the ANS binding affinity resulting from an alteration in the environment of the probe. These results suggest that the cell surface may be sensitive to the intracellular metabolic state.

The interaction of ANS with intact viable HTC cells gave an emission spectrum (Fig. 6) that was only 3 times greater than ANS alone. As opposed to the hepatocyte system, the time course of fluorescence enhancement (Fig. 7) and fluorescence microscopy (Fig. 3c) suggested a greatly reduced transport of ANS into the HTC cell system. This observation suggested a significant alteration in membrane properties between normal hepatocytes and HTC cells. We can interpret these results as deriving from an alteration in the accessibility or in the V and/or K_m of the transport system, possibly due to structural modifications in the plasma membrane such as altered lipid-protein interactions. The uptake of ANS was, however, stimulated by tetracaine, as demonstrated in

Fig. 7 and corroborated by fluorescence microscopy (Fig. 3d). The transport now exhibited properties similar to those observed in the intact hepatocytes. The process was concentration- and temperature dependent (Figs. 7,8) and had a requirement for metabolic energy (Fig. 9), suggesting a carrier-mediated transport mechanism.

The interaction of local anesthetics with plasma membranes from several cell systems has been shown to have profound effects on membrane architecture and dynamics [40—44]. Studies have suggested that anesthetics interact with membrane acidic phospholipids and are able to displace membrane-associated Ca^{2+} [41,45], resulting in part in an increase in the fluidity of the membrane system. Hydrophobic interactions have also been shown to play a significant role [46]. Anesthetics have also been shown to alter the structural organization of membrane-associated proteins such as microfilaments and microtubules which are involved in the transmembrane control of surface components [47,48]. The mechanism by which tetracaine can stimulate the uptake of ANS in HTC cells could thus involve a perturbation of one or more membrane components. Several studies [49—53] have reported that the transport of ANS across lipid bilayers was extremely sensitive to the physical state of the phospholipids, and that the rate of ANS transport in phospholipid dispersions increased 100-fold in the presence of local anesthetics such as procaine. This phenomenon may also play a role in the tetracaine effect described in this report.

Many transport systems have been shown to function at elevated rates following cell transformation. The transport of many sugars has been shown to increase in transformed cells [54,55]. Similar effects have been observed for certain amino acids and amino acid analogs [56,57] and for phosphate [58]. The striking decrease in ANS transport in HTC cells which differs from previously reported transport patterns in transformed cells suggests a significant alteration of membrane function in this cell system. Initial studies (unpublished observations) have also shown that sulfate transport is decreased by 90% in HTC cells compared to the normal hepatocyte system. Studies are underway to characterize the membrane components involved in anion transport in the hepatocyte system and the factors involved in the regulation of their function.

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